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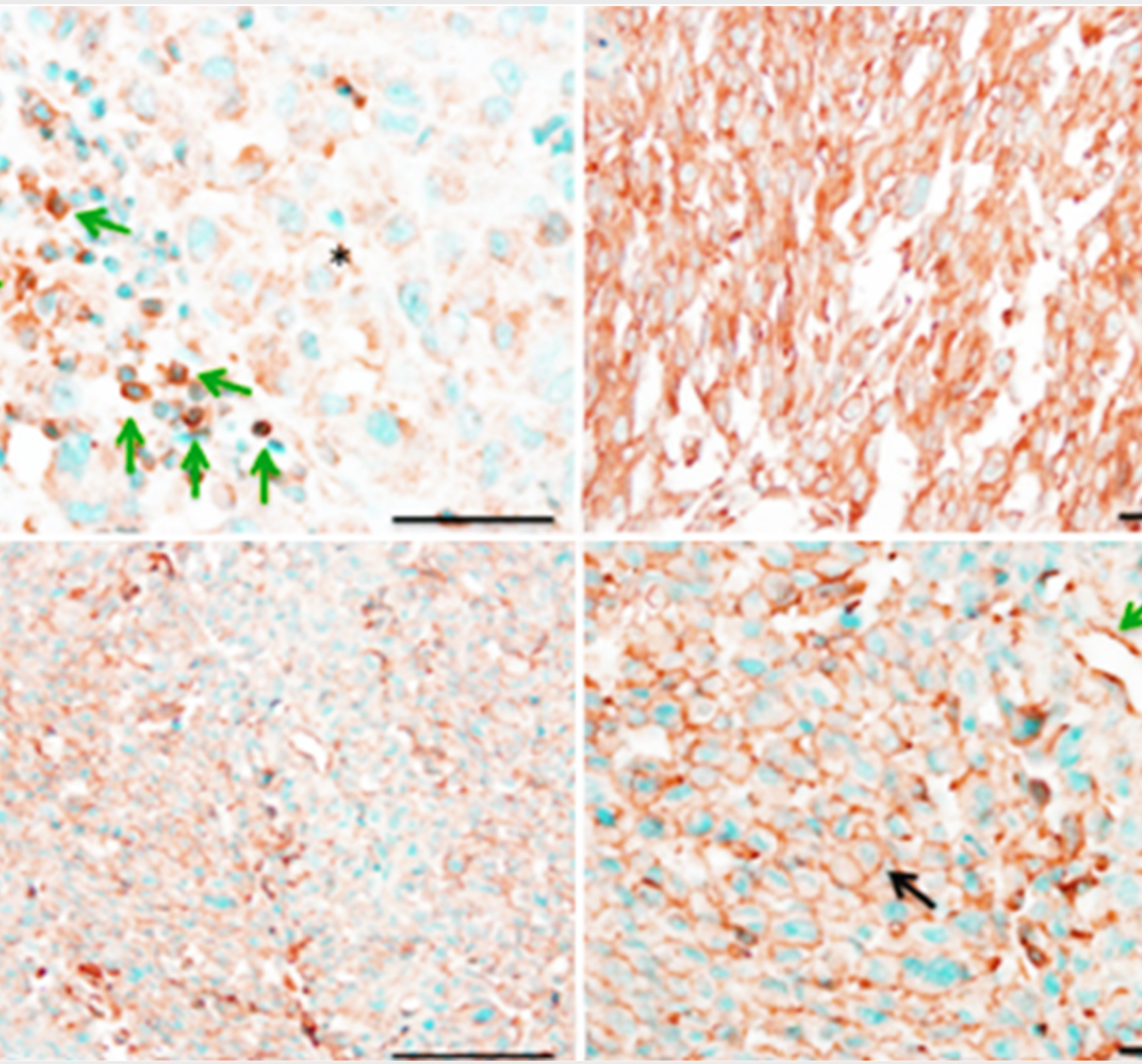

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Editorial

Welcome to *Neuroimmunology and Neuroinflammation*: a new open access journal for neuroscience

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BACKGROUND

The nervous and the immune system are two essential components of the human body. The developing research in neuroscience and immunology demonstrated that these two systems complement and interact with each other.[1] Details of function and cooperation at the molecular level of both systems are not revealed to date.

Inflammation involves the innate and adaptive immune system. It is a normal response to infection. However, uncontrolled progress of inflammation results in the onset of autoimmune or autoinflammatory disorders. Characteristics of inflammation appear in many neurological diseases, including meningitis, encephalitis, multiple sclerosis, Alzheimer's disease, Parkinson's disease, and epilepsy.[2,3] Recent researches in this area focused on the role of immune cells and immune mediators in the initiation and progression of specific neurological diseases.[4,5] Further, pharmacological inhibition of inflammation pathways will be able to safely reverse or slow the course of these diseases.

This hot topic brings together review and opinion articles that describe our current understanding of the crosstalk between the nervous and immune systems, of the immunopathogenesis and of emergent immunotherapies for specific neurological conditions.[3]

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Currently there are more than 600 journals which focus on "neuro". However at present, only six journals focus on neuroimmunology, four of which have been included by Science Citation Index (SCI). To sum up, there was a necessity to found a journal, which is devoted to this field. *Neuroimmunology and Neuroinflammation (Neuroimmunol Neuroinflammation)*, a new journal, is envisioned and founded to represent the growing needs of medical science. It is aimed at publishing high quality articles to promote the development of neuroimmunology.

BRIEF INTRODUCTION OF THE JOURNAL

Neuroimmunol Neuroinflammation is a new peer-reviewed, open access, online journal. The journal is an official journal of the Chinese Society of Neuroinfection and Cerebrospinal Fluid Cytology, and aims to build an open and international academic exchange platform, to promote the exchange of ideas and experiences, and then to improve the academic levels of neuroscience. Primarily focusing on neuroimmunology and neuroinflammation, it extends to biology, pathology, physiology, pharmacology, endocrinology, psychology, oncology, *etc*. The various types of manuscripts, including original articles, review articles, case reports, commentaries, editorials, and letters to the editors are published in *Neuroimmunol Neuroinflammation*.

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The editorial board is composed of 145 experts and scholars from more than 20 countries. Their specialties cover different areas of neuroscience, such as neuroimmunology, neuroinflammation, neurobiology, and neuropharmacology. They are responsible for

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THE AIM OF THE JOURNAL

The plan of the journal is to be indexed by PubMed Central in 1 year and by SCI within 2 years. The objective is great, but every member of the editorial board will try his best. We think in the near future, it will come true!

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Editorial

Pitfalls in clinical diagnosis and treatment of infectious meningitis in China

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INTRODUCTION

Infectious disease of the central nervous system (CNS), such as meningitis, is one of the most important categories of neurological conditions. Such diseases are challenging in terms of diagnosis, treatment, and prognosis. Meningitis occurs most often in young- and middle-aged patients, and the rate of misdiagnosis, especially during the early stages of the disease, is high. Correct diagnosis and treatment can save lives, but many factors, such as difficulty in medications crossing the blood-brain barrier (BBB) can cause difficulty in treatment.

ANALYSIS OF THE MISDIAGNOSIS RATE OF INFECTIOUS MENINGITIS

There are several reasons for the high rate of misdiagnosis in meningitis: (1) the development of this clinical sub-specialty is relatively recent. The professional setting and staffing for research into infectious CNS diseases fall behind those for research into cerebrovascular, demyelinating, neuromuscular, degenerative, and genetic diseases. At present, only a few hospitals in China, such as in Beijing, Shanghai, Xi'an, Shijiazhuang, and Yinchuan, have infection and cerebrospinal fluid cytology (CSFC) as a professional sub-specialty. (2) The content of current textbooks is out of date. It lacks data from large, double-blind, multicenter, case–control studies, as well as basic research data. The data on meningitis in current textbooks are about 40–50 years old, and some are from foreign studies, whereas the chapters on cerebrovascular disease are updated every 5–10 years. (3) The clinical manifestations of meningitis can be atypical. Meningitis has shown the greatest change in clinical manifestations over time compared with other nervous system diseases. "Atypical clinical manifestations" may, in fact, be the current "typical" features, but are different from the manifestations seen 50 years ago. The 50-year fight between the meningitis pathogen and the human immune system, the natural variation of the pathogenic organisms, the misuse of antibiotics and immune suppressants, and the prevalence of drug addiction and AIDS have all produced changes in the clinical manifestations of meningitis. (4) Obtaining samples of the pathogen is difficult. Taking a biopsy from the mater has technical limitations, and it may be difficult for the patient and family to accept. The positivity rate for pathogens in CSF is very low, except for *Cryptococcus neoformans*, which can be 99% in most references.[1,2] The textbooks state that the positivity rate for *Mycobacterium tuberculosis* by smear and culture of bacteria from CSF can be as high as 30%–40%, but clinical reports from most hospitals show a positive rate of below 10%. Of 167 patients with tubercular meningitis who were assessed during the period 1990–2010 in our hospital, only one had a positive result for *M. tuberculosis* by bacterial smear and culture from CSF samples.[3] Using the new acid-fast stain method of The Fourth Military Medical University, the positivity rate of CSF smear can be above 90%, enabling early diagnosis of tubercular meningitis.[4] Identifying the pathogens underlying cases of viral meningitis, weakly pathogenic bacterial meningitis, and parasitic meningitis is also difficult. For these reasons, the misdiagnosis rate in early meningitis is very high.

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DIFFICULTIES IN DIAGNOSIS AND TREATMENT OF INFECTIOUS MENINGITIS

The BBB protects the brain tissue, but is also the greatest obstacle to treatment of infectious meningitis. The focus is often on the effective permeation rate of treatment drugs across the BBB, instead of the sensitivity of the drug, which can influence the efficacy of treatment.[5]

High doses and a long treatment course are needed for therapy of infectious meningitis. For example, an intensive course of treatment for tubercular meningitis requires a dose of isoniazid of 15 mg/kg per day, whereas the general dose in instruction is 0.6 g/day. The treatment course for intracranial tuberculosis is double that for extracranial tuberculosis, that is, 4–6 months of intensive treatment and 18–24 months for the whole course.[6] Such doses and treatment courses pose challenges for both doctors and patients.

Because of the problems of pathogen isolation and difficulty in permeating the BBB, doctors need to perform experimental therapies and choose drugs that can effectively cross the BBB. That means doctors have to break the usage principle of antibiotics or the medical insurance regulations. This is also a great challenge.

From the three points above, we can see that the risk of treatment failure in infectious meningitis is higher than in other infectious diseases. Doctors are extremely concerned about the risk in specific countries and regions (tense physician – patient relationship) or of legal action.

DIAGNOSIS AND TREATMENT OF MENINGITIS

Differential diagnosis of infectious and noninfectious meningitis

A patient presenting with fever, headache, nausea, emesis, meningeal irritation, and abnormal CSF findings (high pressure, increased white cell count, and decreased glucose and chloride levels) is easily misdiagnosed as having meningitis. However, it is necessary to exclude noninfectious causes of meningitis such as chemicals, connective tissue diseases, and tumors. A patient with chemical meningitis usually has a clear history of intrathecal medicine injection such as cytarabine, methotrexate, or analgesics. Because chemical meningitis often occurs during a period of hospitalization or in patients with a clear history of using specific medicines, it is easier to exclude. Connective tissue disease-related meningitis is often ignored. In such cases, we need to clarify if there is a medical history of systemic lupus erythematosus, sarcoidosis, or rheumatoid arthritis; order the appropriate laboratory investigations; and request a rheumatology consultation. Tumor-related meningitis (immunity meningitis or cancerous meningeal disease) can present with fever or other symptoms of meningitis. Detection of tumor markers in blood and CSF, cytology testing of CSF, and scans (computed tomography, magnetic resonance imaging [MRI], and positron emission tomography) can be helpful in the differential diagnosis.

Differential diagnosis of possible infectious meningitis pathogens

Pathogens causing infectious meningitis include bacteria, fungi, and viruses. It is important to distinguish the species of pathogens with no result of CSF smear. Purulent meningitis is easier to identify by observing the CSF appearance, CSF cell number, and the percentage of multinucleate cells.

Viral, tuberculous, and *C. neoformans* meningitis are more difficult to distinguish. The disease course for tuberculous and *C. neoformans* meningitis is over 6 weeks, and may be as long as several months, but that for viral meningitis is often less than 3 weeks. The body temperature of a patient with viral or *C. neoformans* meningitis can be over 39°C, but a patient with tuberculous meningitis often has fever in the afternoon and the body temperature is below 39°C.

With regard to CSF examination, the differences between the various meningitis types are as follows: (1) pressure: in *C. neoformans* meningitis, pressure is above 300 mmH_2O; in tuberculous meningitis, it is more often between 250 and 280 mmH_2O (rarely above 300 mmH_2O unless there is meninges adhesion); and in viral meningitis, it is normal or a little higher, rarely above 250 mmH_2O.[7] (2) Glucose and chloride levels: in tuberculous meningitis, these are both decreased or at least glucose is decreased, sometimes below 1.0 mmol/L; in viral and *C. neoformans* meningitis, glucose is decreased or normal, often between 2.0 and 2.8 mmol/L, while chloride is generally normal, or if decreased, is often between 110 and 118 mmol/L. (3) Protein levels: in tuberculous meningitis, protein is obviously increased at between 1.0 and 2.0 g/L,[8] and may be over 10 g/L, but in viral and *C. neoformans* meningitis, it is rarely more than 1.0 g/L.

Using MRI with enhancement, we can see that the strengthened signals in the meninges are strongest for

tuberculous meningitis, and are sometimes accompanied by ring enhancement of tuberculoma.[9] These signals are weakest for viral meningitis and may sometimes be absent.

How to determine the diagnosis and treatment strategy without identifying the pathogen?

It is difficult to determine the diagnosis and treatment strategy without having pathogen identification. On the basis of that stated above, experimental therapy can be carried out for 2–3 weeks if we have propensity diagnosis, and then the subsequent strategy can be determined based on the effect of treatment. If there is no tendency for diagnosis, my personal experience is as follows.

Step 1: we treat the condition as viral meningitis for 2–3 weeks. This treatment can continue if there is a positive effect on the clinical, CSF, or imaging findings; otherwise, we go on to the next step. Step 2: we treat it as tuberculous meningitis for 2–3 weeks. This treatment can continue if there is a positive effect on the clinical, CSF, or imaging findings; otherwise, we go on to the next step. Step 3: we treat it as *C. neoformans* meningitis for 2–3 weeks, and then assess the effect.

During every treatment step, efforts must continue to identify the pathogen and then re-diagnose. If the pathogen is identified, targeted treatment can commence; otherwise, we can only perform experimental therapy based on the clinical, CSF, or imaging findings.

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Editorial

The M1/M2 immune polarization concept in microglia: a fair transfer?

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Although macrophages were first described in 1882 by Ilya Ilyich Metchnikoff, it was 30 years before the first descriptions of monocytes, their relatives within the blood stream.[1,2] Initially described as a phagocytic cell mainly within the context of tissue inflammation, it took until the turn of the millennium to introduce the alternative myeloid cell (MØ) immune polarization concept, which was provided by Mills *et al*. in 2000.[3] This concept of differential M1-M2 polarization of MØ was deduced from the classical dichotomic activation program of lymphocytes.

However, it was an oversimplified idea to attribute MØ activation to the so-called Th1-lymphocytes producing interferon-γ while MØ inhibition was ascribed to Th2-lymphocytes secreting high levels of interleukin 10, among other cytokines. It was demonstrated that lymphocytes determine the activation state of MØ, and MØ strongly influences the differential activation state of lymphocytes.[4] Because it was shown that MØ stimulated by Th1-lymphocytes secreted high levels of nitric oxide, thereby leading to a reduced proliferative potential, the M1-M2 concept was quickly adapted to other paradigms, such as tumor-associated macrophages (TAM) or tissue repair mechanisms.[5] In general, M1-polarized TAMs were regarded as immune cells with tumor-suppressive capacity, whereas M2-polarized TAMs are tumor promoting.

The M1-M2 concept also quickly attracted the attention of many neuroscientists working in immunological research. Although there was a lack of substantial data, direct transfer of the immune polarization idea to microglial cells was frequently performed without further testing its applicability. More recent studies made it increasingly evident that microglial cells and blood-derived macrophages display considerably different phenotypes upon similar stimulation conditions.[6] In particular, M2 conditions, which typically induce CD163 and CD206 in macrophages, failed to result in a similar phenotype in microglial cells. Furthermore, in central nervous system (CNS) disorders, such as Alzheimer's disease, the immune polarization state of microglial cells is not solely dependent on the microenvironmental immune milieu, it is also strongly related to the amyloid beta deposit subtype (oligomeric or fibrillar forms).[7] A more recent study demonstrated that autopsy cases of Alzheimer's disease display M1-polarized MØs in very early stages, while more severe stages with increased levels of neuritic plaques (and often accompanied with extensive cerebrovascular pathology) displayed a M2a-polarized subtype.[8] Other findings in animal models of neurodegenerative disorders, such as the mutant superoxide dismutase model for amyotrophic lateral sclerosis, revealed that M2-polarized microglia were neuroprotective, but M1-polarized microglia were neurotoxic.[9] Notably, the first experimental treatment approaches revealed that chronic neurodegenerative changes related to microglial activation can be attenuated via minocycline, a substance inhibiting microglial activation, which leads to increased activation of the M2-polarized microglial phenotype.[10] However, there is also opposing data claiming that activated microglia do not specifically up-regulate molecules of either the M1- or M2-polarized phenotype; rather they show an increase on both axes.[11]

Nevertheless, many authors simply classify myeloid immune cells in the CNS under the combined term as

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"microglia/macrophages" without further distinction of their real origin, thereby gaining only a single phenotypic signature for distinct cell types.[12-16] The most recent experimental data from brain injury models even revealed that microglial cells did not transform to classical M1 or M2 phenotypes under circumstances in which their blood-borne-derived counterparts do, thereby suggesting another regulatory function subtype.[17] This study further pointed to a more protective role of microglia upon CNS injury, while blood-borne-derived myeloid cells seem to exert more cytotoxic properties. Because the pioneering authors of the M1-M2 concept in macrophages stated that their proposed views of classifying macrophages in either a M1 or M2 polarized state might be an oversimplification, a fortiori is its transfer to microglia cells.[3] In CNS pathologies, microglial cells are nonclonal and show a high-degree of plasticity, as well as intermixture with peripheral, blood-borne macrophages.

Deciphering the immunological properties of microglial cells under normal and pathological circumstances with regard to the M1/M2 concept requires more functional studies, which will need to take into account the distinct microglial gene expression signature.

More sophisticated scientific neuroimmunological and/or neuroinflammatory research approaches are necessary. Notably, sorting myeloid cell populations might pave the way for deeper insight into the applicability of the M1/M2 immune polarization concept for microglia, the resident immune cells of the CNS.[18] Such studies will impact and broaden the knowledge of basic research and influence further treatment strategies in traumatic, inflammatory, or neoplastic disorders of the CNS.

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Editorial

Diagnosis and therapy of rare central nervous system infections

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INTRODUCTION

Central nervous system (CNS) infection is one of the most disabling and deadly diseases worldwide. According to the World Health Organization, there were about 700,000 cases of meningitis in 2004, with approximately 340,000 related deaths.[1] CNS infection includes infection with bacteria, viruses, fungi and parasites. In most cases, it is difficult for radiologists and clinicians to make a definitive diagnosis. Therefore, we reviewed all the relevant domestic and international clinical research development.

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS OF CENTRAL NERVOUS SYSTEM *MYCOBACTERIUM TUBERCULOSIS* INFECTION

Central nervous system tuberculosis, accounting for 5%–10% of all types of *Mycobacterium tuberculosis* (MTB) infection, is an extra-pulmonary tuberculosis leading to various complications and high rates of morbidity and mortality.[2] CNS tuberculotic patients may show either general manifestations caused by toxins from N/med tuberculosis bacilli, such as low-grade fever, night sweats and headache, or the symptoms of encephalitis and meningitis. Major complications of tuberculous meningitis include hydrocephalus, tuberculous vasculitis and cranial nerve palsies. Intracranial tuberculosis includes tuberculoma tuberculosis, tuberculous abscess, tuberculosis encephalitis, and encephalopathy.[3]

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The clinical symptoms of tuberculous meningitis are relatively obvious. Among them, headache and fever are the most common symptoms. Besides, the symptoms of tuberculous meningitis, tuberculous meningoencephalitis can also show signs of the brain parenchymal involvement, insanity or cognitive impairment, and signs of specific area damage, such as cranial nerve palsies and epilepsy. Typical characteristic cerebrospinal fluid (CSF) findings of CNS tuberculosis include the following: total white cell counts increase (usually [50–200] $\times 10^6$/L, 1000 $\times 10^6$/L in very few cases), and neutrophil predominance presents very early, with lymphocytic-predominant pleocytosis developing later; an elevated CSF protein, typically 1–2 g/L; and a significant reduction in sugar and chloride levels. Tuberculous meningoencephalitis often occurs with atypical clinical features, sometimes with the acute or chronic onset, manifested by fever, neurological symptoms, and nondiagnostic CSF findings. Above all, at present, we need to make a comprehensive judgment-based on both clinical features and image data of the patients. Meningeal enhancement is usually visible at the skull base, lateral fissure, optic chiasma, and brain stem. When CSF protein is increased significantly, computed tomography (CT) images may additionally show enhancement of the suprasellar cistern, optic chiasma cistern, and prepontile cistern due to exudative reaction. Magnetic resonance imaging (MRI) offers a significant advantage for diagnosis of CNS tuberculosis, especially when incorporating magnetization transfer imaging.[3]

Nowadays, the typical fever symptom in tuberculoma patients is rare, and CSF changes may not be typical either. The only abnormal finding is the presence of granulomas in brain CT and MRI images, with nodular and rim enhancement and without obvious meningeal enhancement. CNS tuberculoma must sometimes be

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distinguished from CNS sarcoidosis, which leads to a significant increase in angiotensin-converting enzyme in the serum and most of the patient's CSF.

Tuberculous meningitis can easily be confused with cryptococcal meningitis, which has similar manifestations, such as fever, headache, and signs of meningeal irritation, but cryptococcal meningitis has higher elevated intracranial pressure, lower CSF chloride, and rarely any red blood cells in the CSF. *Cryptococcus* meningitis can be confirmed by CSF India ink capsule staining. By contrast, tuberculous meningitis often has red blood cells in the CSF, a greater number of changes in the WBC count, and higher CSF protein.

Although T–SPOT.TB and Xpert MTB/RIF assays have improved the positive detection rate of MTB in CSF, diagnosis of tuberculous meningitis remains difficult. A modified acid fast stain designed by doctors from the Department of Neurology, Xijing Hospital of the Fourth Military Medical University, has significantly improved the positive detection rate of MTB in CSF.[4] However, there is still an urgent need for more effective testing techniques to improve the detection rate of MTB.

One way to diagnose tuberculosis is by antituberculosis treatment itself. For patients in whom a definitive diagnosis is difficult, antituberculosis treatment can be used to diagnose - through observation - any improvement in clinical symptoms and changes in the CSF. For all tuberculotic patients, both standard treatment and a strict antituberculosis treatment regimen are needed; treatment duration is usually 18–24 months.

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS OF CENTRAL NERVOUS SYSTEM VIRUS INFECTION

In clinical practice, we can make our diagnosis of CNS virus infection based on clinical manifestations, CSF changes, and corresponding serum or CSF virus antibody detection results and neuroimaging observations. Using epidemic encephalitis, which is insect-borne viral encephalitis, for example, it can easily be found in summer and autumn, which are the high-incidence seasons. The main pathological changes are visible on the thalamus, basal ganglia, brain stem and cerebellum, with the corresponding changes visible on MRI scans. High fever, lethargy, coma and epilepsy are the most common symptoms. Herpes simplex viral encephalitis often involves bilateral temporal lobes and frontal lobes, and makes the patient prone to epilepsy and mental symptoms. Herpes simplex viral encephalitis can be diagnosed easily by typical imaging observations [Figure 1]. Rabies encephalitis often has a history of dog or cat bite; some patients may have the first attack 10 years after a bite. Until date, its pathogenesis has not been explained. Beside the clinical manifestations, rabies encephalitis has some notable symptoms, such as laryngeal muscle cramp, hydrophobia, and neck stiffness and opisthotonos. Head imaging generally shows no obvious changes, and some cases of rabies encephalitis progress quickly. Patients may go into coma and then die within a few days.

Many cases of viral meningoencephalitis cannot be confirmed effectively by testing because of the variation in viruses and detection of new viruses. Without typical clinical manifestations, such cases are easily misdiagnosed.

Acyclovir, rather than ganciclovir, should be the first choice of antiviral treatment for viral encephalitis. According to the virus treatment principle, treatment should last at least 3–4 weeks; a few cases may need treatment for 8 weeks or more. When elevated transaminases occur during treatment, we should distinguish between a

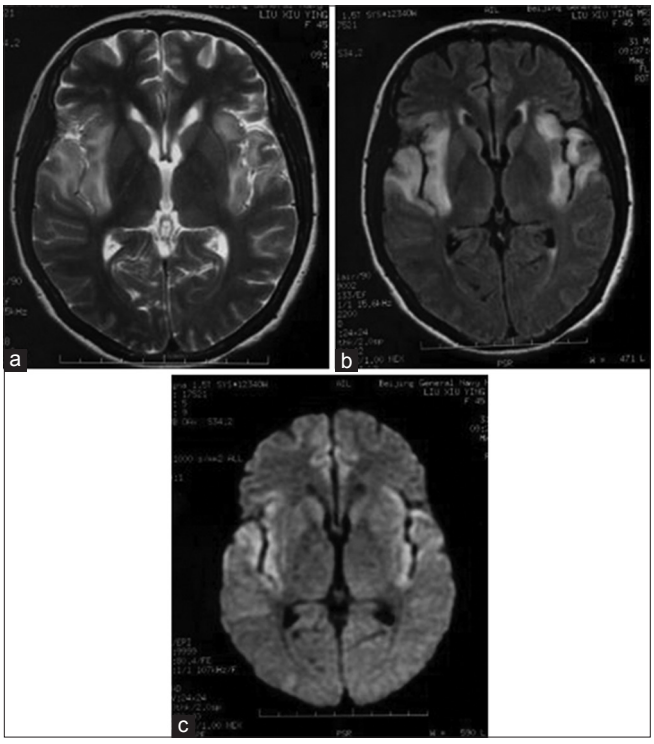

Figure 1: Magnetic resonance imaging representations of a herpes simplex virus encephalitis patient with intelligent obstacle and epilepsy seizure: high signal on T2-weighted image, fluid-attenuated inversion recovery (FLAIR) image and diffusion-weighted imaging (DWI) image on bilateral temporal lobe and insular lobe. (a) T2-weighted image; (b) FLAIR image; (c) DWI image

temporary rise resulting from the virus infection, or the side-effects of the anti-virus medicine, and offer appropriate liver protective medication instead of immediately changing the medication.

DIAGNOSIS OF CENTRAL NERVOUS SYSTEM PARASITIC AND FUNGAL INFECTIONS

Parasitic infection has regional and seasonal features. For example, brain-type lung fluke (infected by eating raw or undercooked crab or crayfish) and Lyme disease (infected by tick bite) are more common in the Northern regions of China, while sparganosis (infected by playing with or eating frog) and amebic meningoencephalitis (infected by often swimming in a warm, muddy or dead freshwater ditch) are more common in the Southern regions of China. Hydatid disease is more common in the pastoral herders. At present, neurocysticercosis is relatively rare because constantly strengthened pork quarantine and dramatically improved local health conditions have greatly diminished pork infected by tapeworm. Different types of parasitic infections bear their own imaging characteristics. For instance, the head section and apparent body wall of a tapeworm could be distinguished clearly in images of cerebral cysticercosis infection, and the migration of "tunnel-like" lesions in the brain parenchyma are visible in images of sparganosis infection. All the above parasitic infections could be definitively diagnosed by specific antibody tests.

Fungal infection has gradually increased in recent years, mainly due to the increase in acquired immunodeficiency syndrome (AIDS) infection, transplant surgery and drug resistance to fungal medication. Certainly, *Cryptococcus* neoformans infection is still the most common while *Aspergillus* and *Mucor* infection of the nervous system is relatively uncommon. Fungal infection is generally an opportunistic infection and is not directly related to contact with pigeons (doctors often ask patients, whether they raise pigeons). Cryptococcal fungus also exists in the nasal passages of healthy subjects, but is usually not pathogenic. Cryptococcal infection usually occurs in subjects with a weakened or deficient immune system, such as in those with cancer or AIDS, or as a result of long-term use of immunosuppressive agents or hormones. Zhu *et al*.[5] have reported that there was no decline in immune function in a patient infected with *Aspergillus*, in spite of the presence of brain-occupying lesions caused by *Aspergillus* infection. The clinical manifestations of this case resemble those of another 93 cases reported by Antinori *et al*.,[6] of which 55.9% (52 cases) showed no decline in immune function and no predisposing factors. Therefore, fungal infections can also occur in people with normal immune function. It is not easy to distinguish deep brain-occupying lesions of granuloma formation from brain tumors and abscesses. Such cases require a diagnostic approach that combines CSF examination with bacteria and fungi examination, analysis of pathogens by incubation, and polymerase chain reaction testing. Brain radiation therapy or excision surgery should not be performed blindly before a clear diagnosis is made, otherwise the outcome will be misdiagnosis or, even worse, the spread of fungi. At present, the main treatment for fungal infections is by use of, for example, liposomal amphotericin B, fluconazole and voriconazole, which exhibit greater efficacy, safety and fewer side-effects than both amphotericin B and allicin.

DIAGNOSIS OF PRION DISEASE

Creutzfeldt–Jakob disease (CJD) is one type of prion disease – a molecular conformational disease caused by deposition of abnormal prion protein (PrP^{Sc}) – in which the structure of the normal prion protein PrPc changes, in neurons. Prion diseases, also known as "transmissible spongiform encephalopathies", are a class of CNS degenerative encephalopathies that can infect both animals and human beings with a long incubation period and a 100% mortality rate. In addition to CJD, human prion diseases include fatal familial insomnia, Kuru and Gisborne Terman-Strauss syndrome (Gerstmann–Sträussler–Scheinker syndrome). The most common human prion disease is sporadic CJD, the incidence of which seems to have increased in recent years.

The typical symptom triad of CJD is progressive dementia, ataxia and myoclonus. Clinical manifestations can be divided into three stages. The early stage is characterized by weakness, fatigue, difficulty in concentrating, and memory loss. The interim stage (dementia-spasticity) is characterized by memory disorders, personality changes and dementia, and it can also be associated with aphasia and agnosia. Two-thirds of patients may exhibit myoclonus, and a series of symptoms may occur successively or alternately in this period owing to cortical, extrapyramidal, pyramidal or cerebellar (alternating or damaged) disease. At the late stage, urinary incontinence, akinetic mutism or decorticate rigidity arise. Diagnosis is confirmed by rapid progression of recent memory impairment, without symptoms of infection. Imaging, especially diffusion-weighted imaging and

fluid-attenuated inversion recovery (FLAIR) pulse sequences, often reveals ribbon-like lesions along the cerebral cortex lesions, which is a very characteristic feature [Figure 2a and b]. However, at the late stage, brain atrophy and ventricular dilatation in patients is extremely severe, and ribbon-like lesions are no longer evident [Figure 2c and d]. White blood cells may also become visible in the CSF of the CJD patients. For instance, a virus or other infectious encephalitis cannot be confirmed until 10–30 white blood cells are found in the CSF. Until date, there has been no case in which the disease has been transmitted between patients, their family members and medical staff in China.

DIAGNOSIS OF CENTRAL NERVOUS SYSTEM SYPHILIS INFECTION

Recently, the incidence of syphilis – a disease caused by infection with *Treponema pallidum* – has escalated, with an increase in the incidence of syphilis infection of the CNS. Nervous system syphilis can be classified as, for example, asymptomatic neurosyphilis, syphilitic meningitis or myelomeningitis, syphilitic brain or spinal cord vasculitis, syphilis of the brain parenchyma (including polyparesis, tabes dorsalis and syphilitic retrobulbar neuritis), syphilitic gumma, or acute inflammatory polyradiculoneuritis. Polyparesis usually occurs in patients aged 35–45 years with a long incubation of between several years and 20 years. With the insidious onset, the main symptom of polyparesis is progressive memory loss, which is easily misdiagnosed as Alzheimer's disease. At the early stage, polyparesis patients experience personality changes, anxiety, and emotional volatility, which can easily be misdiagnosed as depression.

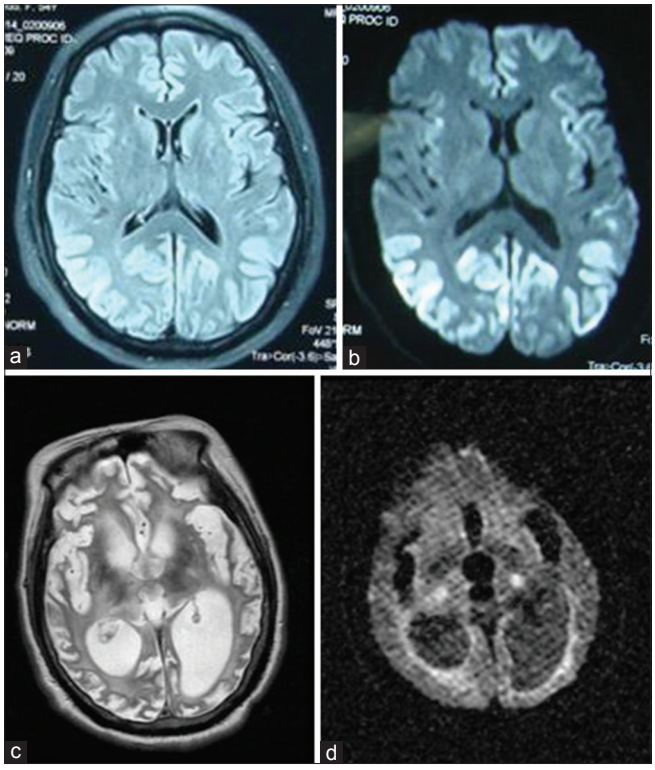

Figure 2: (a and b) MRI representations of a sporadic Creutzfeldt–Jakob disease patient: high signal on both fluid-attenuated inversion recovery image and diffusion-weighted imaging (DWI) (2009–6–16). (c and d) T2–weighted image and DWI image of the same patient in a persistent vegetative state (2011–7–13): serious encephalatrophy with an obvious increase in ventricular volume

In most cases, syphilitic antibody is the positive in serum, the white blood cells in the CSF are moderately elevated, and the protein level is also slightly increased, but a toluidine red unheated serum test and *T. pallidum* particle agglutination assay test can reveal normal results in a few cases. Imaging observations reveal brain atrophy, mainly in the hippocampus of the temporal lobe, and ventriculomegaly.[7] There is a difference in antisyphilitic treatment between polyparesis and general syphilis. For polyparesis patients, treatment duration time is 6 months to 1 year, sometimes even longer, which is longer than that for general syphilis patients. Improvement in symptoms varies considerably between patients depending on when the disease is first diagnosed.

Besides the infections described above, there are other CNS infections with typical clinical characteristics, such as human immunodeficiency virus, Brinell bacillus infection, Whipple's disease, Guangzhou Angiostrongylus disease, and malaria. Such diseases can be diagnosed by the application of appropriate tests. It should be noted that, alongside the development in clinical practice, clinical viewpoints vary. For example, we used to think that parasitic infection in the brain would cause an increase in the eosinophil count in the CSF, but actually, in most parasitic infections of the brain, the eosinophil count does not increase (except for Guangzhou Angiostrongylus disease), and the eosinophil count in the peripheral blood was not elevated or even mildly elevated. A diagnosis of either eosinophilia or Churg–Strauss syndrome should be considered for patients with an elevated eosinophil count in their peripheral blood and fever. Diagnoses should be made with caution for patients with viral meningitis and no identified pathogen, but with normal electroencephalography, brain MRI scan and CSF, with reference to the patient's medical history and a careful consideration of the neurological examination, rather than reaching a conclusion based on only the results of a laboratory examination. Attention should also be paid to the differential diagnosis of immune-mediated

encephalitis or autoantibody-mediated encephalitis (such as N-methyl-D-aspartate receptor encephalitis and anti-voltage-gated potassium channel antibody-associated encephalitis).[8]

CONCLUSION

More attention should be paid to the screening of CNS infection. We need to understand the geographical distribution, epidemic season and living history of all the pathogens, pathogenic pathways and pathogenic mechanisms and so forth. Careful consideration should also be given to the clinical history and the physical examination. Only in this way can we gain a better understanding of all the processes involved in CNS infection and provide a theoretical basis for appropriate treatments in the future.

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Research Highlight

Differences in stroke damage in aged mice may not be due to differential cerebral blood flow dynamics

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Manwani B, Friedler B, Verma R, Venna VR, McCullough LD, Liu F. Perfusion of ischemic brain in young and aged animals: a laser speckle flowmetry study. Stroke 2014;45(2):571-8.

INTRODUCTION

Stroke is the leading cause of death and adult disability.[1-3] Aging is the most important nonmodifiable risk factor for stroke, and aged patients exhibit impaired stroke recovery.[4] Consistent with clinical data, findings from animal models have demonstrated that aged mice have higher mortality and worse outcomes when subjected to same duration of occlusion compared with young mice, yet paradoxically, the infarct damage is smaller in the aged mice.[5,6] Impaired recovery has been linked to several underlying mechanisms, including altered peripheral immune responses, enhanced neuroinflammation, and reductions in neurogenesis.[7-9] However, it was not known whether the discrepancy in histological outcome is associated with age-induced changes in the cerebral vasculature or cerebral blood flow.

Consistent blockage of blood flow is essential to achieve a homogenous ischemic infarct. Previous animal studies use laser Doppler flowmetry (LDF) to confirm blood flow blockage after ischemic occlusion. However, this technique has often been criticized as it not quantitative and only examines blood flow changes in a small region of the brain. As aging leads to several morphological and pathological changes throughout the vasculature, leading to atherosclerosis and small vessel disease, these changes cannot be identified by LDF. These alterations might contribute to significant stroke damage variability in aged animals. Unlike LDF, laser speckle flowmetry (LSF) provides a broader spatial, and temporal pattern of blood flow changes in the brain, allowing investigators to better examine and control cerebral blood flow changes during and following occlusion.

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COMMENT

To determine if the discrepancies in histological outcomes in aged mice were secondary to variability or differences in blood flow dynamics, we first assessed whether aged mice exhibited differential blood flow patterns after stroke compared to young mice using LSF. A structural immunohistochemical analysis of the vasculature was also performed by perfusing blood vessels with fluorescein isothiocyanate-dextran and co-labeling with the CD31 antibody. No significant difference in blood flow dynamics or microvascular density was observed between young (3-month-old) and aged (18-month-old) animals after middle cerebral artery occlusion (MCAO).

Although these results refute the hypothesis that changes in cerebral blood flow or vascular density was responsible for the smaller infarcts in aged mice, the study has provided some very interesting findings. Based on the LSF data, focal ischemia during MCAO induced a dramatic blood flow drop to the ipsilateral hemisphere as expected, but it was also associated with

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reduced blood flow in the contralateral hemisphere. This is an interesting observation as it suggests that even a focal ischemic stroke induces changes throughout the brain.

To further assess, the underlying mechanisms of smaller infarcts seen in aged mice, we investigated differences in blood-brain barrier breakdown after stroke. Somewhat surprisingly, IgG extravasation at both 24 h and 72 h after stroke was lower in aged mice compared with young mice, which likely correlated with the existence of smaller infarcts.

Despite some limitations, this is a very interesting study as it is the first to utilize LSF to investigate blood flow changes in aged animals. The mechanism responsible for the higher mortality and poorer recovery seen in aged and elderly animals remain a mystery. Age-related changes in neuroinflammation and the immune response to stroke are areas under intense investigation by a number of researchers and will hopefully be answered in the future.

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Research Highlight

The expanding phenotype of stroke-induced immune alterations

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Ruhnau J, Schulze K, Gaida B, Langner S, Kessler C, Broker B, Dressel A, Vogelgesang A. Stroke alters respiratory burst in neutrophils and monocytes. Stroke 2014;45(3):794-800.

INTRODUCTION

The clinical outcome of ischemic stroke patients is determined by the extent of the ischemic lesion as well as the occurrence of severe systemic infections. Patients with pneumonia have a three-fold increase in 30-day mortality, and survivors have poorer clinical outcomes.[1]

Pneumonia has long been known to complicate the clinical course of many stroke patients. It was assumed that being bedridden and the occurrence of dysphagia would account for the increased risk of stroke-associated infections (SAI). However, over the last years, it has been demonstrated that stroke-induced immune alterations (SIIA) observed in patients and experimental stroke models are strong predictors of subsequent poststroke infections.[2-4] Furthermore, one study associated insular stroke localization with pneumonia.[5]

These findings led to a new concept in which SIIA are causally related to SAI. Here, we briefly outline the current knowledge on SIIA and SAI and highlight recent studies that investigated cell functions involved in early bacterial defense mechanisms.

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STROKE-INDUCED IMMUNE ALTERATIONS

Stroke-induced immune alterations can be observed in the peripheral blood of stroke patients within the first hour after stroke. Blood samples obtained shortly after stroke already exhibit severe lymphocytopenia. Stress hormones are thought to be closely involved in these rapid changes; stress hormone levels have been shown to be elevated in human stroke patients, blockade of catecholamines prevents SAI in animal models of experimental stroke, and *in vitro* exposure of peripheral blood mononuclear cell to stress hormones mimics some aspects of SIIA.[6,7] Our initial observation in humans that lymphopenia was associated with susceptibility to infection was subsequently confirmed by several groups.[3,8]

It was unexpected that these disease induced immune alterations which reduced lymphocyte counts and induced granulocytosis should account for enhanced susceptibility to infection because the first line of defense against these infections is the innate immune response. In humans, the functional deactivation of monocytes (e.g. reduced human leukocyte antigen expression, reduced release of tumor necrosis factor-α) was detected after cerebral ischemia and was shown to be associated with a higher risk of poststroke infections.[3,9] We investigated in detail bactericidal mechanisms of monocytes and granulocytes after stroke.

Granulocytes home to the site of infection and kill pathogens by degranulation and phagocytosis, via a process termed oxidative burst and the release of web-like structures called neutrophil extracellular traps (NETs). During inflammation and infection, granulocytes and monocytes are recruited by chemoattractants and inflammatory cytokines. The cells adhere and internalize pathogens by endocytosis. Due to the generation of toxic

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radicals, they are able to eliminate pathogens by oxidative burst. In addition, neutrophils can release bactericidal peptides (e.g. α-defensin human neutrophil peptides 1-3 [HNP 1-3]) to fight against extracellular pathogens. Another killing strategy of neutrophils is the release of NETs. These complexes of decondensed chromatin consist of over 30 different neutrophil proteins that can capture, neutralize, or eliminate different pathogens. These structures form a physical barrier to reduce the spread of pathogens and enhance the concentrations of antimicrobial effectors.[10-12]

We recently investigated these granulocyte and monocyte functions in 63 human stroke patients. Our data demonstrated that migration, phagocytosis, and the release of HNP 1-3 were unimpaired in the peripheral blood of stroke patients compared with healthy controls. However, oxidative burst and NET formation were impaired in stroke patients. Our observations are in line with a previous small study that indicated an impaired oxidative burst in 17 patients with hemorrhagic stroke.[13] Furthermore, cells obtained from patients that went on to develop SAI showed a stronger impairment of oxidative burst capacity than patients without subsequent infections. These data suggest that alterations in innate immune functions may be causally related to SAI susceptibility. Alterations in lymphocyte counts in the peripheral blood of stroke patients may indicate SIIA severity, without directly affecting the host's bacterial defense capacity.

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Review Article

Anti-N-methyl-D-aspartate receptor encephalitis in China

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ABSTRACT

N-methyl-D-aspartate receptors (NMDARs) are mainly distributed in the central nervous system, and play important roles in the mechanisms of learning and memory. A newly discovered disease caused by autoantibody to NMDAR has been described, and is called anti-NMDAR encephalitis. Patients with this disease often suffer from mental disorders, seizures and other encephalitis-like symptoms. Accumulated data suggests that the severity of the disease makes early diagnosis very important. Accurately detecting the autoantibody to NMDAR is considered to be the gist of diagnosis. Good prognosis is predicted in most patients, when treated properly. Immunotherapy is preferred in most cases. In China, this disease has been reported only for a few years, but sporadic case reports are also helpful for profiling.

Key words: Encephalitis, N-methyl-D-aspartate, therapy

INTRODUCTION

N-methyl-D-aspartate

N-methyl-D-aspartate (NMDA) is an artificially synthesized amino acid with a similar structure to glutamate, and can activate a certain type of glutamate-receptor located in postsynaptic membrane. High-dose NMDA could cause neuronal death by inducing excitotoxicity, and was used in behavioral neuroscience studies. Subsequently, low-dose NMDA was used to investigate the roles of glutamate and glutamate-receptor pathway in the central nervous system (CNS).[1]

Glutamate-receptor and N-methyl-D-aspartate receptor

Glutamate-receptors in the CNS can be classified into two classes. One class is metabotropic glutamate-receptors coupled with a type of G-protein, whose activation mediates signal transduction cascade based on second message to produce a slow physiological reaction. Another class is ionotropic glutamate-receptors (iGluRs) that constitute ligand-gated ion channel complex and mediate transduction of rapid physiological signals. Based on agonist preference, iGluRs can be categorized into three different groups. Receptors that can be activated by the synthetic agonist NMDA are called NMDA receptors (NMDARs) or NMDA-GluRs to understand their nature better. The other two groups are α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate receptors.[2]

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Structure and physiological features of NMDA receptor

NMDA receptor is a tetramer, which is composed of three subunits: NR1, NR2, and NR3. In mammalian neural tissues, the functional NMDARs contain at least NR1 and NR2 subunits, in which NR1 functions as the ion channel, NR2 possesses regulatory function for the receptor, and NR3 could act as the regulatory subunit.[3] NMDARs are highly permeable for Ca^{2+}, which acts as the second message to regulate some cellular signal transduction pathways. NMDARs are also important for neurons to maintain normal function and morphology. The normal physiological level of Mg^{2+} blocks NMDAR, so its biological activity relies on a certain degree of

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excitatory postsynaptic potentials, which can remove the Mg^{2+} block. Glycine and its analog D-serine can amplify the response of NMDARs to activate them together with glutamate.[4]

Location of NMDA receptor

NMDA receptors are mainly distributed in the CNS, and contribute to excitatory synaptic transmission. However, NMDARs are also expressed in the peripheral nervous system (PNS), for example, in the peripheral terminals of primary afferent nerves innervating the colon.[5] NMDARs in PNS contribute to nociceptive stimulus, pain in facial muscles, and edema. Regions of the brain that prominently express NMDARs include hippocampus, dentate gyri, forebrain cortex, anterior cingulate cortex, and piriform cortex. Other areas such as corpus striatum, thalamus, and granule cells in the cerebellum also express high levels of NMDARs. Early studies had shown that NMDARs were mainly located in the postsynaptic membrane, especially in the postsynaptic density (PSD), for example, in the dendritic spine in excitatory neurons.[6] However, recent research has indicated a more diverse distribution of NMDARs not only in PSD, but also in extra-PSD, presynaptic, and extra-synaptic regions.[7] Typical examples of extra-synaptic NMDARs are in cerebellum astrocytes and retina ganglion. Factors that influence the activation of extra-synaptic NMDARs include the location and activity of neurons, the transporter in astrocytes, and glutamate spillover in synapses.[8]

Functions of NMDA receptor

NMDA receptors are involved in the development of the nervous system, including the survival of neurons, maturation of dendrites and axons, synaptic plasticity, and formation of neural circuits. In addition, long-term potentiation effect mediated by NMDARs is one of the basic mechanisms in learning and memory.[9] It has been proven that over-activation of NMDARs is a potential mechanism for the occurrence of seizures, dementia and stroke, while the under-activation of NMDARs is involved in schizophrenia-like symptoms.[10,11] In addition, NMDARs are known to mediate central sensitization in the pathogenesis of chronic pain after nociceptive stimulus.[12] Therefore, NMDARs play a very important role in CNS.

Factors regulating NMDA receptor activity

The activity of NMDARs is regulated by several factors. Glycine can amplify the response of NMDARs to glutamine through allosteric effect after binding to NR1 subunit. Polyamines can also potentiate the response of NMDARs to glutamine, while zinc ion can block this effect. Several agonists and antagonists have been developed for laboratory research or used as tools in the discovery of new drugs targeting NMDARs such as, D-serine, L-alanine as agonists, and amantadine, ketamine, methoxetamine as antagonists.

Discovery of anti-NMDA receptor encephalitis

Several cases of neural deficiency accompanied with teratoma were seen from 1997 to 2004.[13] In 2005, Vitaliani *et al.*[14] analyzed four patients diagnosed with paraneoplastic encephalitis and five patients who were reported having similar symptoms. They found that all the patients were females, and had teratoma. Most of the patients experienced changes in their personalities, loss of short-term memory, seizures, central hypoventilation, and illusions. Abnormal immune systems were found in all patients.[14] Two years later, Dalmau *et al.*[15] found that serum and cerebrospinal fluid (CSF) samples from these patients showed positive immunochemistry reaction with the rat hippocampus. Finally, they found an antibody that could bind to NMDAR expressed in cultured neurons or human embryonic kidney 293 (HEK 293) cell membranes in an unregulated manner. The term "anti-NMDAR encephalitis" was coined in that year. In 2008, the antibody was shown to bind to the NR1 subunit of NMDAR, resulting in the loss of its ion channel function.[16]

The first case of limbic encephalitis with teratoma was reported in 2009. Since the antibody could not be detected in serum or CSF, it could not be ascertained whether this case was an anti-NMDAR encephalitis.[17]

Probable mechanism of anti-NMDA receptor encephalitis synaptic plasticity

The autoantibody in the patient's CSF could reduce the NMDAR clusters in the synapse by binding, cross-linking and internalization. The reduction was reversible, and dependent on the titer of the autoantibody. Integrality of the neurons, synapses, receptors or proteins in the synapse was not harmed by the antibody. The density of the NMDAR clusters could recover four days after deletion of the autoantibody.[18]

Progress after discovery of anti-NMDA receptor encephalitis

After the discovery of anti-NMDAR encephalitis, the diagnosis of some clinical signs and syndromes such as catatonia, subacute confusion of memory, seizures, abnormal movements and limbic encephalitis had to be changed. Other forms of encephalitis mediated by autoantibodies against synaptic receptors were found later, such as anti-AMPA receptor,

anti-gamma-aminobutyric acid-B receptor and leucine-rich, glioma-inactivated1.[19-21]

Here, we reviewed all the cases reported with details and made a profile about anti-NMDAR encephalitis in China.

MANIFESTATION, DIAGNOSIS, TREATMENT, AND PROGNOSIS OF ANTI-NMDA RECEPTOR ENCEPHALITIS

Epidemiological features

The precise data on the incidence of anti-NMDAR encephalitis is still unknown. However, it is believed to be the most common paraneoplastic syndrome. A retrospective analysis found that anti-NMDAR encephalitis accounts for 1% of all encephalitis cases of unknown origin.[22] A prospective study found that anti-NMDAR encephalitis accounts for 4% of the reasons leading to encephalitis in Britain, and is the second most common immune-related encephalitis after acute disseminated encephalomyelitis, and the most common antibody-related encephalitis.[23] Moreover, the incidence of anti-NMDAR encephalitis is increasing.[23]

In 2008, Dalmau *et al.*[16] analyzed 100 consecutive patients and found that 91 of them were females with a mean age of 23 years. About 59% (58/98) of patients were paraneoplastic, and teratoma was the most common cancer (54/58, 93%). The data were updated 2 years later after the sample size had reached 400. Eighty percent of the patients were females, and the younger the patients were the lower was the percentage of paraneoplastic syndrome. About 60% of the patients above 18 years had cancer.[18]

Incidence of anti-NMDAR encephalitis in China is unknown, too. From all the 32 cases reported with details and 3 patients of ours, we found 80% (28/35) of them are females, and the mean age is 19.8 ± 9.7 years. About 40% (14/35) are reported with teratoma [Table 1]. Maybe, the gender ratio and age pattern of this disease in China described by Ren *et al.*[37] are more precise because of their bigger pool of patients.

Symptoms and signs

The most common symptoms include mental disorders, memory loss, decrease in consciousness, movement disorders, seizures, autonomic nerve symptoms, and hypoventilation.[38] Most of these symptoms are associated with pathological changes in brain function.

Examination

Half of the patients with anti-NMDAR encephalitis had no abnormalities in magnetic resonance imaging (MRI), while others had obvious changes in hippocampus, cerebellum, cortex, frontal lobe, insular lobe, basal ganglia, brain stem, and medulla. Multifocal metabolic abnormalities were found in the cortex or beneath the cortex in positron emission tomography or single-photon emission computed tomography tests. Low perfusion in frontal lobe and brain atrophy could be seen in some patients, which reversed in follow-ups, 5–7 years later.[18,39]

Majority of the patients had slow waves in electroencephalography (EEG), and continuous δ-θ rhythms predominated the tension episode of anti-NMDAR encephalitis. These slow waves were not accompanied by abnormal movements, and did not react to anti-epileptic drugs. Video EEG should be involved in diagnosis and treatment.[18,40]

Cerebrospinal fluid samples of all patients were abnormal. Lymphocytes and quantity of protein in CSF increased in 80% of the patients at an early stage. Oligoclonal bands appeared in 60% of patients, and intrathecal synthesis of anti-NMDAR antibody could be detected in most patients. In the long-term follow-up patients, the titer of anti-NMDAR antibody in serum was still high, while it could not be detected in CSF. Because of the linear correlation between the loss of NMDAR in synapse and the titer of anti-NMDAR antibody in CSF, the latter is considered to be one of the definitive diagnostic criteria.[41,42]

All the reported Chinese patients showed positive results for antibody detection. Some of the patients had a high signal in fluid-attenuated inversion recovery and T2 of MRI, while others showed extreme δ brush wave. Similar findings were observed in patients abroad.[29]

Diagnostic criteria and methods

The emerging neuropsychiatric symptoms and anti-NMDAR antibodies in serum or CSF are accepted as diagnostic criteria for anti-NMDAR encephalitis.[16] Detection of anti-NMDAR antibody includes two necessary methods: immunochemistry with rat hippocampus (and cerebellum) neurons, and genetically engineered HEK 293 cells expressing NMDAR on the surface.[14] Antibody in CSF has higher sensitivity for diagnosis as compared to blood.[41]

Assay kits for detecting anti-NMDAR antibody are provided by EUROIMMUN Corporation. According to

Table 1: Demographics, treatment and prognosis of the patients in China

Patient	Sex	Age	Teratoma	Surgery	Glucocorticoids	IVIg	Serum exchange	Rituximab	Anti-virus	Prognosis	Author
1	Female	13	No	N/A	Yes	Yes	-	-	-	Completely normal	Hu *et al.*[24]
2	Female	13	No	N/A	Yes	Yes	-	-	Yes	Completely normal	
3	Female	25	Yes	Yes	Yes	Yes	-	-	Yes	Completely normal	
4	Female	19	Yes	Yes	-	-	-	-	-	Self-maintenance	Chen *et al.*[25]
5	Female	35	Yes	Yes	-	Yes	-	-	-	Near self-maintenance	Chen *et al.*[26]
6	Female	25	No	N/A	Yes	No	-	-	Yes	Obviously improved	Song and Liu[27]
7	Female	22	Yes	Yes	No	Yes	-	-	No	Obviously improved	
8	Male	18	No	N/A	Yes	No	-	-	Yes	Obviously improved	
9	Male	15	No	N/A	Yes	No	-	-	Yes	Obviously improved	
10	Female	30	No	N/A	Yes	Yes	Yes	-	Yes	Unfavorable	
11	Female	18	No	N/A	Yes	No	-	-	Yes	Obviously improved	
12	Female	26	Yes	Yes	Yes	Yes	-	-	Yes	Death	
13	Female	16	No	N/A	No	Yes	-	-	Yes	Self-maintenance	Xu *et al.*[28]
14	Male	12	No	N/A	Yes	Yes	-	Yes	-	Favorable and no recurrence	Lu *et al.*[29]
15	Male	23	No	N/A	Yes	Yes	-	-	-	Recurrence, but improved after IVIg again	
16	Female	22	No	N/A	Yes	Yes	-	-	-	Continuous stupor-like state	
17	Female	22	Yes	Yes	-	Yes	-	-	-	Favorable and no recurrence	
18	Female	26	Yes	Yes	-	Yes	-	-	-	Favorable and no recurrence	
19	Female	28	Yes	Yes	-	Yes	-	-	-	Favorable and no recurrence	
20	Female	17	No	N/A	No	Yes	-	-	-	Favorable and no recurrence	Wei *et al.*[30]
21	Female	24	Yes	No	Yes	Yes	-	-	-	Favorable and no recurrence	Wu *et al.*[31]
22	Female	30	Yes	Yes	Yes	Yes	-	-	-	Favorable and no recurrence	Peng *et al.*[32]
23	Female	6	No	N/A	-	Yes	-	Yes	-	Favorable and no recurrence	Wang *et al.*[33]
24	Male	9	No	N/A	-	Yes	Yes	-	-	Favorable and no recurrence	
25	Male	15	No	N/A	-	Yes	-	-	-	Favorable and no recurrence	
26	Female	11	No	N/A	-	Yes	-	-	-	Favorable and no recurrence	
27	Male	8	No	N/A	-	Yes	-	-	-	Favorable and no recurrence	
28	Female	7	No	N/A	-	Yes	-	-	-	Favorable and no recurrence	
29	Female	7	No	N/A	Yes	Yes	-	-	-	Favorable and no recurrence	
30	Male	9	No	N/A	-	Yes	-	-	-	Favorable and no recurrence	Zhang *et al.*[34]
31	Female	17	Yes	Yes	-	Yes	-	-	-	Favorable and no recurrence	Shang *et al.*[35]
32	Female	17	Yes	Yes	Yes	Yes	-	-	-	Favorable and no recurrence	Xu *et al.*[36]
33	Female	45	No	N/A	-	Yes	-	-	-	Favorable and no recurrence	Our case

Contd...

Table 1: Contd...

Patient	Sex	Age	Teratoma	Surgery	Glucocorticoids	IVIg	Serum exchange	Rituximab	Anti-virus	Prognosis	Author
34	Female	47	Yes	No	Yes	No	-	-	-	Favorable and no recurrence	Our case
35	Female	17	Yes	Yes	-	Yes	-	-	-	Favorable and no recurrence	Our case

IVIg: Intravenous immunoglobulin; N/A: Not applicable; "-": Not mentioned by author

our investigation, the mean price of an antibody test is about 60 dollars for the reported patients in China.

Treatment

Regarding to the treatment for anti-NMDAR encephalitis, Dalmau *et al.*[18] provided a treatment proposal for this disease in 2011 [Figure 1]. They prefer concurrent intravenous immunoglobulin (IVIg) (0.4 g/kg/day for 5 days) and methylprednisolone (1 g/day for 5 days) to plasma exchange. As for the second-line therapy, they often use rituximab combined with cyclophosphamide in adults. And in children, they often use only one of these drugs – mostly rituximab. In China, anti-NMDAR encephalitis as a new disease, is often confused with viral encephalitis, and is treated with acyclovir or/and virazole. When the diagnosis was uncertain, some doctors gave IVIg as an alternative to the patients who did not respond to anti-viral treatment. Rituximab was seldom used for anti-NMDAR encephalitis patients due to its high cost, and lack of doctor's experience with the drug [Table 1].

Prognosis

Gresa-Arribas *et al.*[41] conducted a 5-year study with 501 patients. Their findings include: (1) 81% of anti-NMDAR encephalitis patients had favorable outcomes from immunotherapy, and factors affecting these outcomes include early diagnosis and nonintensive care unit treatment; (2) risk of recurrence is about 12% within 2 years, of which 67% is less harmful as compared with the first outbreak; (3) normally, the second-line immunotherapy was effective when the first-line therapy had failed.[41] Based on the long-term follow-up, the higher titer of antibody in patients' serum or CSF, worse was the prognosis. There was significant association between CSF antibody titer and the risk of recurrence.[42]

Among the 35 patients with anti-NMDAR encephalitis, one patient was in a continuous stupor-like state, one patient died 4 days after the tumor removal, one patient had an unfavorable prognosis, one patient recurred but improved after IVIg again, and 30 patients (86%) had favorable prognosis without recurrence or sequel [Table 1].

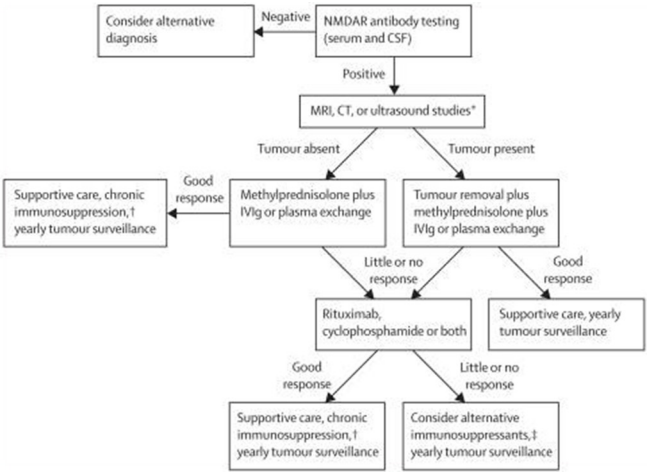

Figure 1: Proposed algorithm for the treatment of anti-N-methyl-D-aspartate receptors encephalitis

OTHER ISSUES RELATED TO ANTI-NMDA RECEPTOR ENCEPHALITIS

Pregnancy

Majority of the patients are females, and the issue of pregnancy is unavoidable. Pregnant patients could deliver a healthy baby if they have no NMDAR antibody in their serum. The curative effect increases significantly after giving birth or after termination of pregnancy.

Synaptic autoimmune encephalopathy

Some types of autoimmune encephalitis, such as anti-NMDAR encephalitis, anti-AMPAR encephalitis, anti-GABABR encephalitis, and anti-LGI1 encephalitis, can be distinguished by the antibodies against the receptors anchored in synapses. There are some common features of these diseases: high incidence in females always associated with tumor, psychiatric disorders, behavioral changes, and refractory seizures. Importantly, these diseases are reversible and curable with immunotherapy and removal of possible tumors, if they are diagnosed at an early stage.

The term of synaptic autoimmune encephalopathy is recommended for labeling these disease, thereby hinting at their favorable prognosis and the necessity for early immunotherapy.

SUMMARY AND FUTURE DIRECTIONS

As compared to patients abroad, Chinese patients normally have a long course of recovery. There are several reasons for this, including inadequate laboratory techniques, absence of standard operating procedures for such type of treatment, insufficient drug treatment, less experience in second-line treatment, and noncompliance of treatment from time to time. This is a big challenge routinely faced by the neurologists in checkups, diagnosis, or treatment.

Viral encephalitis and anti-NMDAR encephalitis have similar clinical symptoms such as headache, fever, mental disorder and seizures, and similar observations in MRI or EEG. It has not been aligned whether glucocorticoid should be used in the early treatment of viral encephalitis. With the incidence of autoimmune encephalopathies rising, we propose that patients with unconfirmed encephalitis could use glucocorticoid once they have a CSF puncture, and IVIg can also be administered.

B-lymphocyte depletion therapy mediated by anti-CD20 monoclonal antibody has been a brilliant breakthrough in the treatment of various antibody-related autoimmune diseases, such as anti-NMDAR encephalitis, rheumatoid arthritis, *etc*. It is important to investigate the possibility of applying this therapy to other higher-incidence neural autoimmune diseases, such as ophthalmoneuromyelitis. The vista is optimistic.

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Original Article

Detection of Epstein–Barr virus infection subtype in patients with multiple sclerosis by indirect immunofluorescence assay

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ABSTRACT

Aim: The aim was to investigate the infectious conditions of Epstein–Barr virus (EBV) in patients with multiple sclerosis (MS). **Methods:** Cerebrospinal fluid (CSF) of 20 patients with MS and 20 with other neurological diseases (OND) were tested with indirect immunofluorescence for anti-EBV capsid antigen (EBV-CA) immunoglobulin G (IgG), IgG affinity for anti-EBV-CA, anti-EBV-CA immunoglobulin M (IgM), anti-EBV early antigen (EBV-EA) IgG and anti-EBV nuclear antigen (EBNA) IgG. According to the pattern of antibodies in CSF, infection rates of acute, chronic, primary, recurrent, and past infections were analyzed in the two groups of patients. **Results:** There were no significant differences in anti-EBV-CA, anti-EBC-EA, and anti-EBNA antigen IgG in CSF between MS and OND patients ($P > 0.05$). The positive rate of low affinity for anti-EBV-CA IgG in MS patients was significantly higher than that for OND patients (75% *vs.* 40%, $P < 0.05$). Furthermore, significant differences in the positive rate of anti-EBV-CA IgM were found between MS and OND patients (70% *vs.* 25%, $P < 0.05$). Of the MS patients, 75% were in an EBV acute infection state compared with 40% of OND patients ($P < 0.05$). **Conclusion:** Acute infection of EBV closely correlates with the occurrence of MS.

Key words: Epstein–Barr virus, fluorescent antibody technique, herpes virus 4, multiple sclerosis

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system, the etiology and pathogenesis of which are currently poorly understood, but are known to be primarily associated with genetic and environmental factors.[1,2] However, recent studies have shown that bacteria and viruses are closely related to the incidence of MS. The characteristic of Epstein–Barr virus (EBV) infection, as a latent infection with periodic recurrence, makes EBV a risk factor for MS.[3] According to an epidemiological survey of MS and infectious mononucleosis patients, the experimental results indicate that the high incidence of MS is correlated with EBV genetic susceptibility in patients. Serum epidemiological and immunological evidences also show that the incidence of MS is significantly higher in serum EBV antigen-antibody-positive patients than in serum antibody-negative patients.[4,5] In addition, EBV capsid antigen (EBV-CA) and Epstein–Barr nuclear antigen (EBNA) antibody titers may be associated with the prevalence of MS.[6] Previous studies were based on enzyme-linked immunosorbent assay (ELISA) to confirm the relationship between serum/cerebrospinal fluid (CSF) EBV antigen-antibody and the occurrence of MS. This method did not, however, show details of the type of EBV infection. In contrast, indirect immunofluorescence assay (IFA) can not only compensate for this ELISA defect, but also has the advantage of using a standardized preparation.[7,8] As a result, our study has used IFA to reveal the correlation of MS with EBV antigen-antibody and thus, provide a better method for diagnosis and treatment of MS.

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METHODS

Participants

A total of 20 patients with MS, of which 6 males and 14 females were examined at Beijing Friendship Hospital from 2002 to 2010 and were enrolled in this study. MS was defined using the 2010 McDonald criteria for MS.[9] Among the patients, 14 had relapsing remitting MS, 2 had progressive remitting MS, 3 had primary progressive MS, and 1 had secondary progressive MS. None of the patients had been treated with corticosteroids or immunosuppressive agents 2 months before hospital admittance. Demographic data were collected from the patients by retrospective review of their medical records.

Twenty further patients were recruited with other neurological diseases (OND), including 9 with peripheral neuropathy, 4 with Parkinson's disease, 2 with headache, 2 with neurosis, 2 with motor neuron disease and 1 with cerebellar ataxia. Any OND patients with immunological diseases were excluded. There was no statistically significant difference ($P > 0.05$) in age and gender composition of the MS and OND patients.

This study was approved by the Capital Medical University Affiliated Beijing Friendship Hospital Ethics Committee. Written informed consents were obtained from individuals who participated in this study.

Anti-EBV antibody assay

The presence of anti-EBV antibodies, including anti-EBV-CA immunoglobulin G (IgG), anti-EBV-CA IgG affinity, anti-EBV-CA immunoglobulin M (IgM), anti-EBV early antigen (EBV-EA) IgG and anti-EBNA IgG, were assayed as described previously. Green florescence indicated that CSF had related antigen-antibody (positive reaction). Anti-EBV-CA IgG antibody affinity was assayed according to the green florescence intensity of urea-treated CSF parallel with physiological saline-treated CSF.[10] The intensity of florescence was depicted as follows: 0 referred to no florescence, 1 to very weak florescence, 2 to weak florescence, 3 to moderate florescence, 4 to strong florescence and 5 to very intense florescence. High antibody affinity indicated that the rate difference between urea-treated CSF florescence intensity and physiological saline-treated CSF florescence intensity was lower than two grades, while low antibody affinity was equal or higher than two grades. Antibody affinity is unable to be assayed when florescence intensity of physiological saline-treated CSF is lower than two grades.

The different EBV infection subtypes are shown in Table 1.[11]

Table 1: EBV infection type

Infection type	Anti-EBV-CA		Anti-EBV-EA IgG antibody	Anti-EBNA IgG antibody
	IgG antibody	IgM antibody		
Acute infection	Low antibody affinity	Positive	-	Negative
Chronic infection	High antibody affinity	-	Positive	Negative
Primary infection	Negative	-	Positive	Negative
Recurrence after infection	High antibody affinity	-	Positive	Positive
Past infection	High antibody affinity	-	Negative	Positive

EBV: Epstein-Barr virus, CA: Capsid antigen, EA: Early antigen, IgG: Immunoglobulin G, IgM: Immunoglobulin M, EBNA: Epstein–Barr nuclear antigen

Statistical analysis

SPSS for Windows version 15.0 (SPSS Inc., Armonk, NY, USA) was used for statistical analysis. The difference between two means was tested by χ^2 and Fisher's exact probability test. $P < 0.05$ was considered as statistically significant.

RESULTS

Detection of CSF EBV related antibody in MS/OND patients

Figure 1 shows the positive/negative reactions of the antibodies of anti-EBNA IgG, anti-EBV-CA IgG, anti-EBV-CA IgM, and anti-EBV-EA IgG. The anti-EBV-CA IgG antibody affinity is indicated in Figure 2. There was no significant difference between MS and OND patients whose CSF had antibodies of anti-EBNA IgG, anti-EBV-EA IgG or anti-EBV-CA IgG ($P > 0.05$). However, there was a statistical difference between MS and OND patients whose CSF displayed high/low anti-EBV-CA IgG antibody affinity or positive anti-EBV-CA IgM antibody ($P < 0.05$) [Table 2].

Composition of EBV infection type in MS and OND patients

The MS group had 15 patients who suffered from EBV acute infection, while the OND group had only 5 patients. This difference between the groups was statistically significant. One patient in the MS group had a recurrence after an EBV infection, while this was not detected in anyone in the OND group. EBV past infection existed in 4 MS and 12 OND patients, which was statistically different ($P < 0.05$). We failed to find an EBV chronic or primary infection in either MS or OND group.

DISCUSSION

EBV is a ubiquitous human DNA herpes virus. More than 90% of the world's population has been infected with EBV. EBV infection is closely related to

the occurrence of nasopharyngeal carcinoma, Burkitt lymphoma, Hodgkin disease, and immunoblastic lymphoma. Children infected with EBV often display invisible symptoms. Adolescents and adults with EBV infection frequently suffer from infectious mononucleosis syndrome. In addition, EBV infection may correlate with the occurrence of some autoimmune diseases, such as systemic lupus erythematous[12] or MS.[4] Epidemiological investigations with patients having infectious mononucleosis syndrome and MS show that MS often occurs in populations with high EBV genetic susceptibility. Serum epidemiological and immunological evidence also suggests that the probability of occurrence in MS patients with EBV antigen-antibody-positive serum is significantly higher than in the serum antibody-negative population.[5] EBV-CA and EBNA antibody titer has also been associated with the prevalence of MS.[6] Although previous studies using ELISA have confirmed the relationship between serum/CSF EBV antigen-antibody and MS, this assay fails to distinguish the EBV infection subtype. As a result of ELISA's poor reproducibility and the specificity of the antigen preparation and complexity, we used IFA in this experiment owing to IFA having merit with a conjugate-standardized preparation and in EBV-infection type differentiation.[7,8]

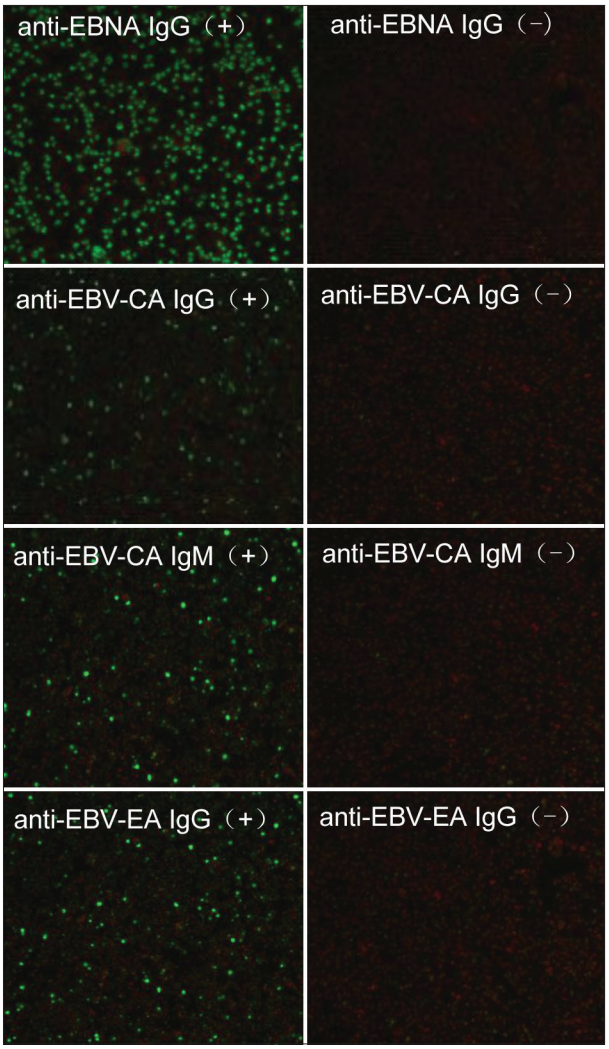

Figure 1: Detection of anti-EBNA IgG, anti-EBV-CA IgG, anti-EBV-CA IgM and anti-EBV-EA IgG in CSF. EBV antigen immunoglobulin antibody in CSF is indicated by green fluorescence. Red fluorescence reveals that EBV antigen immunoglobulin antibody is absent in CSF. EBV: Epstein–Barr virus; CA: Capsid antigen; EA: Early antigen; IgG: Immunoglobulin G; IgM: Immunoglobulin M; CSF: Cerebrospinal fluid; EBNA: Epstein–Barr nuclear antigen

The IFA assay was used in 20 MS and 20 OND patients to detect the CSF antibodies of anti-EBNA IgG, anti-EBV-CA IgG, anti-EBV-CA IgG antibody affinity, anti-EBV-CA

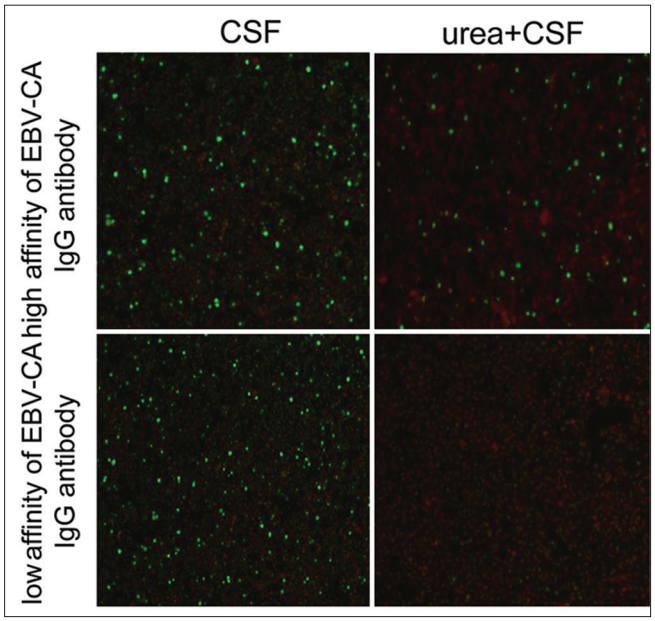

Figure 2: Detection of affinity of EBV-CA IgG. EBV-CA IgG antibody in CSF is indicated by green fluorescence. High affinity of antibody reveals that green fluorescence still exists after CSF is treated with urea. Disappearance of green fluorescence after CSF was treated with urea demonstrates low affinity of the antibody. EBV: Epstein–Barr virus; CA: Capsid antigen; EA: Early antigen; IgG: Immunoglobulin G; IgM: Immunoglobulin M; CSF: Cerebrospinal fluid; EBNA: Epstein–Barr nuclear antigen

Table 2: Percentage of EBV antibody in MS and OND patients

Group	Anti-EBNA IgG	Anti-EBV-CA IgM	Anti-EBV-EA IgG	Anti-EBV-CA IgG	Anti-EBV-CA IgG antibody affinity	
					High	Low
MS	5 (25)	15 (75)*	1 (5)	18 (90)	3 (15)*	13 (65)*
OND	6 (30)	5 (25)	0 (0)	19 (95)	12 (60)	8 (40)

Data are shown as *n* (%). *$P < 0.05$ *vs*. OND. EBV: Epstein-Barr virus, CA: Capsid antigen, EA: Early antigen, IgG: Immunoglobulin G, IgM: Immunoglobulin M, MS: Multiple sclerosis, OND: Other neurological diseases, EBNA: Epstein–Barr nuclear antigen

IgM, and anti-EBV-EA IgG. The results showed that the anti-EBNA IgG antibody-positive rate of the MS group was 25% compared with 30% in the OND group, but the difference was not statistically significant. Similarly, no statistically significant difference in the anti-EBNA IgG antibody-positive rate was found by Villegas *et al*.[13] (6.6% in MS patients and 17.0% in OND) and by Castellazzi *et al*.[14] (6.3% MS and 1.3% in OND). In addition, Pohl *et al*.[15] showed that the anti-EBNA IgG antibody-positive rate of MS patients was 8%, similar to observations by Sargsyan *et al*.[16] and Jafari *et al*.[17] Our results support the above conclusions, but we found the anti-EBNA IgG antibody-positive rate was much higher than in these previous research reports. This difference may be explained by (1) different sample sizes, (2) the IFA used in our experiment which has a higher sensitivity than the ELISA assay in the previous studies, and (3) the EBV infection rate in China is higher than in European and American countries with better sanitary conditions. In our study, the positive rate of the CSF anti-EBNA IgG antibody in the MS group was lower than that in the OND group, which contrasts with the reports of Jaquiéry *et al*.[18] and Cepok *et al*.[19] This difference may be explained by the smaller sample size in our study and the different living environments and the genetic susceptibility of the European and American populations as compared to the Chinese.

Positive anti-EBV-CA IgG antibodies in human CSF suggest a past history of EBV infection. Our data showed that the positive rate of anti-EBV-CA IgG antibodies in MS patients was 90% compared with 95% in OND patients. This observation is also supported by other reports.[20,21] The detection of anti-EBV-EA IgG antibody in CSF in our study represents either an acute or chronic EBV infection, suggesting that EBV reproduces. This has also been observed in a previous study.[22] The high affinity for anti-EBV-CA IgG antibodies in MS and OND patients is characterized by EBV chronic or past infections, but a low affinity was found where there was an acute or a recurrent infection.[23] The data revealed that the positive rate of low affinity antibodies was higher in the MS group than in OND patients, which paralleled with the results reported by Robertson *et al*.[10] and Gray.[23] According to the positive/negative reactions of various EBV antigen-antibodies in the analysis of the EBV infection types, we found that the acute EBV infection rate in MS patients was significantly higher than that of the OND group (75% *vs*. 40%) while having a previous infection the rate was significantly lower than in the OND group (20% *vs*. 60%). This may suggest that acute EBV infections may be associated with the onset of MS. The EBV-specific super-antigens activate $CD4^+$ T cells, which produce a cross-reaction with myelin protein through interaction with B and NK cells.[24] In addition, EBV can directly cause acute myelin oligodendrocyte glycoprotein-specific cellular and humoral immune responses,[25] and simultaneously activate $CD8^+$ T cells. Moreover, $CD8^+$ T cells react with B cells infected with EBV for anti-myelin associated protein antibody production.[26] However, our data are different with the results reported by Kiriyama *et al*.[27] Further investigations are required to establish the pathogenesis of MS affected by EBV acute infections.

In summary, our study suggests that acute EBV infection is closely associated with the pathogenesis of MS, and that inhibition of EBV infection is beneficial to the prevention and treatment of MS. However, the prevalence of EBV infection is high in the general population, but the prevalence of MS is relatively low, which suggests there may be other MS causative factors, such as genetic predisposition, EBV primary infection, age and other microbial infections. As a result, further studies are necessary to investigate MS pathogenesis of EBV infections.

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Original Article

Imaging and cytological analysis of 92 patients with Japanese encephalitis

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ABSTRACT

Aim: Japanese encephalitis (JE) is caused by a mosquito-borne flavivirus and demonstrates high mortality and serious sequelae. Imaging and cytological examinations are important for the diagnosis of JE. We performed this study to analyze the imaging and cytological characteristics of JE. **Methods:** This study enrolled 92 JE patients with 108 cerebral spinal fluid (CSF) samples. Diagnosis was based on clinical features and positive immunoglobulin M antibodies against JE virus, which were measured using enzyme-linked immunosorbent assay. All patients received detailed neurological examinations, relevant cerebrospinal fluid tests, and brain neuroimaging (computed tomography, magnetic resonance imaging, or both). **Results:** Prominent involvement in the hippocampus was observed in 10 patients on neuroimaging, in addition to classic involvement in the thalamus and basal ganglia. Lumbar puncture pressure was normal in 61 CSF samples. White cell count increased in 81.19% of CSF samples, 67.65% and 83.33% of CSF samples demonstrated normal chloride and glucose concentrations, respectively, and 82.52% of CSF samples demonstrated > 0.4 g/L protein content. JE patients demonstrate mixed-cell reaction on cerebrospinal fluid cytology in the early phase, which subsequently mainly develop as mainly lymphocyte reaction or typical lymphocyte reaction. **Conclusion:** JE imaging is characterized by bilateral thalamic involvement, and the basal ganglia and hippocampus are also commonly affected. The mixed-cell reaction in JE lasts longer than in general viral encephalitis. This may facilitate the differential diagnosis of JE.

Key words: Cerebrospinal fluid, cytology, Japanese encephalitis, neuroimaging

INTRODUCTION

Japanese encephalitis (JE), which is caused by infection with the JE virus (JEV), is one of the most important viral encephalitis in the world, especially in East and Southeast Asia. Approximately 35,000–50,000 people develop JE each year, demonstrating annual mortality of 10,000–15,000.[1-3] About one-third of patients die, and half of all survivors develop severe sequelae.[4-6] JE is characterized by high fever, conscious disturbance, seizures, focal neurological deficit, signs of meningeal irritation, *etc*. JE is regarded as a pediatric disease in endemic areas. The morbidity of JE has substantially decreased due to the wide application of the JE vaccine, though outbreaks still occur in some districts and the number of adult infections is increasing.[7] Bilateral thalamic lesions developed in endemic areas during susceptible seasons should be considered as encephalitis. Cytological analysis of the cerebrospinal fluid (CSF) may reflect the clinical course of JE. Here, we analyze the radiological and cytological features of JE.

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METHODS

This study enrolled 92 JE patients who were diagnosed and treated at the Second Hospital of Hebei Medical University between August 2013 and October 2013. Diagnosis was positive confirmation of immunoglobulin M (IgM) antibodies to JEV in sera using IgM antibody capture enzyme-linked immunosorbent assay by the center for disease control and prevention in Hebei province. All CSF samples were examined at Second Hospital of Hebei Medical University to determine the cellular, glucose, protein, chloride, and cytological characteristics. Ethics approval was given by the review board of the Second Hospital of Hebei Medical University.

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Cranial computed tomography (CT) scans were performed using a high-resolution CT scanner (GE OPTIA 660), and 10 mm axial sections were obtained parallel to the orbitomeatal plane. Magnetic resonance (Philips Achieva 3.0T) imaging (MRI) of the head was performed to obtain 3.0 T1-weighted spin-echo (repetition time [TR] = 2000 ms, echo time [TE] = 20 ms) and T2-weighted spin-echo images (TR = 1500 ms; TE = 80 ms) in the axial, coronal, and sagittal planes. Section thickness was 6 mm.

RESULTS

Ninety-two patients were included in this study, including 50 males and 42 females (male:female = 1.19:1), and the mean age was 42.32 ± 15.47 years. All patients (100%) had fever, 53 patients (57.61%) had headache, 74 patients (80.43%) occurred consciousness disorder, 21 patients (22.83%) demonstrated twitching, 34 patients (36.96%) demonstrated dystonia, and 25 patients (27.17%) demonstrated respiratory failure. Fifty patients (54.35%) had sequelae at discharge following treatment.

Nineteen patients received CT examination of head between 2 and 25 days after onset, which were abnormal in 7 patients (36.84%). Abnormalities included 1 patient (5.26%) with thalamic hypodensity accompanied by basal ganglia and temporal lobe hypodensity, 4 patients (21.05%) with basal ganglia hypodensity, 2 patients (10.53%) with brain swelling, and 1 patient (5.26%) with frontal lobe hypodensity.

Sixty-five patients received MRI of head within 2–32 days after onset. Lesions appeared hyperintense on fluid attenuated inversion recovery (FLAIR) and T2-weighted images, and isointense to slightly hypointense on T1-weighted images. MRI showed no lesions in 16 patients (24.62%) within 2–22 days after onset. MRI findings of 30 patients with clear inflammatory focus included varying degrees of thalamic lesions except two patients. MRI lesions were also noted in the basal ganglia in 11 patients, mid-brain and hippocampus in 10 patients, pons in 1 patient, and cerebral cortex in 5 patients [Table 1].

Seventy-five patients underwent lumbar puncture, and 34 patients required puncture more than once. Symptom duration prior to lumbar puncture ranged between 3 and 33 days after onset. Hence, we identified 108 CSF samples (3 in the initial phase, 53 in the acute phase and 52 in convalescence). The mean lumbar puncture pressure was 173.41 ± 77.87 mmH_2O, and

Table 1: Different lesions identified using MRI

Features	CT (*n*=20)	MRI (*n*=65)
Abnormal	7	30
Thalamic	1 (1*)	28 (20*)
Basal ganglia	4 (3*)	11 (8*)
Hippocampus	0	10 (6*)
Mid-brain	0	10 (4*)
Pons	0	1
Cortex	2	5 (2*)
Insult	0	2
White matter lesions	1	6 (2*)

*Number of bilateral lesions. CT: Computed tomography, MRI: Magnetic resonance imaging

Table 2: Different kinds of cells in different JE phases

Cell type	Initial phase %	Acute phase %	Convalescence %
Lymphocytes	35.67	71.06	89.68
Monocytes	19.33	12.27	4.7
Activated monocytes	15.67	9.51	2.82
Neutrophils	29.67	5.84	2.49
Plasma cells	0.67	1.14	0.16
Lymphoid cells	0	0	0.12
Eosinophils	0	0.04	0.04
Basophils	0	0.08	0

JE: Japanese encephalitis

27 samples (25.71%) demonstrated high lumbar puncture pressure and 61 samples (58.10%) demonstrated normal lumbar puncture pressure, 3 samples weren't tested for pressure. The mean number of white cells in CSF was (42.50 ± 71.31) × 10^6/L, and 81.19% of samples demonstrated an increase in their white cell count. Mean chloride, glucose, and protein values were 122.26 ± 9.02 mmol/L, 3.77 ± 1.79 mmol/L, 0.64 ± 0.28 g/L, respectively. In total, 67.65% and 83.33% of samples demonstrated normal chloride and glucose samples (normal chloride content: 120–130 mmol/L; normal glucose content: 2.5–4.4 mmol/L), while 82.52% of samples demonstrated >0.4 g/L protein content. The cytological examination of CSF confirmed mixed-cell reaction in 11 (10.19%) samples, an increase in activated monocytes in 40 (37.04%) samples (lymphocytes were the main cells in 35 (32.41%) samples), mainly lymphocyte reaction in 41 (37.96%) samples (neutrophil were found in 15), typical lymphocyte reaction in 12 (11.11%) samples, and <100 cells in 4 (3.7%) samples. We also analyzed the proportion of various cells in different JE phases [Table 2].

DISCUSSION

The imaging findings obtained using CT and MRI showed the pathological changes that occur in JE patients. JE patients typically demonstrate hypodense lesions in the thalamus and basal ganglia on CT. MRI is more sensitive than CT for revealing abnormalities,[8]

and MRI demonstrates a higher diagnostic value than CT, especially when assessing site, range, quantity, and extent of infection. Thalamic changes, especially bilateral involvement, could be used to help diagnosis JE in endemic areas.

Lesions can also be observed in the substantia nigra, brain stem, cerebellum, cerebral cortex, and white matter.[8-10] One study reported changes in the thalamus (94%), basal ganglia (35.5%), midbrain (58%), cerebellum (25.8%), pons (19%), and cerebral cortex (19%) on MRI.[8] MRI lesions are generally hypointense on T1 and hyperintense on T2 and FLAIR images. FLAIR is the most useful sequence for detecting lesions and defining the extent of supratentorial lesions. On FLAIR images, cerebral lesions can be better observed, but T2-weighted imaging is better for evaluating the midbrain and brain stem.[11] Thalamic lesions may demonstrate mixed intensity on T1 and T2 imaging in the subacute phase, which may be suggestive of hemorrhagic changes.[12] The involvement of temporal lobe has also been observed in some studies,[8,13,14] but all reported patients also demonstrated involvement of the thalamus and substantia nigra.[14] Temporal lobe involvement is fairly characteristic and mostly involves the hippocampus, usually sparing the rest of the temporal lobe. Because JEV takes a hematogenous route during the invasion and infects the blood supply in parts of the thalamus, cerebral peduncles, hippocampus, and uncus,[14] this may explain concurrent involvement in the medial temporal lobe along with the thalami and substantia nigra. The hippocampal involvement was most commonly in the tail and body, occasionally in the head and amygdala.[14,15] The presence of typical JE lesions in the thalami, substantia nigra, and basal ganglia, along with temporal lobe involvement, may help differentiate JE from herpes simplex virus encephalitis (HSE). Sawlani[11] has reported that MRI techniques such as FLAIR and diffusion weighted imaging (DWI) can be used to evaluate HSE and JE. DWI and apparent diffusion coefficients mapping (ADC) can differentiate cytotoxic edema from vasogenic edema.[16] Significantly restricted diffusion and low average ADC values have been observed in the acute phase lesions in HSE patients, whereas JE lesions do not show restricted diffusion or significantly low ADC values in the acute phase (whereas chronic phase lesions show restricted diffusion and high ADC values).[11] However, Prakash *et al*.[17] have reported restricted diffusion and low ADC values in the acute phase JE. Coinfection of neurocysticercosis (NCC) and JE has also been observed by MRI and CT, which may result in poor clinical outcomes.[13,18] A significantly higher proportion of abnormal CT scans and abnormal MRI has been reported when evaluating NCC/JE-coinfected lesions.[18] JE in association with cerebral venous sinus thrombosis has also been reported with the help of MRI and MR venography.[19]

In this study, 65 patients took MRI examination in 92 patients. Thirty patients (46.15%) presented with inflammatory lesions between 2 and 32 days after onset, 3 patients (10.00%) presented in the initial phase, 19 patients (63.63%) in the acute phase, and 8 patients (26.67%) in convalescence. Handique[15] has reported 90.00% MRI sensitivity during the 1st week of JE infection. However, in this study, 13 of 30 (43.33%) patients presented with lesions on MRI during the 1st week of JE infection. Our patients were first treated at other hospitals, and some patients were too ill for early examination, so their MRI examinations were not timely, which may explain the low sensitivity in this study.

However, this study does show changes in the thalamus (93.33%), basal ganglia (36.67%), hippocampus (33.33%), mid-brain (33.33%), pons (3.33%), and cerebral cortex (16.67%) on MRI [Figure 1]. Except for one patient, all patients demonstrated hippocampal involvement on MRI that was accompanied by thalami involvement. The gray matter areas of the brain, including the thalamus and hippocampus, were primarily affected by JEV on autopsy.[20] These areas are associated with increases in activated and phagocytic microglia.[21] Srivastava *et al*.[22] have reported that JEV RNA load in different brain regions of rat with higher affinity of JEV to thalamus and mid brain compared to other regions. These may explain the commonly affected areas observed on imaging. Three patients were examined using both CT and MRI, but only MRI revealed lesions. One MRI reexamination reported lesion reduction following treatment, while another reported enlarged lesions. That may be the first study that used early MRI examination and did not observe the peak of brain injury. Three patients showed inflammatory lesions in combination with ischemic infarction on MRI. All of these patients demonstrated risk factors that are also shared with severe encephalitis, such as hypertension and age. Basumatary *et al*.[4] have reported that changes on MRI or CT in combination with thalamic involvement are significantly related with dystonia. However, other clinical symptoms, such as behavioral abnormalities, seizure, coma level, and death, do not demonstrate a significant correlation with radiological abnormalities.

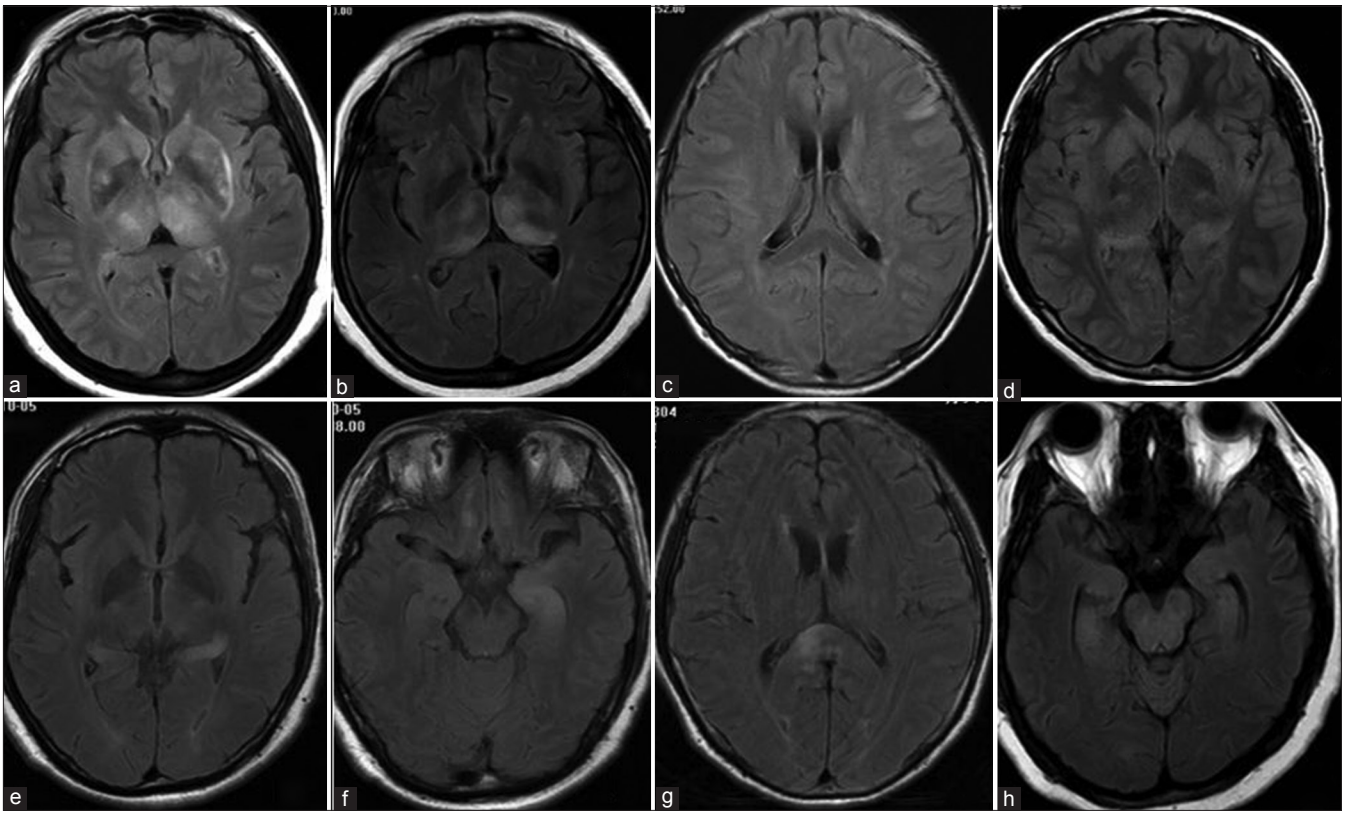

Figure 1: Axial fluid attenuated inversion recovery images of involvement in different areas: (a) Involvement in the bilateral thalamus and basal ganglia; (b) Involvement in the bilateral thalamus; (c) Involvement in the left prefrontal cortex; (d) Involvement in the bilateral frontal lobe, temporal lobe, insula cortex, thalamus, and basal ganglia; (e) Involvement in the bilateral hippocampal tail; (f) Involvement in the bilateral hippocampal head and body and uncus; (g) Involvement in the corpus callosum; (h) Involvement in the bilateral hippocampal head and body and midbrain

We did not observe NCC and JE coinfection in this study, but there was a forty-year-old woman who got JE associated with cerebral venous sinus thrombosis. The MRI displayed the swelling of the left frontal, parietal lobes and the right occipital with hypointense on T1 and hyperintense on T2 and FLAIR images. The superior sagittal sinus, left sigmoid sinus and transverse sinus filling defect were observed, indicating venous thrombosis. MRV scan further demonstrated thrombosis in the superior sagittal sinus, left sigmoid sinus and transverse sinus, confirming the diagnosis of CVST. Twenty-six patients presented with dystonia, including 16 patients (61.54%) who demonstrated MRI changes with thalamic involvement. Another 16 JE patients did not show lesions on MRI within 6-22 days after onset. This may indicate that thalamic abnormalities suggest JE, but absence does not exclude it. In additional, 11 of 30 patients with MRI abnormalities also received DWI examinations, and 9 of these patients demonstrated lesions with abnormal signals on both T2-weighted imaging and DWI within 5-12 days (mean = 5.9 days) after onset. Due to the decrease in the vasculitis component and perivascular cuffing, the proportion of diffusion restriction decreased and ADC began to rise in the late-acute and early-subacute phases.[17] In this phase, cytotoxic edema is accompanied by the vasogenic collection of fluid that allows the lesion to become visible on both T2W and DWI.

CSF parameters may change in JE patients when the brain tissues or meninges are involved. To summarize, lumbar puncture pressure was normal or slightly increased in most patients. White blood cell count and protein content increased slightly, similar to related reports,[23,24] while the glucose and chloride content were normal in most JE patients. This increase in protein reflects the increase of endothelial cell permeability, which indicates that the blood-brain barrier is damaged in JE patients. The cytological features of CSF are associated with the clinical course of JE, and performance varies in different phases of disease. We analyzed 108 CSF samples and observed the mixed-cell reaction in the early phase of JE. Neutrophils were the major inflammatory cells (mean = 35.67%), though neutrophil predominance is not uncommon in other viral central nervous system infections.[25] We also identified activated monocytes and plasma cells during the disease course. In the acute phase of JE, the proportion

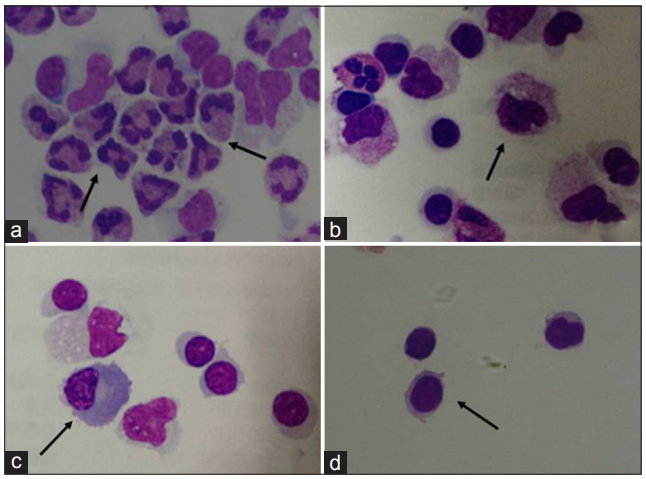

Figure 2: May–Grünwald–Giemsa staining results (×1000) of the cerebral spinal fluid in Japanese encephalitis patient at different phases: (a) Initial phase – Neutrophils (black arrows) are the main inflammatory cells in the background; (b) Acute phase – A decrease in the number of neutrophils and an increase in the number of lymphocytes were noted. Furthermore, activated monocytes (black arrows) are apparent; (c) Acute phase – Plasmocytes developed in the acute phase (black arrows); (d) Convalescence – Cytology mainly shows the lymphocyte reaction (black arrows)

of neutrophils dramatically declined to 5.82%, while lymphocytes significantly increased. Monocytes and activated monocytes gradually decreased, but plasma cells peaked. At convalescence, mainly lymphocyte reaction or typical lymphocyte reaction were observed on CSF cytological examination. Neutrophils, monocytes, activated monocytes, and plasma cells all rapidly decreased or even disappeared [Figure 2]. In addition to typical cytological characteristics of viral encephalitis, CSF cytological examination of JE patients also showed that the mixed-cell reaction existed for long periods of time, and we observed neutrophils existed for longer than one week. The rates of neutrophils decrease and lymphocyte increase are fast during treatment, and this may facilitate the differential diagnosis of JE. Misra and Kalita[26] have reported that the 3-month clinical outcomes were not significantly related to CSF abnormalities. Further studies are required.

Based on the results of routine biochemical examinations of CSF, white blood cell and protein content slightly increased while glucose and chloride content remained normal in most JE patients. The mixed-cell reaction was noted in the early phase of JE and existed longer than in general viral encephalitis. At convalescence, cytological examinations of CSF demonstrated mainly lymphocyte reaction or typical lymphocyte reaction. The mixed-cell reaction in JE lasts longer than in general viral encephalitis. JE imaging is characterized by bilateral thalamic involvement, and involvement of the basal ganglia and hippocampus are also common. In endemic areas and epidemic season, the patients considered as CNS infection with bilateral thalamic involvement should be highly suspected as JE.

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Original Article

Neonatal immune activation during early and late postnatal brain development differently influences depression-related behaviors in adolescent and adult C57BL/6 mice

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ABSTRACT

Aim: Immune challenge during early and late neonatal periods can induce robust alterations in physiological and behavioral functions, resulting in greater risk for the development of neuropsychiatric disorders, such as anxiety and depression, later in life. In addition, previous studies concluded that increasing age correlates with increased depression behaviors in humans and rodents. This study aimed to investigate for the first time whether immune challenge with a viral mimic, synthetic double-stranded ribonucleic acid (Poly I: C) during different neonatal periods can differently affect depression-related behaviors in adolescent and adult mice. **Methods:** Male C57BL/6 mice were treated with either saline or Poly I:C (1 mg/kg and 4 mg/kg) on postnatal days (PND) 3–5 (early neonatal phase) or PND 14–16 (late neonatal phase), and then subjected to behavioral tests, including tail suspension test and forced swimming test, during adolescence (PND 35 or 40) and adulthood (PND 85 or 90). **Results:** The results demonstrated that early neonatal immune activation increases depression-related behaviors in both adolescent and adult mice, but late neonatal immune activation only increases depression in adult mice. In other words, these findings indicated that the nature of the offspring's neuropathology can depend on the severity of the insult, the pup's age at the time of the insult, and offspring age at the time of behavioral testing. **Conclusion:** These findings suggest that dose and timing of neonatal insult and offspring age may be important factors for evaluating neuropsychiatric disorders in adults who experienced early life infection.

Key words: Age, depression, hypothalamic-pituitary-adrenal axis, mice, neonatal infection, Poly I:C

INTRODUCTION

Many studies have recently demonstrated the importance of early life infection on the brain and behavior development, and how such infections can increase susceptibility to the onset of neuropsychiatric disorders, such as anxiety and depression, later in life.[1-7] Lipopolysaccharide (LPS, mimics bacterial infection) and polyinosinic-polycytidylic acid (Poly I:C, mimics viral infection) administration during the neonatal period are known as two animal models of neonatal infection, and both can stimulate the immune and endocrine systems.[8] We and others have shown that LPS- or Poly I:C-induced neonatal infection can lead to hypothalamic-pituitary-adrenal (HPA) axis abnormalities and results in increased anxiety- and depression-like behaviors in adult rodents.[6,7,9]

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Scientific reports have indicated that the nature of the offspring's neuropathology can depend on the nature and severity of insult and the pup's age at the time of insult. This argument is supported by previous studies in which we and others showed that the time points of prenatal and neonatal immune activation, the offspring's age, and the dose of immunogen can be important factors for assessing neuropsychological alterations such as anxiety- and depression-related behaviors that persist until adulthood in mice and rats.[3,6,10,11] For instance, a study conducted by Walker *et al*.[11] indicated that postnatal immune challenge had no impact on anxiety levels in adolescence, but did lead to increased levels of anxiety in adulthood and senescence in male rats. Furthermore, previous studies concluded that older age correlates with an increased level of depression behavior in humans and rodents.[10,12] However, limited information is available regarding the relationship between the timing of neonatal immune challenge and age in development of depression-related behaviors in adulthood.

Therefore, the aim of the current study was to investigate the impacts of neonatal immune activation with equal Poly I:C doses at two time points, postnatal days (PND) 3–5 (early neonatal phase) or PND 14–16 (late neonatal phase), on depression-related behaviors in adolescent and adult male mice.

METHODS

Animals

Male and female C57BL/6 mice (70–80 days) were obtained from the animal house of Pasteur Institute (Tehran, Iran). Mice were housed in standard polycarbonate cages in a room with a 12:12 h light/dark cycle (lights on 08:00–20:00), controlled temperature (23 ± 1°C), and had free access to food and water. These conditions were kept as a standard housing condition in all stages of experiments. Following a 2-week period of acclimatization to the new animal housing room, male and female mice were kept together one-by-one in a cage to facilitate mating. Female mice were visually monitored daily for confirmation of pregnancy. Once confirmed, the female mice were removed from the breeding cages and housed individually in standard cages.[10,13] All pregnant mice were allowed to have a normal delivery, and the first day of birth was considered PND 0.[6] One day after the birth, all litters were culled to four pups per mother. On day 21, litters were weaned by removal of the mother, and only male pups were used in this study. A total of 30 litters were used during this study in three stages, each of which included 10 litters. Only one mouse per dam was selected for each of the groups to avoid the litter effect. All procedures of the study were performed in accordance with the ethical guidelines set by the Research and Ethics Committee of the Tabriz University of Medical Sciences, which completely adhere with the National Institutes of Health NIH Guide for the Care and Use of Laboratory Animals (NIH; Publication No. 85–23, revised 1985).

Neonatal immune activation

A summary of the experimental design is shown in Figure 1. The pups were divided based on treatment conditions into two clusters: group 1 – saline-injected mice and group 2 – Poly I:C-injected mice (each group only used for two tests with a 5-day interval between each test; $n = 8$ per group). The dams were removed from their pups for approximately 5 min, and the pups were weighed and received a subcutaneous (in the interscapular region) injection of Poly I:C (Sigma Aldrich, St Louis, MO, USA; 1 mg/kg and 4 mg/kg) or saline (1 mL/kg) during the early neonatal phase (PND 3–5, which corresponds to the third trimester of human pregnancy when major brain growth occurs) or the late neonatal phase (PND 14–16, which corresponds to 1–2 years old humans).[6,14] The doses and timing of Poly I:C treatment were chosen based on previous studies.[6,8,15] The Poly I:C was dissolved in sterile saline (0.9% NaCl), and injections were performed between 10:00 and 11:00. Each injection was performed through a 27-gauge needle connected by polyethylene tubing to a 10-μL Hamilton syringe. Newborn mice were returned to their housing immediately after injections.

Behavioral tests

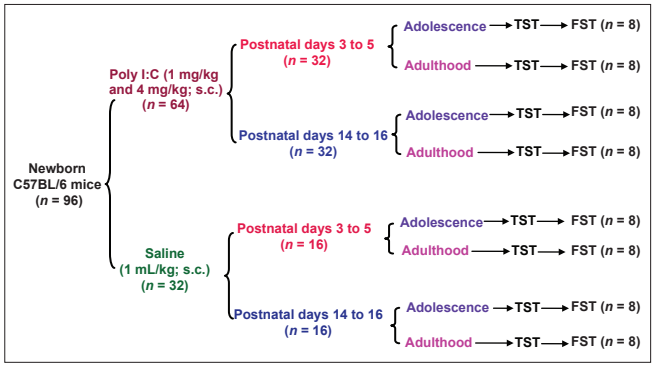

Figure 1: Experimental design: The effects of early and late neonatal immune activation with Poly I:C on depression-related behaviors during adolescence and adulthood in the tail suspension test (TST) and forced swimming test (FST) in C57BL/6 mice

All behavioral parameters were recorded by observers who were blind to the treatment. In addition, all behavioral tests were conducted in a quiet room during the light period (between 13:00 and 18:00) under bright and moderate illumination, and the mice were kept in the room for at least 1 h before the assessment. Depression-related behavior was separately studied in adolescent (PND 35: tail suspension test [TST]; PND 40: forced swimming test [FST]) and adult (PND 85: TST; PND 90: FST) male mice.

TST: The TST was performed according to the previously described procedure.[6] At the beginning of the experiment, each mouse was individually suspended by its tail using a clamp, 2 cm from the distal end, for 5 min in a gray wooden box (40 cm high, 30 cm wide, and 20 cm deep), with the head about 25 cm above the floor. The total duration of immobility was recorded (in seconds). All animals that climbed their tails during the TST were excluded from the further analyses. Immobility was defined as the lack of whole-body motion, whereas mobility was defined as hind leg movement.[16]

FST: The FST remains one of the most widely used tools for measuring behavioral despair in rodents. To describe this behavioral model in mice, the following procedure was adopted: mice were individually placed into the transparent glass cylinders (height: 25 cm, diameter: 10 cm) filled with water to a height of 15 cm and maintained at 25 ± 1°C. The water was replaced between each test. The total duration of immobility was recorded during the last 4 min of the 6-min testing period. At the end of swimming session, the animals were removed from the cylinder, dried with towels, and gently placed near an electric heater for 15–30 min. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in the duration of immobility time is considered indicative of depression-like behavior in mice.[6,10]

Statistical analysis

The statistical analysis was performed using Statistical Package for Social Sciences software (Version 21, IBM, Armonk, NY, USA). The depression results were analyzed using three-way analysis of variance, with age, treatment, and neonatal infection timing as the main factors. All data are presented as the mean ± standard error of the mean. Further analysis was carried out using Tukey's honest significant different *post-hoc* tests for multiple comparisons. $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of early and late neonatal immune activation on depression-related behaviors during adolescence and adulthood in the TST

The three-way analysis revealed the significant effects of the time of neonatal immune activation ($F_{1,84} = 5.65$, $P < 0.03$), age ($F_{1,84} = 43.03$, $P < 0.001$), and treatment ($F_{2,84} = 11.57$, $P < 0.001$) on the immobility time in the TST. Significant interactions existed between age × treatment ($F_{2,84} = 4.66$, $P < 0.02$) and the time of neonatal immune activation × age × treatment ($F_{2,84} = 3.37$, $P < 0.04$). However, there was no significant interaction between the time of neonatal immune activation × age and the time of neonatal immune activation × treatment. These results indicate that neonatal immune activation with Poly I:C can influence depression-related behaviors in dose-, age-, and time-dependent manner in mice. Therefore, the dose of immunogen, the timing of immune activation, and age may be important factors for evaluating the consequences of neonatal immune activation on affective disorders, like depression, later in life.

The data analysis indicated that early neonatal immune activation with Poly I: C increased the total duration of immobility at the dose of 4 mg/kg in adolescence [Figure 2a; $P = 0.037$] and at both doses in adulthood [Figure 2b; $P = 0.042$ and $P = 0.002$], indicating high levels of depression-related behaviors in Poly I:C-treated mice in comparison with the saline-treated group.

As shown in Figure 3, late neonatal immune challenge with Poly I:C resulted in an increase in the total duration of immobility time at the dose of 4 mg/kg in adulthood [$P = 0.03$], but not in adolescence [Figure 3].

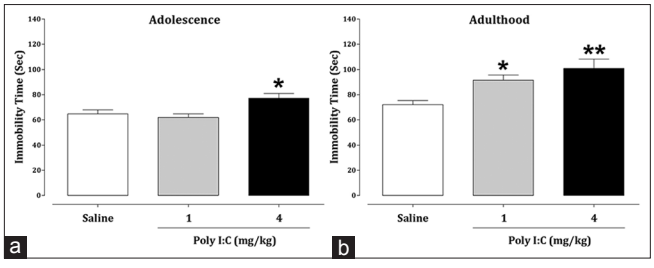

Figure 2: Effects of early neonatal immune activation on depression-like behavior during adolescence (a) and adulthood (b) in the tail suspension test. The data are presented as mean ± standard error of the mean ($n = 8$). *$P < 0.05$ and **$P < 0.01$ compared with the saline-treated group

In addition, we found high levels of depression-related behaviors in adulthood in Poly I:C-exposed mice compared with saline-injected mice.

Effects of early and late neonatal immune activation on depression-related behaviors during adolescence and adulthood in the FST

The three-way analysis indicated the significant effects of the time of postnatal immune activation ($F_{1,84} = 34.69$, $P < 0.001$), age ($F_{1,4} = 20.37$, $P < 0.001$), and treatment ($F_{2,84} = 20.53$, $P < 0.001$) on the immobility time in the FST. Considerable interactions existed between the time of neonatal immune activation × treatment ($F_{2,84} = 5.02$, $P < 0.009$) and age × treatment ($F_{2,84} = 5.77$, $P < 0.005$). However, there was no interaction between the time of neonatal immune activation × age and the time of neonatal immune activation × age × treatment. These data demonstrate that immune activation with Poly I:C during postnatal brain development can affect depression-related behaviors in dose-, age-, and time-dependent manner in adult mice. Thus, these different factors may affect the effects of neonatal immune activation on affective disorders later in life in animal models.

The results of the FST assessment showed that early postnatal immune activation with Poly I:C increased immobility time at the dose of 4 mg/kg during adolescence [Figure 4a; $P = 0.015$] and at both doses in adulthood [Figure 4b; $P = 0.002$ and $P = 0.000$]. Higher levels of depression-related behaviors were measured in Poly I:C-treated mice in comparison with the saline-treated group.

Our data also showed that immune activation with 4 mg/kg Poly I:C during late neonatal brain development increased the total duration of immobility in adulthood ($P = 0.019$), but not in adolescence [Figure 5], indicating high levels of depression-related behaviors during adulthood in Poly I:C-exposed mice relative to the saline-treated group. These results confirmed that time of postnatal immune challenge, age and the dose of immunogen can be important factors for evaluating depression-related behaviors in mice.

DISCUSSION

We recently showed that early postnatal immune challenge with the bacterial endotoxin and LPS can lead to increased levels of corticosterone (COR) and depression-like symptoms in adult male and female NMRI mice.[6] In addition, early postnatal immune challenge has been shown to have adverse outcomes on

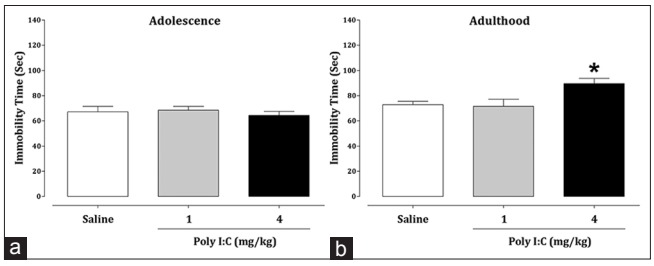

Figure 3: Effects of late neonatal immune challenge on depression-like behavior during adolescence (a) and adulthood (b) in the tail suspension test. The data are presented as mean ± standard error of the mean ($n = 8$). *$P < 0.05$ compared to the saline-treated group

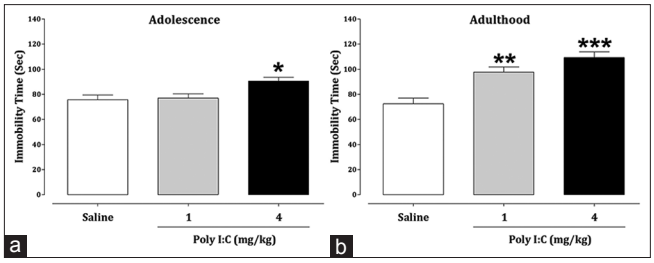

Figure 4: Impacts of early postnatal immune activation on depression-like behavior during adolescence (a) and adulthood (b) in the forced swimming test. The data are presented as mean ± standard error of the mean ($n = 8$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to the saline-treated group

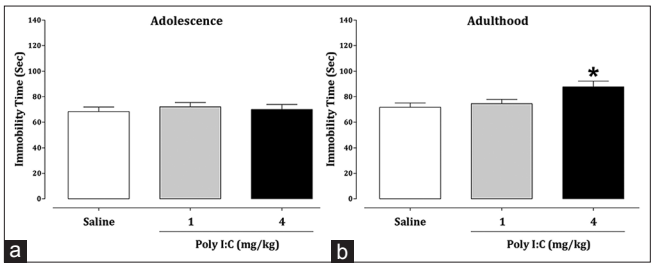

Figure 5: Effects of late neonatal immune activation on depression-like behavior during adolescence (a) and adulthood (b) in the forced swimming test. The data are presented as mean ± standard error of the mean ($n = 8$). *$P < 0.05$, compared to the saline-treated group

physiological, behavioral, and neuroendocrine systems in adulthood.[6,8,9] For instance, it has been reported that bacterial and viral infections during early[9,11] and late[15] neonatal periods results in increased anxiety-like behaviors and disrupted HPA axis activity in adult rodents. Our results demonstrated that early neonatal immune activation led to increased depression-related behaviors in both adolescent and adult mice, but late neonatal infection only increased depression in adult mice. In this regard, Konat *et al.*[15] showed that anxiety levels in rats following late postnatal immune activation were much larger than those observed by Ibi *et al.*[17] in mice following early neonatal immune challenge using a similar behavioral testing. Previous studies demonstrated that early postnatal immune challenge increased baseline COR levels during adolescence and adulthood, while late neonatal immune activation did not alter baseline COR levels in adulthood.[18] It was

also found that COR suppressed cell proliferation and neurogenesis in the hippocampus, which can induce depression-like behaviors.[19] Moreover, the important role of the hippocampus in depression-related behaviors has been shown in humans and rodents.[13] In line with this, we demonstrated that adolescent fluoxetine treatment reduced depression-like behaviors induced by early neonatal infection in adult mice.[6] It has been reported that chronic fluoxetine and imipramine treatments prevent the COR-induced reduction in cell proliferation and activates neurogenesis in the hippocampus.[19] It seems reasonable to speculate that an increase in depression-related behaviors or baseline COR during adolescence following early neonatal immune activation may further suppress cell proliferation and neurogenesis in the hippocampus in comparison with late neonatal immune activation, and these effects can increase the severity of depression in adulthood. Moreover, we observed an interaction between treatment, age, and the time of neonatal immune activation on depression-related behaviors in mice. Poly I:C at the dose of 4 mg/kg during early postnatal brain development increased depression-related behaviors in adolescent mice, while the same dose during late neonatal phase had no significant effect on depression in adolescent mice. Notably, the mice treated with Poly I:C at both doses during early neonatal period exhibited elevated depression-related behaviors as adults, while only the dose of 4 mg/kg during the late neonatal phase increased depression in adult mice. Taken together, the findings of this study suggest that the effect of neonatal immune activation on depression-related behaviors in mice is dependent on the timing of the immune challenge and the dose of immunogen.

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Case Report

POEMS syndrome associated with Castleman disease: a case report and literature review

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ABSTRACT

Polyneuropathy, organomegaly, endocrinopathy, M proteins, and skin changes (POEMS) syndrome is a multisystemic disorder that clinically manifests as paraneoplastic and monoclonal plasma cell dyscrasia. Its acronym is derived from its principal characteristics: polyneuropathy, organomegaly, endocrinopathy, M proteins, and skin changes. Here, the authors reported a case of POEMS syndrome that was also associated with Castleman disease. A 53-year-old female patient was admitted to our hospital with limb weakness, numbness, edema, abdominal distention, and fever. Physical examination revealed tetraplegia, paraesthesia, and hyporeflexia in all four limbs, in addition to lymphadenectasis, splenomegaly, skin hyperpigmentation, hypertrichosis, and pitting edema. Laboratory tests and imaging revealed thrombocytosis, hypothyroidism, diabetes, hydropericardium, hydrothorax, splenomegaly, and lymphadenectasis. Electromyography showed the characteristic patterns of both demyelinating disease and axonal degeneration. Serum protein electrophoresis revealed monoclonal immunoglobulin G-lambda paraproteins. Histological examination clearly diagnosed the disease as the hyaline vascular subtype. The final diagnosis in this case was POEMS syndrome in association with Castleman disease.

Key words: Castleman disease, hyaline vascular variant, M protein, polyneuropathy, POEMS syndrome

INTRODUCTION

Polyneuropathy, organomegaly, endocrinopathy, M proteins, and skin changes (POEMS) syndrome, also known as Crow–Fukase syndrome, osteosclerotic myeloma, and Takatsuki syndrome,[1-4] is the paraneoplastic clinical manifestation of monoclonal plasma cell dyscrasia. POEMS syndrome is a multisystemic disorder, and its acronym is derived from its principal characteristics: polyneuropathy, organomegaly, endocrinopathy, M proteins, and skin changes.[5,6] Other important clinical features include fever, papilledema, extravascular volume overload, sclerosis, bone lesions, thrombocytosis, erythrocytosis, elevated vascular endothelial growth factor (VEGF) levels, abnormal pulmonary function, predisposition toward thrombosis, *etc*.[2,3,6-13] Early diagnosis is a challenge because of the diverse clinical manifestations that are often accompanied with multiple organ injury. Here, we reported a patient with the Castleman disease variant of POEMS syndrome, which we hope will prompt the universal recognition of POEMS.

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CASE REPORT

A 53-year-old Chinese woman was admitted to the Neurology Department of our hospital because of progressive limbs weakness, numbness, and edema.

Approximately 7 months before admission, she began to develop limb weakness and numbness. Twenty days before admission, the patient developed fever and edema. She had been diagnosed with diabetes and treated with insulin i.h. for 3 months prior to admission. Her past medical history was unremarkable, with no history of smoking, alcohol use, HIV infection, tuberculosis, or tumor.

On examination, the patient was suffering from progressive tetraplegia, paraesthesia, and

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edema. Physical examination revealed fever, skin hyperpigmentation, hypertrichosis, multiple small peripheral lymph nodes, splenomegaly, pitting edema in the lower extremities, tetraplegia, paraesthesia, and hyporeflexia in all four limbs. Routine blood examination revealed thrombocytosis (527×10^9 platelets/L; normal = $(101–320) \times 10^9$ platelets/L). Blood biochemistry analysis revealed low albumin and globulin. Laboratory tests on admission were positive for hepatitis B surface antigen, hepatitis B core antibody, and hepatitis B e-antibody, but serum hepatitis B virus (HBV)-DNA was within normal limits. Fibrinogen was 4.72 g/L (normal = 1.8–3.5 g/L). Thyroid function test revealed hypothyroidism. C-reactive protein was 22.60 mg/L, and erythrocyte sedimentation rate was 40 mm/h. Lumbar puncture was performed on admission, and cerebrospinal fluid testing revealed increased protein and pressure levels, but the cell count was normal. Serum protein electrophoresis revealed monoclonal immunoglobulin G-lambda (IgG-λ) paraprotein, while serum immunoglobulin levels were within normal limits [Figure 1]. Ultrasonic examination indicated hydropericardium, right hydrothorax, splenomegaly, and lymphadenectasis (including the anterior cervical, axillary, and inguinal lymph nodes). At the same time, ultrasound revealed multiple hemangiomas and splenomegaly. Needle electromyography confirmed diffuse, symmetrical, demyelinating, and axonal lesions in the sensorimotor fibers that affected all four limbs. Meanwhile, a portion of the F-waves in the peripheral nerves had reduced and disappeared. Electromyography revealed a pattern that is characteristic both of demyelinating disease and axonal degeneration.

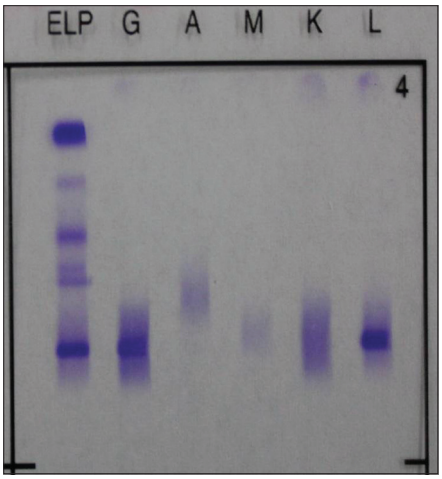

Figure 1: Immunofixation, a monoclonal immunoglobulin G-lambda paraprotein, showing deep dyeing belts (Band L)

Biopsy of the lymph nodes from the right cervical chain revealed vascular, follicular, and lymphoid hyperplasia, thickening of the mantle zone, and the formation of concentric lymphocytes surrounding the germinal center, which were hyalinized, atrophic, and surrounded by blood vessels. Vascular proliferation was found, in addition to sinus histiocytosis in the interfollicular parenchyma [Figure 2]. Meanwhile, immunohistochemical analysis revealed positive staining for CD3, CD4, CD8, CD20, CD21, CD138, kappa, lambda, Pax-5, and Ki-67. These histological findings are consistent with the hyaline vascular variant of Castleman disease. Patient improvement was not apparent after treatment with dexamethasone for 10 days. The patient was transferred to the Hematology Department for further treatment.

DISCUSSION

POEMS syndrome was first reported by Scheinker in 1938.[2,5] The first Chinese case of POEMS syndrome was described in 1986.[14] POEMS syndrome is a rare multisystemic disorder that is related to underlying plasma cell dyscrasia. The important traits of POEMS syndrome including polyneuropathy, organomegaly, endocrinopathy, M proteins, and skin changes.[6] The other important features include Castleman disease, sclerotic bone lesions, VEGF elevation, *etc*. The diagnosis of POEMS syndrome is based on having both polyradiculoneuropathy and monoclonal plasma cell disorder, at least 1 of 3 other major criteria (Castleman disease, sclerotic bone lesions, or elevated VEGF), and at least 1 minor criterion [Table 1].

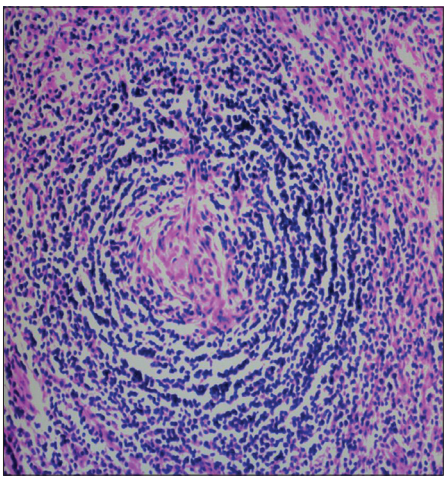

Figure 2: Right cervical lymph node biopsy showing hyaline vascular Castleman disease, follicular, lymphocytes, vascularity hyperplasia, and the formation of concentric of lymphocytes surrounding the germinal center. H and E staining (original magnification, ×200)

Our patient had almost all the features of POEMS syndrome, including multiple peripheral neuropathy, splenomegaly, and multiple enlarged peripheral lymph nodes, diabetes, hypothyroidism, monoclonal IgG-λ paraprotein, skin hyperpigmentation, and hypertrichosis. Meanwhile, our patient also presented with systemic signs such as fever, abdominal distention, pitting edema of the lower extremities, hydropericardium, hydrothorax, thrombocytosis, hypoalbuminemia, and increased fibrinogen levels. In brief, this patient is consistent with the standard diagnostic standard of POEMS syndrome.

Castleman disease (CD, or angiofollicular lymph node hyperplasia) is also a rare lymphoproliferative disorder.[15] Castleman disease was first described by Castleman *et al*.[16] in 1956. According to previous studies, the pathological feature of Castleman disease is reactive proliferation in the lymphoid tissues.[17] The clinical features of Castleman disease are classified into two categories: localized and multicentric.[18] There are also three histological forms of CD: (1) hyaline vascular form, (2) plasma cell form, and (3) mixed. Multicentric Castleman disease (MCD) is generally the plasma cell type, but the hyaline vascular type has been described in some patients.[19] Localized Castleman disease usually presents as masses in young adults (20–30 years of age). Systemic symptoms are rare in localized Castleman disease patients. In contrast, MCD develops in old patients (40–50 years of age). The involvement of multiple lymph nodes and organs is frequent.[20] Our older patient presented with systemic symptoms and multiple enlarged lymph nodes. The histological findings in this case are consistent with the hyaline vascular form of Castleman disease.

Table 1: Criteria for the diagnosis of POEMS syndrome[11]

Criteria/other symptoms and signs	Affected, %*
Mandatory major criteria (both required)	
Polyradiculoneuropathy (typically demyelinating)	100
Monoclonal plasma cell disorder (almost always λ)	100†
Other major criteria (1 required)	
Castleman disease‡	11–25
Sclerotic bone lesions	27–97
VEGF elevation§	
Minor criteria (1 required)	
Organomegaly (splenomegaly, hepatomegaly, or lymphadenopathy)	45–85
Extravascular volume overload (edema, pleural effusion, or ascites)	29–87
Endocrinopathy (adrenal, thyroid, pituitary**, gonadal, parathyroid, pancreatic**)	67–84
Skin changes (hyperpigmentation, hypertrichosis, glomeruloid hemangiomata, plethora, acrocyanosis, flushing, white nails)	68–89
Papilledema	29–64
Thrombocytosis/polycythemia***	54–88
Other symptoms and signs	
Clubbing, weight loss, hyperhidrosis, pulmonary hypertension/restrictive lung disease, thrombotic diatheses, diarrhea, low vitamin B_{12} values	

The diagnosis of POEMS syndrome is confirmed when both of the mandatory major criteria, 1 of the 3 other major criteria and 1 of the 6 minor criteria are present. *Summary of frequencies of POEMS syndrome features based on largest retrospective series,[2,3,6-9] †Takasuki and Nakanishi series are included, even though only 75% of patients had a documented plasma cell disorder. Because these are among the earliest series describing the syndrome, they are included, ‡There is a Castleman disease variant of POEMS syndrome that occurs without evidence of a clonal plasma cell disorder that is not accounted for in this table. This entity should be considered separately, §A plasma VEGF level of 200 pg/mL is 95% specific and 68% sensitive for a POEMS syndrome,[12] **Because of the high prevalence of diabetes mellitus and thyroid abnormalities, this diagnosis alone is not sufficient to meet this minor criterion, ***Approximately 50% of patients will have bone marrow changes that distinguish it from a typical monoclonal gammopathy of undetermined significance or myeloma bone marrow.[24] POEMS: polyneuropathy, organomegaly, endocrinopathy, M proteins and skin changes, VEGF: Vascular endothelial growth factor

Castleman disease and POEMS syndrome are closely related. An association with MCD was reported in about 50% of patients with POEMS.[6,19,20] Of 113 patients with MCD, 32% presented with criteria sufficient for a diagnosis of POEMS syndrome.[21] Here, our patient presented with POEMS syndrome in association with Castleman disease.

The pathogeny of POEMS syndrome remains unclear. It is assumed that hepatitis B antigen may play a role in the etiology of this lymphatic disorder.[22] Our patient was positive for HBV. A previous study confirmed that increased levels of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and VEGF in patients of POEMS syndrome are correlated with disease activity.[23] However, we did not assess TNF-α, IL-6, or VEGF. Therapy for POEMS syndrome should include radiation, chemotherapy, peripheral blood stem cell transplant, targeting therapy, intravenous gamma-globulin therapy, plasmapheresis, corticosteroids, *etc*.[11] The clinical course of POEMS syndrome is also chronic. A previous study revealed that the median survival time of patients with POEMS syndrome is 165 months.[6] Another study reported that the prognosis of MCD patients was poor, demonstrating a median survival time of 30 months.[20]

The diagnosis of POEMS syndrome is often delayed because the syndrome is rare and can be mistaken for other neurological disorders. Thus, we hope our patient with the Castleman disease variant of POEMS syndrome will prompt the universal recognition of this disease.

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Review Article

Human leukocyte antigens-immunogenetics of neuromyelitis optica or Devic's disease and the impact on the immunopathogenesis, diagnosis and treatment: a critical review

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ABSTRACT

Neuromyelitis optica (NMO) is an autoimmune demyelinating disorder, predominantly characterized by severe optic neuritis, transverse myelitis and the high level of antibodies against aquaporin-4 (AQP4) or NMO-immunoglobulin G (IgG). Researches trying to correlate NMO with specific human leukocyte antigen (HLA) alleles took place in a limited extend in the last few years. Nevertheless, it has become clear that HLAs play a crucial role in the genetic risk of NMO, in the understanding of its pathogenesis and the differential diagnosis mainly from multiple sclerosis (MS), and also from other demyelinating diseases. In this study, we retrieved all the available data in the MEDLINE concerning the distribution of HLA frequencies in NMO and NMO-spectrum diseases, in all available ethnic groups, and compared them with those of MS. The results suggest that, the well-established HLA-DRB1*15:01 allele, associated with MS, plays rather a protective role for NMO. HLA-DRB1*03 allele is highly frequent in the NMO-IgG positive Caucasian patients, while HLA-DPB1*05:01 is the predominant allele in Japanese patients. The HLA-genotype and anti-AQP4 presence are the common immunological components in cases of comorbidity of NMO and other autoimmune diseases. The authors aim to summarize in the critical review the results of these researches worldwide, create a workable table including all this information for an easier reading approach and highlight the importance of these results in therapeutic decision making, using the HLA profile as biomarker in patients' stratification.

Key words: Diagnosis, human leukocyte antigens-immunogenetics, immunopathogenesis, neuromyelitis optica, treatment

INTRODUCTION

Since its very first discovery and disease association studies in early 1970's, the major histocompability complex (MHC) with its polymorphisms has been the "gold standard" and the primer genetic locus in attributing genetic burden for certain autoimmune diseases, like multiple sclerosis (MS). Initial studies, using serological techniques, showed an association of MS with human leukocyte antigens (HLA) class I, especially HLA-A3 and HLA-B7.[1] Multiple recent researches which used current molecular methods (sequence specific oligonucleotide-polymerase chain reaction [PCR], single specific primer-PCR and single-nucleotide polymorphisms, genome-wide association study, *etc.*)[1] and which were conducted in many MS cohorts, made clear that HLA-DRB1*15:01 is by far the main independent, responsible allele for attributing genetic risk in different MS ethnic groups.[1] In addition, co-existence of certain alleles probably leads to an increase or decrease of the overall risk, via epistatic mechanisms (i.e. HLA-DRB1*15:01 and HLA-DQ1*01:02).[1] Moreover, a Vitamin D response element has been found in the promoter region of HLA-DRB1*15:01, changing the expression of the allele and the risk for the disease. Thus, an environmental factor, the sunlight, via the metabolites of Vitamin D, has been linked to the genome, especially to HLA-DRB1*15:01 and finally to the disease phenotype.[2] The interaction

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between HLA-DRB1*15:01 and Epstein-Barr virus,[3] as well as the estrogen receptor,[4] has also been very well established.

Neuromyelitis optica (NMO) is an autoimmune demyelinating disorder, predominantly characterized by severe optic neuritis (ON) and transverse myelitis (TM).[5] It was considered as a variant of MS, but the discovery that most NMO patients have antibodies against aquaporin-4 (AQP4) or NMO-immunoglobulin G (IgG), dramatically changed our perception of the disease and brought NMO and its spectrum in the center of interest.[6] Researches trying to correlate NMO with specific HLA alleles took place in a limited extend in the last few years, especially in Japanese population, in which NMO appears its greatest frequency. Nevertheless, it has become clear that HLA play a crucial, and maybe the primer role, in the genetic risk of NMO and provide great insight in the profound understanding of its pathogenesis and the differential diagnosis mainly from MS and other demyelinating diseases as well.

In this study, our aim was to summarize in a critical review the results of these researches worldwide and shed light on the contribution of HLA alleles in NMO immunopathogenesis, given the total absence of such a review.

HISTORICAL NOTES AND EPIDEMIOLOGY

Neuromyelitis optica was first described in 1870 by Allbutt, who reported an association between myelitis and unilateral optic nerve disorder, but it was in 1894 when the term "neuromyelite optique aigue" (acute optic neuromyelitis) was coined by Devic[7] in order to describe patients who first suffered unilateral or bilateral loss of vision and within weeks developed severe spastic para- or tetraparesis, loss of sensation and sphincter control. In 1999, Wingerchuk *et al*.[8] proposed the first diagnostic criteria for NMO. Current revised criteria for diagnosing NMO were defined in 2006 by the same group [Table 1],[9] because in 2004, the AQP4 protein was identified for NMO, which was the first molecular target described for any type of demyelinating diseases of the central nervous system (CNS).[6]

Neuromyelitis optica represents < 1% of demyelinating diseases of the CNS in Caucasians[10] and it is certainly more common in Asians. It has been reported to account for up to 30% of West Indian cases of CNS demyelination,[11] 20-30% of Japanese cases,[12] 48% of East Asian cases,[13] 23% of Indian cases[14] and 15% of Afro-Brazilian cases.[15] Japanese patients with opticospinal MS (OSMS) or Asian MS represent a distinct entity from western MS. The equal detection of

Table 1: Revised diagnostic criteria for NMO

Definite NMO
Optic neuritis
Acute myelitis
At least two of three supportive criteria
1. Contiguous spinal cord MRI lesion extending over three vertebral segments
2. Brain MRI not meeting diagnostic criteria for multiple sclerosis
3. NMO-IgG seropositive status

NMO: neuromyelitis optica; MRI: magnetic resonance imaging; IgG: immunoglobulin G

NMO-IgG in the sera of Japanese patients with OSMS and NMO, as well as the similar clinical and pathological characteristics, indicate that both syndromes may belong to the same clinical entity.[16] NMO is more common in women than men (> 2/3).[17,18] More than 80% present the relapsing form of the disease,[17] while the median age of onset is the late 30s,[18] with few reports of NMO occurrence in children[19] or elderly.[20] Familial cases of NMO are estimated to account for 3% of all NMO cases.[21]

BASIC CLINICAL FEAUTURES AND IMMUNOPATHOGENESIS

Neuromyelitis optica is characterized by ON, which is often bilateral (simultaneously or sequentially), and longitudinally extensive TM with a well-defined sensory level, as well as sphincter dysfunction, pain and tonic spasms of the trunk and extremities.[8] Involvement of the brain stem may cause hiccups, nausea and even respiratory failure,[22] while hypothalamic-pituitary axis dysfunction commonly manifests as hyponatremia, hyperthermia and hyperprolactinemia.[23] Encephalopathy mimicking posterior reversible encephalopathy syndrome has also been described.[24]

Clinical attacks generally progress over days, with variable recovery within months. Most patients endure some residual disability, which accumulates over time.[8]

Even though a review of the complicated mechanisms of pathogenesis of NMO is far from the purpose of our review, in order to explore in which ways HLA may contribute to it, we refer to some important aspects, providing the basics for this purpose. AQP4-antibodies have a decisive role in the pathogenesis of NMO, by complement-mediated astrocyte damage, cascading to leukocyte infiltration, oligodendrocyte death and neuronal cell damage.[25] They are present in up to 80% of NMO cases. AQP4 is highly expressed in astrocytic end-feet in the blood-brain barrier, nodes of Ranvier and neuronal synapses.[25] AQP4 is also expressed in a sub-population of CNS ependymal cells associated with the pia, subfornical organ and

to a lesser extent in other ependymal cells (not in the choroid plexus), *in situ* in lipopolysaccharide-activated microglia and on retinal astrocytes (Müller cells).[26] It is abundant in the grey matter of the spinal cord, the periventricular and periaqueductal area. Outside the CNS, it is found on epithelial cells of the kidney collecting ducts, airways, parietal cells of the stomach, skeletal muscle sarcolemma and colon.[27] AQP4-IgG serum levels are found to correlate with NMO disease activity, distinct phenotypic features (gender, course and co-existing autoimmunity), severity and response to treatment.[25] In patients with isolated ON or isolated longitudinally extensive TM, AQP4-antibodies have been shown to predict conversion to NMO.[25] Recent researches have shown that patients' sera with MS, acute disseminated encephalomyelitis, systemic lupus erythematosus (SLE) and Sjögren syndrome (SS) were negative for AQP4-antibodies.[28,29] However, Alexopoulos *et al*.[28] managed to demonstrate that, despite the negativity of the serum in antibodies, 13% of the sera with relapsing-remitting MS reacted with the epitope AQPaa252-275 (NMO-positive sera exhibited reactivity against the intracellular epitope AQPaa252-275 in this study, confirming previous observations).

Additional immunological components participate.[25] NMO lesions contain large numbers of macrophages, eosinophils and neutrophils, on which AQP4-IgG acts by binding to Fc receptors, as well as on B-cells, which produce interleukin (IL)-6. IL-17 and IL-6 are the main pro-inflammatory cytokines which are found to be elevated in the serum and cerebro-spinal fluid of patients with NMO.[25] T-cells, though fewer, are also certainly relevant, as T-helper cells are involved in B-cell isotype switching and affinity maturation. The possible role of natural killer cells and glutamate-mediated excitotoxicity has also been discussed.[25] Of course, it has become clear that NMO is associated with certain HLA alleles, which are extensively described below.

NEUROMYELITIS OPTICA AND OTHER AUTOIMMUNE DISEASES

There is a strong association between NMO and other autoimmune diseases, especially in NMO-IgG positive patients: co-existence with autoimmune thyroid disease, SLE, SS, celiac disease, sarcoidosis, or myasthenia gravis (MG) has been described in a higher frequency than it could be by chance.[30] This co-association could be due to common genetic factors, such as HLA and non-HLA genes, including *PTPN22*, a tyrosine phosphatase associated with type-1 diabetes, rheumatoid arthritis (RA), SLE, Crohn's disease and MG;[31,32] *IL-23R*, associated with SLE, Crohn's disease and psoriasis; and finally, *TNFAIP3*, involved in control of unbiquitination, associated with RA, SLE, Crohn's disease and psoriasis.[31] As far as MG is concerned, despite of the well-established link between the thymus gland and MG (human thymus tissue has been shown to express AChR, which is widely thought to be a triggering mechanism in early-onset AChR-MG), recent evidence suggests that AQP4 is also expressed in human thymus suggesting a similar and early involvement of the thymus in NMO-spectrum diseases (NMOSD).[33-36] HLA-DRB1*03 and especially the whole haplotype, HLA A1-B8-DR3-DQ2, is the most commonly attributed to MG in the Caucasians, an haplotype common and in other autoimmune diseases.[32] HLA-DRB1*03 allele is highly frequent in the NMO-IgG positive patients, as we explain in detail below and it could be one of the links between MG and NMO.[37]

NEUROMYELITIS OPTICA AND HUMAN LEYKOCYTE ANTIGEN

According to the results of the researches that have been conducted in Japanese, conventional MS is associated with the HLA-DRB1*15:01 allele,[12,38,39] while OSMS, which is now accepted as a component of the NMO spectrum, is associated with the HLA-DPB1*05:01.[40-45] HLA-DPB1*05:01 is the most common DPB1 allele in Japanese,[46] which may explain the frequent occurrence of anti-AQP4 antibody in Japanese OSMS.[42] Nevertheless, Fukazawa *et al*.[44] in 2006 came to the conclusion that HLA-DPB1*05:01 plays an important role in the development of MS in general, but not in OSMS. The strong association of HLA-DPB1*05:01 with OSMS may be due to the over-representation of the HLA-DPB1*03:01 allele among individuals in the non-OSMS group, a question that needs further investigation. The observed protective effect of HLA-DRB1*01 in anti-AQP4-negative MS patients is in accordance with findings in Caucasians.[47,48]

It is also estimated that there is a protective effect of HLA-DRB1*09 in anti-AQP4-negative MS patients, probably by reducing the susceptibility attributed to HLA-DRB1*15, as individuals with a HLA-DRB1*09/HLA-DRB1*15 genotype have a decreased risk of anti-AQP4-negative MS.[39] Recently, HLA-DRB1*09 was also shown to be negatively associated with ulcerative colitis.[49] Thus, it is assumed that HLA-DRB1*09, or some genes in linkage disequilibrium with it, protect against certain autoimmune diseases, at least in Japanese, as it is quite rare in Caucasians. Moreover, a predisposing effect of HLA-DRB1*12 in anti-AQP4-positive MS has been found.[39] Interestingly, HLA-DRB1*12 has been reported to increase the risk of

allergic disorders, such as asthma,[50] urticaria,[51] and food allergy.[52] Finally, HLA-DRB1*04/HLA-DRB1*04, HLA-DRB1*04/HLA-DRB1*14, and HLA-DRB1*04/HLA-DRB1*15 genotypes increase the risk of non-NMO MS, probably by interaction with DRB1*15 allele.[39]

In contrast, researches in Caucasian populations have come to the conclusion that the HLA-DRB1*03 allele is highly frequent in the NMO-IgG positive patients, while DPB1*05:01 is quite rare both in patients and healthy controls.[37] We should also highlight the negative association between HLA-DRB1*15:01 and NMO, which indicates a possible protective role.[53,54] In this point, the observation that NMO-IgG-positive and negative patients differ mostly in terms of gender and the association of other autoimmune diseases, could imply that HLA-DRB1*03 is associated with the NMO-IgG presence, but not with NMO *per se* and raise the question of whether NMO-IgG is epiphenomenon or pathogenic.[37] In reply to this, Arellano *et al*.[55] found that hAQP4281-330 is the dominant linear immunogenic determinant of hAQP4 in the context of HLADRB1*03:01. Within hAQP4281-330 are two dominant immunogenic determinants that induce differential Th phenotypes. In recent times, Asgari *et al*.[27,53] reported a high frequency of HLA-DQB1*04:02 in NMO patients, an allele described to be associated with autoimmune diseases such as primary biliary cirrhosis, type-1 diabetes and juvenile idiopathic arthritis, but he didn't show any correlation with HLA-DRB1*03.

In Brazilian cohorts, NMO patients present a high frequency of the HLA-DRB1*03 allele and extremely low frequency of the HLA-DRB1*15. In addition to this, the same study showed that HLA-DRB1*01 allele is associated with NMO and benign MS, a correlation that indicates that this allele may influence the outcome of these demyelinating disorders.[56] We would like to emphasize once more that HLA-DRB1*01 has a protective effect in anti-AQP4-negative MS patients in Japanese and Caucasians.[47,48] In African-Americans, none OSMS patient carries the HLA-DRB1*1501 allele,[57] while in Afro-Carribeans, NMO has been associated with the HLA-DRB1*03 allele [Table 2].[58]

DISCUSSION

To the best of our knowledge, this is the first review aiming at summarizing all the results concerning HLA allelic frequencies in NMO and NMOSD, worldwide. Apart from a detailed description of HLA allelic frequencies in all genotyped NMO ethnic groups, we created a workable table including all this information, for an easier reader's approach.

As a conclusion, it is clear that quite different HLA-alleles are correlated to NMO/NMOSD compared to MS patients, reflecting different underlying immunopathogenic mechanisms. Particularly, the well-established and most frequent HLA-DRB1*15:01 allele, associated with MS, plays rather a protective role for NMO. In addition, rare alleles, HLA-DRB1*12, like HLA-DRB1*01 and especially HLA-DRB1*09, play a core role in NMO risk or protection respectively and obviously in immonopathogenesis, in some ethnic groups. On the other hand, it is clear that different HLA alleles are associated with different ethnic groups, like Eastern NMO (association with the HLA-DPB1*05:01), which in turn are specifically associated with certain clinical/paraclinical features.

Moreover, the comorbidity of NMO with other autoimmune diseases is still under further investigation, although it seems that so far this comorbidity is highly reflected in HLA profile and anti-AQP4 antibody presence, suggesting common pathways in their immunopathogenesis.

In MS there is also comorbidity with other autoimmune diseases, like SLE, Hashimoto's thyroiditis, *etc*., However, this co-existence presumes rarer than in NMO, although more investigation studies are warranted to prove this notion.

In this paper, we tried to focus only on the HLA-immunogenetics of NMO, since the HLA molecule is a core component of the trimolecular complex, which is involved in antigen-presentation, as the first step of the immune response. However, as in MS, similarly in NMO, many non-MHC genes are candidates for the overall genetic burden. First, genes correlated to immune system and immunogenetics, namely IL-7 receptor polymorphisms,[59] IL-2 receptor a chain gene polymorphisms[60] and CD6, interferon regulatory factor 8 and tumor necrosis factor receptor superfamily[61] polymorphisms and secondly, the polymorphisms of the promoter region of cytochrome-P450-7A1 gene[62,63] and AQP4 genetic variations[64] are involved.

Regarding MS, it has been shown that specific alleles, in particular HLA-DRB1*04:01, HLA-DRB1*04:08 and HLA-DRB1*16:01, are associated with an increased risk of antiinterferon beta antibody development.[65] As a result, the poorer therapeutical outcome highligths the importance of the stratification of patients to responders and nonresponders, according to HLA-genotyping.[1] Similarly, Warabi *et al*.[66] concluded that patients carrying the NMO-specific HLA allele DPB1*05:01 showed a poor prognosis following interferon beta-1b treatment. The crucial role of the AQP4-antibodies in the pathogenesis of NMO has been

Table 2: The distribution of HLA alleles in different ethnic groups				
Ethnic group	**HLA**	**Alleles**	**Findings**	**References**
Caucasians	HLA class I		No correlations found	[37]
	HLA-DR	HLA-DRB1*01	High frequency in NMO-IgG positive patients	[37]
		HLA-DRB1*0301	High frequency in NMO-IgG positive patients Not demonstrated association	[27,37,53]
		HLA-DRB1*1501	Not associated with NMO	[37,54]
	HLA-DQ	HLA-DQB1*0402	Higher frequency in NMO compared to HCs Increased in NMO	[27,53]
		HLA-DQA1*0102	High frequency in NMO-IgG negative patients No significant differences noticed	[37,53]
	HLA-DP	HLA-DPB1*0501	Rare allele in Caucasians. No correlations found	[37]
Japanese-Chinese	HLA class I		No data	No data
	HLA-DR	HLA-DRB1*01	Protective effect on anti-AQP4 negative MS patients	[39]
		HLA-DRB1*04	Increases the risk of non-NMO MS, especially HLA-DRB1*04/04, 04/14, 04/15	[39]
		HLA-DRB1*09	Protective factor for anti-AQP4 negative MS, especially HLA-DRB1*09/15. Decreased the risk of anti-AQP4 positive MS in monovariate studies NMO/NMOSD patients showed a significantly lower frequency	[39,40]
		HLA-DRB1*12	Increased frequency in anti-AQP4 positive MS, especially HLA-DRB1*12/15	[39]
		HLA-DRB1*15	Common in MS patients. Probable in correlation with *04, *09, *12 alleles Association with common MS	[12,38,39]
		HLA-DRB1*1602	Higher frequency in anti-AQP4 positive patients in Han Chinese Risk factor only for anti-AQP4 positive NMO/NMOSD	[40,41]
	HLA-DP	HLA-DPB1*0501	Strong positive association with OSMS Associated with opticospinal MS Increased frequency in anti-AQP4 positive patients Risk factor only for anti-AQP4 positive NMO/NMOSD patients Susceptibility in anti-AQP4 positive NMO in Han Chinese Important role in the development of MS in general, but not in OSMS. The strong association of DPB1*0501 with OSMS may be due to the over-representation of the DPB1*0301 allele among individuals in the non-OSMS	[12,38,40-46]
		HLA-DPB1*0301	The most strongly associated allele with conventional MS, complete lack in OSMS Possible protection against the development of OSMS	[38,44]
Brazilians	HLA-class I		No data	No data
	HLA-DR	HLA-DRB1*01	High frequency in NMO	[56]
		HLA-DRB1*03	High frequency in NMO	[56]
		HLA-DRB1*15	Low frequency in NMO, possible protective role	[56]
African-Americans and Afro-Caribbeans	HLA class I		No data	No data
	HLA-DR	HLA-DRB1*1501	None OSMS African-American patient	[57,58]
		HLA-DRB1*03	Highly noticed in NMO Afro-Caribbean patients	

HLA: human leukocyte antigens; NMO: neuromyelitis optica; IgG: immunoglobulin G; MS: multiple sclerosis; OSMS: opticospinal multiple sclerosis; AQP4: aquaporin-4; NMOSD: NMO-spectrum diseases

in our consideration for a few years only, in contrast to the 30 years of worldwide research regarding MS and HLA. We expect this to be the new field of extensive future research, in correlation with the accumulated knowledge on the pathogenesis of NMO.

Finally, the HLA profile in a patient with a CNS demyelinating disease tends to highlight different backgrounds in immunopathogenesis and clinical phenotype, components which are very important in the diagnosis and disease therapeutic decision making, which is strongly requested. To this direction and in order to use HLA alleles, as a biomarker, in patients' early stratification, more HLA-genotyping studies are needed, in different ethnic groups, in order to clarify, replicate or even expand the already existed results.

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Review Article

Targeting glioma stem cells via the Hedgehog signaling pathway

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ABSTRACT

Cancer is one of the leading causes of death worldwide. Gliomas are among the most devastating tumor types, and current clinical therapies are unsatisfactory. Recent reports revealed the importance of glioma-propagating cells in the malignancy of gliomas. These cells, also referred to as glioma stem cells (GSCs), share similarities with neural stem cells (NSCs). The Hedgehog (Hh) signaling pathway controls tissue polarity, patterning maintenance, and maintenance of NSCs during embryonic development. Aberrant activation of the Hh pathway resulting from mutation and deregulation has recently been recognized to cause tumorigenesis in a wide variety of tissues, including gliomas and GSCs. In this review, we explore the role of the Hh signaling pathway in GSCs and its potential as a therapeutic strategy.

Key words: Cancer stem cells, glioma, Hedgehog, microRNA

INTRODUCTION

Cancer is estimated to be the leading cause of death worldwide by the World Health Organization (WHO).[1] Gliomas are one of the most lethal adult brain tumors. Although substantial progress has been made, there is still a lack of effective therapy. It is reported that patients with low-grade gliomas can survive for years, while patients with glioblastoma (WHO grade IV) survive for only 1-2 years after diagnosis.[2]

Tumors are composed of a heterogeneous group of cells. Some tumor cell fractions have the ability to initiate tumors in xenograft models, whereas other fractions do not.[3] These cells, capable of sphere-like growth *in vitro* and tumor formation *in vivo*, are defined as cancer stem cells (CSCs) and share similarities with normal neural stem cells (NSCs). It has been hypothesized that these cells are involved in radio- and chemo-resistance, as well as tumor recurrence.

The Hedgehog (Hh) signaling pathway plays a pivotal role in embryonic development, including the formation and maintenance of glioma stem cells (GSCs).[4] Since GSCs are important biological factors responsible for cancer invasion, metastasis, drug resistance, and relapse, Hh signaling is believed to be an important target for cancer therapy. Recently, both natural and synthesized small-molecule inhibitors of Hh signaling have been investigated as potential cancer treatments. However, targeting only one molecule may be insufficient. Therefore, strategies using a combination of natural products and chemotherapeutics with different targets may improve the overall survival of patients with gliomas.

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CANCER STEM CELLS IN GLIOMA

Cancer stem cells and the niche

The concept of CSCs has been in use for over 50 years. In the 1990s, Lapidot *et al*.[5] identified leukemia stem cells capable of generating human acute myeloid leukemia after transplantation. Within gliomas, stem-like cells with the ability to self-renew, differentiate into multiple lineages, and initiate tumors are known as GSCs. Subsequently, GSCs capable of self-renewal and producing glioma-initiating cells were identified using a limiting dilution analysis.[6] The presence of GSCs and the increasing evidence of radio-resistance and chemo-resistance indicated that GSCs may contribute to tumor maintenance and recurrence, and that targeting GSCs may be a promising therapeutic intervention.[7,8] Stem cells reside in specialized niches, which could regulate their proliferation and differentiation.[9] More than one

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study has demonstrated the presence of GSCs near blood vessels, consistent with the perivascular niche for NSCs.[10,11] Alternatively, Li *et al*.[12] demonstrated that GSCs could also be found in regions of necrosis, which are hypoxic, suggesting that there may be more than one niche. Hypoxia promotes the self-renewal capability of both the stem cell and nonstem cell populations, and also promotes the conversion of nonstem cells into stem cells by up-regulating the expression of important stem cell factors,[13] indicating that this niche may be important for GSCs. Therapies that target cytokines, such as hypoxia-inducible factor-1α (HIF-1α) and HIF-2α, or cells in the niche, such as glia cells and endothelial cells, may, therefore, show promise.

Glioma stem cells and resistance to therapy

Surgical resection, radiation, and chemotherapy are still the mainstay treatments for gliomas and are associated with a clear improvement in overall survival in patients with high-grade gliomas. However, recurrence of the tumor is common following conventional therapy. Antiangiogenic therapy against vascular endothelial growth factor is another frequently used therapeutic treatment, but drug resistance is common.[14] The mechanisms of resistance are not understood in complete detail and may be multi-factorial. The proposal that GSCs are a prerequisite for tumor formation suggests that chemo- and radio-resistant GSCs are the main cause of recurrence.[15,16] Liu *et al*.[17] found that $CD133^+$ cells were resistant to chemotherapeutic agents, whereas $CD133^-$ cells sorted from the same primary glioma cultures were not. Furthermore, Bao *et al*.[7] determined that ionizing radiation treatment enriched the $CD133^+$ population in human gliomas. Although CD133 does not identify all GSCs, these data suggest that GSCs play a key role in resistance to traditional therapies. The mechanism for resistance is complicated. Bao *et al*.[7] demonstrated that $CD133^+$ cells contribute to glioma radio-resistance via preferential activation of DNA damage checkpoints, and that their resistance could be partially reversed by inhibitors of Chk1 and Chk2. Other researchers demonstrated that the bone morphogenetic proteins and cannabinoids inhibit the tumorigenic potential of GSCs and promote their differentiation.[18] Radiation treatment may expand the GSC population, enhance the aggressiveness of tumors, and induce expression of GSC marker proteins, such as CD133 and nestin, as well as proteins involved in self-renewal, such as Notch2 and Sox2.[19] The GSC niche may enhance the radio-resistance of GSCs as well.[20] Besides their relative radio-resistance, GSCs can express high levels of multiple genes associated with drug resistance[21] and show significant resistance to chemotherapeutic agents.[17] Temozolomide (TMZ) treatment, which could eliminate O6-methylguanine-DNA methyltransferase-negative cells, increased the stem population.[22] Increased expression of drug transporters, such as ATP-binding cassette (ABC) transporters, could lead to chemotherapeutic agents being pumped out of tumor cells and may be another mechanism of chemo-resistance. Recent studies have found that the expression of ABC transporters is increased in stem cells.[23] Therefore, focusing on the connection between GSCs and their niche may help to elucidate the mechanisms behind the treatment-resistant phenotype of GSCs, which may lead to solutions that reduce the resistance of gliomas to therapy and improve clinical outcome. This information may also be useful in the pursuit of effective therapeutic strategies for the treatment of radiation-associated injuries.

Cell sorting and culture

Several methods may be used in order to obtain purified GSCs for study. In some studies, fluorescence-activated cell sorting and magnetic activated cell sorting have been used to separate GSCs from other cell types.[12,24] Glioma cell lines and clinical samples have also been used to isolate and culture GSCs.[25,26] However, there is currently no standard definition of what constitutes a GSC. Specific markers for identifying GSCs are, therefore, required. The current definition of GSCs is based on their capacity for self-renewal, long-term proliferation, and tumor formation *in vivo*. The ideal marker, therefore, is a molecule that is specifically expressed on GSCs and functionally associated with GSC maintenance.

CD133 has been widely used as a marker for GSC sorting. However, NSCs also express CD133, which limits its utility and reliability as a target. Some studies have also suggested that $CD133^-$ cells have the capability to act as GSCs.[13] Moreover, $CD133^-$ glioma cells can give rise to $CD133^+$ GSCs.[13] Other surface molecules such as CD15 (SSEA-1),[27] A2B5,[28,29] and L1CAM[30] have been used as markers but are not widely accepted. Interestingly, both $CD133^+$ $A2B5^-$ and $CD133^-$ $A2B5^+$ cells have been shown to exhibit characteristics of GSCs.[31] Therefore, these markers may only label specific sub-populations of GSCs. In recent times, one group exploited the intrinsic auto-fluorescence properties and distinctive morphology of a subpopulation of cells ($FL1^+$) in order to isolate them from human gliomas. $FL1^+$ cells are capable of self-renewal *in vitro*, tumorigenesis *in vivo*, and preferentially express stem cell genes, but expression of FL1 did not correlate with the expression of other proposed GSC markers.[32] This finding deserves special attention as it may provide a new way to identify GSCs.

HEDGEHOG IN GLIOMA

Hedgehog signaling pathway

Hedgehog is a highly conserved signaling pathway and a key regulator of embryonic development, including the processes of cell differentiation, proliferation, and tissue patterning.[33,34] In adults, Hh plays an important role in the maintenance of stem cells, tissue repair, and regeneration. The Hh family consists of three secreted proteins, sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and desert Hedgehog. Two molecules that are important for Hh signaling are patched (Ptch) and smoothened (Smo). In the absence of Hh, Ptch inhibits the activity of Smo, a receptor-like protein with seven transmembrane domains. In the presence of activated Smo, a complex consisting of the glioma-associated oncogene family zinc finger (Gli) and the suppressor of fused (Sufu), an important negative regulator of Hh signaling, enters the nucleus, leading to nuclear translocation and activation of the Gli1 and Gli2 transcription factors, as well as degradation of Gli3. Activated Gli subsequently promotes the transcription of Hh target genes [Figure 1]. Three types of Gli transcription factors, Gli1, Gli2, and Gli3, have been identified in mammals. Gli1 and Gli2 are activators of Hh target genes, while Gli3 mainly appears to be a repressor. The function of Hh signaling is very complicated and critical. Thus, it is important to elucidate the function and molecular regulation of Hh signaling, especially in GSCs.

Functional studies of Hedgehog in glioma stem cells

Aberrant activation of Hh signaling has been shown to be associated with the formation of gliomas. Several studies have investigated the role of Hh-Gli signaling in GSCs and found that it regulates self-renewal and tumorigenic potential in GSCs.[22,35-37] Importantly, inhibition of Hh-Gli signaling enhances the ability of TMZ to inhibit GSC proliferation and induce cell death.[38] Several studies have demonstrated that inhibition of Hh signaling blocks tumor growth and influences both proliferation and malignancy.[39] The

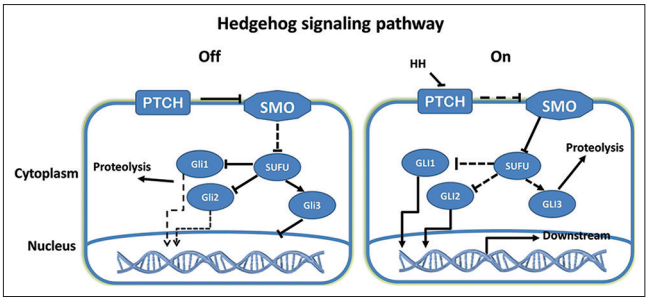

Figure 1: The off and on states of the Hedgehog (Hh) signaling pathway. Patched (Ptch) inhibits the activity of smoothened, which prevents the suppressor of fused-Gli1/2 complex from entering the nucleus and promotes Gli3 nuclear accumulation, leading to low expression of Hh target genes. Hh binding to Ptch activates the Hh signaling pathway by promoting Gli1/2 expression

Shh pathway plays an important role in the migratory ability of cells derived from $CD133^-$ glioblastoma cells.[35] Furthermore, the Hh inhibitor cyclopamine has been shown to improve the effect of radiation on GSCs. All of the studies mentioned above suggest that Hh signaling is one of the critical pathways for the maintenance, proliferation, migration, and tumorigenic potential of GSCs. Thus, targeting this pathway with pharmacologic inhibitors may inhibit GSC growth and improve the efficacy of conventional therapies.

The regulatory role of microRNA

MicroRNAs (miRNAs) are a class of small noncoding cellular RNAs that bind to cis-regulatory elements located primarily in the 3' untranslated regions of target mRNAs, resulting in their translational inhibition or degradation. The function of some miRNAs has been determined to be important for neural development.[40] Other studies have indicated that miRNAs play a potentially important role in glioma biology. The relationship between the Hh pathway and miRNA is currently being investigated.

It has been shown that stable miR-302-367 cluster expression is sufficient to suppress the stem cell-like signature, self-renewal, and infiltration of cells by inhibition of the CXCR4 pathway. Furthermore, inhibition of CXCR4 leads to disruption of the Shh-Gli-Nanog network, which is involved in self-renewal and expression of the embryonic stem cell-like signature.[41,42] Wu *et al*.[43] suggested that miR-5 can specifically suppress Hh signaling by directly targeting Smo in Drosophila. In addition, miR-125b and miR-326 have been identified as suppressors of Smo, and miR-324-5p targets the downstream transcription factor Gli1. Down-regulation of these miRNAs allows high levels of Hh-dependent gene expression leading to tumor cell proliferation.[44] Furthermore, functional analyses have shown that miR-326 may be regulated by Shh activation and act as a negative modulator of Shh signaling by directly targeting Smo and Gli2.[45] Other studies have demonstrated that miR-214 can inhibit Sufu, allowing maximal activation of Gli-mediated transcription,[46] and miR-941 targets key components of the Hh-signaling pathway, including Smo, Sufu, and Gli1.[47] Moreover, miR-212 was found to be involved in tumorigenesis, and the oncogenic activity of miR-212 was partly due to suppression of Ptch1.[48] Although many miRNAs have been shown to regulate the Hh pathway as upstream factors, the Hh pathway in turn has been shown to regulate the mir-29b-1/mir-29a promoter [Table 1 and Figure 2].[49]

The studies mentioned above show that miRNAs affect the expression of numerous genes involved in glioma

Table 1: MicroRNAs and their targets in Hh signaling pathway	
MicroRNA	**Target**
miR-302~367	CXCR4 Shh-Gli-Nanog
miR-125b	Smo
miR-326	Smo, Gli2
miR-324-5p	Smo, Gli1
miR-214	Sufu
miR-941	Smo, Sufu, Gli1
miR-212	Ptch1

Hh: Hedgehog; Shh: sonic Hedgehog; Ptch: patched; Smo: smoothened; Sufu: suppressor of fused

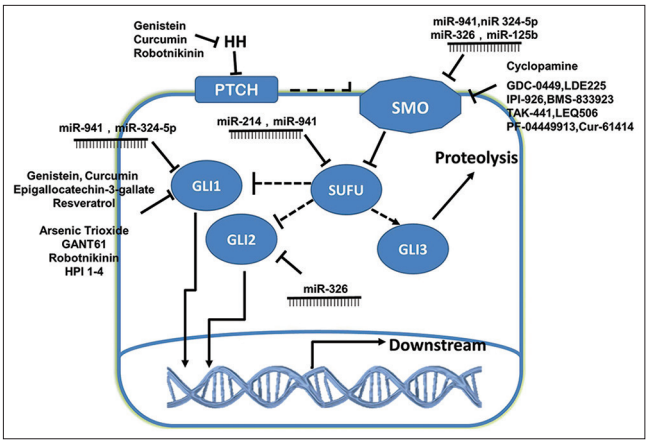

Figure 2: Inhibition of the Hedgehog signaling pathway. A selection of currently known inhibitors, including microRNAs and small molecular inhibitors (both natural and synthetic) are shown, along with their corresponding targets

biology. Identifying the roles that different miRNAs play may help to understand the mechanisms leading to glioma-propagation and provide new therapeutic strategies. In particular, miRNAs that affect the Hh pathway should be investigated in more detail.

Cross-talk with other pathways

Cross-talk between the Hh signaling pathway and other embryonic signaling pathways, such as the Notch and Wnt pathways, has been reported not only in glioma cell lines, but in other cancers as well. Cross-talk between signaling pathways has the potential profoundly to add to the complexity of cellular responses to external stimuli. Wnt signaling directs the development of a variety of organ systems during embryogenesis. In adults, Wnt signaling has a key role in the regulation of tissue self-renewal. Over the past several years, various discoveries have suggested that there are fundamental similarities between the Wnt and Hh signaling pathways.[50] Both pathways are activated by a membrane protein (Frizzled or Smo) and prevent phosphorylation-dependent proteolysis of key effector (β-catenin or cubitus interruptus), which converts a DNA-binding protein from a repressor into an activator of transcription. In addition, silencing of both pathways in the absence of ligand requires Slimb-β-TRCP-FWD-1, which is a component of the SCF ubiquitin ligase complex, and the protein kinases glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). It has been observed that molecules involved in Wnt signaling, such as GSK-3 β, regulate the Hh signaling pathway.[51] In turn, activation of Gli may stimulate the transcription of Wnt ligands. It has been found that GSK-3 β phosphorylates and stabilizes Sufu, leading to inhibition of Hh activation.[52] Moreover, the Hh pathway was found to inhibit Wnt signaling, as a result, of Gli1 induction through up-regulation of secreted frizzled-related protein 1.[53]

Notch signaling is another conserved developmental signaling pathway that is important for embryogenesis, cellular homeostasis and stem cell renewal.[54] Notch receptor activation induces the expression of hairy and enhancer of split 3 (Hes3) and Shh through rapid activation of cytoplasmic signals, including the serine/threonine kinase Akt, the transcription factor STAT3, and the mammalian target of rapamycin (mTOR), leading to the promotion of NSC survival.[55] Simultaneously, Shh induces the expression of another specific target gene, Hes1, and Smo function has been found to be necessary for Shh-induced up-regulation of Hes1.[56] Moreover, inhibition of Shh and Notch may enhance the sensitivity of $CD133^+$ GSCs to TMZ;[38] and the Shh, Notch, and Wnt pathways combined may regulate self-renewal and differentiation of breast CSCs and progenitor cells.[57]

Aberrant activation of epidermal growth factor receptor (EGFR) signaling has been implicated in a number of human malignancies, which has made EGFR a prime molecular target in chemotherapy.[58] Several studies suggest that the combination of specific EGFR and Hh inhibitors may provide a therapeutic benefit. For instance, the combination of the selective EGFR inhibitor gefitinib and the Smo antagonist cyclopamine, with or without the chemotherapeutic drug docetaxel, inhibits cell growth and induces apoptosis.[59] EGFR synergizes with Gli1 and Gli2 to selectively activate transcription of Gli target genes via stimulation of RAS/RAF/MEK/ERK signaling.[60] Moreover, EGF has already been shown to have the capability to stimulate the proliferative activity of Shh on NSCs and to enhance the invasive properties of epidermal cells expressing Shh.[61,62] Further investigation is required, however, in order to understand these interactions in more detail.

Hedgehog signaling may also be involved in cross-talk with other pathways, such as transforming growth factor-β (TGF-β) and AKT signaling. TGF-β has been shown to promote Gli2-mediated expression of parathyroid hormone-related protein.[63] Other studies have indicated that co-activation of the Hh and AKT pathways promote tumorigenesis.[64] The results of these

studies, in addition to those mentioned above, indicate the existence of cross-talk between Hh signaling and several other pathways. It is, therefore, possible that combination therapies targeting both Hh and other pathways may provide additional benefits to patients in comparison with individual treatments.

Hedgehog as a therapeutic target in glioma

In recent times, several inhibitors of Hh signaling have been synthesized or discovered for use in studies of cancer treatment *in vitro* and *in vivo*. By targeting important molecules in the Hh signaling pathway, Hh inhibitors down-regulate the activity of Hh signaling in cancer cells, resulting in the inhibition of cancer cell growth and tumor progression. GDC-0449 (vismodegib) is a small-molecule inhibitor specifically designed to target Smo. In preclinical experiments, GDC-0449 has been shown to inhibit the activation of the Hh pathway, leading to the inhibition of tumor growth initiated by mutations of Ptch or by increased levels of Hh ligands.[65] A search of the clinicaltrials.gov database identified 38 clinical trials of GDC-0449 focused on the treatment of different malignancies. In one trial, for example, GDC-0449 was tested in combination with Avastin (bevacizumab) and traditional chemotherapy in metastatic colorectal cancer. Treatment with GDC-0449 resulted in a reduction of symptoms, and the data suggests that GDC-0449 may be safely used in combination with conventional agents. Unfortunately, mutations in Smo and its downstream targets are common, and may lead to GDC-0449 resistance. However, it has been shown that resistant medulloblastomas are sensitive to PI3K inhibition, which may indicate that combined therapy is necessary.[66]

LDE225, another Hh inhibitor specifically targeting Smo, has also been shown to reduce Hh-dependent proliferation. The main side effects include nausea, vomiting, anorexia, fatigue, muscle cramps, and dysgeusia. During the course of LDE225 treatments, resistance to the drug was observed. Possible mechanisms for this resistance include Gli2 amplification and Smo mutations, leading to reactivation of Hh signaling. Similar to the GDC-0449 study, a combination treatment of LDE225 with PI3K inhibitor delayed the development of resistance.[67] Thus, combined therapy targeting multiple pathways needs more investigation.

IPI-926 (saridegib) is a unique, selective, and potent molecule that inhibits Smo. IPI-926 is orally bioavailable and has demonstrated biological activity in multiple preclinical animal models of cancer. IPI-926 appears to down-regulate Hh signaling, leading to inhibition of the potential for self-renewal. Drug resistance was observed after extended treatment periods, but was primarily caused by increased expression and activity of P-glycoprotein drug transporters rather than the emergence of genetic mutations that prevent drug-target interactions.[68] BMS-833923 is another Hh inhibitor that acts by binding to Smo. Clinical trials have been conducted to evaluate the effects, safety, tolerability, and pharmacokinetics of BMS-833923 alone or in combination with other drugs. Resistance to this drug and the mechanisms behind it still need to be studied, however. In addition, other synthetic Smo antagonists including TAK-441, LEQ506, PF-04449913, and Cur-61414 have already been tested in clinical trials in order to determine dosage levels and evaluate safety [Table 2 and Figure 2].

Currently, most drugs targeted against the Hh pathway function by inhibiting Smo and thus lead to the suppression of tumor proliferation. However, Hh signaling could also be altered by targeting components located downstream of Smo. Accordingly, several groups are attempting to develop agents that target Gli or other molecules in the Hh pathway. For example, GANT61 is an Hh inhibitor targeting Gli1 and Gli2. GANT61 has been shown to effectively down-regulate Gli expression, inhibit cell proliferation and migration, and induce G1 arrest and apoptosis.[69] GANT61 may also decrease cell invasiveness by inhibiting Gli2 in human bladder transitional cell carcinoma.[70] Another potential therapeutic agent is arsenic trioxide (ATO). ATO has been proposed to block the accumulation of Gli2, resulting in reduced protein levels,[71] and to bind directly to Gli1, inhibiting its transcriptional function.[72] Furthermore, four Hh pathway inhibitors (HPIs) have been identified that act downstream of Sufu to modulate Gli processing, activation, and/or trafficking, including small molecule antagonist of ciliogenesis. HPI-1 has been shown to inhibit activation of the Hh pathway induced by overexpression of Gli1. HPI-2, on the other hand, inhibits Hh target gene expression in cells lacking Sufu function or overexpressing Gli2, but is less effective against exogenous Gli1. HPI-3 likely blocks activation

Table 2: Synthetic inhibitors of Hedgehog signaling pathway

Synthetic inhibitors	Target
GDC-0449 (Erivedge, vismodegib)	Smo
LDE225	Smo
IPI-926 (Saridegib)	Smo
BMS-833923	Smo
TAK-441	Smo
LEQ506	Smo
PF-04449913	Smo
Cur-61414	Smo
Arsenic trioxide	Gli
GANT61	Gli
Robotnikinin	Shh
HPI 1-4	Gli

HPI: Hedgehog pathway inhibitor; Shh: sonic Hedgehog; Smo: smoothened

by Gli2 as well, albeit through a different mechanism. HPI-4 appears to act by perturbing ciliogenesis.[73] Another synthetic molecule, named Robotnikinin, specifically binds to extracellular Shh and blocks Shh-signaling in cell lines, human primary keratinocytes and a synthetic model of human skin,[74] which may represent an alternative treatment for tumors resistant to Smo inhibitors.

In addition to synthetic drugs, several natural molecules have been found to target the Hh pathway. Genistein is an isoflavone found in soybeans and most soy-protein products. Both *in vitro* and *vivo* studies have demonstrated that genistein may inhibit Gli1 mRNA expression and down-regulate Gli reporter activity, leading to significant inhibition of prostate cancer cell proliferation.[75] Cyclopamine, derived from Veratrum californicum, has been showed directly to bind to Smo, blocking the Hh pathway and preventing transcription.[76] In addition, cyclopamine reduces neurosphere formation in glioblastoma cell lines.[77] Epigallocatechin-3-gallate (EGCG) is one of the most potent anticarcinogenic compounds known as catechins found in green tea. Studies indicate that EGCG could decrease the expression of Gli1 mRNA and inhibit Gli reporter activity.[75] Another study found that EGCG down-regulates Ihh, Gli1, and Bcl-2 expression, which may inhibit cell proliferation and induce apoptosis.[78] Another natural inhibitor of the Hh pathway is resveratrol, a stilbenoid found in the skin of red grapes and peanuts. Experimental studies have shown that resveratrol suppresses cancer cell proliferation and induces apoptosis partially through the down-regulation of Gli1 mRNA expression and the inhibition of Gli reporter activity.[75] Curcumin (diferuloylmethane), derived from *Curcuma longa*, inhibits the nuclear factor-κB, PI3K/Akt, and activator protein-1 signaling pathways, resulting in antiinflammatory, antioxidant and anticancer effects. Curcumin also regulates Hh signaling by down-regulating Shh and Gli1.[79] In addition, curcumin reduces the protein levels of β-catenin and its downstream targets, c-Myc and cyclin D1, suggesting that curcumin could interrupt the cross-talk between Hh and Wnt signaling.[79] In an advanced model of pancreatic cancer, cyclopamine alone was not sufficient to deplete the number of CSCs. Following treatment with a combination of the conventional chemotherapy agent gemcitabine and the mTOR inhibitor rapamycin, however, CSCs were virtually undetectable both *in vitro* and *in vivo*.[80] Natural molecules targeting a variety of different pathways involved in cancer proliferation have been identified, and may lead to effective therapies for glioma or other malignancies. It is possible, however, that combination therapy may be needed for treatment of gliomas. Therefore, the exact mechanism of each inhibitor should be investigated, and the effects and defects of combined therapy should be evaluated in clinical trials [Table 3 and Figure 2].

Table 3: Hh related natural products

Natural compounds	Target
Cyclopamine	Smo
Genistein	Gli1
Curcumin (diferuloylmethane)	Shh, Gli1
Epigallocatechin-3-gallate	Ihh, Gli1
Resveratrol	Gli1

Hh: Hedgehog; Ihh: indian Hedgehog; Shh: sonic Hedgehog; Smo: smoothened

CONCLUSION

the Hh signaling pathway plays a pivotal role in the process of embryonic development. Aberrant activation of Hh signaling contributes to cancer development, progression, and the processes of cancer invasion and metastasis, leading to the formation of gliomas. GSCs are at the core of glioma biology and play an important role in cancer invasion, metastasis, drug resistance and tumor recurrence. Thus, strategies specifically targeting Hh signaling in GSCs could lead to promising therapies that inhibit tumor initiation and progression. A variety of synthetic molecules and natural products are currently under investigation in both fundamental research studies and clinical trials. Although some benefits have been observed, there are still problems that remain to be solved. In particular, further studies are needed to (1) identify more effective methods of differentiating GSCs from NSCs; (2) evaluate the benefits of combination therapy with HPIs and conventional chemotherapeutic agents; and (3) determine the mechanisms behind nutraceuticals that inhibit the Hh pathway for the prevention of human malignancies *in vitro*, *in vivo*, and in clinical trials.

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Review Article

Pyroptosis and neurological diseases

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ABSTRACT

Pyroptosis is a new process of programmed cell death, which has been discovered and confirmed in recent years. Its cardinal features include activation of caspase-1 and a massive release of inflammatory cytokines (interleukin (IL)-1β, IL-18), *etc*. The morphological characteristics, occurrence and regulatory mechanisms of the pyroptosis greatly, differ from other cell death mechanisms such as apoptosis and necrosis. It has already been proven that pyroptosis participates and plays an important role in a wide range of neuronal diseases. Here, we review the current understanding of the pyroptosis and its roles in neurological diseases.

Key words: Caspase-1, inflammasome, interleukin-1β, interleukin-18, neurological diseases, pyroptosis

INTRODUCTION

Cell death is a critical and inevitable phase common to all cell types. A deeper understanding of cell death in its form and nature is critical to shed new light on the emergence, development and treatment of diseases. Many different types of cell death patterns have been discovered in the last years; among that pyroptosis is one of the most recent. It is now widely accepted that this mechanism contributes to the development of neurological diseases. In this review, we first describe the definition of the pyroptosis and its basic mechanisms and discuss how pyroptosis and its relevant molecules participate in neurological diseases and their progression.

THE HISTORY OF PYROPTOSIS AND ITS CHARACTERISTICS

The understanding of cell death has changed a lot through decades. Nowadays we believe that cell death can be roughly divided into necrosis and programmed cell death, the latter one, including apoptosis, oncosis, autophagy, *etc.*, as well as pyroptosis that will be discussed in this review.

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Pyroptosis was first observed in 1992 when Zychlinsky *et al*. described that *Shigella flexneri* can induce programmed cell death in macrophage, but this process was mediated by a caspase-1, and the iconic molecule in apoptosis, caspase-3, was not apparently involved. This observation suggested that such programmed cell death was different from apoptosis.[1] Subsequent studies confirmed that in *S. flexneri* specific caspase-1 blocker Ac-YVAD-CHO inhibited programmed cell death of macrophages, whereas caspase-1 knockout could protect macrophages from death following *S. flexneri* infection.[1,2] In contrast, caspase-3 specific blockers and caspase-3 knockout macrophages did not show any effects.[3] Then, in 2001, Cookson and Brennan found a type of caspase-1 dependent cell death in *Salmonella* infected macrophages, and for the first time named it "pyroptosis", its meaning deriving from the Greek root pyro (fireworks) and ptosis (to-sis) (death).[4]

In the process of the pyroptosis, activated caspase-1 mediates massive generation of pro-inflammatory cytokines, interleukin (IL)-1β, IL-18,[5] leading to cell morphological changes similar to apoptosis, such as nucleus pycnosis, DNA fragmentation and TUNEL staining positivity, *etc*. However, in contrast to apoptosis, in pyroptotic cell, the integrity of the cell membrane is not preserved and micro-pores with diameter about 1-2 nm are formed on it, resulting in potassium efflux, intracellular and extracellular ion imbalance, cell swelling and rupture. Meanwhile, the pro-inflammatory cytokines and cytoplasmic components are released to the extracellular space, causing focal inflammation and cell death.[6]

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Lately, researchers realized that a variety of bacterial and nonbacterial stimuli (e.g. substance related to autoimmune diseases and cardiovascular and cerebrovascular diseases[7-9]) can drive programmed cell deaths similar to pyroptosis. Meanwhile, in addition to macrophages, there are a variety of cells (such as dendritic cells, *etc.*) undergoing programmed death involving caspase-1 activation, thereby different from caspase-3 mediate apoptosis. Moreover, cells undergoing pyroptosis exhibit a series of cellular changes from complete necrosis to complete the apoptosis.[10-12]

MECHANISMS OF PYROPTOSIS

Pyroptosis and caspase-1 are closely associated. Caspase family is a group of proteases with high homology, and it can be divided into two categories according to its relationship with apoptosis and pyroptosis. One includes apoptosis-related proteases, for instance caspase-3, the executor of apoptosis, and also caspase-2, 6, 7, 8, 9, 10 *etc.* Caspase-3 is activated by co-action of caspase-2, 6, 7, 8, 9, 10 *etc.*, and activated caspase-3 could induce DNA dissolution, proteolysis, and downstream events leading to apoptosis. The second is inflammation-associated proteases, including caspase-1 and caspase-4, 5, 11, 12, 13, 14 *etc.*, taking part in cytokines-mediated inflammatory response.[13] Caspase-1 is not involved in apoptosis, but represents the key factor in pyrotosis. Caspase-1 is an IL-1β converting enzyme. Pro-caspase-1 does not have biological activity when produced. Its molecular weight is 45 kDa, constituted by the three domains, including the caspase activation and recruitment domains (CARD) structure in NH_2-terminal, a large subunit about 20 kDa, and a small subunit about 10 kDa. Then, pro-caspase-1 is converted into heterodimer in the cytoplasm, and further assembled into biologically active tetrameric caspase-1.[13] This activation process is regulated by a multi-protein complex in the cytoplasm named inflammasome.[14]

Inflammasome is a multi-protein complex composed by NOD-like receptors (NLRs), proteins containing NATCH, leucine-rich repeat and PYD domains (NLRP1 NLRC4 NLRP3 NAIP5 and NLRC5), or absent in melanoma 2 (AIM2), or Caspase-1 *etc.* Some of the inflammasomes also contain apoptosis-associated speck-like proteins containing CARD (ASC). Recent studies have confirmed that retinoic acid-inducible gene I (RIG-I), one of the receptors of some RNA viruses, can form inflammasome with ASC, without the participation of NLRs.[15] Under the regulation of inflammasome, pro-caspase-1 is activated, promoting the processing and maturation of pro-inflammatory factors such as IL-1 and IL-18.

NOD-like receptors are one of the pattern recognition receptors (PRR); they can be assembled into inflammasome under the stimulation of pathogens or other dangerous signals. According to different NLRs, the inflammasome can be classified into four types, NLRP-1, 3, 4 Ice Protease-Activating Factor (IPAF), 5. During pathogen stimulation, effector domains of NLRs are exposed to activating caspase-1 through CARD-CARD and PYD-PYD interactions or with the help of ASC directly. Different types of NLRs respond to different stimuli. NALP3 is sensible to perforin, extracellular adenosine triphosphate (ATP), urate crystals, DNA and RNA in virus and ultraviolet. IPAF is sensible to extracellular pathogens, such as Pseudomonas, and intracellular pathogens such as *Salmonella, Listeria, Shigella, Legionella bacteria*. *Legionella* also needs the help of NALP5-5 to activate caspase-1.[16,17] AIM plays an important role in viral infections; its function is to identify DNA cytoplasm. It is a cytoplasmic DNA transducer, one of PRRs sensible to extrinsic DNA. It belongs to HIN-200 family, with a PYD domain in amino-terminal and an HIN-200 domain in carboxy-terminal.[18] In virus-infected cells, AIM2 and caspase-1 can form inflammasome to induce innate immunity and resist intracellular bacteria and DNA viruses.[19] RIG-I also binds to the adaptor ASC to trigger caspase-1-dependent inflammasome activation by a mechanism independent from CARD and NLRP3 in RNA infection.[15] The effects of ASC are to combine caspase-1 and NLRP1, NLRP3, AIM2, RIG-I together. The mechanism is mediated by the PYD domain in the carboxyl terminus of ASC combined with PYD domain in NLRP1 NLRP3 and AIM, with the CARD domain in N-terminal of ASC combined with pro-caspase-1's CARD domain. In addition, ASC can be assembled into ASC dimer without the participation of NLRs, and ASC dimer can activate caspase-1 directly. This ASC dimer has been named Pyroptosome recently.[20]

The activators of the inflammasomes can be divided into two categories: pathogen associated molecular patterns activate a host-defense reaction, and damage associated molecular patterns activate a self-defense mechanism in response to danger signals.[21] Activators include bacteria, virus, fungus, protozoa, microbial proteins, crystalline urea, RNA, Alum, ATP, potassium efflux, fatty acids, Aβ, and most recently, degraded mitochondrial DNA.[22-24] Overall the assembly and activation of inflammasomes are cell-type and stimulus-specific.[25,26]

With inflammasome, pro-caspase-1 is activated to caspase-1. Its function includes conversion of the pro-IL-1β and pro-IL-18 into active IL-1β and IL-18. When bound to their receptors, IL-1R and IL-18R, they lead to nuclear factor-κB dependent gene transcription.[27,28] IL-1β is a key molecule in inflammasome initiation and IL-18 can

regulate the function of interferon-γ in T-cell and natural killer cell.[27,28] Finally, they can recruit and activate other immune cells and induce the synthesis of other inflammatory cytokines, chemokines, and adhesion molecules, expanding local inflammation response.[13] Moreover, cell membrane integrity is destroyed by micro-pores formation on it, which is caused by caspase-1, IL-1β and IL-18. These micro-pores lead to a series of pyroptoic processes such as cytoplasm release, cell osmotic lysis and inflammatory reaction.[13,29] In addition, during the process of the pyroptosis, caspase-1 is involved in chromosomes and DNA degradation. A specific endonuclease is activated by caspase-1. Once activated, this endonuclease can mediate degradation of DNA, which differs from the DNA degradation occurring in apoptosis.[29] More experiments have confirmed that the degradation of cytoskeletal proteins is also associated with pyroptosis and that this process is related with treatment and processing of substrates by caspase-1.[30]

PYROPTOSIS AND NEUROLOGICAL DISEASES

Pyroptosis is closely related to neurological diseases. Pyroptosis and its relative mechanisms participate in acute and chronic aseptic inflammation in the nervous system. Our immune system could recognize disease-associated molecules through PRR. In the central nervous system (CNS), PRR are expressed mainly on microglial, macrophages and astrocytes. They are distributed on the surface of membranes to recognize extracellular signals (i.e. toll like receptors), or in the cytoplasm to transmit intracellular signal (i.e. NLR receptor).

There are several NLRP1 and NLRP3 inflammasomes expressed in the nervous system.[31] Mouse microglial cells could express NLRP3 and NLRP4 inflammasome, and they can respond to stimulation of dangerous signals.[32-35] Additional evidences indicate that inflammasomes can be expressed in nonmyeloid cells of the nervous system. Meanwhile, many studies have proven that caspase-1, IL-1β and IL-18 could be activated and NLRs inflammasomes can be assembled in neurons under stress conditions.[36-40] In addition, recent studies have also shown that NLRP2 inflammasomes can be expressed in astrocytes.[41,42] In the CNS, microglia, astrocytes and neurons can all undergo pyroptosis and express its related downstream molecules and receptors, thus taking part in the immune reaction to local inflammation.[27,28,43] In fact, in diseases such as viral encephalitis, stroke, Alzheimer's disease (AD) and multiple scleroses (MS), many studies have shown massive expression of IL-1β and IL-18 *etc.*, in the nervous system.[39,44-46] However, further investigation is required to elucidate mechanisms.

Pryoptosis and infection diseases in the nervous system

Pyroptosis and its related molecules may participate in the development of nervous system encephalitis and meningitis. These phenomena have a different prognosis in bacterial and virus infection. For example, in *Streptococcus pneumoniae* meningitis participation of NLRP3 inflammasome aggravate the damage caused by the disease. IL-1β and IL-18 are not involved in growth inhibition of bacteria, but contribute to exacerbate the inflammatory response in the nervous system.[47-49] Some studies indicate that mouse microglia and peripheral macrophages infected with *Staphylococcus aureus*, *Mycobacterium tuberculosis* and *Legionella pneumophila in vitro* may activate the NLRP3 or NLRP4 inflammasome thus inducing pyroptosis.[32,50,51]

However, in viral encephalitis caused by West Nile virus (WNV), influenza A virus, and herpes simplex virus, IL-1β and IL-18 can increase survival rate of neurons by inhibiting viremia.[39,52-54] In WNV encephalitis, it was observed that the production and release of IL-1β increased in neurons, and IL-1β inhibited the replication of WNV. The survival rate decreased in NLRP3 and ASC knockout mice infected by WNV. ASC knockout mice can experience excessive immune response after WNV infection, and this will contribute to neuronal damage.[39] Japanese encephalitis virus can activate NLRP3 inflammasome in microglia, promote the release of IL-1β and IL-18.[34] In CMV retinitis, it was also observed microglia death through pyroptosis pathway.[55] But their influence on prognosis is not yet clear.

Pyroptosis and acute aseptic disease in the nervous system

In acute aseptic nervous system damage (such as stroke or traumatic brain injury), local autoimmune activation can cause nerve injury. Studies have demonstrated that mice with caspase-1 defection may have a certain resistance to stroke, which indicated that pyroptosis and its relative mechanisms exacerbate brain damage in stroke.[56] IL-18 knockout mice didn't show any kind of protective effects in stroke. In contrast, some IL-1 receptor antibodies could still have a protective effect(s) to neurons, even after the occurrence of stroke. This suggests that the protective effect is not only dependent on IL-1β, but also IL-1α. IL-1β and IL-1α defected mice have a better resistance to stroke.[57] Although IL-1α and caspase-1 do not have a direct relationship, caspase-1 may have an indirect protective effect (s) by influencing IL-1R2 and caspase-1 dependent nonclassical secretion system.[58,59] Meanwhile, inflammasome also been observed in a study of excitotoxic injury in kainate model.[38]

Similarly, in the rodent model, antibodies for ASC or NLRP1 can reduce injury of brain trauma or stroke.[60,61]

A study demonstrated that MCAO could induce NLRP1 and NLRP5 inflammasome expression in rat neurons.[62] Traumatic brain injury patients with higher NLRP1 level in cerebrospinal fluid may have a worse prognosis.[63]

Pyroptosis and chronic aseptic disease in the nervous system

Chronic aseptic diseases have a great influence on the structure and function of CNS. MS is a typical one. In MS, T cells and macrophages move into CNS. A study of NLRP3 and ASC knockout mice found that autoimmune encephalitis depends on the NLRP3 inflammasome.[64,65] Inhibition of NLRP3 expression and subsequent reduction of IL-1β and IL-18 secretion can restrain the activation of T cell and its migration into CNS, so as to mitigate the autoimmune encephalitis.[64-66]

In cuprizone-induced CNS autoimmune inflammation and demyelination model, IL-1β and IL-18 play a different role in demyelination. IL-1β knockout mice have a similar MS phenotype to wild-type animals, but the process of remyelination is delayed. This suggests that IL-1β may promote recovery from MS.[67] In contrast, in IL-18 knockout mice, the disease is reduced, and the speed of myelination is faster.[68] In NLRP3 knockout mice, the onset is delayed in cuprizone induced demyelination, but the extent of remyelination is identical to those of wild-type.[68] Therefore, the pyroptosis and its relative mechanisms are involved in the pathological process, and IL-1β and IL-18 have opposite effects on the recovery of the disease.

Besides, accumulating evidences suggest that the immune system participates in the process of amyotrophic lateral sclerosis (ALS), AD, Parkinson's disease and Huntington's disease.[69] Amyloid beta is the main components of senile plaques in AD, it is also one of the first molecules found to be involved in the relationship between chronic aseptic diseases and inflammasome.[33] LPS sensitized macrophages exposed to fibrillar amyloid-beta activate caspase-1 and induced the release of IL-1β. This process is dependent on NLRP3, endosomal rupture and cathepsin B release.[33] A similar phenomenon was found in α-synuclein in Parkinson's disease and prion protein.[70,71] However, to elucidate the function of IL-1β, different studies have reached different conclusions. Some indicate that in Il-1α knockout mice, injecting human amyloid beta into encephalocoele would activate microglia, so as to reduce neuron survival rate.[72] However, other experiments show that over-expression of IL-1β in hippocampus could reduce senile plaque formation by recruiting macrophage.[73]

In ALS, mutation of superoxide dismutase 1 (SOD1) leading to accumulation of toxic protein is one of the main pathogenic factors. Mutant SOD1 in cultured microglia activates caspase-1 and the amount of subsequent IL-1β is proportional to the concentration of mutant SOD1 added. In this process, the activation of inflammasome requires endosomal rupture and participation of ASC. However, it is not clear which specific inflammasome is involved. Caspase-1 or IL-1β defect would improve the survival rate of mice expressing toxic SOD1, which indicates that pyroptosis and its relative mechanisms could exacerbate ASL.[74]

CONCLUSION

Recent findings of the pyroptosis and inflammasome have provided insight into a new mechanism that may contribute to neuronal and glial cell death during neurological diseases. Multiple potential targets upstream and downstream of pyroptosis signaling and targeting its expression, assembly, activity and products, may pave the way for newly therapeutic drugs that may rescue inflammation in neurological diseases. However, it is important to note that although some aspects of the inflammatory response will not only exacerbate brain injury, it is also likely that other components will provide a beneficial contribution to brain recovery. Elucidating the role of these components will represent a challenge for future research. Unquestionably, still a lot needs to be done to clarify the role of the inflammasome during the recovery phase following neurological diseases.

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Review Article

Chronic inflammation drives glioma growth: cellular and molecular factors responsible for an immunosuppressive microenvironment

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ABSTRACT

This review examines glioma disease initiation, promotion, and progression with a focus on the cell types present within the tumor mass and the molecules responsible for the immunosuppressive microenvironment that are present at each step of the disease. The cell types and molecules present also correlate with the grade of malignancy. An overall "type 2" chronic inflammatory microenvironment develops that facilitates glioma promotion and contributes to the neo-vascularization characteristic of gliomas. An immunosuppressive microenvironment shields the tumor mass from clearance by the patient's own immune system. Here, we provide suggestions to deal with a chronically-inflamed tumor microenvironment and provide recommendations to help optimize adjuvant immune- and gene therapies currently offered to glioma patients.

Key words: Astrocytoma, glioma, immunotherapy, inflammation, microenvironment

INTRODUCTION

The World Health Organization (WHO) grade IV glioma is called glioblastoma, and is formerly termed glioblastoma multiforme (GBM) because its appearance can take on a variety of morphologic forms. GBM is the most common and lethal of all primary malignant brain tumors, and is responsible for over 13,000 deaths per year in the United States.[1] The median survival is approximately 15 months.[2] The first line of treatment for this disease is usually surgical resection followed by concurrent chemo-radiotherapy. Importantly, the invasive tumor cells that remain after surgery and survive these aggressive treatments are likely responsible for tumor recurrence. Thus, alternative novel therapies that enhance or work in conjunction with conventional treatments are being actively pursued. While immunotherapies seemingly provide viable, theoretically effective options, in practice they have produced mixed results. The glioma microenvironment is highly immunosuppressive, thereby inhibiting the efficacy of immune treatments. Microenvironmental factors allow glioma cell evasion from the immune system. The source of the factors is not solely derived from the tumor mass, but rather is also a consequence of chronic inflammation present in the tumor microenvironment.

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GLIOMAS AND CHRONIC INFLAMMATION

Chronic inflammation can influence a wide range of ailments including heart disease, stroke, Crohn's disease, rheumatoid arthritis, multiple sclerosis, asthma, Alzheimer's, depression, fatigue, neuropathic pain, and - relevant to our discussion - cancer.[3-13] Indeed, it is thought that around 15% of all cancer-related deaths are in some form linked with inflammation as a result of bacterial or viral infections.[14] Further, chronic inflammation occurring within the microenvironment of tumor lesions is now thought to either drive the first malignant-conferring genetic mutations and/or induce them as a result of oncogene expression.[14]

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GBM TYPES AND STAGES IN DISEASE PROGRESSION

GBM is classified as one of two types: primary or secondary GBM. Primary GBM arises as the *de novo* high grade disease that has no discernible stages of progression. Secondary GBM, on the other hand, arises as low grade and over time progresses to a higher-grade of malignancy. Therefore it is extremely difficult - if not impossible - to analyze the changes that arise in a step-wise manner for primary GBM, while the progression of secondary GBM can be closely followed. However, recent genomic analysis of primary resected GBM tissue has allowed for a second dimension of their grouping by gene expression/mutations patterns: neural, pro-neural, classical, and mesenchymal.[15]

Contemporary theory links the phenotypic characteristics observed in the tumor microenvironment to each of three stages of glioma pathology: initiation, promotion and progression.[16] A defect in the switch for wound repair may play a major role in glioma development because it leads to "type 2" chronic inflammation that fails to shut off and this may drive gliomagenesis. Further, "type 2" chronic inflammation, which is propagated indefinitely in the tumor microenvironment, may be critical in triggering tumor initiation. The effect of chronic inflammation that develops in the tumor microenvironment is far reaching beyond the initiating effects, and it may also drive the second stage of disease, glioma promotion. The third and last stage of the disease, glioma progression, is a stage of the disease that loops back adding to the intensity of the underlying inflammation.

The initiating event

Embracing the cell of origin model, the theoretical consideration of the inflammatory-initiating event beginning with a specific mutation in the cancer stem cell may occur either through oncogene over-expression/stimulation or production of an inflammatory onco-metabolite. A traumatic event at a specific site in the brain may activate all the necessary signal transduction pathways to initiate inflammation. The site of injury could be a source of micro-damage induced by chronic stress, depression, or some other factor extrinsic to the host.[17]

As another consideration, the source of the micro-damage and neural-degeneration may be the cancer stem cell itself. Recently, a novel genetic mutation encoding the isocitrate dehydrogenase 1 (IDH1) protein known to be present in a large number of low-grade to secondary GBM tumors has been identified as one of the possible first events in disease initiation.[18] Subsequently, following the identification of this mutated oncogene, its function was revealed. Cells encoding the mutant IDH1 protein were found to convert, in an irreversible reaction, α-ketoglutarate to 2-hydroxyglutarate (2-HG).[19] The function of the accumulation of 2-HG and its role in disease progression is not yet fully elucidated. The fundamental role of 2-HG has been difficult to assign partly because its role is unlike conventional onco-metabolites characterized thus far. Rather than promoting disease progression by conferring itself with a proliferative or selective advantage, 2-HG initiates development of the cancer stem cell niche within the frontal lobe. Thus, the onco-metabolite 2-HG is the first example demonstrating that although the derivation of cancer is mono-cellular in nature at initiation, much like tumor promotion and progression, complicated heterogeneous interactions involving multiple cell types occurs. The cellular interplay also offers an explanation to the challenges associated with establishing a tumor model containing solely the IDH1 mutation both *in vivo* and *in vitro*.[20] This cancer stem cell niche may in turn provide the mutant IDH1 stem-like cell with the necessary factors to promote self-renewal, further genetic mutations, and ultimately disease progression. Indeed, previous studies in patients suffering from the genetic disorder known as D-2-hydroxyglutaric aciduria, where the accumulation of 2-HG is observed, show ROS-mediated neural excito-toxicity upon NMDA receptor chronic potentiation by 2-HG.[21,22] The neural excito-toxicity fueled by the $IDH1^{mut}$ stem-like cell may initiate a neural-inflammatory cycle of wound repair ultimately leading to pro-creation of a cancer stem cell niche that promotes glioma formation and immune evasion. This niche provides the cancer stem cell with growth factors to sustain proliferation and an environment that promotes the emergence of more genetic mutations. Indeed, a connection between excitotoxicity and inflammation has been proposed to be linked by interleukin-1β.[23]

Tumor promotion

Inflammation is the first line of defense in response to tissue injury and/or infection. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, and IL-6 are synthesized to initiate the inflammatory cascade.[24] IL-1 has been shown to be a key mediator in the proliferation of "reactive astrocytes".[25] Next, either of two types of inflammatory processes may be activated depending on the stimulus. In the presence of microbial infection or necrotic cell death classical "type 1" inflammation ensues, characterized by the appearance of activated T helper (Th) 1 lymphocytes.[26] In the presence of parasites, allergens, or phosphatidylserine positive early apoptotic cells the Th2 inflammatory cascade is activated. Interestingly, Th2 inflammation is closely related to wound repair. Indeed, the principal mode of

action against helminth infection is the "walling off" of large bodies through granuloma formation which bears resemblance to the glial scar encountered in central nervous system (CNS) repair.[27]

The initiation of the classical inflammatory response is marked by the localization and subsequent activation of blood circulating monocytes into M1 macrophage. The M1 macrophage are activated by cytokines produced by Th1 cells, like interferon-γ (IFN-γ), TNF-α, or after recognition of pathogen-associated molecular pattern molecules, through toll-like receptors (TLRs) or C-type lectin receptors. Upon activation, the M1 macrophage promote a proinflammatory environment by releasing cytokines such as TNF-α, IL-1, IL-6, IL-12, IFN-γ, and IL-23. IL-12 stimulates IFN-γ production in T lymphocytes and natural killer (NK) cells.[28] Phenotypically, the M1 phenotype is associated with cell mediated cytotoxicity, tissue injury and destruction. Thus, the presence of the M1 macrophage is counter-productive once the invading threat is neutralized and tissue repair is in order. The resolution of the inflammatory response and transition into wound repair is facilitated by the M2 macrophage. One of the key events leading to immunosuppression and activation of "type 2" inflammation is apoptotic cell death of recruited neutrophils.[26] The apoptotic neutrophils signal to close classical inflammation and thus modulate immunosuppression after their engulfment by macrophages. In response, the macrophage upregulate expression of the Th2 anti-inflammatory cytokine IL-10, while significantly downregulating the pro-inflammatory cytokines TNF-α, IL-1β, and IL-12.[29]

Several subtypes of the M2 macrophage exist depending on the inflammatory program that is activated and required.[30] The M2a or alternative macrophage is activated by the cytokines IL-4 and IL-13, and these macrophages are specialized to carry out the allergic response and the killing and encapsulation of parasites. The M2b macrophage is activated by ligation of TLRs + immune complexes and the IL-1 receptor. This macrophage subset is primarily responsible for immune regulation and activation of the Th2 program. The M2c macrophage, activated by the cytokine IL-10, is primarily responsible for matrix deposition and tissue remodeling. Recently, a fourth and distinct subtype, termed the M2d subset, has been identified. This subset is activated by IL-6 and is thought to aid in tumor metastasis and progression.[31] The primary cells responsible for the synthesis of those cytokines are eosinophils, basophils, and $CD4^+$ Th2 cells, and tumor cells.[32,33] M2 macrophage down-modulate the release of IL-1, IFN-γ, IL-12, and TNF-α.[34,35]

Also, the recruitment and/or activation of T regulatory lymphocytes are thought to play a key mediating role in the "type 2" inflammatory process.[36-38] Indeed, regulatory T cells have been found not only to be present in the peripheral circulation of glioma patients in increased percentages compared to controls, but also to infiltrate glioma tissue in a tumor grade-dependent manner.[39,40] Interestingly, some encouraging anti-tumor responses have been obtained in attempts to neutralize the substantial peripheral regulatory T cell populations encountered in glioma patients with systemic administration of TLR ligands.[41,42] For example, systemic administration of a TLR9 ligand enhanced survival, decreased the number of peripheral regulatory T cells and enhanced the antigen-presenting capacity of infiltrating microglia.

Recently, there have been many studies documenting a decreased risk of glioma development in individuals with asthma, which is also thought to be driven by "type 2" inflammation.[43,44] Reduced immunoglobulin E levels have been found in patients who developed glioma. Further, additional studies have found that specific polymorphisms in genes encoding IL-4RA and IL-13, both factors that induce IgE production in immune cells, are found to be inversely correlated with glioma development. This apparent contradiction can be reconciled by considering the macrophage subtype that predominates in each pathology. The M2a macrophage are induced by IL-4 and IL-13, express Fc-epsilon receptors, and are involved in the allergic response. On the other hand, the M2b macrophage are induced by engagement of the IL-1 receptor and/or ligation of TLR +/- immune complexes, they express Fc-gamma receptors and are involved in immune regulation.[45,46] It appears that patients developing asthma, as a result of hyperactive IL-13 or IL-4 receptor signaling, are at lower risk of developing gliomas; this may be due to the preferential activation of the M2a subset, which may not be as advantageous to the developing glioma mass that is dominantly populated by the M2b-d macrophage subtypes. IL-10, damage associated molecular pattern molecules, and IL-6 are highly expressed in GBM tissue, where they localize to the macrophage/microglia population.[31,47-51] Further, it has been shown that the presence of IL-4 or IL-13 inhibit the proliferation of astrocytes and low-grade astrocytomas, but not GBM.[52]

In glioma tissue, macrophages/microglia can account for up to 30% of the total lymphocytic infiltrate present in the tumor mass.[53,54] It is now accepted that the macrophage and microglia populations found within glioma originate from distinct progenitor cell populations. Infiltrating macrophages are derived from the bone marrow, whereas microglia are brain-resident;

they originate from primitive progenitors in the yolk sac and migrate into the CNS during early embryo development (days 8.5 to 9.5).[55,56] It has also been clearly demonstrated, using parabiosis (a technique that surgically connects the circulatory system of two organisms) and experimental auto-immune encephalomyelitis models, that circulating monocytes do not invade the CNS unless the CNS is preconditioned with irradiation or the blood-brain barrier is compromised/damaged.[57-60] Interestingly, a key distinction between "type 1" and "type 2" inflammation is that the latter activates bone-marrow derived macrophage in the CNS and/or brain resident microglia.[61,62] Taken altogether, microglia are probably recruited to the glioma microenvironment at all stages of malignancy, whereas a majority of the macrophages accumulate only after insult or blood-brain barrier breakdown, when chronic "type 2" inflammation is dominant in the glioma microenvironment.

Convertibility of macrophage from an M1 to an M2 polarized state is driven by factors produced by the local glioma microenvironment. Indeed, secreted or displayed glioma factors are capable of manipulating macrophage and microglial behavior that favor tumor survival and growth. Resting microglia are characterized by a ramified morphology; they display extensive branched projections that aid in continuous surveillance of the CNS microenvironment.[63] Glioma cells secrete key immunomodulatory factors that suppress "type 1" immune activity, such as IL-10, IL-4, IL-6, transforming growth factor (TGF)-β, and prostaglandin E2.[64-66] The cytokines IL-10, IL-4, and IL-6 have been shown to induce an M2 rounded morphology that is typical of activated microglia, whereas the T helper (Th) 3 cytokine, TGF-β, is known to inhibit microglial cell proliferation and the expression of pro-inflammatory cytokines *in vitro*.[67] Due to the dominant effect that glioma cells and their secreted factors have on the surrounding cells, it is likely that glioma-recruited microglia preferentially adopt an M2 phenotype. Studies that delineate the interactions between glioma cells and macrophages/microglia are still warranted.

Inflammation status temporally may play a pivotal role in cancer development. The "type 1" pro-inflammatory process cannot be sustained in the absence of proper stimulation. In brain trauma, "type 1" monocyte recruitment from the blood becomes negligible over time, but in low grade gliomas, constant neuronal damage from continuous 2-HG expression may prevent the Th1 inflammatory process from subsiding. Eventually both "type 1" and "type 2" immune responses are both activated leading to chronic inflammation. The strength of "type 2" *vs.* "type 1" inflammation, which is generally reflected by the serum Th2/Th1 cytokine ratio(s), has been positively correlated with the grade of glioma malignancy.[68] As another example, patients displaying genetic polymorphisms of the IL-1, IL-10, and TNF-α genes are at higher risk for developing gastric cancers.[69,70] Studies with human glioma tissues and patient sera indicate Th1, Th2, and Th3 cytokine deregulation as evidenced by increased Th2 associated cytokines such as IL-10 and the Th3 associated cytokine TGF-β. This increase is offset by a concomitant decrease in Th1 cytokines such as IL-12, IFN-γ, TNF-α, IL-2, and many of their corresponding receptors.[71,72]

Tumor progression and invasion

Cancer cells become "self-sufficient" once they have accumulated the proper genetic mutations to support their own growth. Some of the key findings associated with this stage in disease development include independence from external growth factors, the ability to bypass cell senescence, and dysfunctional apoptotic pathways. In order to develop glioma subtypes, two combinations of genetic mutation may prevail that involve the mutation of the IDH1 gene and p53, resulting in astrocytoma formation, or 1p/19q loss of heterozygosity (LOH) leading to formation of oligoastrocytomas or oligodendrogliomas.[73] Such mutations increase the proliferative rate of cancer stem cells, which allows them to grow outside of their niche. This concept was confirmed in studies using an IDH1 mutant model both *in vitro* and *in vivo*.[74] Tumor samples derived from WHO grade II and III gliomas were successful in retaining the mutation in neurosphere culture. The lower grade gliomas proliferate slowly and are difficult to utilize in standard *in vivo* xenograft models.

The late stages of "type 2" inflammation primarily consist of extracellular matrix deposition, angiogenesis, and tissue remodeling. Once gliomas becomes "self sufficient", these late stage processes are aberrantly used by the proliferating glioma mass to fuel and sustain proliferation. In particular, myeloid derived suppressors cells are now thought to play a large role in facilitating glioma angiogenesis, neo-vascularization, and invasion [Figure 1].[75] Recent studies have shown that the Tie2-expressing monocyte population is pro-angiogenic, expressing relevant gene transcripts [e.g. matrix metalloproteinase 9, vascular endothelial growth factor (VEGF), cyclooxygenase 2, and wingless-type MMTV integration site family, member 5A] necessary for angiogenesis and neo-vascularization.[76,77] Some myeloid derived suppressor cells also seem to contribute to the integrity of neo-endothelium of tumor vessels because they express endothelial markers, such as CD31 and VEGF receptor and can morphologically resemble endothelial cells.[78] Microglia also localize near the invasive border of the glioma mass at three-fold higher

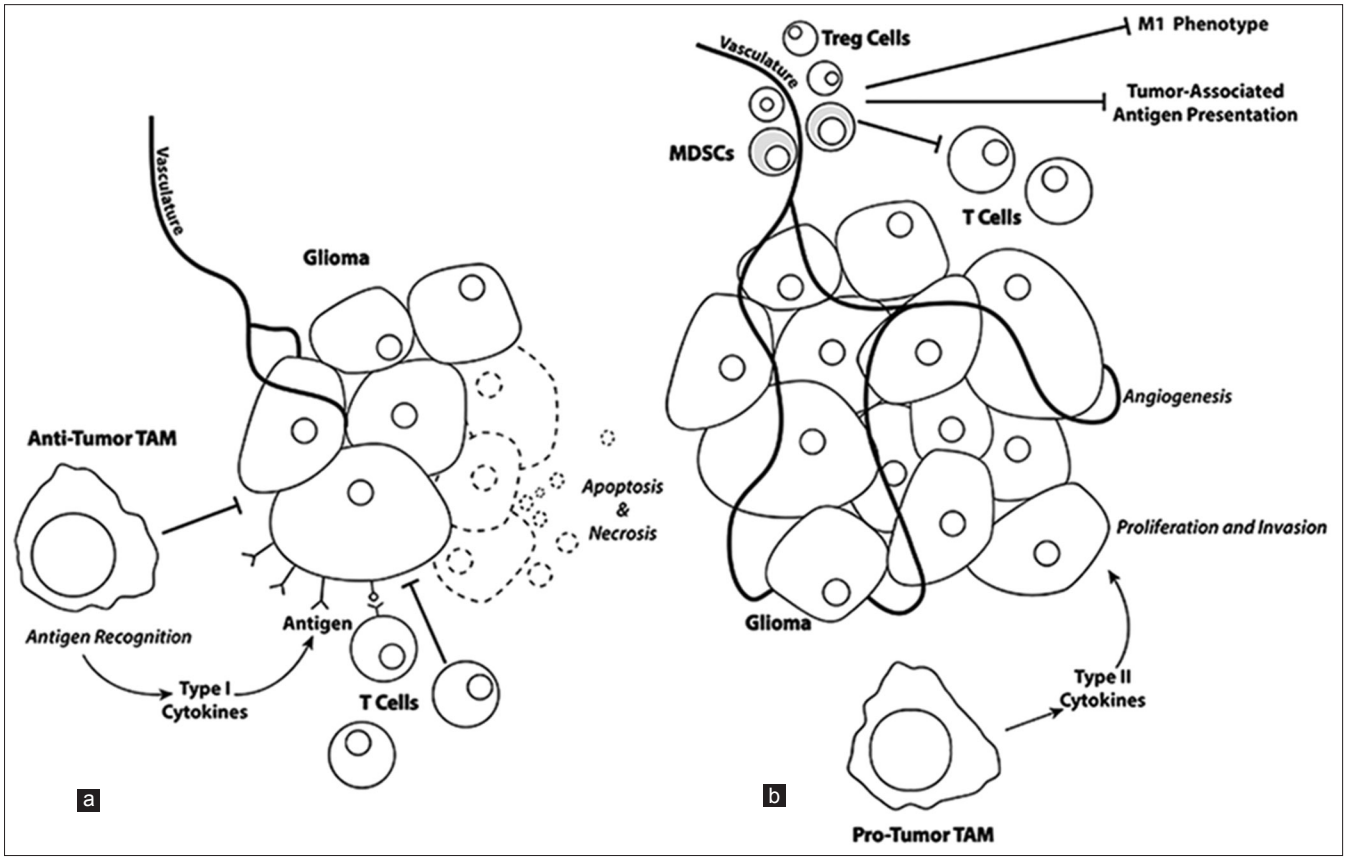

Figure 1: (a) Glioma cell proliferation and invasion is negatively affected when T cells recognize tumor-associated antigens resulting in recognition and tumor cell injury that reduces the tumor mass. (b) Mobilization of T regulatory (Treg) cells and myeloid-derived suppressor cells (MDSCs) to the tumor mass, as well as changes in the phenotypes of tumor-associated macrophages (TAM) result in pro-tumorigenic regulation with increases in tumor cell proliferation, angiogenesis, and invasion

numbers than tumor associated macrophages, suggesting that they might play a key role in glioma invasion.[79]

Recent genetic microarray analyses of glioma patient tumors have revealed variations between glioma subtype progression, invasion, and response to therapy. In patients enrolled in a phase I dendritic cell (DC) vaccine therapy clinical trial, we identified significant trends in the mesenchymal glioma subtype, including its progression and its particular responsiveness to treatment.[80] It may be worth exploring if the mesenchymal subgroup of GBM patients have tumor cells carrying LOH in the neurofibromatosis-1 (NF-1) gene, also have NF-1 heterozygous microglia populating the GBM tumor microenvironment. NF-1 heterozygous microglia are essential in driving optic nerve astroglioma with NF-1 LOH.[81] Further, NF-1 heterozygous microglia drive optic nerve glioma by facilitating a relatively more "type 1" chronic inflammatory microenvironment through increased c-Jun-NH_2-kinase (JNK) signaling leading to the constitutive expression of higher levels of pro-inflammatory cytokines and proteins TNF-α, IL-1, iNOS, and Cox2.[82] The JNK and ERK1/2 pathway is not only responsible for the expression of pro-inflammatory cytokines, but also for the repression of the transcriptional potential of Smad3 activated by TGF-β as well.[83-85] Conversely, TGF-β1 mediates its effects through inhibition of the ERK pathway.[86] Among its many effects, TGF-β1 in the tumor microenvironment is an important regulator of glioma invasion.[87,88] The overactive JNK signaling in NF-1 heterozygous microglia may lead to a constitutively active state of microglia based on morphology and expression profiles. The existence of activated macrophage/microglia within the GBM tumor mass may facilitate a relatively more favorable immunogenic microenvironment that maintains T cell activation once they are mobilized to tumor by DC vaccination. This theory underscores the crucial role that microglia may play in the tumor microenvironment by potentiating the immune responses against tumor cells. Indeed, it has been proposed that modified microglia may have benefit for glioma treatment.[89,90]

Indeed, modulating the microglia in the tumor microenvironment of wild type NF-1 patients may prove to be an important aspect to glioma therapy. IL-10-mediated inhibition of NF-κB heterodimer (p50/p65) formation leads to an over-expression of the NF-κB homodimer (p50/p50), which prevents transcription elongation of various genes encoding pro-inflammatory cytokines. This is predominantly responsible for the tolerant M2 macrophage phenotype encountered in the microenvironment of wild type NF-1 patients.[91-93] Interestingly, IL-10 is a cytokine translated in tandem with other pro-inflammatory

cytokines in response to lipopolysaccharide stimuli.[94] This attribute is most likely an evolutionarily hard-wired negative feedback mechanism to preserve the cyclic response curve characteristic in NF-κB signaling.[95] We propose that the presence of IL-10 in the glioma microenvironment substantially dampens the transient pro-inflammatory activating pulse delivered by tumor-lysate activated DCs and booster injection of TLR agonist. This mechanism is circumvented in NF-1 heterozygous microglia through deregulated Ras/Rac1/JNK/c-Jun/AP-1 signaling, which operates in parallel and independent of the NF-κB signaling pathway [Figure 2]. Deactivating antibodies against IL-10 may restore the formation of the NF-κB heterodimer ultimately leading to a M1 microglia phenotype without overshooting the pro-inflammatory response, which may have detrimental effects on patients. Then, effectors cells mobilized by the vaccine can operate and maintain functionality by encountering a skewed microenvironment to a "type 1" pro-inflammatory state. Ultimately tumor regression may lead to a natural resolution of the inflammatory phase mediated in large part by IL-10.

IMMUNE AND GENE THERAPEUTICS THAT ENGENDER INFLAMMATION

Our translational immunotherapy research team has a long-standing interest in the development of novel therapeutic options for brain tumor patients. Our group and others have preclinically explored active and passive immune and gene therapy approaches, some of which are translated to the clinic.[96,97] The therapies are generally designed as adjuvant treatments and entail tumor resection followed by administration

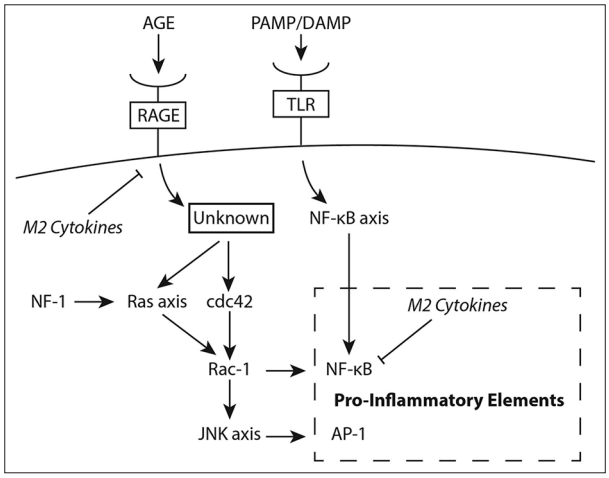

Figure 2: Signaling imparted through interactions of advanced glycation end products (AGE) with the receptors for AGE (RAGE) versus interactions of pathogen associated molecular pattern (PAMP) or damage associated molecular pattern (DAMP) molecules with Toll like receptors (TLR). The schematic shows the two signaling pathways that operate in parallel but independent to each other; both lead to expression of pro-inflammatory cytokines. The presence of anti-inflammatory (M2) cytokines inhibits the expression of pro-inflammatory cytokines of both inflammatory response pathways. NF: Neurofibromatosis; JNK: Jun-NH2-kinase

of the experimental agent. Surgical resection serves multiple purposes. Importantly, resection reduces tumor burden and the immunosuppressive factors present in the tumor microenvironment that will enhance the effectiveness of the immunotherapy. Also, the degree of the mobilized inflammatory response is minimized. The tumor specimens are valuable since they serve as a source of tumor associated antigens to make vaccines. Likewise, tumor specimens can be processed and placed into culture where the cells can serve in *in vitro* studies and as target cells for cytotoxicity testing.[98]

We have successfully used tumor-lysate pulsed DC vaccines that are given with or without TLR agonist; they represent an active immunotherapy strategy designed to enhance cell-mediated immunity.[99] The conclusion of a phase II clinical trial has shown the vaccine treatment to extend median survival to 34 months. It appears that the treatment has a relevant role in flagging the tumor cells remaining after surgical resection.[100] We have also examined a passive immunotherapy approach that utilizes effector alloreactive cytotoxic T lymphocytes (alloCTL) that are intratumorally implanted with low doses of Interleukin-2.[101] The allogeneic CTL are trained *in vitro* to target patient human leukocyte antigens that are present on glioma cells but not on normal neuroglia. A pilot clinical study described at www.clinicaltrials.gov (NCT00068510),[102,103] suggested a clinical response in recurrent WHO grade III gliomas. These studies led to a second phase I dose escalation trial that is currently open for patient enrollment at University of California, Los Angeles (www.clinicaltrials.gov; NCT01144247).

In another gene therapy approach, transduction of glioma cells with retroviral replicating vectors (RRV) coding for pro-drug activating enzymes followed by their exposure to non-toxic pro-drug has also proven to be another potent cancer therapy strategy. Non-cytolytic RRV are particularly well suited for the treatment of primary or metastatic brain tumors. In the CNS, normal brain neuroglial cells are relatively quiescent, thus, the dividing glioma cells are selectively targeted by the RRV. After achieving genomic integration, the viral constructs can stably seed the tumor mass and replicate within the tumor cells even as they infiltrate *in vivo*. Pro-drug administration results in targeted destruction of the cells harboring the RRV. Such an approach utilizes RRV coding for yeast cytosine deaminase. Upon administration of the pro-drug, 5-fluorocytosine, the drug is converted to its toxic form, 5-fluorouracil. If sufficient time is allowed for RRV spreading, the administered prodrug converts to a cytotoxic form, killing infected cells and providing tumor cytoreduction. Predicated upon successful and extensive preclinical testing,[104-108] phase I clinical trials

are testing the RRV suicide gene therapy in recurrent glioma patients (www.clinicaltrials.gov, NCT01156584; NCT01470794; NCT01985256).

Most recently, our attention has turned to preclinical studies examining a more aggressive combined immunogene therapy approach. RRV-transduced alloCTL have effector and delivery functions. If combined with pro-drug administration, the immunogene therapy is more efficacious *in vivo* than the individual therapies and control groups. Better extension was obtained in the survival of mice bearing orthotopic intracranial implants of breast carcinoma.[109,110] The immunogene therapy is similarly being tested in a syngeneic mouse glioma model. If the data look as promising in this model after optimizing doses and timing, combining the therapies should be easily translatable since both are being individually tested now in the clinic.

Challenges in immunotherapy

Immunotherapeutics do not always robustly provide efficacious treatment for gliomas. This may be due to the concurrent activation of both pro- and anti-inflammatory responses and this may have clinical and therapeutic consequences [Figure 3]. Clinically, immunotherapy entails protracted treatments. While manageable in theory, maintaining patients on immune treatments over the extended period necessary to effect a cell-mediated immune response has proven difficult.[102] Furthermore, inflammation associated with immune therapy is indistinguishable from tumor progression on follow-up magnetic resonance images; a clinician must give benefit of doubt and recommend other treatments inhibiting possible tumor growth. The immunotherapy is unfortunately either interrupted or incompletely tested. With the inability to distinguish pseudo- from tumor- progression, completion of trials is difficult, especially with the availability of drugs such as Avastin, Temodar, or other chemo- or radio-therapeutics for use at recurrence. Developing an appropriate set of neuroimaging parameters to distinguish inflammation from tumor growth would help advance this field. Perhaps one solution would be to offer immunotherapy upfront, or integrate it with standard of care treatments.

Therapeutically, the chronic inflammation that develops and worsens in correlation with glioma grade promotes a skewed "type 2" inflammatory state, both in the local tumor microenvironment and systemically.[40] Once gliomas are in the progression phase (i.e. pro-wound repair) deactivation of T cell-mediated immune response occurs. To effectively mount a host-generated, anti-tumor response immune homeostasis must be "reset" and skewed towards a "type 1" inflammatory state. An interesting possibility to generate a (Type 1) inflammatory response is the administration of attenuated microbes. Indeed, Bacillus Calmette Guérin (BCG) is effectively used for immunotherapy of superficial bladder cancer.[111] The success of BCG as a therapeutic modality for low-grade bladder cancer can be effectively attributed to two characteristics: immunogenicity and anti-tumor targeting. In BCG tumor models, the initial presence of both Th1 and Th2 inflammatory cytokines was also observed, but then later skewed towards Th1 cytokines that in particular involved the up-regulation of IFN-γ.[112] However, the situation is complicated for the treatment of high-grade glioma. Studies of immunosuppression have shown that once "type 2" inflammation has been activated, challenge with a bacterial lipopolysaccharides fails to skew the cytokine expression towards "type 1" in a time-dependent manner.[29] Thrombospondin receptor (CD36) expressed on macrophages among other cell types formed a "molecular bridge" between anionic sites on apoptotic cells and CD36. This cell-cell signaling interaction was sufficient to signal the resolution of inflammation and activation of "type 2" inflammation. Further, antibodies against thrombospondin prevented its binding to CD36 receptor leading to a decrease of IL-10 and restored TNF-α, IL-1β, and IL-12 in the presence of apoptotic cells. Thus, it appears that immune homeostasis must first be restored for high-grade tumors that are driven by "type 2" inflammation before further intervention

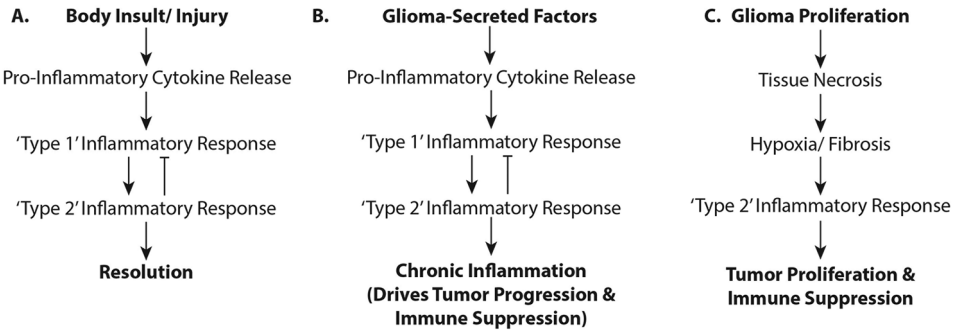

Figure 3: Activation of pro- and anti-inflammatory responses in glioma patients. The flowcharts illustrate (a) the "normal" physiologic processes in the inflammatory response and its resolution; (b) the physiologic processes occurring when glioma-secreted factors influence a state of chronic inflammation resulting in glioma progression; and (c) how rapid glioma growth creates a necrotic/hypoxic environment supporting tumor proliferation and immunosuppression

to activate the "type 1" inflammatory response can be implemented. Enhancing the endogenous immune response by deactivating the ensuing chronic inflammatory tumor microenvironment might provoke an immune response potent enough to activate and mobilize endogenous CTL and NK cells to eliminate the threat posed by high-grade cancerous masses.

CONCLUSION

It has long been held that tumor cells outwit the host's defenses by altering their own cellular signaling pathways. The pathway exploited to achieve malignancy may be a combination of unique derivations. Gliomas are known to exhibit compensatory activity in that when supplied with selective pressure from one treatment, they readily adapt with other mutations to survive. Other mounting evidence now suggests that some of the pathways exploited by cancer cells adopt a more malignant phenotype and are simply responses to the stimuli created by the rapidly dividing tumor cells rather than novel re-circuited pathways exploited by neoplastic cells for growth. One of the crucial responses facilitating and nurturing cancerous transformation is inflammation. A chronically active inflammatory microenvironment provides the developing cancerous mass with proliferative and mutational factors necessary to realize "self-sufficiency". It is evident that some tumors can bypass this "nurturing stage" as might be expected with primary GBM. Regardless, once this "self-sufficiency" is realized, the tumor is able to survive outside of the cancer stem cell niche. Empowered with constant proliferative cues the tumor mass divides uncontrolled. The increased proliferation results in necrosis and the resultant environment is skewed more strongly towards the Th2 inflammatory response. Thus, for high-grade gliomas a higher Th2/Th1 cytokine ratio supports the production of other immunosuppressive factors. To mount a successful cytotoxic anti-tumor response, it is crucial to restore a balanced Th2/Th1 cytokine ratio of 1:1 or less. This should decrease the proliferative rate of the tumor mass as well, since it is the Th2 response that ultimately works with the tumor cell to drive the angiogenic response. Ultimately, successful brain tumor immunotherapy should leave patients with intact immunosurveillance function and the ability to enact a cell-mediated response in the event of recurrence.

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Original Article

Effect of conduction block in classification and prognosis of Guillain-Barre syndrome

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ABSTRACT

Aim: The aim was to investigate the electro-physiological characteristics in disease progression of Guillain-Barre syndrome (GBS) and observe the effect of conduction block (CB) in classification and severity of the disease. **Methods:** Two hundred and ninety-four patients with GBS were divided into acute inflammatory demyelinating poly-neuropathy (AIDP) group, acute motor axonal neuropathy (AMAN) group and equivocal group according to their electro-physiological results and then reclassificated after electro-physiological review. All of the patients were followed for 6 months since their attacks. **Results:** Bad prognosis is more pronounced in AMAN group than in AIDP group ($P < 0.05$). Most of the patients classificated as AIDP transformed into AMAN when CB occurred in the early phase of the disease. There is a positive relationship between CB in the early phase of the disease and severity of illness ($P < 0.05$), but CB showed no correlation with prognosis of the patients ($P > 0.05$). **Conclusion:** CB in the early phase of GBS indicates the probability of AIDP transforming into AMAN; it suggests that patients with CB in the early phase of the disease might be in serious conditions in a certain extent.

Key words: Clinical features, Guillain-Barre syndrome, nerve conduction block, nerve electrophysiology

INTRODUCTION

Gillan-Barre syndrome (GBS), which is also known as acute inflammatory demyelinating polyradiculoneuropathy, is an autoimmune disease in which the typical clinical symptoms are rapidly progressing symmetrical weakness, areflexia and cerebrospinal fluid (CSF) protein levels elevated without accompanying pleocytosis. It usually affects spinal nerve roots especially the anterior roots, ganglions and peripheral nerves, sometimes affects the cranial nerves.

According to its clinical manifestation, laboratory examinations and electro-physiological characteristics, GBS can be classified as acute inflammatory demyelinating poly-neuropathy (AIDP), acute motor axonal neuropathy (AMAN), acute motor sensory axonal neuropathy, Miller-Fisher syndrome (MFS), acute autonomic neuropathy, and acute sensory neuropathy.[1] GBS is single-phase process and self-limiting, so most of the GBS patients have a good prognosis, but some may have a bad prognosis clinically. As the disease progresses, some of the AIDP patients may turn into AMAN; some patients without obvious curative effect may progress into chronic inflammatory demyelinating polyneuropathy; and some patients may transform into relapsing GBS. Neural electro-physiological examination, an irreplaceable auxiliary examination for diagnosis of GBS, can provide an important basis for the diagnosis and classification of the disease. In this study, the clinical data of 338 GBS patients who were hospitalized during the period of August 2008 to February 2013 were analyzed retrospectively.

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METHODS

Patients

This study retrospectively includes 338 GBS patients who were hospitalized during the period of August 2008 to February 2013. All the cases were diagnosed under the diagnosis and treatment guidelines of GBS published in 2010 by Chinese medicine association. Excluding 20 cases without neural electro-physiological examination and 24 patients who were diagnosed with MFS, 294 cases were included in this study eventually, with 186 male patients (63.3%) and 108 female patients (36.7%). Patient's ages ranged from 4 to 82 years (mean 40.4 ± 18.3 years), and the average hospital stay was 18.8 days. There

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were precursor events emerging 1-4 weeks before in some of the patients: respiratory infection or fever in 126 cases (42.9%), digestive tract infection in 71 cases (24.1%), flu vaccination in 3 patients (1.0%), influenza vaccination in 2 cases (0.7%), rabies vaccination in 2 cases (0.7%), pregnancy in 1 case (0.3%), and the rest cases with no precursor events. The first symptom was limb weakness in 276 cases (93.9%), sensory disturbance in 98 cases (33.3%), and cranial nerve symptom in 54 cases (18.4%). There were 76 cases (25.9%) with clinical symptoms of cranial nerve lesions, including 36 cases with facial nerve paralysis, 8 cases with ophthalmoplegia, 7 cases with diplopia and 62 cases with drinking water choking. There were 102 cases (34.7%) with clinical symptoms of disturbance of sensation, 260 cases (88.4%) with upper-limb weakness, 276 cases (93.9%) with lower limb weakness. There were 192 cases (65.3%) with tendon hyporeflexia, 66 cases (22.4%) with disappearing and 11 cases (3.7%) with hyperreflexia. 261 cases had a lumbar puncture while they were hospitalized, and there were 205 cases (78.5%) with albumincytological dissociation. Two hundred and eighteen cases had CSF immunoglobulin test, and there were 178 cases (81.7%) with high levels of IgG, 36 cases (16.5%) with high levels of IgM, 137 cases (62.8%) with high levels of IgA. The average time to disease peak period was 10 days; 65 cases (22.1%) suffered from the lung infection while 16 cases (5.4%) suffered from urinary tract infection.

Electro-physiological examination

Keypoint electromyography made by the Danish Dandi Company was used. Since early stage of the disease, the electro-physiological result showed abnormal F-wave only, and the result of nerve conduction showed reduction of compound muscle action potential in most cases, so all patients were performed at least one electro-physiological examination 2 weeks after the onset of disease. Of the patients, 132 cases had their first electro-physiological examination 2 weeks after the onset of disease and had another check within 10-14 days after the first check: (1) All patients had nerve conduction test by using surface electrodes to record and stimulate. We detected motor conductive test on bilateral median nerve, ulnar nerve, peroneal nerve, and tibial nerve recording the motor nerve conduction velocity, distal latency, and amplitude, *etc*., and observed whether there was a nerve conduction block (CB) or not. Sensory conduction test were performed at median nerve, ulnar nerve, sural nerve, and phil shallow nerve recording the sensory nerve conduction velocity; (2) all patients had F-wave detection on the median nerve, ulnar nerve peroneal nerve and tibial nerve by recording the wave rate, latency, *etc*.; (3) needle electromyography were performed on thenar muscle, hypothenar muscle, deltoid, quadriceps, pretibial muscle, and gastrocnemius by observing whether there was a spontaneous activity or not on the resting moment and by testing the motor unit potential and recruitment order of slight and strong muscle contraction.[2,3]

Diagnostic and evaluation criteria

(1) GBS and nerve block were diagnosed under the diagnosis and treatment guidelines of GBS published in 2010 by Chinese Medicine Association; (2) the neural electro-physiological types were classified into AIDP and AMAN according to the electro-physiological diagnostic standard [Table 1];[4] (3) normal patients and the patients not satisfying the diagnostic criteria of AIDP and AMAN were included into the unclear type group; (4) the disease classification and follow-up results were marked according to rating scales designed by Hughes *et al*. Based on patients' ability to walk with the help, patients were classified into mild type and serious type. Patients with Hughes score equal or lesser than two points is the mild type, and equal or more than three points is a serious type; (5) the prognostic evaluations were marked according to rating scales designed by Hughes *et al*. Based on the patients' sequelaes (whether can walk without help or not), patients were classified into favorable prognosis type and poor prognosis type. Patients with Hughes score equal or lesser than two points is the favorable prognosis type and equal or more than 3 points is the poor prognosis type; (6) follow-up: 294 cases of GBS patients included in this study were follow-up by telephone for 6 months after being discharged from hospital and 103 cases were lost to follow-up, so the response rate was 65.0%. Among the remaining 191 cases, 3 died of non-GBS cause. There were 188 cases with efficient results, which were included in this study eventually.

Statistical analysis

Data analysis was carried out using SPSS 17.0 Statistical Analysis Software (Polar Enginneering and Consulting. http://www.winwrap.com/). We compared clinical symptom, grading and prognosis between groups with Chi-squared test, and considered statistical significance at $P < 0.05$.

Table 1: Electro-physiological types diagnostic standard

Types	Electro-physiological index	Diagnostic standard %
AIDP	Conduction velocity far-end lurk waveform disperse F wave	At least 2 nerves have 1 or more electro-physiological behaviors < 90 LLN; if CMAP < 50 LLN; < 85 LLN; > 110 ULN; if CMAP < LLN; > 120 ULN; clear waveform disperse > 120 ULN
AMAN		No electro-physiological behaviors of AIDP; 2 or more nerves CMAP< 20

AIDP: acute inflammatory demyelinating poly-neuropathy; AMAN: acute motor axonal neuropathy; LLN: lower limit of normal; ULN: upper limit of normal; CMAP: compound muscle action potential

RESULTS

Neural electro-physiological results

There were 102 cases (34.7%) in AIDP group and 81 cases (27.6%) in AMAN group [Figure 1]. Based on the first electro-physiological testing, 132 patients were classified into: 58 cases (43.9%) of AIDP, 24 cases (18.2%) of AMAN, 50 cases (37.9%) of unclear [Figure 2]. Cases belonged to AMAN group based on two different testing results were fewer than the cases in AIDP group and unclear classification cases group.

Relationship between early nerve conduction block and its electro-physiological changes

The first electro-physiological results for 132 cases with rechecks were: 58 cases (44%) in AIDP group, 24 cases (18%) in AMAN group, 50 cases (38%) in unclear classification group [Figure 3]. A total of 36 cases in AIDP group had CB, and cases transforming into AIDP and AMAN were 19 and 17, respectively.

Relationship between different types and the prognosis

The first electro-physiological results and the recheck results all demonstrated that comparing to AIDP, AMAN had more cases with poor prognosis [Tables 2 and 3] (all $P < 0.05$).

Relationship between early nerve conduction block and the severity of the illness

Results demonstrated that the severity of the illness was related to the development of CB in early stage in AIDP group and unclear classification group (all $P < 0.05$) [Table 4].

The results of Chi-squared test within each type of group were: in AIDP the value was 11.334, $P = 0.001$, in unclear classification the value is 8.408, $P = 0.004$, both with statistical significance; in AMAN group the value is 3.472, $P = 0.062$, with no statistical difference.

Relationship between early nerve conduction block and prognosis

Results demonstrated that irrespective of the severity of the disease, poor prognosis was not related to the development of CB (all $P > 0.05$) [Tables 5 and 6].

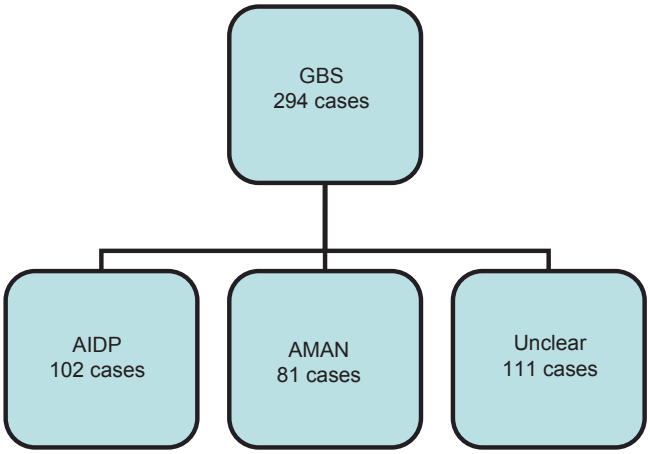

Figure 1: Neural electro-physiological results. GBS: Gillan-Barre syndrome; AIDP: acute inflammatory demyelinating poly-neuropathy; AMAN: acute motor axonal neuropathy

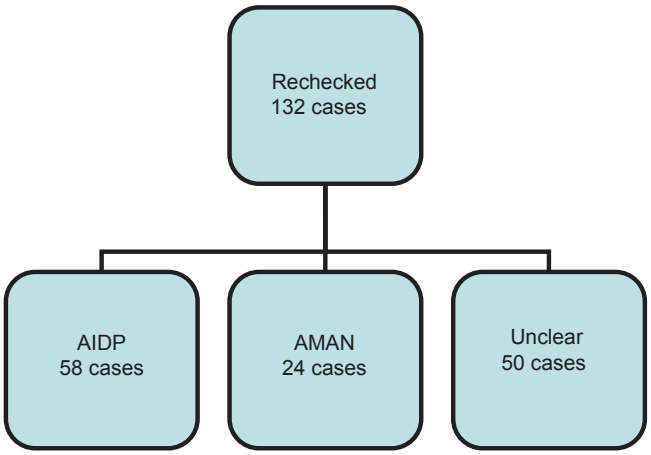

Figure 2: One hundred and thirty-two rechecked cases' first classification. AIDP: acute inflammatory demyelinating poly-neuropathy; AMAN: acute motor axonal neuropathy

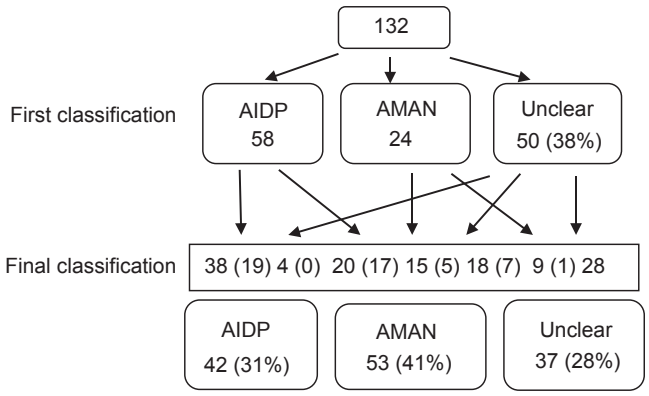

Figure 3: One hundred and thirty-two Gillan-Barre syndrome patients' first and final neural electro-physiological classifications (conduction block numbers in brackets clear to arrows). AIDP: acute inflammatory demyelinating poly-neuropathy; AMAN: acute motor axonal neuropathy

DISCUSSION

In this study, more male than female patients were included. Respiratory tract and intestinal infections were the most common precursor events. A few patients had influenza vaccine, H1N1 influenza vaccine and rabies vaccine before the onset of the illness. It has been reported that H1N1 vaccine maybe is a risk factor of GBS, but season influenza vaccine was not related to it.[5,6] In our data, there is no evidence that H1N1 influenza vaccine was related to GBS. The most common symptoms were symmetrical limb weakness and numbness. Sensory disturbance is usually milder than motor disturbance with reducing or disappearing tendon reflex. The common cranial nerve damages are facial nerve paralysis, drinking water choking, hoarseness, and ophthalmoplegial. All the cases were followed by telephone for 6 months after hospital

discharge. The response rate was 65.0%. Twenty-one percent of the patients had serous sequel which was the same as those reported in the literature. The mortality was 10.6% which was higher than previously reported.[6]

The most common subtypes of GBS are AIDP and AMAN with only motor fiber damage. According to the literature, in North America and Europe more than 90% of the patients is classified as AIDP,[4] but in China, the most common subtype is AMAN, and 65% of the GBS are AMAN.[7] In this study, most cases among the 294 GBS patients were AIDP. Of these, 132 cases were classified as AIDP based on their first electro-physiological examination. These findings are in contrast with what previously reported.

Because the classification based on early stage GBS electro-physiological results is inaccurate, electrophysiology testing was repeated after the illness developed and the percentage of AIDP and AMAN cases changed comparing to the early stage results.[8] We classified patients into AIDP and AMAN groups and unclear classification group which included patients whose electro-physiological testing were normal and those who did not meet the diagnostic criteria of AIDP and AMAN. We found that during disease progression electro-physiological results, as well as the electro-physiological classification, changed. The percentage of AMAN patients after recheck increased significantly compared to the first check (from 18% to 41%), which is the same as reported literature,[8,9] and AIDP reduced from 44% to 31%. We also found that AMAN had a worse prognosis than AIDP, and this finding is consistent with the literature,[10,11] thus suggesting a poor prognosis in more patients. The classification based on early stage GBS electrophysiologic results only may lead to inaccurate judgment of the patients' diagnosis and prognosis, instead continuous electrophysiology recheck can reflect the change of patient's condition without delay.

Among the patients transforming from AIDP into AMAN, CB occurred in 17 cases in the early onset of the illness: 5 and 7 cases in AMAN and unclear classification group, respectively. CB is a blockage in a nerve that prevents impulses from being conducted across a given segment although the nerve beyond is viable and is one of the important electro-physiological parameters of peripheral nerve functional status. CB is one of the physiological results caused by demyelination and is also the basic physiological mechanism of most clinical manifestations.[12] Most studies of CB published before are on multifocal motor neuropathy and amyotrophic lateral sclerosis, peroneal muscular atrophy and peripheral neuropathy caused by pressure.[13] Though conventional wisdom holds that the main cause of CB is demyelination and CB is the typical characteristics of demyelinating, recent studies demonstrated that demyelination is not the only reason for CB. It can caused by demyelination, depolarization on node of ranvier nearby axolemma, hyperpolarization and sodium channel damage.[14] The damage of nearby axolemma may cause CB, electro-physiological manifest as decreased amplitude, discretized waveform. If the illness continues to progress, reversible CB will turn into irreversible CB, and axonal degeneration. This might explain why some of the CB cases transformed into AMAN in electro-physiological classification.

Our study demonstrated that CB not only occurred in AIDP patients, but also in AMAN and unclear classification patients. In recent years, other groups found that CB plays an important role in axon damaged AMAN.[15,16] Kuwabara *et al*.[17] thought that the possible cause of CB in AMAN was axonal degeneration. The bridge type union of GM-1 antibody-mediated inflammatory cells and axons, the release of inflammatory mediators, local acidosis, damage on sodium ion channel of Axonal membrane and tight junction of axon myelin (some authors believe that this is a different type of demyelination from AIDP) resulted in a further decline of the safety factors, eventually leading to CB.[18]

Table 2: All cases classifications and prognosis

Prognosis	AIDP	AMAN	Total
Good prognosis	59	27	86
Poor prognosis	12	19	31
Total	71	46	117

$\chi^2 = 8.535$, $P = 0.003$. AIDP: acute inflammatory demyelinating poly-neuropathy; AMAN: acute motor axonal neuropathy

Table 3: Rechecked cases classifications and prognosis

Prognosis	AIDP	AMAN	Total
Good prognosis	35	8	43
Poor prognosis	6	9	15
Total	41	17	58

$\chi^2 = 9.197$, $P = 0.002$. AIDP: acute inflammatory demyelinating poly-neuropathy; AMAN: acute motor axonal neuropathy

Table 4: Relationship between different classifications nerve CB and the severity in 294 cases

Severity	AIDP			AMAN			Unclear		
	With CB	Without CB	Total	With CB	Without CB	Total	With CB	Without CB	Total
Slight	6	18	24	2	17	19	2	24	26
Severe	50	28	78	20	42	62	32	53	85
Total	56	46	102	22	59	81	34	77	111

AIDP: acute inflammatory demyelinating poly-neuropathy; AMAN: acute motor axonal neuropathy; CB: conduction block

Table 5: Early CB and prognosis of 140 severe GBS cases			
Prognosis	**With CB**	**Without CB**	**Total**
Good prognosis	46	54	100
Poor prognosis	15	25	40
Total	61	79	140

$\chi^2 = 0.840$, $P = 0.360$. CB: conduction block; GBS: Gillan-Barre syndrome

Table 6: CB and prognosis of 47 slight GBS cases			
Prognosis	**With CB**	**Without CB**	**Total**
Good prognosis	7	39	46
Poor prognosis	0	1	1
Total	7	40	47

$\chi^2 = 0.179$, $P = 0.672$. CB: conduction block, GBS: Gillan-Barre syndrome

Our results suggested that the severity of the illness was related to the development of CB in early stage in AIDP group and unclear classification group but in the AMAN group is limited by small sample size. As mentioned previously, damage factors of the axonal membrane may the cause of CB, and then reversible CB will turn into irreversible CB and axonal degeneration. According to the report of Kokubun *et al*.[9], the proportion of the two outcomes is 1:1 on AMAN patients who had CB. Patients in this group with CB in early stage were not related to the severity of the illness may be because some patients had reversible CB. In addition, the use of immunoglobulin in early stage of disease in patients with serious conditions may improve the prognosis and the finding that development into CB in early stage correlates with the severity of the illness might be another factor.

Due to the objective condition limit that our patients mostly come from surrounding cities and counties, and even other provinces, it is difficult to diagnose these patients' neurological recovery face-to-face after they are discharged from our hospital. We have to perform telephone follow-up for most of them. At the same time, many patients filled temporary numbers in the contact information form when hospitalized, which were no longer used after they went back. This also limited the follow-up results, and the response rate was only 65%. Furthermore, through telephone follow-up, we can't evaluate the neural function of patients completely and clearly. We will try to improve this in future work and research.

In conclusion, reversible CB might be the cause of changes in patients' electro-physiological classification. CB is not only a typical electro-physiological manifestation in AIDP, but also a manifestation of axonal degeneration for AMAN in the early stage. CB and axonal degeneration are caused by immune-mediated damage factors which attack axon membrane on the motor fiber. To a certain extent, CB is very helpful in classifying the severity of the illness.[14] Electrophysiology recheck can be very meaningful to reveal change of patient's condition, classification alteration and severity of the nerve damage in time.

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Original Article

A novel method for evaluating microglial activation using ionized calcium-binding adaptor protein-1 staining: cell body to cell size ratio

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ABSTRACT

Aim: The aim was to validate a newly developed methodology of semi-automatic image analysis to analyze microglial morphology as marker for microglial activation in ionized calcium-binding adaptor protein-1 (IBA-1) stained brain sections. **Methods:** The novel method was compared to currently used analysis methods, visual characterization of activation stage and optical density measurement, in brain sections of young and aged rats that had undergone surgery or remained naïve. **Results:** The cell body to cell size ratio of microglia was strongly correlated to the visual characterization activation stage. In addition, we observed specific surgery and age-related changes in cell body size, size of the dendritic processes and cell body to cell size ratio. **Conclusion:** The novel analysis method provides a sensitive marker for microglial activation in the rat brain, which is quick and easy to perform and provides additional information about microglial morphology.

Key words: Image analysis, immunohistochemistry, ionized calcium-binding adaptor protein-1, microglia, neuroinflammation

INTRODUCTION

Microglia, the primary immune cells in the central nervous system, are highly plastic cells.[1] Under resting conditions, microglia have a ramified morphology, characterized by small cell bodies and numerous long branching processes.[2] The ramified microglia continuously scan the environment for danger signals associated with pathogens or injury.[2,3] When a danger signal is detected microglia undergo a rapid change in morphology and function, a process that has been termed activation.[4] Microglial activation is classically described as a graded process, in which the processes retract and thicken, cell body size increases and the cell starts excreting cytokines and radical species.[1,4,5] Eventually, the microglia may become amoeboid cells capable of phagocytosis.[5] More recently, it has become apparent that depending on the signal detected and local environment microglia can undergo various changes in morphology and function.[2,6] Although microglial activation provides a defense against injury and infection, chronic or excessive activation is considered to be detrimental and has been implicated in many neurodegenerative and psychiatric disorders.[2,5,7]

Ionized calcium-binding adaptor protein-1 (IBA-1) is a 17-kDa actin-binding protein that is specifically and constitutively expressed in all microglia.[8,9] It is widely employed as an immunohistochemical marker for both ramified and activated microglia.[9,10] IBA-1 is shown to have a function in the actin-crosslinking involved in membrane ruffling of microglia.[11] Since membrane ruffling is essential for the morphological changes from quiescent ramified microglia to activated amoeboid microglia, microglial activation is associated with increased IBA-1 expression.[8,11] Densitometry can, therefore, be utilized for measuring microglial activation, especially when microglia is strongly activated and/or microglia number is substantially increased. However, morphological changes of microglia can occur without significantly affecting the optical density (OD) in IBA-1 stained brain areas and densitometry does not provide any specific information on the nature of the morphological changes.

Alternatively, the stadia of microglial activation described by Kreutzberg[4] are often used visually to determine the activation stage of all individual

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microglia in an area of interest, and presenting activation as the percentage of microglia that score above a certain level. This technique provides a more specific and sensitive measurement for microglia activation than densitometry, but is very labor intensive and rather depends on a subjective interpretation of the activation stage. Moreover, this analysis method is solely based on the classical view on microglial activation and as such does not take alternatively morphological changes, such as hyper-ramification into account.[12,13]

In the past years several other methods have been developed that involve morphological characteristics, such as morphological complexity,[14] the extent of ramification[13,15,16] or the length of the dendritic processes.[13,15,16] Although these methods allow a more detailed and accurate analysis of microglial morphology, they still tend to be time consuming and depend on specialized equipment (e.g. a confocal microscope) and software.

Considering the disadvantages of the aforementioned methods, we were in search of a method to determine changes in microglial morphology that is quick and easy to perform, reproducible, and reliable as a microglial activation marker. In this paper, we describe a newly developed semi-automatic image analysis method for IBA-1 stained brain sections of rat. We compare this novel method to the most commonly used methods today, namely densitometry and visual characterization of the activation stage, using IBA-stained sections of rat brains from a previously published experiment.[17]

METHODS

Animals and interventions

To investigate whether our method could be applied to rat brain sections with a range of activation stadia we re-analyzed IBA-1 stained brain sections from a previously performed experiment in young and aged rats.[17] Male Wistar rats (HsdCpb: WU) of 3 months (young, $n = 10$) and 18-20 months (old, $n = 10$) were obtained from a colony of the Semmelweis University (Budapest, Hungary). In each age group, 5 rats were subjected to abdominal surgery under anesthesia (3% sevoflurane in O_2 at 0.7 L/min) and buprenorphine analgesia (0.003 mg/kg s.c.) and the other 5 remained naïve as control, yielding 4 experimental groups (young control, young surgery, old control and old surgery). Between week 1 and 6 following surgery, the rats underwent several behavioral tests.[17] Animals were sacrificed 6 weeks after the surgical intervention. All experiments were approved by the local animal experiment and welfare committee (Dier Experimenten Commissie, Groningen, The Netherlands).

Immunohistochemistry

Transcardial perfusion sacrificed animals with saline containing 0.1% ethylene diamine tetraacetic acid, under pentobarbital anesthesia (6%, 2 mL/kg). Half of each brain was immersion fixed in 4% paraformaldehyde for 4 days followed by cryoprotection with 30% sucrose in 0.01 mol/L phosphate-buffered saline (PBS). Free floating sections of 30 μm containing the prefrontal cortex (PFC) and the dorsal hippocampus were pretreated with 0.3% H_2O_2 for 20 min. Sections were incubated for 3 days with 1:2500 rabbit-anti IBA-1 (Wako, Neuss, Germany) in 2% bovine serum albumin, 0.1% triton X-100 at 4°C, followed by a 1 h incubation with 1:500 goat-anti rabbit secondary antibody (Jackson, Wet Grove, USA) at room temperature. The sections were then incubated for 2 h with avidin-biotin peroxidase complex (Vectastain ABC kit, Vector, Burlingame, USA) at room temperature. Labeling was visualized by using a 0.075 mg/mL diaminobenzidine (DAB) solution activated with 0.1% H_2O_2. All dilutions were made in 0.01 mol/L PBS. All sections were thoroughly rinsed 4 times with 0.01 mol/L PBS between staining steps. Sections were mounted onto glass slides in a 1% gelatin solution and dehydrated through gradients of ethanol and xylol solutions.

Analysis of microglial activation

Three immunohistochemical labeled sections per area for each rat were analyzed by an operator blinded to the treatment. Average microglial activation was determined in the apical dendritic field (stratum radiatum) of the dentate gyrus inner blade (DGib) and the cornu ammonis 1 (CA1) hippocampal region and in layer III of the PFC (Zilles's Cg1).

Densitometry

The OD of the IBA-1 stained sections was measured using quantitative imaging software (Leica QWin, Leica Microsystems, Rijswijk, The Netherlands), at ×100 magnification. The OD was corrected according to the background staining measured in the corpus callosum of each individual section. The resulting average OD in each brain area was taken as a measure for microglial activation.

Visual characterization

Images were taken of the DGib, CA1 and PFC of the IBA-1 stained sections at ×400 magnification using quantitative imaging software (Leica QWin, Leica Microsystems). In each image, a circle was drawn which covered 0.06 mm^2 of the original section. The microglia in this area were counted and morphologically characterized based on the activation stages 1-2 or 3-5 as described by Kreutzberg.[4] Microglia characterized as being in stages 3-5 were considered to be activated. The number of activated

microglia expressed as a percentage of the total number of microglia was used as a measure for microglial activation.

Quantifying morphological characteristics (cell size, cell body size, size dendritic processes and cell body size to cell size ratio)

As described in the previous section, images were taken of the DGib, CA1 and PFC of the IBA-1 stained sections. Several morphological characteristics of the stained microglia were analyzed by using image analysis software (Image-Pro Plus 6.0.0.26, Media Cybernetics, Inc. Rockville, USA). Figure 1 shows an overview of the method in microglia with a more quiescent morphology and microglia with a more activated morphology (for clarity purposes only a small section of the original picture are shown).

The area of interest was selected. The image analysis software automatically analyzes the picture and bases on the color intensity and distribution determines the background and intensity range of this area. The total cell size of all microglia in the area of interest was determined by counting all pixels that were darker than the background using the "automatic dark objects" function. The total cell body size was determined by applying an intensity threshold (histogram based manual intensity range selection) and size filter (area filter range) (similar to the method described by Tynan *et al.*[18]). With the intensity threshold only pixels with a staining intensity above a certain value in the intensity range scales, (0-255) are counted. Since IBA-1 staining gives a higher staining intensity in the cell bodies than most of the dendritic processes applying an intensity threshold will filter out most dendritic processes from the analysis. In addition, the size filter excludes all pixel clusters that are smaller than a certain size from the analysis, filtering out any dendritic processes with a high-staining intensity. The intensity threshold and size filter depend on the overall intensity of the staining. In our case, the intensity threshold was 150-170 and the size filter was 100 pixels.

In addition, the number of cell bodies was counted to give the number of microglia in the area of interest. The total size of the dendritic processes was determined by subtracting the cell body size from the cell size. The total cell size, total cell body size and total size of dendritic processes were corrected for the number of microglia in the sample area to gain the average size, cell body size and size of dendritic processes for each microglial cell in the sample. Finally, the cell body to cell size ratio (%) was determined and utilized as a measurement for microglial activation.

As an alternative to the analysis with Image-Pro (Image-Pro Plus 6.0.0.26, Media Cybernetics, Inc.), we performed a similar analysis by using ImageJ (ImageJ 1.48v, http://imagej.nih.gov/ij), a publically available image analysis package. The area of interest was selected. Using the adjusted threshold and analyze particles functions the intensity thresholds and size filter were applied. To measure the total cell size the threshold was maintained at the level that was automatically provided by the program, and no size filter was applied. To measure the total cell body size the threshold was lowered 40 points, and size filter of 150 pixels was applied.

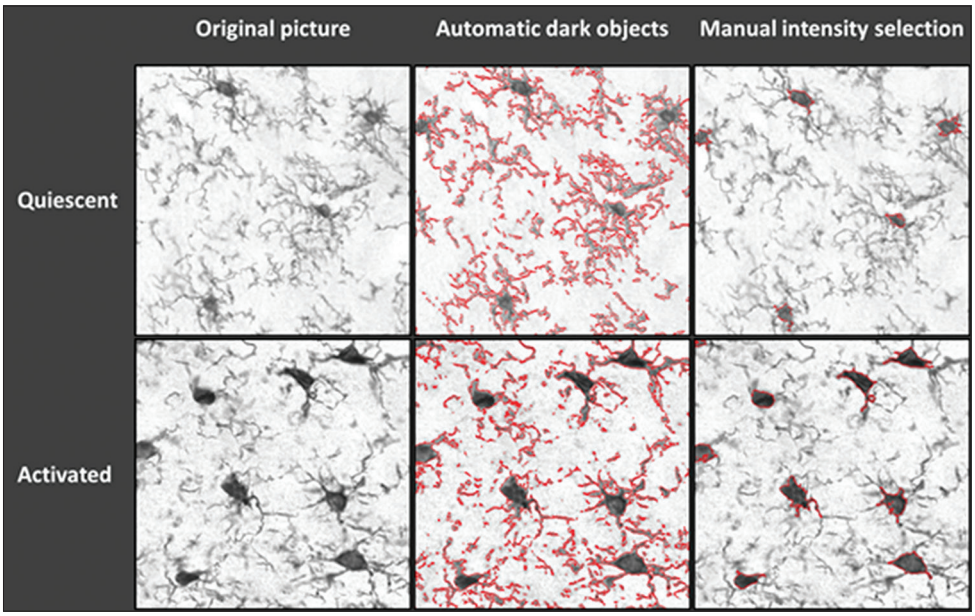

Figure 1: Illustration of the image analysis method used to quantify the morphological characteristics of microglia in ionized calcium-binding adaptor protein-1 stained sections (for clarity purposes only a small section of the original picture is shown). Left: the unprocessed pictures. Middle: all the pixels that are darker than the background are traced (red lines) to determine the total cell size of all microglia combined. Right: pixel-clusters that are above an applied staining threshold and size-filter are traced (red lines) to determine the total cell body size of all microglia combined, as well as the number of microglia

Statistical analysis

All statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows, 20.0.0.2, New York, USA). Means and confidence intervals are shown. Figures were prepared by using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, California, USA). Pearson's correlation coefficients were determined to examine the relationship between the various measures for microglial activation for all individual brain areas, and all brain areas combined. The significance level for the Pearson's correlation coefficients was corrected by using Bonferonni method.

In addition, group comparisons were made for microglial activation in the CA1, DGib and PFC combined, using an analysis of variance with the experimental group as between subject factor followed by Tukey *post-hoc* analysis. Outcomes were considered to be significant when $P < 0.05$.

RESULTS

Table 1 shows the average values of the outcome measures used to analyze microglia activation.

The image analysis by Image-Pro and ImageJ yielded highly comparable results as indicated by a strong correlation of the main outcome parameters cell body size ($r = 0.792$, $P = 0.000$), size of dendritic processes ($r = 0.499$, $P = 0.029$) and cell body to cell size ratio ($r = 0.918$, $P = 0.000$). Therefore, we have only used the outcomes of the analysis with Image-Pro for the rest of the study.

There is no significant correlation between OD and the other parameters used to measure microglia activation. The Pearson's correlations between the percentages of activated microglia based on visual characterization and the quantified morphological characteristics are shown in Table 2. In all separate brain area, the parameter of the cell body to cell size ratio is significantly and strongly correlated with microglial activation based on visual characterization. Therefore, this outcome may be a more accurate morphological characteristic to represent a marker of (classical) microglial activation.

Figure 2 shows a scatter plot of microglia activation based on morphological characterization and the cell body to cell size ratio for the three analyzed brain areas combined.

A comparison of the study outcome using the different analysis techniques is presented in Figure 3, which displays the average of densitometry measurements [Figure 3a], visual characterization [Figure 3b] and our novel measurement, cell body to cell size ratio [Figure 3c]. Whereas the OD does not differ significantly between experimental groups, both the percentages of activated microglia based on visual characterization and the cell body to cell size ratio shows a significant difference between groups (visual

Table 1: Outcome measures microglial analysis

	n	Mean (95% CI)
Activated microglia (stages 3-5) (%)	60	40.4 (37.0-43.9)
Optical density	60	0.17 (0.16-0.18)
Number of microglia	60	13.3 (12.7-13.8)
Average cell size (pixels)	59	2341 (2227-2454)
Average cell body size (pixels)	59	328 (302-355)
Average size dendritic processes (pixels)	59	2013 (1910-2116)
Cell body to cell size ratio (%)	60	14.2 (13.2-15.1)

Mean ± SEM of the outcome measures used to analyze microglial activation. Average outcomes are representing an area of 0.06 mm × 0.06 mm in the CA1, dentate gyrus inner blade and prefrontal cortex. The visual characterization outcomes and number of microglia have been previously published by Hovens *et al.*[17] CA1: cornu ammonis 1; CI: confidence interval, SEM: standard error of the mean

Table 2: Correlation between the percentages of activated microglia based on visual characterization and the quantified morphological characteristics of microglia

	Morphological characteristics				
	Number of cells	Cell size	Cell body size	Processes size	Cell body/cell
Visual characterization					
DGib	0.137	0-0.449	0.625#	0-0.495	0.839*
CA1	0.001	0-0.282	0.796*	0-0.524	0.890*
PFC	0-0.390	0-0.029	0.680*	0-0.288	0.944*
Total	0-0.104	0-0.170	0.619*	0-0.343#	0.855*

Correlation (Pearson's R) between the percentage of activated microglia as determined by visual characterization according to Kreutzberg and the quantified morphological characteristics of microglia in the DGib ($n = 20$), hippocampal CA1 region ($n = 20$), PFC ($n = 20$) and all three brain areas combined (total, $n = 60$). The morphological characteristics are: the number of microglia (number of cells), the average size of microglia (cell size), the average cell body size of microglia (cell body size), the average size of the dendritic processes of microglia (processes size) and the cell body to cell size ratio (cell body/cell) in the area of interest. *$P < 0.0013$, #$P = 0.01$. PFC: prefrontal cortex; CA1: cornu ammonis 1; PFC: prefrontal cortex; DGib: dentate gyrus inner blade

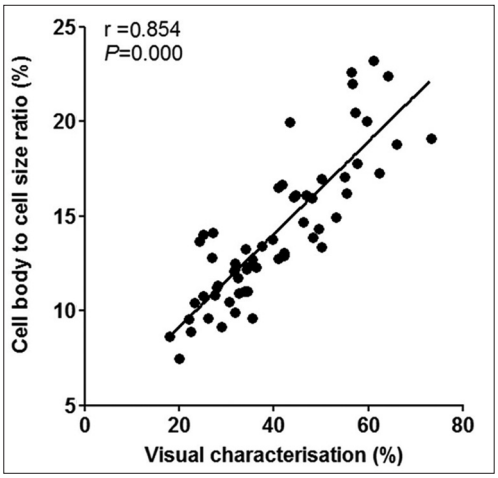

Figure 2: Relation of the cell body tot cell size ratio (cell body size as percentage of total cell size) with the percentage of activated microglia (activation stages 3-5) based on visual characterization for all rats in all analyzed brain areas. A regression line with associated Pearson's *r* is shown

characterization: $F_{3,56} = 23.86$, $P = 0.000$; cell body to cell size ration: $F_{3,56} = 22.48$, $P = 0.000$). Both of these measures for microglial activation show a similar pattern, with both groups of old rats showing increased microglial activation when compared to their young counterparts and further increase of microglial activation observed only in operated old rats.

Additional morphological characteristics of microglia in the IBA-1 stained sections are presented in Figure 4. Total cell size [Figure 4a] does not differ significantly among groups ($F_{3,56} = 1.88$, $P = 0.144$). The total cell body size [Figure 4b] differs significantly among groups ($F_{3,56} = 5.66$, $P = 0.002$), with a significant increase in cell body size only in old rats after surgery. The total size of the dendritic processes [Figure 4c] differs significantly among groups ($F_{3,56} = 4.33$, $P = 0.008$) with a significant decrease in the size of dendritic processes only in old rats compared to their young counterparts; however no effect of surgery was observed.

The number of cell bodies in the areas of interest did not differ significantly in our experiment (young control: 14.0 [12.6-15.3]; young surgery: 13.9 [12.3-15.5]; old control: 12.5 [11.7-13.3]; old surgery: 12.8 [12.0-13.6]; $F_{3,56} = 0.89$, $P = 0.454$).

DISCUSSION

The aim of the current study was to validate a newly developed semi-automatic image analysis method to analyze microglial morphology as marker for microglia activation in IBA-1 stained brain sections of rat. For this purpose, we adapted a method previously developed by Vinet *et al.*,[10] using image analysis with an intensity threshold and size filter, to obtain the cell body size and the total cell size of microglia. We used these parameters as a measure for morphological changes and presented cell body to cell size ratio as validated measure of microglial activation.

In this study, we compare different analysis methods for IBA-1 stained brain sections of young and aged rats with or without surgery.[17] Surgery has been associated with the development of postoperative cognitive dysfunction (POCD), including impairment of memory, attention, and executive functions.[19-22] Accumulating evidence indicates that surgery-induced (neuro) inflammation plays an important role in POCD development.[19,20,22-25] Although patients of all ages can experience POCD, persisting and more severe problems are mainly seen in elderly surgical patients.[22,26] Aging itself is also associated with neuroinflammatory changes in several brain regions, which may contribute to the increased incidence of POCD in elderly patients.[1,19,27] Hence, our study design allows us to investigate whether different analysis methods can distinguish the effects of an intervention associated with pathophysiological changes, and the effects of aging, which could be considered to be a more physiological process.

The cell body to cell size ratio shows a very strong positive correlation with microglial activation determined by the visual characterization method. Both visual characterization and the newly developed method reveal similar alterations related to aging and surgery, validating the cell body to cell size ratio as a marker for (classical) microglial activation. However, no correlation was observed with densitometry, nor did densitometry measurements distinguish microglial changes related to aging and surgery could be obtained. Seemingly densitometry may have insufficient sensitivity to distinguish the relatively modest changes in IBA-1 protein expression that accompanies the microglial activity associated with aging and surgery.[19,20,23] In addition, our new method provides outcome parameters that can be used for a more detailed analysis of microglia in an area of interest. Firstly, changes in the number of microglia can be determined. Second, by studying

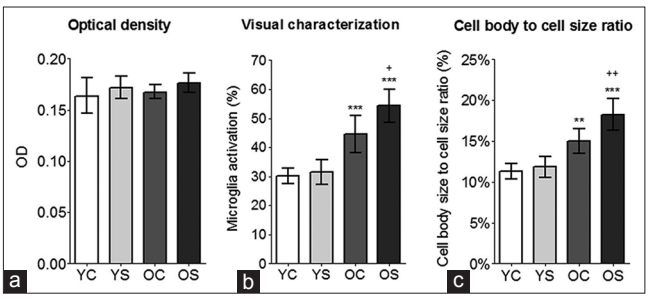

Figure 3: Potential markers for microglial activation in young and old rats with or without surgery. (a) The corrected optical density as marker for microglia activation; (b) The percentage of activated microglia (with an activation stage ≥ 3) based on visual characterization. (c) The cell body to cell size ratio (%) as marker for microglia activation. YC: young control; YS: young surgery; OC: old control; OS: old surgery. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to young rats. $^{+}P < 0.05$ and $^{++}P < 0.01$ compared to age matched controls

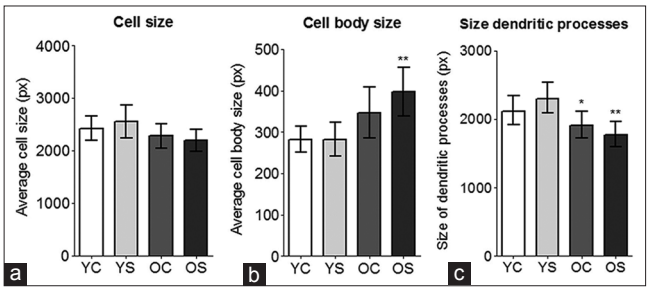

Figure 4: Morphological characteristics of microglia in young and old rats with or without surgery. (a) The total cell size (pixels) in the area of interest; (b) The total cell body size (pixels) in the area of interest; (c) The total size of the dendritic processes (pixels) in the area of interest. YC: young control; YS: young surgery; OC: old control; OS: old surgery. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to young rats

the total microglial cell size, cell body size and size of the dendritic processes separately, the specific characteristics of the morphological changes can be investigated. In our experiment for instance, it became apparent that (without significantly changing the number of microglia or cell size) there is a reduction of the size of the dendritic processes. Surgery, on the other hand, led to increased cell body size, specifically in the aged rats. Accordingly, a dystrophic deramificated morphology of microglia has been observed in old rats and humans,[6,28] whereas surgery was associated with a more classical microglial activation.[20,29]

The method proposed in this article has several limitations. Firstly, in this study we validated our method on relatively small areas (0.06 mm^2) in order to compare it to microglial activation based on visual characterization in those same areas. Therefore, only approximately 13 microglia/area were analyzed. However, we successfully explored the method on substantially larger areas (data not shown), further confirming its benefits.

Second, although we aim for an objective marker for microglial activation, the staining threshold and size filter have to be subjectively determined by researchers based on the staining intensity of the dendritic processes and cell bodies. A possible solution is to standardize the value of the intensity threshold as compared to the intensity threshold of the background (provided by the program).

Third, the outcome of the analysis is largely dependent on the quality of the staining. Currently, we do not have a method automatically to filter out artifacts. To circumvent this problem, it is possible to analyze only those sections with good quality by manually indicating the area of interest. However, this makes the analysis more time consuming and may lead to bias.

We developed this method to analyze IBA-1 stained microglia in DAB stained sections. Theoretically the analysis could be applied to fluorescent staining. However, the analysis depends substantially on a clear visibility of the dendritic processes and with fluorescent staining this is not always the case. If dendrites are not clearly visible, only the cell body size could be measured as an alternative to our method.

Finally, in this study we only looked at age-related and surgery-induced microglial activation. Microglial activation in these cases is expected to be relatively subtle. Therefore, we currently cannot confirm whether our method is applicable for other conditions in which microglia activation occurs, such as the most extensive neuroinflammation in brain trauma.

In conclusion, the method for analyzing microglial morphological changes as marker for microglial activation by semi-automatic image analysis of IBA-1 stained brain sections, described in this article, provides a novel sensitive marker for microglial activation in rats, which is quick and easy to perform and provides additional information about the morphological characteristics of microglia. Although conclusions about microglial activation based on morphological characteristics alone should be drawn with great caution, this novel analysis method could, combined with other markers related to microglial activation, contribute to research in the field of neuroimmunology.

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Case Report

Posterior reversible encephalopathy syndrome due to seronegative systemic lupus erythematosus

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ABSTRACT

Posterior reversible encephalopathy syndrome (PRES) is a neurotoxic state coupled with a unique computed tomography or magnetic resonance imaging (MRI) appearance. Recognized in the setting of a number of complex conditions (preeclampsia/eclampsia, allogeneic bone marrow transplantation, organ transplantation, autoimmune disease and high-dose chemotherapy) in the imaging, clinical and laboratory features of this toxic state are becoming better elucidated. We are presenting a case of PRES due to seronegative systemic lupus erythematosus, with MRI findings of diffuse vasogenic edema.

Key words: Posterior reversible encephalopathy syndrome, seronegative systemic lupus erythematosus, vasogenic edema

INTRODUCTION

Posterior reversible encephalopathy syndrome (PRES) is a clinicoradiological entity that was well-described by Hinchey *et al*.[1] in 1996. This condition has been designated by a variety of names (reversible posterior leukoencephalopathy syndrome, reversible posterior cerebral edema syndrome, and reversible occipital parietal encephalopathy). PRES is now the accepted term but has been challenged recently based on the risk of neurological impairment and up to 15% mortality rate.[2] The most common clinical symptoms and signs are headache, altered alertness and behavior ranging from drowsiness to stupor, seizures, vomiting, mental abnormalities including confusion and diminished spontaneity and speech, and abnormalities of visual perception.[1] Hypertension is the most commonly identified cause of PRES, followed by medications, eclampsia and other systemic factors. The pathophysiology of hypertension related to PRES is due to a failure of cerebrovascular autoregulation, which in turn results in vasogenic edema. Nonhypertensive PRES may be due to an autoimmune or immune response to various stimuli.

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Usually, it is a reversible phenomenon, as indicated by the name, but if not recognized early and treated appropriately, permanent brain injury may ensue.[3]

CASE REPORT

A 55-year-old female, known case of hypertension, hypothyroidism, old treated pulmonary tuberculosis (12 years back) admitted with the chief complaints of generalized swelling, recurrent vomiting and weakness of all 4 limbs for 3 months and altered sensorium for 1-week duration. Patient was initially evaluated outside and following studies elicited no abnormalities: complete blood count, kidney function tests, liver function tests, blood glucose, and electrolytes. Abdominal ultrasonogram detects bilateral pleural effusion (right > left side) and free fluid in pelvis. Nerve conduction studies of all 4 limbs revealed mild asymmetrical large fiber, motor axonal neuropathy in lower limbs > upper limbs, whereas electromyography revealed acute denervation in proximal and distal muscles of both lower limbs. Magnetic resonance imaging (MRI) of spinal cord showed spondylotic changes with mild disc dehydration and posterior disc bulge at L4-L5 level. Vasculitic profile, including antinuclear antibody (ANA) three was negative. She was managed with intravenous (i.v.) fluids, levothyroxine, antihypertensive, and i.v. methylprednisolone (1 g daily for 5 days). She improved clinically to some extent and was discharged. Soon within 2 days after discharge, she became confused and agitated and was brought to our department as a case of encephalopathy.

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A detailed history revealed past history of arthralgias and photosensitivity, for which she was not on any treatment. On clinical examination, she was in grade 3 encephalopathy with Glasgow Coma Scale (GCS) of 8/15, hemodynamically stable, with generalized areflexia. Rest of the systemic examination was normal. Serum urea and creatinine levels were on higher side (serum urea = 88, serum creatinine = 1.9), whereas rest of the baseline investigation and biochemistry were within the normal limits. Ultrasound abdomen showed bilateral raised cortical echogenicity, with mild ascites. Repeat vasculitic profile including ANA, anti-ds-DNA, Perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA), cytoplasmic antineutrophil cytoplasmic antibodies (C-ANCA), anticentromere were negative. Noncontrast computed tomography head was suggestive of diffuse brain edema [Figure 1], while MRI brain showed diffuse bilateral edema in white matter predominantly in occipital region [Figures 2-4]. Cerebrospinal fluid was acellular with raised protein (92 mg/dL) and glucose (126 mg/dL). She was restarted with i.v. methylprednisolone (1 g daily) for 5 days and followed by oral prednisolone (1 mg/kg body weight). After 2 weeks, the patient's condition had improved to the point that she was conscious with GCS of 15/15, could ambulate without assistance and showed nearly normal strength in all 4 limbs. Follow-up MRI [Figure 5] showed significant resolution of white matter edema. Hence, on the basis of arthralgias and photosensitivity in past and features of polyserositis, renal impairment and neurological dysfunction in the form of encephalopathy, a likely diagnosis of seronegative systemic lupus erythematosus (SLE) presenting first time as PRES, was established. Patient is presently on oral tapering dose of steroids along with supportive treatment.

DISCUSSION

The exact pathophysiological mechanism of PRES remains uncertain. To date, three hypotheses have been proposed, which include: (1) cerebral vasoconstriction with subsequent infarcts of the brain, (2) failure of cerebral autoregulation with consequent vasogenic edema, and (3) endothelial damage with disruption of the blood-brain barrier (BBB) causing fluid and protein transudation in the brain. The pathophysiology of PRES in SLE is also less well understood. In most cases of SLE-related PRES, immunosuppresants used to treat the SLE were suggested as causative factors, though lupus itself or SLE-related hypertension, antiphospholipid antibodies or renal failure might also be contributive. Abnormal endothelial activation, dysfunction and leukocyte tracking have recently been documented to

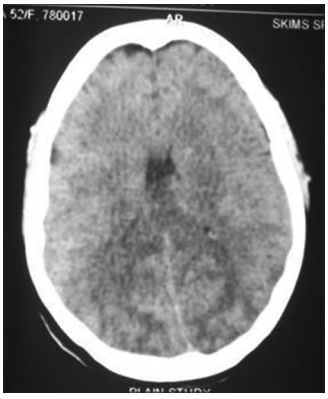

Figure 1: Noncontrast computed tomography head suggestive of diffuse brain edema

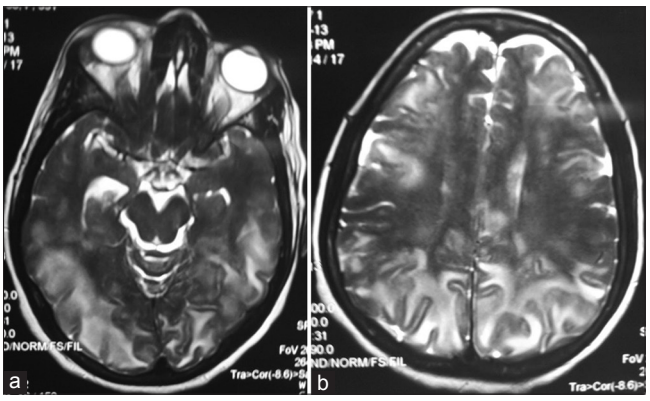

Figure 2: T2-weighted magnetic resonance imaging showing. (a) edema in posterior circulation region and; (b) diffuse white mater edema

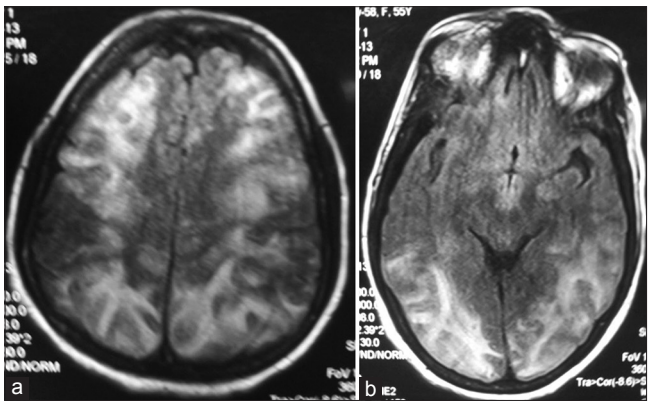

Figure 3: Fluid attenuation inversion recovery image showing (a) suggestive of diffuse white matter edema and (b) edema in posterior circulation region

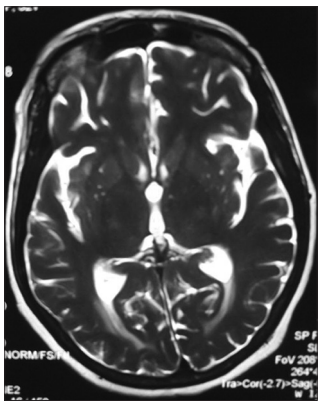

Figure 4: Repeat T2-weighted magnetic resonance imaging showing significant reduction in brain edema

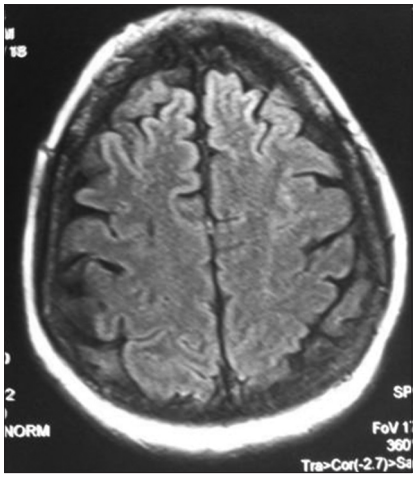

Figure 5: Repeat fluid attenuation inversion recovery image showing resolution of white matter edema in bilateral cerebral hemisphere

cause brain and systemic hypoperfusion, which may be causative factors for PRES in SLE. On the other hand, endothelial cell activation is one of the pathogenic hallmarks of neuropsychiatric SLE (NPSLE). It usually occurs after exposure to interleukin 1 (IL-1) and tissue necrotic factor-α (TNF-α), and may be enhanced by local release of IL-1 and IL-6. SLE patients with high SLE disease activity index have increased serum levels of TNF-α and other pro-inflammatory cytokines that may stimulate endothelial cells of intracranial vessels and astrocytes to produce nitric oxide, causing BBB damage and plasma leakage. In some cases the endothelial dysfunction together with hemodynamic factors may allow the leakage of blood plasma and large amounts of red blood cells resulting in secondary parenchymal hematoma. Histopathology showed the PRES manifestation result from NPSLE were due to focal cerebral edema associated with blood vessel injury and ischemic changes, although in many cases histopathology did not demonstrate specific lesions. SLE patients might develop reversible focal neurological deficits, which responded to steroid therapy.[4]

Even though the classical neurolupus includes seizures and psychosis, a number of other features such as myelopathy, optic neuropathy, meningitis, cognitive dysfunction, and cerebral infarction could be seen in SLE. PRES has been claimed as a particular form of neurological manifestation of SLE with characteristic MRI findings and a usual good outcome. Antihypertensive, antiepileptic, and supportive care are the mainstay of treatment.[5]

In some cases, the diagnosis of PRES remains in doubt. In this situation, regression of the clinical and radiological abnormalities with appropriate treatment supports the diagnosis. Thus, repeated brain imaging is beneficial of diagnosis.[6] Radiographically, PRES is heralded by relatively symmetric, reversible T2 hyperintensities affecting the posterior aspects of the brain, namely the occipital and parietal lobes. It is now known that this description is more of a general rule, and those asymmetric images can be seen, and can involve the deep grey matter as well as the frontal and temporal lobes. The advent of diffusion weighted imaging helped clarify that the MRI changes were not due to ischemia or cytotoxic edema, but due to vasogenic edema.[7]

In our case, as per diagnostic criteria for SLE,[8] more than four well documented features were present, that is, history of arthralgias, photosensitivity, polyserositis, renal impairment and nervous system involvement. Although her vasculitic profile was negative, but her brain imaging was suggestive of diffuse white matter edema, she was treated as seronegative SLE presenting as PRES. She received pulse therapy of i.v. methylprednisolone followed by oral steroids as per body weight. Patient improves clinically and her repeat imaging, done after 6 weeks, was almost normal.

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Case Report

Toluene-induced leukoencephalopathy with characteristic magnetic resonance imaging findings

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ABSTRACT

Toluene-induced leukoencephalopathy is a frequently seen medical condition worldwide; however the lack of specific clinical manifestations and laboratory tests makes it difficult to diagnose. Neuroimaging and medical history are often crucial to diagnosis of this disorder. In this report, a case is presented of a patient suffering from toluene-induced leukoencephalopathy with deteriorating cognition impairment and characteristic magnetic resonance imaging (MRI) findings, typified by a "sunflower-like" change in T2-weighted imaging. In addition, the pharmacokinetic properties of toluene are reviewed, as well as the clinical manifestations, typical MRI findings, neuropathology, possible mechanism, and treatment of toluene-induced leukoencephalopathy.

Key words: Leukoencephalopathy, neuroimaging, occupational protection, toluene

INTRODUCTION

Leukoencephalopathy is a broad term used to describe a number of leukodystrophy-like diseases. Impairment of the nervous system due to toluene inhalation is a frequent source of toxin-induced leukoencephalopathy. Reported symptoms are mostly nonspecific, such as headache, dizziness, and impaired cognition.[1] Toluene-induced leukoencephalopathy may, therefore, go undiagnosed by doctors and neurologists, especially in patients without hematopoietic system impairment. We present here an interesting case of toluene-induced leukoencephalopathy with characteristic magnetic resonance imaging (MRI) findings.

CASE REPORT

A 44-year-old male patient presented to the clinic with dizziness, headache located primarily in the occipital region, memory decline, and a dull response, occurring over a period of 1-month with gradual deterioration. The patient had been a shoemaker for 3 years and had daily contact with toluene-containing glue for around 10 h a day. The patient had no history of vascular risk factors such as hypertension, diabetes, smoking, or alcohol, and there was no family history of similar symptoms. No other features of note were present in the patient's medical history. Physical and neuropsychological examination revealed memory decline, impaired calculation, and visuospatial impairment, with a mini-mental state examination (MMSE) score of 23. Blood tests showed increased levels of total cholesterol (6.7 mmol/L) and triglycerides (3.6 mmol/L). Blood cell counts, fasting glucose levels, liver and renal function, and levels of folic acid, vitamin B_{12}, and ferritin were all normal. The possibility of syphilis or human immunodeficiency virus infection was also excluded. Intracranial pressure was 180 mmH_2O as measured by lumbar puncture; however no abnormalities were observed upon further examination of the cerebrospinal fluid. Levels of protein (0.37 g/L), glucose levels (3.67 mmol/L), chloridion (124.4 mmol/L), aspartate transaminase (15.2 U/L), lactate dehydrogenase (15.9 U/L) and adenosine deaminase (15.18 U/L) were all normal. The total cell count was 0.008 × 10^9/L, and the white blood cell count was 0.001 × 10^9/L. Pandy's test was negative. No evidence of bacterial infection was

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detected using Gram stain, ink stain, acid-fast stain, or *Mycobacterium tuberculosis* culture. DNA-based tests for cytomegalovirus, Epstein-Barr virus, and herpes simplex virus were all negative. Chest X-rays, electrocardiogram, and ultrasonography of the heart and abdomen detected nothing unordinary; however, a slight abnormality was observed on the electroencephalogram. Most importantly, cranial MRI of the patient showed diffuse cerebral white matter hyperintensity [Figure 1], which resembled a characteristic "sunflower-like" change in T2-weighted images. The patient was diagnosed with toluene-induced leukoencephalopathy according to the "Diagnostic Criteria of Occupational Acute Toluene Poisoning" of China. After 2 weeks of treatment with mannitol, hyperbaric oxygenation, and neurotrophic medicine including intravenous ganglioside GM1, Vitamins B12, B1, and B6, huperzine-A tablets, and Oxiracetam capsules, the patient's symptoms were mostly resolved, with an MMSE score of 26 on discharge.

DISCUSSION

As a common component of experimental organic solvents and industrial products such as glues, inks, paints, and paint thinners, toluene is a ubiquitous solvent. The number of people suffering from medical conditions caused by toluene exposure is growing rapidly, particularly among early adolescents experimenting with volatile substances or inhalants and workers in developing countries who lack occupational protection. After inhalation, toluene is quickly absorbed by the lungs, then enters the brain due to its high lipophilicity and accumulates. Following metabolization in the liver, it is excreted by the kidneys mostly in the form of hippuric acid.[2] Chronic toluene inhalation primarily damages the central nervous system (CNS), especially the white matter, and causes toluene-induced leukoencephalopathy and psychosis.[2-4] Although cognitive impairment is the most prominent symptom, additional clinical manifestations may differ depending on the brain regions that are affected, including ataxia, tremors, psychiatric disorders, Parkinson's disease, and temporal lobe epilepsy.[5] The diagnosis of toluene-induced leukoencephalopathy is primarily based upon the history of contact with toluene, clinical manifestations caused by damage to the nervous system, and exclusion of other medical conditions with similar symptoms. Neuroimaging and medical history are crucial to the diagnosis of toluene-induced leukoencephalopathy, as opposed to other types of encephalopathy such as heroin-related encephalopathy or hypoxic-ischemic encephalopathy.

Several studies have focused on the characteristic features of toluene-induced leukoencephalopathy

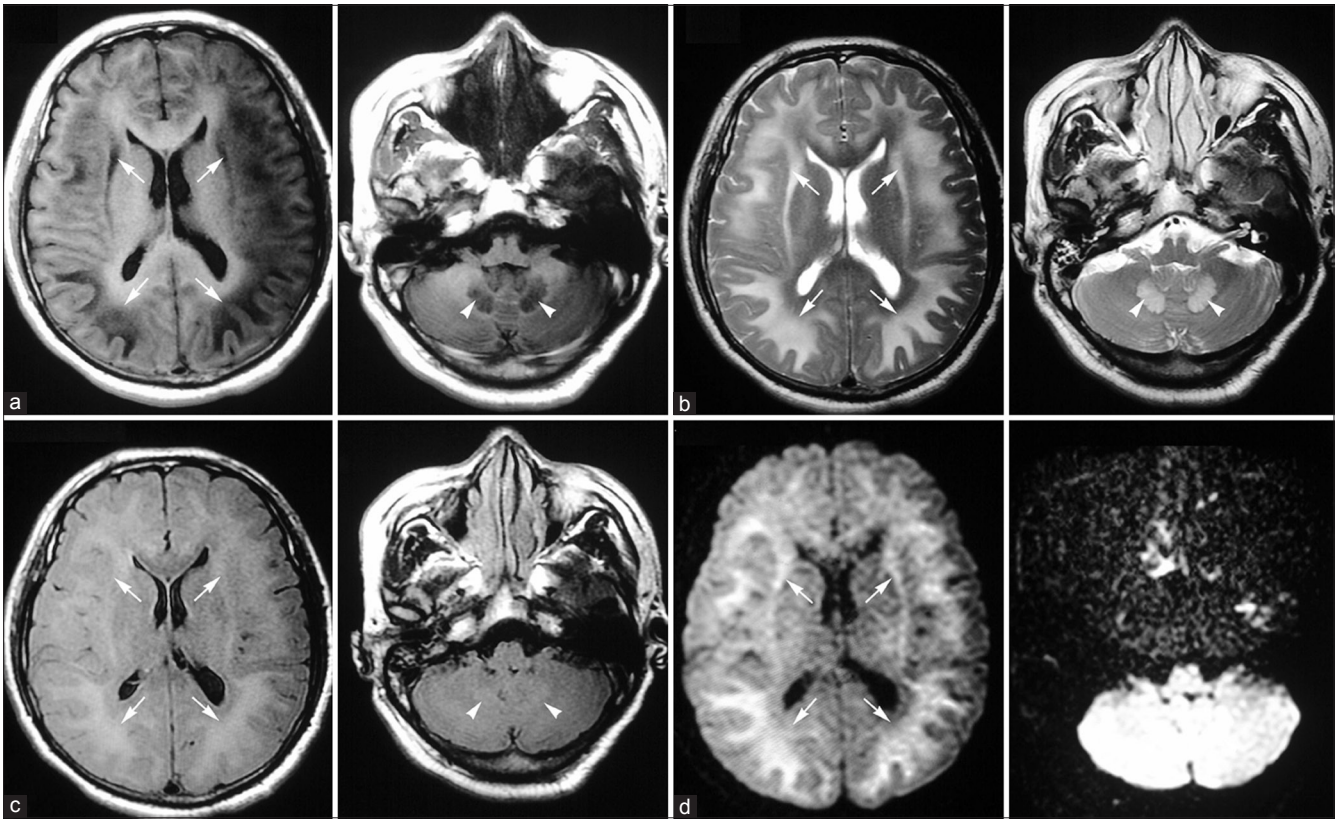

Figure 1: Magnetic resonance imaging shows symmetrical periventricular white matter hyperintensity (arrows) in axial T2-weighted (b), fluid-attenuated inversion recovery (c), and diffusion-weighted imaging (d) images, along with corresponding changes in T1-weighted images (a). The characteristic "sunflower-like" change is readily apparent, particularly in the T2-weighted image. Increased signal is also visible in the cerebellar dentate nuclei (arrowheads). Gray matter-white matter differentiation is preserved

detectable by neuroimaging. It has been reported that in chronic toluene abusers, MRI often reveals diffuse atrophy of the cerebrum, cerebellum, and brainstem (also discernible with computed tomography), increased periventricular white matter signal on T2-weighted images, and loss of gray matter-white matter differentiation across the cortex. The severity of these abnormalities is considered to be associated with the duration of abuse and concentration of toluene.[2,5] In this case, MRI shows a characteristic "sunflower-like" change and abnormal signal in the cerebellar dentate nuclei [Figure 1], which can be explained by the lipid-dependent distribution of toluene within the CNS and its resulting neuropathology.[1,2,6] Unfortunately, follow-up cranial images were not available for this patient, but according to a previous study, no significant improvement was found in the cranial MRI of a chronic toluene abuser following 5 months of abstinence.[7]

Previous neuropathological studies have reported that the brains of chronic toluene abusers show thinning of the corpus callosum and prominent pathology in the periventricular regions. Microscopically, diffuse and ill-defined myelin pallor is seen in cerebral and cerebellar white matter, along with evidence of neuronal loss in the cerebral cortex, basal ganglia, and cerebellum, intense, reactive gliosis, and giant axonal degeneration in the long tracts of the spinal cord.[1,2,6] The degree of myelin and axonal loss is considered to be associated with the extent of exposure, age of onset of toluene abuse, concurrent abuse of other substances, and polymorphisms in the gene encoding the enzyme aldehyde dehydrogenase, which is the main hepatic enzyme responsible for metabolizing toluene.

Although not fully understood, impairment of NMDA, GABA receptor signaling, and mitochondrial function may be involved in the mechanism of toluene-induced leukoencephalopathy.[8,9] Damage to white matter caused by toluene appears to be irreversible, and no treatment is currently known other than abstinence or general neurotrophic compounds. Melatonin was recently reported to reverse the reduction in dendritic branching in animals exposed to toluene, which might be a promising treatment for toluene-induced leukoencephalopathy.[10] Further studies are needed in order to develop more effective therapeutic strategies for this disease.

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Case Report

Malignant middle-cerebral artery territory infarction in tuberculous vasculitis

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ABSTRACT

Intracranial large vessel involvement is an unusual complication of tuberculous meningitis. The authors report a 39-year-old female presenting with an episode of seizure, followed by rapid decline in sensorium without prominent systemic features. An initial cranial magnetic resonance imaging revealed tuberculomata and patchy infarcts. Despite antituberculous therapy, she progressively worsened. A cranial computed tomography scan done following the worsening revealed a massive middle-cerebral artery (MCA) infarct. Unfortunately, the patient died in spite of decompressive craniotomy. Malignant MCA territory infarct is a rare and potentially fatal complication of tuberculous meningitis.

Key words: Arteritis, malignant middle-cerebral artery territory infarct, tuberculous meningitis

INTRODUCTION

Involvement of small and medium sized vessels of the intracranial vasculature is well recognised in tuberculosis. Large artery involvement is however a rare manifestation of tuberculous vasculitis. Here, we present the first reported case in the literature of a patient who presented with a malignant middle-cerebral artery (MCA) territory infarct as a manifestation of tuberculous vasculitis.

CASE REPORT

A 39-year-old female had presented to her general practitioner 15 days prior to this admission with a history of cough with expectoration and fever. Sputum stained with Ziehl-Neelsen stain revealed the acid fast bacilli morphologically characteristic of mycobacterium tuberculosis. A few days prior to this admission, she had had an episode of generalized tonic-clonic seizure followed by progressive decline in sensorium. Cranial magnetic resonance imaging (MRI) [Figure 1a-c] done at the referring hospital revealed multiple heterogeneously enhancing nodular lesions of varying sizes involving right posterior parietooccipital and frontal subcortical and cortical regions with significant thickening of adjacent leptomeninges and exudates. These features were classical of focal cortical and subcortical tuberculomata with cerebritis and leptomeningitis, especially on a background of acid-fast bacilli detected in sputum. There was also cerebral edema with effacement of the ventricular system, sulci and cisterns and focal vasogenic edema around the right parieto-occipital lesion with patchy areas of diffusion restriction representing arteritis-induced-infarcts. Antituberculous drugs (isoniazid 300 mg/day, rifampicin 600 mg/day, pyrazinamide 1500 mg/day and ethambutol 800 mg/day), dexamethasone 8 mg twice a day and phenytoin (300 mg/day) were administered. Sensorium had however gradually deteriorated, and she had become unresponsive since a day when she was transferred to our hospital.

On examination, she was hemodynamically stable, pyrexial and anemic. Neurologically she was comatose with decerebrate movements of right upper limb to pain. There were no meningeal signs. On the oculocephalic maneuver, there was failure of adduction of right eye and upgaze was absent. Pupils were 5 mm on the right side and 2 mm on the left side. Optic fundi were unremarkable. Tendon reflexes were brisk bilaterally, and plantars were extensors on both sides. Investigations revealed: hemoglobin

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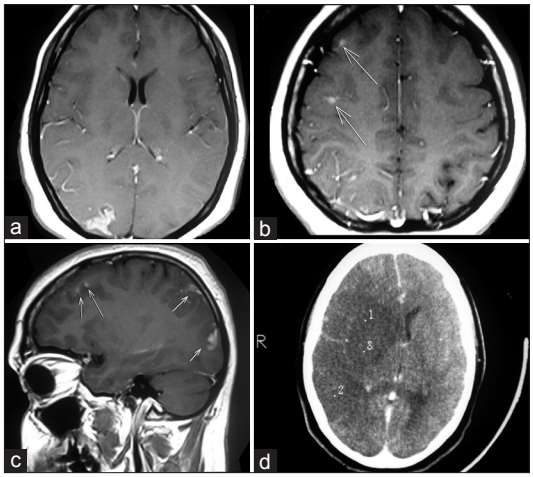

Figure 1: Tuberculous meningitis with arteritis leading to malignant middle cerebral artery (MCA) infartion. Gadolinium enhanced axial T1-weighted magnetic resonance images shows (a) enhancing leptomeningeal exudates with subcortical, juxta cortical and cortical granulomata in the right posterior parietooccipital region; (b) fronto-parietal cortical granuloma with leptomeningeal enhancement; (c) Fronto-temporo-parieto-occipital cortical, juxta cortical granuloma with leptomeningeal enhancement. (d) contrast enhanced computerized tomography (CT) axial image shows malignant infarct involving the entire right MCA territory with compression of the right lateral ventricle and midline shift to the left side. One and three marked regions in CT represent basal ganglionic region and two represent temporo occipital region with no significant hemorrhagic component

12.9 g/dL; total white cell count 14.8 × 10^9/L; differential count-polymorphs 84%, lymphocytes 13%, myelocytes 1% and stab forms 2%; platelet count 19.0 × 10^9/L and erythrocyte sedimentation rate 29 mm/h. A whole list of investigations including blood sugar, renal and liver functions, electrolytes, coagulation profile, urinalysis, human immunodeficiency virus test, antinuclear antibodies, ds-DNA, rheumatoid factor, venereal disease research laboratory, hepatitis B surface antigen, C-reactive protein, antineutophil cytoplasmic antibodies, lupus anticoagulant and antiphospholipid antibody tests were noncontributory. Cranial enhanced computed tomography scan [Figure 1d] revealed an acute nonhemorrhagic complete right MCA territory infarct and few enhancing lesions in and around the sulci in the right occipital, posterior parietal and high frontal lobes with severe right ventricular compression. Marked cerebral edema and midline shift were observed. Decompressive surgery in the form of right fronto-parieto-temporal craniectomy was done as for malignant MCA infarct. Histopathological evaluation of leptomeningeal tissue obtained during surgery revealed features of chronic meningitis with dense lymphohistiocytic infiltrate forming microgranuloma surrounding the meningeal blood vessels. Clinically she deteriorated with bilateral pupillary dilatation on day 3 of admission with hypotension. She, unfortunately, succumbed to the illness on day 4 of admission.

DISCUSSION

Tuberculous vasculitis is an important cause of stroke in the young in developing countries. Stroke in tuberculous meningitis occurs in 15-57% of patients especially in advanced stage and severe illness and are usually multiple, bilateral and located in the basal ganglia, especially the tubercular zone, which comprises of the caudate, anterior thalamus, anterior limb and genu of the internal capsule. Cortical stroke can also occur due to the involvement of the proximal portion of the middle, anterior and posterior cerebral arteries, as well as the supraclinoid portion of the internal carotid and basilar arteries.,[1] While pathological changes suggestive of intracranial vasculitis are common in tuberculosis even without corresponding clinical features, to our knowledge, this is the first reported case of malignant MCA territory infarct in tuberculous meningitis. The initial MRI features [Figure 1a-c] were fairly typical of tuberculosis, especially in the context of positive acid-fast bacilli in the sputum. In a pathological study of 23 postmortem cases of tuberculous meningitis, phlebitis was found in 22 and arteritis of varying degrees in 20. Thrombosis in the territory of MCA with infarction was seen in one of these patients. Both hemorrhagic and nonhemorrhagic infarcts were visualized.[2] Tuberculous vasculitis usually involves vessels that traverse the basal exudates or are located within the brain parenchyma.[3] Arteries running through the subarachnoid space may show obliterative endarteritis with inflammatory infiltrates in their walls and marked intimal thickening.[4] Various stroke syndromes are known with involvement of different regions of the brain including basal ganglia, thalamus, cerebral hemispheres and cerebellum with varying outcomes.[5,6] In our patient, the infarct was extensive with significant mass effect and transtentorial coning. The neuro-ophthalomological findings noted were suggestive of midbrain involvement (right pupillary mydriasis, right medial rectus involvement and paralysis of upgaze). The course of the disease was rapid and malignant despite antitubercular and steroid therapy.

Elective hemicraniectomy has been advocated as a life-saving therapeutic option in patients with complete MCA infarction.[7] Young age, involvement of the nondominant hemisphere and progressively worsening neurological status despite aggressive medical therapy warranted consideration of the surgical procedure. However, we were unsuccessful as the patient deteriorated and died despite aggressive treatment. Clinical deterioration despite surgery is well known to occur in malignant cerebral infarction.

In conclusion, we report a patient with malignant MCA infarct as a consequence of tuberculosis. Such a manifestation may portend a poor prognosis despite aggressive life-saving measures.

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Case Report

Primary diffuse large B-cell non-Hodgkin lymphoma of the cranial vault

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ABSTRACT

Primary non-Hodgkin lymphoma of the cranial vault with extra and intracranial extension in a nonimmunocompromised patient is extremely uncommon. Until date, only limited number of such cases has been reported in the literature and none was the lesion located as a diffuse swelling in the forehead. Imaging of the present case showed in a homogenous contrast enhancement mass involving the scalp of bifrontal supraorbital compartment and intracranial extra axial extension through the frontal bone with extension to the right orbit and right ethmoidal sinus. The intracranial mass was excised along with involved dura. Histopathology of the mass showed diffuse large B-cell non-Hodgkin lymphoma.

Key words: B-cell, cranial vault, non-Hodgkin lymphoma

INTRODUCTION

Primary cerebral non-Hodgkin lymphoma (NHL) is a rare group of neoplasm and are mostly intraaxial with frequent invasion of pachy and leptomeninges.[1] Primary malignant lymphoma arising from skull vault or scalp without involvement of the cerebral parenchyma and systemic or skeletal manifestation is also rare. Only few cases have been reported in the literature.[2-4] Involvement of bone is commonly seen with secondary NHL including that of the scalp.[1] NHL originating primarily in the skeletal location is seen only up to 4% of cases. Initial involvement of the skull is extremely rare at presentation, and primary cranial vault lymphoma constitutes only 0.2% of lymphoma cases.[5] However, primary NHL arising only in the cranial vault or scalp without involving the cerebral parenchyma or without systemic involvement is extremely rare. Here, we are reporting this case because of its rare occurrence.

CASE REPORT

A 37-year-old male patient was admitted from our outpatient department with a history of a progressively growing painless scalp swelling over the frontal region for 2 years, headache for 1 year. He had progressive loss of vision for 8 months and behavioral changes for the last 6 months. He had no past history of fever and head injury. He was normotensive and nondiabetic. The clinical examination revealed a firm and well-defined nontender immovable solitary subcutaneous swelling, measuring 10 cm × 8 cm without any bruit or pulsation in the frontal region [Figure 1]. He had no lymphadenopathy or hepatosplenomegaly. Physical examination of other systems was normal. On ocular examination, visual acuity in both eyes was of no perception of light. Fundoscopy revealed bilateral atrophic papilledema. Hematological examination showed hemoglobin level of 11.0 g/dL, white blood cell count of 5,840/mm^3 with a normal differential and platelet count of 234,000/mm^3. Blood biochemistry was also normal. Antihuman immunodeficiency virus antibody was negative. Magnetic resonance imaging (MRI) of the head showed a fairly large mass with mixed signal intensity of the skull vault within homogenous contrast enhancement involving the scalp of bifrontal supraorbital compartment. The mass was extending into the right orbit and right ethmoidal sinus through its anterosuperior part [Figure 2]. However muscle cone, eyeball and optic nerves were not involved. Left orbit and left ethmoid was not involved. The mass was located within diploe of the frontal bone with intracranial extension through frontal bone with extraaxial extension into bifrontal compartment. The

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frontal lobes were displaced posteriorly, and there was reactive edema in both frontal lobes. Contrast-enhanced computed tomography of the neck, chest, abdomen and pelvis was normal.

The patient underwent surgery after other preoperative investigations were completed. Bicoronal skin incision was made, and skin flap was dissected off the frontal extracranial mass which was then excised. The frontal bone was found to be moth eaten, and bifrontal craniectomy of the involved bone was performed. The intracranial portion of the mass was found to be extraaxial with involvement of the underlying dura. The intracranial mass was excised along with involved dura; however, there was no involvement of underlying cortex. The mass along with the involved bone and dura were sent for histopathological examination.

Histopathology and immunohistochemistry was conducted. Histopathology showed a highly cellular tumor composed of cells in lobules separated by fibrovascular septae. Cells had a moderate cytoplasm, cleaved nuclei and brisk mitotic activity [Figure 3]. On immunohistochemistry tumor cells were immunoreactive for leucocyte common antigen, CD20, CD10, CD3, and CD5 highlighting background T-lymphocytes. The tumor cells were immunonegative for cytokeratin, epithelial membrane antigen, desman, synaptophysin, CD21, CD30 and S-100. The Mib-1 labeling index was approximately 60%. The impression was NHL; diffuse large B-cell phenotype.

After receiving the histopathology and immuno-histochemistry report lumbar puncture and bone marrow biopsy, was performed to prove that was negative for lymphoma cells.

The patient was referred to Oncology Department for radio- and chemotherapy. Whole brain was irradiated with 45 Gy in 25 fractions, involving field irradiation with 10.8 Gy in 6 fractions over 35 days. After completion of radiotherapy, he was treated with 4 cycles of systemic chemotherapy (rituximab + cyclophosphamide, doxorubicin, vincristine, and prednisolone). He was administered 500 mg/m^2 of rituximab on day 1 and 750 mg/m^2 of cyclophosphamide, 50 mg/m^2 of doxorubicin, 2 mg/m^2 of vincristine on day 2 and oral 100 mg tab of prednisolone on 1-5 days. The cycles were repeated after 3 weeks.

Physical examination of the patient, complete hemogram test and serum lactate dehydrogenase level was followed-up at 2 months interval for 1 year. MRI was repeated at 6 months interval. After 12 months of follow-up, no signs of recurrence have been observed.

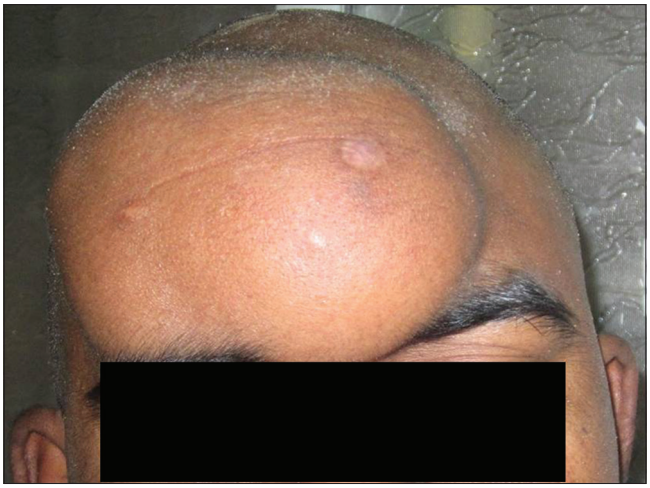

Figure 1: Clinical photograph shows a well-defined swelling over the frontal region

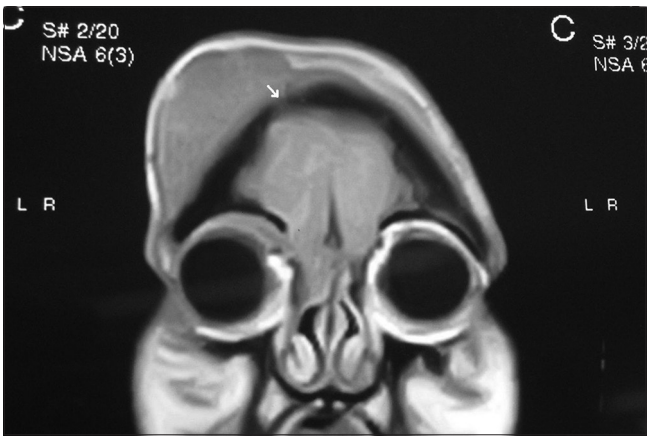

Figure 2: Magnetic resonance imaging of the brain showing a fairly large mass with mixed signal intensity involving the scalp of bifrontal supraorbital compartment with extension into the right orbit and right ethmoidal sinus through its anterosuperior part (marked with a white arrow)

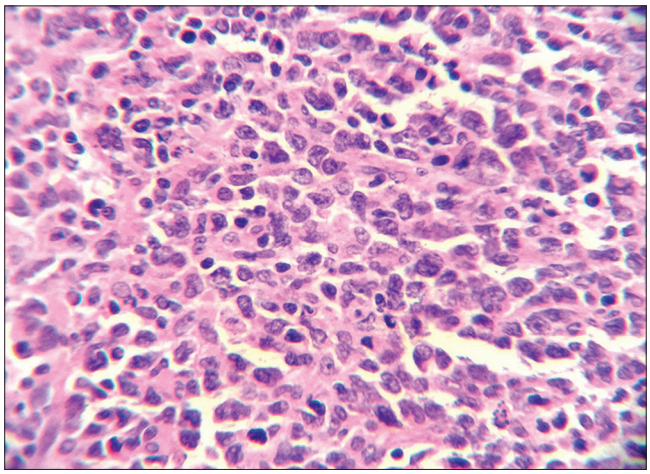

Figure 3: Mature nonneoplastic lymphocytes admixed with atypical lymphoid population of cells, latter having abundant cytoplasm, round to convoluted nuclei with prominent nucleoli (HE, ×400)

DISCUSSION

Primary lymphoma of the skull vault is extremely rare neoplasm and NHL originating in bone has been

reported in only 4% of cases, which is typically seen in femur, tibia, pelvis, spine mandible, and scapula.[1,6] Skull vault lymphoma needs special attention from other bony lymphomas because the cerebral structures may get involved by direct invasion. Usually these are intraaxial with pachy- and leptomeningeal involvement being frequent. True primary malignant lymphoma of the bone is defined as a solitary mass lesion without any evidence of disease at another site and no systemic dissemination within 6 months of tumor detection.[7,8] The present case thus fulfills the criteria for primary malignant lymphoma of bone. Primary cranial vault lymphomas have been reported in immunocompromised or trauma patients;[9,10] however primary NHL of the cranial vault with extra- and intra-cranial extension without systemic or skeletal manifestation in a nonimmunocompromised or nontraumatic is extremely uncommon.[4] Only 19 cases have been reported in the literature out of which six cases had tumor localization in the frontal region.[11] The incidence of lymphomas in the central nervous system are increasing nowadays due to the increasing incidence of immunocompromised patients. Primary malignant lymphomas involving only the cranial vault and scalp without any systemic or cerebral parenchymal involvement is rare, and only a few cases have been reported in the literature.[5-9] The initial symptoms and signs of cranial vault lymphoma include a painless lump in the scalp, headache because of bone destruction or tumor infiltration of meninges and focal neurological deficits secondary to tumor infiltration of the cerebral cortex.[11] Lymphoma cells may infiltrate the spaces within the diploe and along the emissary veins extend to infiltrate the soft tissues on either side of the bone. Malignant lymphomas of the skull bone may present with bony changes at first and by infiltration may lead to complete destruction of the skull. In our case, there was destruction of frontal bone and tumor mass infiltrated the underlying dura. Furthermore, there was no focal neurological deficit, and postoperative cerebrospinal fluid cytology was negative for lymphoma cells. Involvement of the meninges has been speculated due to the extension through the diploic spaces along the emissary veins and nerves that pass through the dura to the leptomeninges.[12] Being rare only a high index of suspicion can diagnose these cases prior to histopathology.

The incidence of large cell lymphomas reported in the literature is approximately 4.68 cases/100,000 persons per year.[3,10,11] These tumors may extend into the brain or the scalp mimicking a meningioma.[4] Most of these reported cases were in the age group of 50s and 60s whereas age of our patient was only 37 years. Differential diagnosis of cranial vault NHL with intra and extracranial extensions include metastatic or primary skull tumors and intraosseous meningioma. There is widespread cortical destruction in these cases, while little cortical destruction is noted in lymphomas. Total surgical excision is rarely possible in lymphomas and treatment of these tumors is surgical removal, followed by radiotherapy and chemotherapy. Treatment with irradiation and corticosteroids often produces a partial response. Therefore, tumor recurs in > 90% of patients. Median survival is 10-18 months in immunocompetent patients and less in immunocompromised patients.[13-16] In our case, there was no sign of recurrence after 12 months of follow-up.

The prognosis of primary cranial vault malignant lymphoma is unknown, and any involvement of cerebral structures and systemic involvement suggests an unfavorable prognosis. Until date, no optimum treatment for cranial vault malignant lymphoma has been established. Therefore, a thorough search is necessary to improve the treatment of cranial vault lymphoma treatment and its prognosis.

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Review Article

Role of the neuromuscular ultrasound in the diagnostic of the multifocal motor neuropathy

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ABSTRACT

Multifocal motor neuropathy (MMN) is the one of the most common acquired immune-mediated inflammatory disorders of the peripheral nervous system. The diagnosis is based on the distribution pattern of the neurological semiology and the pathological changes of nerve conduction studies (NCS) in classical cases. However, in cases with subtle clinical presentation, an extended diagnostic workup may be needed, such as cerebrospinal fluid examination, laboratory tests, and nerve biopsy. NCS remain nowadays fundamental not only for the diagnosis, but also for the follow-up and measurement of response to immune-treatment in MMN. New challenges arose though, on how best to acquire a static and dynamic imaging of the peripheral nerves, aiming to provide a holistic approach to the nerve impairment. According to the literature, neuromuscular ultrasound is able to detect in MMN patients thickened or swollen cervical roots, peripheral nerves or brachial plexus, findings that suggest ongoing inflammation. This review provides a timely update on the nerve ultrasound findings in MMN.

Key words: Brachial plexus, conduction block, immune-mediated neuropathies, multifocal motor neuropathy, nerve hypertrophy, nerve ultrasound

INTRODUCTION

Multifocal motor neuropathy (MMN) is an intriguing peripheral nerve disease with a prevalence of 1-2/100,000 adults.[1] Several diagnostic criteria have been proposed, mainly summarizing the slowly progressive, asymmetric weakness, with a striking predilection for the upper extremities, whereas sensory fibers and upper motor neuron involvement fail in the disease course.[2] Although the detection of conduction block remains the electrophysiological hallmark of the disease, it is important to recognize that it may not be possible to demonstrate this finding even after careful studies, because blocks may be activity-dependent, and the site of pathology may be very proximal in the brachial plexus or nerve root level.[2-5]

The first papers defined conduction block as a 20-30% amplitude or area reduction in the distal compound muscle action potential (CMAP) if the CMAP duration did not exceed 15% greater than normal. Computer modeling of conduction block and temporal dispersion in an animal model has demonstrated that up to 50% area reduction of the proximal to distal CMAP can be due entirely to interphase cancellation. Similar studies in human have shown that distal CMAP duration and proximal CMAP duration prolongation are important factors for the definition of conduction block in the median nerve segment over the forearm: the shorter the distal duration and proximal duration prolongation the less CMAP amplitude reduction is needed to diagnose a conduction block.[2] The association between MMN and immunoglobulin M (IgM) antiganglioside GM1 (anti-GM1) antibodies have already been suggested in the literature, however, the diagnostic accuracy of anti-GM1 testing in diagnosing MMN is unclear. The literature reports the presence of anti-GM1 IgM antibodies in between 30% and 80% of MMN patients.[2]

Meanwhile, neuromuscular ultrasound is an easily applicable and safe method for studying structural changes in peripheral nerves. Various ultrasound studies have reported pathological ultrasound changes in MMN patients, reporting consistently an asymmetric, inhomogenous increase of the nerve cross-sectional area (CSA).[6-9] Three studies have

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reported controversial findings on the correlation between sonographic and electrophysiological results in MMN patients.[6,8,10] In view of the severe functional disability of MMN patients, it remains unknown that of these two methods could better highlight the functional and clinical status of these patients.[11]

The aim of this review is to provide a timely update on the role of the neuromuscular ultrasound in the diagnostic of the MMN.

QUANTIFICATION OF NERVE ULTRASOUND FINDINGS

Cross-sectional area reference values for peripheral nerves and brachial plexus have been already reported in the literature.[7,12,13] The difficulty, however, to differentiate normal from a pathologic heterogeneity of CSA changes in peripheral nerves, especially in cases of immune-mediated neuropathies, remains an important limitation of the neuromuscular ultrasound in clinical application. CSA enlargement can be the result either of edema (usually accompanied by disturbed fascicular echostructure) or hypertrophy (usually accompanied by preserved fascisular echostructure).

Novel ultrasound measures, aiming to quantify pathologic ultrasound changes of peripheral nerves in immune-mediated polyneuropathies, have been recently introduced in the literature:[7,9,11-13] (1) the intranerve CSA variability (for each nerve), defined as maximal CSA/minimal CSA; (2) the internerve CSA variability (for each patient), defined as nerve with maximal intranerve CSA variability/nerve with minimal intranerve CSA variability; (3) the side to side difference ratio of the intranerve CSA variability (for each nerve), defined as side with maximal intranerve CSA variability/side with minimal intranerve CSA variability; and (4) the intraplexus CSA variability defined as maximal CSA of the brachial plexus/minimal CSA of the brachial plexus [Table 1].

Table 1: Equations for calculating the intranerve-, internerve-, intraplexus CSA variability and side to side difference ratio of the intranerve CSA variability

Variability	Calculating equation
Intranerve CSA variability (for each nerve)	Maximal CSA/minimal CSA
Intranerve CSA variability (for each subject)	Peripheral nerve with the maximal intranerve CSA variability/ peripheral nerve with the minimal intranerve CSA variability
Side to side difference ratio of the intranerve CSA variability (for each nerve)	Side with the maximal intranerve CSA variability/side with the minimal intranerve CSA variability
Intraplexus CSA variability (for each brachial plexus)	Maximal CSA of brachial plexus/ minimal CSA of brachial plexus

CSA: Cross sectional area

Using the intranerve CSA variability, the sonographer may differentiate a focal (higher values) from diffuse (lower values) nerve hypertrophy while the internerve CSA variability may reveal possible distribution patterns of peripheral nerve impairment.[7] On the other hand, the side to side difference ratio of the intranerve CSA variability may be useful in detecting any lateralization of pathologic changes and the intraplexus CSA variability in differentiating focal (higher values) from diffuse (lower values) brachial plexus hypertrophy.[9,13]

RESULTS

Currently, 6 studies (evaluating a total of 55 cases) of nerve sonography in MMN patients have been published [Table 2].[6-10,14] The first description of pathological ultrasound findings in MMN was published by Beekman *et al*.[6] In this report, the authors documented at least one anatomical site with pathological hypertrophy of the median or ulnar or radial nerves and/or brachial plexus in 90% of the cases. The authors concluded that the neuromuscular ultrasound may allow the detection of pathological signs to a greater extent than nerve conduction tests in MMN. In a later study of 12 MMN patients, nerve hypertrophy was documented in the median (forearm), ulnar (Guyons' canal, forearm, elbow, upper arm) and tibial nerve (ankle), but not in brachial plexus, when compared to controls [Figures 1 and 2].

Considering the morphology of peripheral nerve hypertrophy (focal *vs.* diffuse), Padua *et al*.[7] have reported the inhomogenous CSA enlargement, mainly of the median, ulnar and fibular nerve in a small group of MMN patients. A second study on two MMN patients not only confirmed the focal type of CSA enlargement, but also documented the significant lateralization of ultrasound findings.[8] Another MMN study has documented a focal type of CSA enlargement in the median nerve, when compared with controls. In addition, the higher values of the internerve CSA variability and "side to side difference ratio of the intranerve CSA variability" of the median, ulnar and fibular nerve, were attributed by the authors to the possible striking predilection of MMN to certain peripheral nerves and the asymmetry of findings respectively.[10]

A possible explanation for the CSA enlargement in MMN cases could derive from pathological studies at sites of conduction blocks. According to these studies, perivascular areas contain scattered demyelinated axons, which are often surrounded by small onion bulb formations.[15] These onion bulb formation may lead to a consecutive CSA enlargement of the nerve. In addition, pathological CSA changes are usually detected at

Table 2: An overview of the existing nerve ultrasound studies on MMN and their pathological findings									
Authors	**Patients (*n*)**	**Controls (*n*)**	**Median nerve**	**Ulnar nerve**	**Brachial plexus or cervical roots**	**Sciatic nerve**	**Femoral nerve**	**Fibular nerve**	**Tibial nerve**
Beekman *et al.*[6]	21	20	x	x	x	-	-	-	-
Padua *et al.*[7]	2	63	x	x	-	-	-	x	-
Kerasnoudis[8]	2	30	x	x	-	-	-	x	-
Kerasnoudis *et al.*[9]	1	-	x	N	N	-	-	-	-
Kerasnoudis *et al.*[10]	12	80	x	x	N	-	-	N	x
Zaidman *et al.*[14]	17	-	x	x	-	-	-	-	-

MMN: Multifocal motor neuropathy; x: The concrete nerve was reported with pathological findings; N: The concrete nerve was reported with normal findings; -: The concrete nerve was not studied at all; *n*: Absolute number

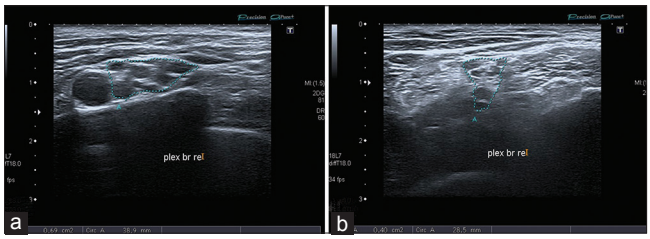

Figure 1: Axial scan of the brachial plexus in a multifocal motor neuropathy (MMN) patient in supraclavicular (a) and interscalene space (b) in a MMN patient. In this case both the cross sectional area and the intraplexus cross sectional area variability of the brachial plexus are within the reference values of our lab[13]

several proximal and distal sites in the anatomic course of the peripheral nerves in MMN patients. This finding may reflect the immune-mediated patchy multifocal demyelination occurring along the motor nerve fibers in this type of immune-mediated injury.[10,11]

Another important aspect in the field of sonography in MMN is the possible use of this method for identifying nerve conduction blocks. The localization of the nerve conduction block is often difficult to be identified in the nerve conduction studies (NCS), especially when dealing with proximal parts of the nerves. By overlooking the electrophysiological hallmark of the disease, delay in the diagnosis and therefore delayed treatment can occur.[2] Beekman *et al.*[6] documented pathological ultrasound findings not only at sites with electrophysiological impairment, but also at sites with normal functioning in NCS. An absolute correlation between site of nerve hypertrophy and site of conduction block has been reported only in one case in the literature.[8] Another study on 12 MMN patients showed a significant correlation between sonographic and electrophysiological findings only between the CMAP and CSA of the median nerve at the upper arm.[10] Systematic prospective studies on the sensitivity of ultrasound in detecting focal immune-mediated nerve lesions fail in the literature.

An interesting point of future study is the applicability of the nerve ultrasound as screening method for immune-therapy in dysimmune neuropathies. Nerve ultrasound and NCS failed to highlight functional disability in post-Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy patients

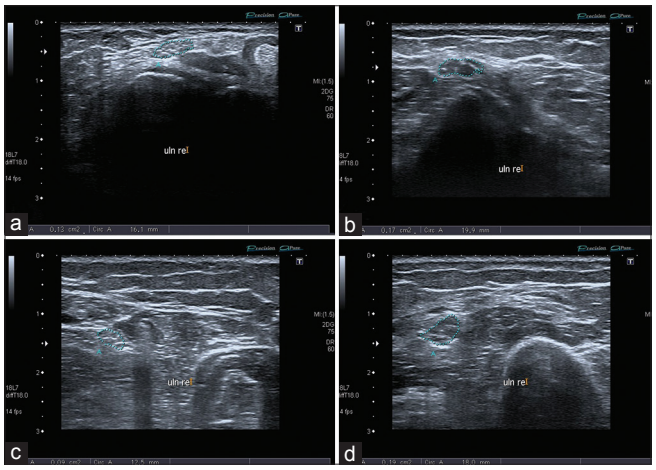

Figure 2: Axial scan of the ulnar nerve in a multifocal motor neuropathy (MMN) patient in Guyon's canal (a), forearm (b), elbow (c) and upper-arm (d) showing a pathological cross sectional area enlargement at all anatomic sites, when compared to the reference values of our lab.[13] This finding may reflect the immune-mediated patchy multifocal demyelination occurring in MMN

in the literature.[16,17] In a later study on MMN patients, neither sonography nor electrophysiology correlated with the Medical Research Council sum score, Rasch-built Overall Disability Scale score or Rasch-built fatigue severity scale.[10] These studies have shown that the already known ultrasound biomarkers (CSA, echogenity, intranerve CSA variability) are not able to highlight the effectivity of immune-therapy.[11]

CONCLUSION

To summarize, the currently available ultrasound studies show that mainly a focal type of asymmetrical peripheral nerve enlargement is expected in MMN. Nerve ultrasound findings seem to show no significant correlation to electrophysiological findings at most anatomical sites. In addition, prospective studies on the applicability of ultrasound as screening method of immune-therapy fail in the literature, while various retrospective studies failed to highlight any significant correlation between ultrasound findings and functional disability.

As the main uncertainties regarding the diagnostic criteria of MMN are steadily resolved, new challenges continuously arise on how to acquire the best static and dynamic imaging of the relevant nerve structures

in this type of immune-mediated disease, aiming to provide a complementary and holistic approach to nerve impairment. The first nerve ultrasound studies on MMN have shown that ultrasound seems to be a reliable and easily applicable method to detect pathological structural changes in peripheral nerves. The quantification of ultrasound changes and highlighting the distribution patterns of pathological findings remains a challenging aspect of future study. The recently proposed measurements in the literature may help to achieve this goal, but multicentre prospective validation is needed.[7,8,12,13]

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Review Article

Positron emission tomography imaging in gliomas

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ABSTRACT

Glioma, the most frequent primary brain tumor in adults, is a highly infiltrative tumor exhibiting resistance to most treatments and associated with short survival of patients. Positron emission tomography (PET) imaging using various tracers takes advantage of the increased metabolic rate of neoplastic cells, in order to detect tumors and validate the treatment response. The most frequently used PET tracer, the (18)F-fluorodeoxyglucose (FDG), is useful during the initial and follow-up assessment of patients with gliomas because it can assist in the selection of the initial biopsy site and to assess early response to a given therapeutic intervention. Furthermore, when there is tumor re-growth after an initial remission, FDG-PET can differentiate between true tumor recurrence versus necrosis from radiation therapy. Newly developed PET tracers may exhibit better sensitivity than FDG to diagnose primary brain tumors, but may occasionally produce false positive results in various conditions. In any event, PET is a useful tool in patients with central nervous system cancer during both initial assessment and follow-up.

Key words: Brain tumor, cancer, glioma, positron emission tomography

INTRODUCTION

Gliomas represent the most common primary brain tumors, with poor prognosis even with aggressive therapies such as various combinations of surgery, radiation therapy and chemotherapies.[1,2] Earlier response and progression criteria in recurrent glioma relied on changes in the contrast enhancing magnetic resonance imaging (MRI),[3,4] however, the dramatic response rates seen in therapies involving antiangiogenic therapies as well as other insufficiencies of the previous criteria resulted in development of updated response criteria that take into account the nonenhancing component of the tumor as well as other critical parameters.[4,5] The newly described response assessment in neuro-oncology (RANO) criteria includes comprehensive recommendations to assess response to a therapy taking into account various issues in gliomas, such as imaging changes postsurgical resection of a tumor or locally delivered therapies, issues-related to contrast enhancement of previously unenhanced areas as well as clinical parameters.[6] This field is still evolving since in a recent report the change in ADC histogram skewness may be more sensitive than the response assessment in RANO criteria for evaluation of antiangiogenic therapy.[7]

Nuclear medicine imaging such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) combined with CT are useful for diagnosis and management of a variety of neurological diseases and cancers.[8] SPECT and PET scans may be utilized to assess brain tumor biologic behavior,[9] distinction of radiation-induced necrosis from tumor recurrence and estimation of overall prognosis.[10] Increased tumor uptake of (99 m) Tc-tetrofosmin in SPECT correlated with aggressive behavior and may be an independent prognostic factor in patients with malignant glioma.[11]

In this article, we present an evidence-based practical approach for the use of PET/CT during evaluation and therapy of patients with a malignant primary brain tumor. We reviewed published papers during the last decade and included some older key references and our own experience.

(18)F-FLURODEOXYGLUCOSE POSITRON EMISSION TOMOGRAPHY

(18)F-flurodeoxyglucose (FDG) PET takes advantage of the increased glucose uptake, a characteristic of

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tumor cells, in order to detect tumors and validate the treatment response [Table 1].

Hypometabolism on FDG PET in brain lesions and stability over a period is indicative of nonmalignancy.[24] When it is difficult to differentiate preoperatively a primary brain tumor from metastasis,[25] FDG PET may be helpful in depicting areas of systemic involvement,[26] or localizing the primary cancer site.[27,28] Occasionally, patients may present with brain lesions, radiologically compatible with brain metastases that after biopsy are proven to be multifocal gliomas.[29,30] In such cases, FDG PET may aid in pinpointing the area of stereotactic biopsy,[31,32] assist in tumor delineation during radiotherapy planning[33] and assessment of treatment response.[34]

In a study of 81 recurrent glioma patients studied by FDG PET, it was found that the higher the FDG uptake by the tumor it was associated with worse survival.[35] In addition, pretreatment uptake of FDG in 25 patients with recurrent gliomas subsequently

Table 1: Representative studies on utility of FDG PET and comparison with other tracers in patients with primary brain tumors

Study	No. of patients	Reason for the exam	Results (%)	Study conclusion
Colavolpe *et al.*[12]	25 patients with recurrent glioma	To assess utility of FDG PET/CT in patients receiving bevacizumab and irinotecan therapy	FDG uptake was the most powerful predictor of both PFS and OS using the RANO criteria	Pretreatment FDG PET predicts survival in recurrent glioma patients following anti-angiogenic therapy
Santra *et al.*[13]	90 patients with possible recurrent glioma	To compare FDG PET/CT with contrast MRI	PET sensitivity: 70 Specificity: 97 MRI sensitivity: 95 Specificity: 23	FDG PET/CT was an accurate modality to detect glioma recurrence
Borbely *et al.*[14]	59 patients with primary and recurrent brain gliomas (50 had MET PET; 33 had FDG PET)	To compare FDG PET with MET PET for *in vivo* grading of malignant gliomas	FDG PET superior to MET PET for grading of gliomas	FDG PET recommended for grading but MET PET may be used for assessing the extent of the tumor
Singhal *et al.*[15]	102 patients with confirmed gliomas were followed for an average of 34.6 months after PET	To compare FDG PET with MET PET and MRI	MET PET superior to FDG PET and MRI in predicting survival in low-grade gliomas	For low grade gliomas MET PET preferred to FDG PET
Yamaguchi *et al.*[16]	26 patients with untreated or recurrent adult gliomas had preoperative FDG (n = 25) and/or MET (n = 22) PET	To compare FDG PET with MET PET	FDG better for tumor grade MET better for delineating the extent of the tumor	Both tracers complement each other to plan the extend of tumor resection
Tripathi *et al.*[17]	15 patients with untreated or recurrent low grade gliomas	To compare FDG PET with FDOPA PET and FLT PET	FDOPA PET superior to both FDG and FLT PET for detection of low grade gliomas	FDOPA PET should be the radiotracer of choice for low grade glioma
Chen *et al.*[18]	25 patients with with untreated or recurrent adult gliomas	To compare FDG PET with FLT PET	FLT PET better to image recurrent high-grade tumors, to correlate with Ki-67 values, and predict tumor progression and survival	FLT a promising tracer of proliferation in high-grade gliomas
Enslow *et al.*[19]	15 recurrent glioma patients	To compare FDG PET with FLT PET	Both FDG PET and FLT PET could differentiate between tumor recurrence and radiation necrosis	FLT PET offers no advantage over FDG PET
Karunanithi *et al.*[20]	28 patients with recurrent gliomas	To compare FDG PET with FDOPA PET for diagnosis of recurrence	FDG sensitivity: 47.6 FDG specificity: 100 FDOPA sensitivity: 100 FDOPA specificity: 85.7	The difference between FDOPA and FDG PET was significant for low grade glioma but not for high grade tumors
Tripathi *et al.*[21]	35 patients with recurrent glioma	To compare FDG PET with MET PET	FDG sensitivity: 81.2 FDG specificity: 88.9 MET sensitivity: 94.7 MET specificity: 88.9	MET should be the radiotracer of choice for recurrent gliomas
Potzi *et al.*[22]	28 patients with recurrent GBM	To evaluate FDG and MET PET for recurrent glioma		FDG PET of limited value; MET PET not superior to conventional imaging
Nihashi *et al.*[23]	Meta-analysis of 26 heterogenous studies	To evaluate the diagnostic accuracy of PET and compare it with conventional imaging modalities	FDG PET and MET PET with acceptable accuracy for diagnosing recurrent glioma	Prospective studies with direct comparisons between various imaging modalities required

PET: Positron emission tomography; CT: Computed tomography; MRI: Magnetic resonance imaging; RANO: Response assessment in neuro-oncology; FDG: (18)F-flurodeoxyglucose; FET: O-(2-(18)F-fluoroethyl)-l-tyrosine; GBM: Glioblastomamultiforme; MET: (11)C-methionine; FDOPA: (18)F-FDOPA; FLT: 3'-Fluoro-3' deoxythymidine; PFS: Progression-free survival; OS: Overall survival; HGG: WHO grades III or IV; LGG: WHO grades I or II

treatment with bevacizumab and irinotecan predicted response to the treatment and correlated with overall survival.[12] Similar predictive value of FDG-PET was reported with other therapies in glioma patients.[36] FDG PET compared to MRI scans with and without contrast enhancement had much higher specificity (97% *vs*. 23%) for detection of recurrence in 90 glioma patients clinically suspicious of tumor growth.[13]

OTHER POSITRON EMISSION TOMOGRAPHY TRACERS AND COMPARISON WITH (18)F-FLURODEOXYGLUCOSE

During the last several years, new PET tracers have been developed for a wide range of biological targets [Table 2].[37]

PET of amino acid transport and metabolism could be a reliable method in assessing a metabolic response after treatment of a tumor or in establishing a treatment-related effect, depending on the rate of the tracer uptake by tumor. Employment of imaging amino acid transport may prove to have an important clinical role in the management of brain tumor patients since it may result in changes in therapeutic management.[62]

For example, application of O-(2-(18)F-fluoroethyl)-L-tyrosine (FET) PET/CT in newly diagnosed brain tumors could predict their biologic behavior in most of the cases.[48,52,63] FET represents an artificial amino acid not incorporates into proteins but transports into active glioma cells.[46] FET-PET may be more accurate than FDG-PET for differentiation of malignant gliomas from low-grade gliomas,[64,65] by their low FET uptake on PET in the low-grade tumors.[66,67] Thus, in a study of 88 patients with an intracerebral lesion observed by MRI, FET PET was performed, followed by biopsy in 60 patients. The sensitivity of FET PET for high-grade tumors (WHO III-IV) was reported 94% and for low-grade tumors (WHO I-II) 68%. However, there were

Table 2: Other PET tracers for patients with gliomas

Tracer	Mechanism	No. of studies	Untreated or recurrent glioma	Advantages	Disadvantages
AMT[38]	Amino acid PET tracer not incorporated into proteins but transported into gliomas via the kynurenine pathway	1	Recurrent	AMT PET could differentiate between tumor and XRT necrosis	False positive results can occur in cortical dysplasia with epileptic focus[39]
MET PET[40]	MET is transported by the LAT1 amino acid transporter into glioma and is incorporated into proteins[41]	5	Upfront[15] Recurrent[41-44]	MET uptake correlated with prognosis[15] MET PET could differentiate between tumor and XRT necrosis[40,42] Correlate with OS and outcome[43,44]	Short half-life (20 min) requiring on site production; MET may accumulate in brain abscesses or inflammation[45]
FET PET	FET is an artificial amino acid transported into active glioma cells but incorporated into proteins[46]	5	Upfront[47,48] Recurrent[49-51]	FET PET could differentiate glioma from nonneoplastic tissue FET PET distinguished active tumor from radiation necrosis;[50,51] dynamic FET uptake could differentiate between high and low grade tumors[49]	Rare false positive in granulomatous conditions and reactive astogliosis[52] or false negative cases[53]
FDOPA PET: (18)F-FDOPA	l-DOPA is the precursor of dopamine and is transported physiologically into the brain and abnormally into the brain tumors[54]	2	Upfront[55] Recurrent[55,56]	Correlation of FDOPA uptake, tumor proliferation and grade Diagnostic accuracy of recurrence similar to MRI[56]	Diagnostic usefulness mostly in upfront gliomas; limited data
FLT PET[57,58]	FLT is an analog of deoxythymidine, which is composed of deoxyribose and the pyrimidine base thymine and phosphorylated by thymidine kinase 1 during DNA synthesis[59]	2	Upfront[57] Recurrent[58]	FLT PET could differentiate between high and low grade tumors FLT-PET responses correlated with OS	FLT may accumulate in benign lesions with BBB disruption[45]
CHO: (18)F-fluoromethylcholine	During glioma cell proliferation choline is trapped into the cells to produce phosphatidylcholine, a necessary constituent of the plasma membrane[60]	1	Various brain lesions (tumors or nontumors)	Higher uptake in malignant tumors	It may also accumulate in various inflammatory processes[61]

PET: Positron emission tomography; MRI: Magnetic resonance imaging; XRT: Radiation therapy; BBB: Blood brain barrier; MET: (11)C-methionine; AMT: Alpha-(11)C-methyl-l-tryptophan; FDG: (18)F-flurodeoxyglucose; FET: O-(2-(18)F-fluoroethyl)-l-tyrosine; FDOPA: (18)F-FDOPA; FLT: 3'-fluoro-3' deoxythymidine; PFS: Progression-free survival; OS: Overall survival

two false-positive cases with postischemic lesions.[52] A study on differences in the dynamics of FET uptake in gliomas could differentiate between recurrent high and low-grade tumors.[49] In another study, it was shown that an FET-PET with a receiver-operating-characteristic curve analysis, a mean tumor-to-brain ratio of 2.5 was highly specific for tumor rather than nontumor tissue.[47] In 10 patients with recurrent glioma treated with a combination of bevacizumab and irinotecan FET PET could predict treatment failure, thus provided additional information from that obtained by MRI response assessment based on RANO criteria.[50] A meta-analysis of 13 studies with 462 newly diagnosed untreated patients with primary brain tumors indicated that FET-PET may be an excellent tool for differentiating tumor for non tumoral lesions.[48]

Another PET tracers that may be employed for evaluation of brain tumors are (18)F-labeled fluoromethylcholine (18F-FCho)[60] and (11)C-methionine (MET) PET.[31,40,50] MET-PET may aid in the differential diagnosis of tumor recurrence versus radiation necrosis although its specificity and sensitivity have been reported both as 75%.[42] In patients with glioma, clinical stability induced by temozolomide chemotherapy correlated to a decline or stability of tumor MET uptake on PET.[43] Furthermore, although the standard MET PET did not correlate with survival, a voxel-wise parametric response map analysis of MET PET correlated with OS in 14 patients with recurrent malignant gliomas treated with specific immunotherapy targeting the Wilms tumor 1gene product.[44] The short half-life (20 min) of (11)C limits its use of MET PET to institutions with an onsite cyclotron. A comparison of MET PET with FET-PET (half-life of 120 min) in 29 patients with recurrent gliomas showed that both tracers differentiated tumor tissue and treatment-related changes with high sensitivity and specificity suggesting that FET PET could be used in places where an onsite cyclotron is unavailable.[68] FET PET may provide more accurate information in respect of treatment response or failure compared with response assessment based on conventional MRI and RANO criteria,[69] and could reliably distinguish between posttherapeutic treatment related effects and tumor recurrence independently on the employed treatment modality.[51]

In addition, there is evidence that FET PET in the management of patients with recurrent glioma treated with a combination of bevacizumab and irinotecan may be cost-effective since it can prevent overtreatment and additional costs, as well as potential side effects to patients.[70]

A comparison study between FDG-PET and MET PET in 59 patients with either untreated or recurrent gliomas demonstrated that FDG-PET was a superior test to *in vivo* predict histologic grade of the tumor compared with MET PET [Table 1].[14] However, in respect to the low-grade gliomas, MET PET appears to better correlate with overall prognosis and survival rather than FDG PET or conventional MRI, suggesting that both tracers may be complementary during evaluation of gliomas before or after therapies.[15] Similar results in another study suggested that both FDG and MET PET provide useful complementary information assisting surgeons to determine the extent of the surgical resection.[16] In a recent study of 35 patients with suspected recurrent gliomas FDG PET and MET PET were performed during the same day and correlated with subsequent histopathology or MRI/modified Rankin scale and clinical follow-up. The results of this study suggested that MET PET should be preferred over FDG PET when available since it demonstrated higher sensitivity for detection of recurrence (94.7% *vs*. 81.2%) and the same specificity.[21] However, one study found that neither FDG PET or MET PET add any additional information over the conventional MRI regarding prognosis of patients with malignant gliomas.[22] A meta-analysis of 26 heterogeneous studies about several PET tracers for diagnosing recurrent gliomas found that FDG-PET had a summary sensitivity of 0.77 and specificity of 0.78 for any glioma histology, and MET PET had a summary sensitivity of 0.70 and specificity of 0.93 for high-grade glioma. Data were limited for FET and 3'-deoxy-3'-[18F] fluorothymidine (FLT) PET. The authors concluded that apart from FDG and MET PET that seem to have utility during evaluation of glioma recurrence, further studies using direct comparisons between PET tracers and imaging modalities are needed.[23]

DOPA: 3,4-dihydroxy-6-(18)F-fluoro-l-phenylalanine (FDOPA) PET tested in a 59 glioma patients (22 with new untreated gliomas and 37 with recurrent tumors) showed that FDOPA uptake was higher in high-grade than in low-grade tumors in newly diagnosed, but not recurrent tumors, suggesting that its usefulness as a noninvasive tumor grading procedure can be only in previously untreated tumors.[55] In recurrent gliomas, FDOPA was able to diagnose the recurrence with a sensitivity of 100% and specificity of 85.7% in contrast to 47.6% and 100% of FDG PET.[20] In that study, the analysis showed superiority of FDOPA PET compared with FDG-PET to diagnose recurrence in low-grade tumors but no statistical difference in high-grade gliomas.[20]

Comparison of FDOPA PET with contrast enhancing MRI scan for detection of tumor recurrence in 35 glioma patients revealed that although both examinations had high sensitivity (100% *vs*. 92.3%), FDOPA PET had much higher specificity (88.9% *vs*. 44.4%) than

MRI.[56] Furthermore FDOPA PET fused with MRI for anatomic localization provides accurate localization of tracer uptake taking advantage of both techniques.[71]

3'-deoxy-3'-[18F]-fluorothymidine is a PET tracer developed for imaging cellular proliferation. In patients with histologically diagnosed primary brain tumors the FLT uptake by the primary tumor could correlate with the grade of malignancy and proliferation index,[72] but occasionally it could result in false positive diagnoses, especially in cases of benign lesions with blood-brain barrier disruption, for example postoperative granuloma.[57] Comparison of FLT PET to MRI with and without contrast in 19 patients with recurrent glioma treated with bevacizumab in combination with irinotecan indicated that both early (1-2 weeks post treatment) and late FLT PET responses (6 weeks) were more significant predictors of overall survival compared with the MRI responses. In this study, metabolic response was defined as more than 25% reduction in tumor FLT uptake compared with baseline.[58] Furthermore, when compared to FDG PET, FLT PET was reported better in imaging recurrent high-grade tumors, correlating with Ki-67 values, and predicting tumor progression and patient survival.[18] Similarly, comparison of FDG with FDOPA and FLT PET in 15 patients with untreated or recurrent low-grade gliomas demonstrated that clearly FDOPA was the tracer of choice for tumor delineation compared with the other 2 tested tracers.[17] However, another small study in 15 patients with recurrent gliomas reported no advantage of FLT PET compared with FDG PET in discriminating between tumor recurrence and radiation necrosis.[19]

FDOPA or FLT PET uptake after bevacizumab treatment may be a useful biomarker for predicting progression-free survival in recurrent gliomas.[73-76]

Alpha-(11)C-methyl-l-tryptophan (AMT) PET utilizes the AMT as PET tracer that accumulates into gliomas through the kynurenine pathway, which leads to the production of nicotinamide adenine dinucleotide (NAD^+) from the degradation of the essential amino acid, tryptophan.[77] In 22 patients with possible recurrent glioma on MRI scan tracer uptake by the tumor could differentiate between recurrent glioma and radiation injury.[38] The (18)F-labeled glycosylated Arg-Gly-Asp peptide is a PET tracer that images the integrin alpha (v) beta (3) expression, which may be important considering the integrin inhibitors as potential therapy for glioblastomas.[78]

LIMITATIONS

(18)F-flurodeoxyglucose although represents the most common radiotracer for PET cancer imaging, it is not tumor-specific, since it shows high uptake in benign conditions such as infections and nonspecific inflammatory tissue.[45,79] In viral encephalitis FDG PET usually demonstrates hypermetabolism but focal areas of hypometabolism may also be observed.[80] Brain abscess may also exhibit FDG hypermetabolism making the differential diagnosis between a metastatic tumor and abscess in a patient with systemic cancer impossible with only this test.[81,82] Tuberculomas may also exhibit FDG hypermetabolism in the periphery and hypometabolism in the center.[83]

Even though, most of the newer PET tracers demonstrated enhanced tumor-specificity compared with FDG, they also had certain limitations; for example, (11)C-choline can be accumulated in various inflammatory processes, MET in brain abscesses and (18)F-FLT in nonmetastatic reactive lymph nodes.[45]

CONCLUSION

(18)F-flurodeoxyglucose PET, as well as PET with other tracers, may be useful for diagnosis of cerebral gliomas in patients that present with a brain mass and no involvement of other organs in conventional imaging. In addition, PET/CT is helpful in selecting the appropriate site for stereotactic biopsy and in monitoring response to various therapeutic interventions. Finally, upon re-growth of the tumor after the initial treatment, PET/CT can differentiate between glioma recurrence *vs*. necrosis from the employed radiation therapy and guide further therapeutic management.

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Review Article

Clinicogenetics of Parkinson's disease: a drawing but not completed picture

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ABSTRACT

Parkinson's disease (PD) is a prevalent neurodegenerative disorder mainly affecting the population over the age of 60 years. The past decade has seen rapidly emerging data supporting a major importance of genetic factors in the development of PD. Increasing number of large-scale and replicating association studies has facilitated the confirmation of the possible predisposing factors to PD and the selection of genetic variants for risk prediction. While evidences are accumulating that variations within the *SNCA*, *LRRK2*, *MAPT* and *GBA* genes increase the individuals' vulnerability to PD, inconclusive or negative results have been reported for an association between PD and variants of the *parkin*, *PINK1*, *DJ-1*, *UCH-L1*, *Omi/HtrA2*, *GIGYF2*, *PLA2G6*, *VPS35*, *EIF4G1* and *BST1* genes. However, our understanding of the genetic picture of PD remains preliminary. Molecular diagnosis of the disease is only recommended for cases with clear family history, and currently, there is no ideal genomic biomarker available to predict the disease onset and progression, or to make a molecular classification of the disease. Efforts are expected to identify more genetic predisposing factors and to further clarify their roles in the mechanisms of PD.

Key words: Association, biomarkers, genetic variants, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is a prevalent neurodegenerative disorder affecting 1-2% of the population over the age of 60 years.[1] The disease results mainly from progressive and profound degeneration of dopaminergic neurons in the substantia nigra with the presence of Lewy bodies containing aggregates of α-synuclein and other substances.[2,3] Although the etiology and mechanisms of PD remain largely unclear, the development of the disease is believed to be the combined results of three interactive events: genetic susceptibility, environmental exposures and the aging process.[4-8] The relative role of genetic and environmental factors has been debated for many years, however, evidences are rapidly accumulating that genetic risk factors are of major importance in the sporadic form of the disease, accounting for at least 10% of the general PD population.[1,9-11]

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One important conceptual update of the genetic profiling of PD is that mutations or variations within causative genes for a minority of monogenic familial PD are also associated with sporadic PD. Studies in PD families have identified 11 (*α-synuclein*, parkin, *UCH-L1*, *PINK1*, *DJ-1*, *LRRK2*, *ATP13A2*, *OMI/HTRA2*, *FBX07*, *VPS35*, *EIF4G1*) causative genes and 4 loci of linkage across the genome (PARK3, PARK10, PARK12 and PARK16) pending characterization. Analysis of mutations or variations in many of these genes has been performed in recent years among diverse ethnic populations. In addition, the newly emerged genome-wide association studies (GWAS) have been used to identify novel genetic associations with the disease at the whole-genome level.[12-15] More recent progress has been made by the powerful technique of next-generation sequencing.[16,17] Further, more and more large-scale and multi-center collaborative analyses have been completed thanks to the improving analytic tools and the increasingly close international cooperation. The results published so far are consistent or conflicting with each other, reflecting confirmative, inconclusive or negative associations between genetic variants and PD. In this review, we give an up-to-date view of the genes that may have associations with the risk for PD and their implications in clinical

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practice, with emphasis on large-scale and multiethnic evidences, as listed in Table 1.

GENETIC VARIATIONS WITH WELL-EVIDENCED ASSOCIATIONS WITH PARKINSON'S DISEASE

SNCA

Genetic variability within the SNCA gene encoding α-synuclein is arguably the most reliable association of a common genetic risk factor with PD identified to date. Although mutations in this gene account for < 1% of PD in the general population, abnormal aggregation of the SNCA-encoding protein, α-synuclein, the principal component of Lewy bodies, is present in all patients with idiopathic PD.[16] In addition, association studies have repeatedly suggested the link of the SNCA variations to both familial and sporadic PD. Further, several most recently completed GWAS consistently showed strong linkage of the SNCA locus to PD across Western and Oriental populations.[12-15]

Although three missense mutations in SNCA were reported in families with PD inheritance[18-20] and thought to increase the aggregation of SNCA protein, point mutations have not been identified in sporadic PD,[21,22] and no several nonsynonymous (SNPs) have been found in the coding region, suggesting that disease-related amino acid changes in SNCA are unlikely in sporadic PD.[23] In contrast, multiplication, in particular triplication, of SNCA was revealed in both familial[24-29] and sporadic PD cases.[30] Due to the absence of point mutations in any of the copies of SNCA in these patients, the cause of PD appears to be the mere increase in α-synuclein levels. In support of this dosage effect, PD patients from families with two extra copies of SNCA have a more severe phenotype than PD patients with only one extra copy,[25,27,31] and SNCA mRNA levels in the brain from sporadic patients are increased.[32-35]

The pathogenicity of multiple SNCA gene copies and the apparent dosage effect of α-synuclein levels in both sporadic and familial PD highlight the clinical significance of the regulation of SNCA gene expression, which can take place at both transcriptional and posttranscriptional levels. Transcription of genes is mainly regulated by the promoter sequence. The first promoter variant reported in association with PD was the mixed dinucleotide repeat sequence (REP1), which resides approximately 10 kb 5′ to the translation start site of SNCA. Despite some negative results of association, the majority of individual studies[36-39] and a meta-analysis of data[40] from 18 sites across multiple ethnic populations have confirmed an association between risk for PD and the longer REP allele. In addition, variants other than REP1 in the promoter region, such as SNPs flanking the core promoter at the -770 and -116 positions, rs2583988, rs2619364, and rs2619363, were also reported to increase the susceptibility to PD in European population.[41] Posttranscriptional regulation of gene expression can be mediated by several elements, many of which are located in the 3'-untranslated region of mRNAs.[42,43] A series of studies reported an association of polymorphisms at the 3'-end of

Table 1: Genes and loci related to Parkinson's disease

Locus	Gene	Chromosome	Inheritance	Type of parkinsonism	Mutation/varaint type	Association with PD
PARK1/PARK4	SNCA	4q21	AD	LOPD/EOPD, dementia	Multiplication, point	Convinced
PARK2	Parkin	6q25-27	AR	EOPD	Re-arrangement, point	Re-arrangement: convinced; point: unconvinced
PARK3	Unknown	2p13	AD	LOPD	Point	Unconvinced
PARK5	UCHL1	4p14	AD	LOPD	Deletion, point	Unconvinced
PARK6	PINK1	1p36	AR	EOPD	Deletion, point	Unconvinced
PARK7	DJ-1	1p36	AR	EOPD	Deletion, point	Unconvinced
PARK8	LRRK2	12q12	AD	LOPD	Point	Convinced
PARK9	ATP13A2	1p36	AR	EOPD, Kufor-Rakeb syndrome	Point	Unconvinced
PARK10	Unknown	1p32	Unknown	LOPD	Point	Unconvinced
PARK11	GIGYF2	2q37	AD	LOPD	Point	Unconvinced
PARK12	Unknown	xq21-25	X-linked	LOPD	Point	Unconvinced
PARK13	HTRA2	2p12	AD	LOPD	Point	Unconvinced
PARK14	PLA2G6	22q13	AR	EOPD, dystonia-parkinsonism	Point	Unconvinced
PARK15	FOXB7	22q12-13	AR	EOPD, pallido-pyramidal syndrome	Point	Unconvinced
PARK16	Unknown	1q32	Risk	LOPD	Point	Unconvinced
PARK17	VPS35	16q11	Risk	LOPD	Point	Unconvinced
PARK18	EIF4G1	3q27	Risk	LOPD	Point	Unconvinced
-	*GBA*	1q21	Risk	LOPD	Point	Convinced
-	BST1	4p15	Risk	LOPD	Point	Unconvinced
-	MAPT	17q21	Risk	LOPD	Haplotype	Convinced
-	ATXN2	12q12	Risk	LOPD	Trinucleotide expansion	Unconvinced
-	ATXN3	14q32	Risk	LOPD	Trinucleotide expansion	Unconvinced

AD: autosomal dominant; AR: autosomal recessive; EOPD: early-onset Parkinson's disease; LOPD: late-onset Parkinson's disease; PD: Parkinson's disease

SNCA (e.g. rs356165 and rs356219) with sporadic PD, especially those from Southern Germany and Asian.[44-47] Although molecular details are not clear, the 3'-variants have been shown to increase the expression of α-synuclein.[48]

LRRK2

The discovery of mutations in the LRRK2 gene as the cause of PD in the families linked to the PARK8 locus (12q12) was probably the most important step forward since the α-synuclein discovery.[49,50] LRRK2 is a very large gene that contains 51 exons, and over 30 sequence variants have been linked to autosomal-dominant Parkinsonism. However, only five (R1441C, R1441G, Y1699C, G2019S, and I2020T) have been shown or be clearly pathogenic, and two substitutions (G2385R, R1628P) have been associated with an increased risk for sporadic PD.

The most common LRRK2 mutation is Gly2019Ser. It is detected in about 5% of familial and 1-2% of sporadic European PD patients[51,52] and up to 30% of patients with PD from North African and 10-40% of Middle Eastern populations.[53-56] One intriguing feature of this mutation is its association with both familial and sporadic PD. However, the penetrance of this mutation is relatively low. By analyzing the pooled data of 24 populations worldwide (including 19,376 unrelated patients with PD), the International LRRK2 Consortium reported the risk of PD for a person who inherits the LRRK2 G2019S mutation was 28% at age 59 years, 51% at 69 years and 74% at 79 years. Although motor and nonmotor symptoms of LRRK2-associated PD were more benign than those of idiopathic PD, the core features (asymmetrical, tremor predominant parkinsonism with bradykinesia and rigidity that responded to dopamine) of the patients with LRRK2 G2019S-associated PD are indistinguishable from patients with idiopathic PD, again implying a critical contribution of LRRK2 to the PD pathogenesis.[57]

Although the G2019S is prevalent in PD patients in the above-mentioned populations, it does not occur at appreciable frequency in control cohorts from these populations and is strikingly rare in Chinese[58,59] and South African.[60] Therefore, it is more a population-specific mutation than a popular susceptibility variant. In contrast, two variants reported from Asian populations appear to be true risk variants for PD. The first G2385R was initially described in a Taiwanese family.[61] Assessment of this variant in large Asian populations showed association with risk for disease in Taiwanese, Japanese, Hong Kong Chinese and mainland Chinese populations.[61-63] In general this variant is present in about 10% of PD populations and 0.5-5% in controls and confers at least two-fold risk for the chance of PD. Given that this association appears robust across Asian populations, this risk allele is an underlying factor in a very large number of PD cases worldwide. More recently a second LRRK2 risk allele, also identified within Asian PD populations has been described.[64,65]

Microtubule associated protein tau

The microtubule associated protein tau (MAPT) gene encodes MAPT. Tau modulates the assembly, dynamic behavior, and spatial organization of microtubules, and is a major protein component of neurofibrillary tangles, a hallmark lesion of Alzheimer's disease (AD). Mutations in the MAPT gene were identified to cause autosomal dominant frontotemporal dementia with parkinsonism linked to chromosome 17.[66] In addition to rare causal mutations, common variability in MAPT has been linked to disease such as progressive supranuclear palsy, AD and PD. The most frequently reported variation is caused by a common genomic inversion within a large block (approximately 1.6 Mb in length) containing the MAPT locus that shows reduced recombination and high levels of linkage disequilibrium. This phenomenon results in two common Caucasian haplotype groups across this locus, often termed H1 and H2. Association between MAPT H1 and risk for PD has been tested by many groups,[67,68] and the results in general show a consistent association with the disease. Moreover, patients carrying the H1 allele present in their fifth decade either with behavioral/cognitive changes or with rapidly progressive and poorly levodopa-responsive parkinsonism. A recent follow-up study demonstrated that 17% of incident PD patients developed dementia over 5 years, and the MAPT H1/H1 genotype was an independent predictor of dementia risk (odds ratio = 12.1).[69] The results also suggested that Lewy body deposition in posterior cortical areas, which is influenced by MAPT genotype and the aging process are associated with subsequent global cognitive decline and dementia. However, the H1 haplotype may not be a universal risk allele because the H2 haplotype is almost exclusively Caucasian in origin, and its prevalence in other populations is essentially zero.[70]

In addition to the H1 haplotype, a subhaplotype within the H1 clade composed of two "H1-SNPs" (rs242562 and rs2435207) spanning MAPT exons 1-4 was also significantly overrepresented in cases versus control subjects.[71,72] However, except for in one Greek and one Nowegian study, the association of H1-subhaplotype with PD was not well replicated in other Caucasian studies, and Taiwan Chinese,[68,73,74] and it has not been tested whether this subhaplotype is associated with PD in populations that possess merely the H1 clade.

Glucocerebrosidase

The glucocerebrosidase (GBA) gene encoding a lysosomal enzyme called glucocerebrosidase that hydrolyses the beta-glycosidic linkage of glucosylceramide, a ubiquitous sphingolipid present in the plasma membrane of mammalian cells.[75] Over 200 mutations have been described in GBA, including point mutations, deletions and recombination alleles derived from the pseudogene sequence.[76] These mutations usually cause a recessive lysosomal storage disorder - Gaucher disease (GD), which is characterized by macrophages enlarged with deposits of glucosylceramide.

The initial recognition of an association between PD and GBA mutations came from the clinical observations of parkinsonian manifestations in genotypically heterogeneous patients with GD.[77] Moreover, brain samples from autopsy-confirmed PD cases revealed significantly higher carrier frequencies (14%) than the estimated GBA mutation carrier frequency in the general population (0%).[78] The frequency and distribution of GBA mutations in PD vary among populations. Ashkenazi Jewish PD patients have the highest carrier frequency with a range 13.7-31.3%, compared with 4.5-6.2% in controls.[79,80] It was lower in nonAshkenazi-Jewish populations, ranging 2.8-12%, compared with 0.2-5.3% controls from the same populations.[81-83] Among all the mutations, L444P and N370S turned out to be the most frequently identified in PD patients. Although N370S is also common in European populations, it has not been encountered among Asians.[84] In contrast, L444P was believed as a panethnic mutation associated with PD.[85,86] A most recent multi-center study including 5691 patients and 4898 controls from 16 centers revealed that either mutation was found in 15% of patients and 3% of controls among Ashkenazi Jewish subjects, and in 3% of patients versus 1% of controls among nonAshkenazi Jewish subjects.[87] The odds ratio for any GBA mutation in patient's versus controls was 5.43 across centers, which is the highest effect size conferred by the known risk variants for PD. There is preliminary evidence that, overall, mutation carriers have an earlier age at onset (AAO), more atypical clinical manifestations, more cognitive changes and more likely to have affected relatives.[82,88]

GENETIC VARIANTS IN INCONCLUSIVE OR NEGATIVE ASSOCIATION WITH PARKINSON'S DISEASE

Parkin

The most frequent mutations in early-onset PD (EOPD) (AAO ≤ 50 years) patients are those identified in the parkin gene, which account for up to 50% of autosomal recessive juvenile parkinsonism (AR-JP) and 15-20% of sporadic EOPD.[89-91] Over 100 types of mutations including sequence substitutions, insertions and exonic deletions/duplications (or dosage mutations) in the parkin gene have been described in diverse ethnic groups.[92] While homozygous or compound heterozygous mutations are causative, heterozygous mutations have been suggested to increase the risk for PD.[93,94] The predisposing effects of heterozygote were, however, soon questioned by other studies in which they were reported as common in control subjects as in PD patients.[95,96] These conflicting observations, as suggested by some studies, may come from the heterogeneous effects of different types of mutations, which may have different origins and pathogenic effects. For example, a haplotype analysis for a European EOPD family series demonstrated that exonic rearrangements occurred independently whereas point mutations may have been transmitted by a common founder.[97] In addition, some studies suggested that dosage mutations are more pathogenic than sequence mutations in the development of familial PD.[98,99] However, these results remain to be confirmed by large-scale studies, and it is unclear whether the dosage mutations are associated with typical sporadic PD.

PINK1

Mutations in the PTEN-induced putative kinase 1 (PINK-1) gene are the second common cause of autosomal recessive EOPD after parkin. The gene resides on chromosome 1p35-36 (PARK6) and encodes a protein locating on mitochondria.[100-102] Evidences are gathering that PINK-1 is crucial for the normal functions of mitochondria and might participate in the detoxification of proteins.[103] Different PINK-1 mutations including missense, nonsense, splice site mutations and entire PINK-1 gene deletion have been identified in both familial[104] and sporadic EOPD cases,[105,106] with a frequency ranging from 1% to 8%. However, single heterozygous PINK-1 mutations have also been reported in healthy controls and large-scale case-control studies confirming the association between PINK-1 mutations, and sporadic PD are not available.

DJ-1

The DJ-1 gene (PARK7) encodes a protein belonging to the DJ-1/Thi/PfpI protein super family. It was initially described in association with oncogenesis and male rat infertility,[107,108] and later found to be associated with autosomal recessive EOPD.[109,110] DJ-1 is proposed to play a role in protecting neurons from oxidative stress and protecting against mitochondrial damage.[111] A few PD-causing mutations have been identified, including exon deletions, truncations, homozygous and heterozygous point mutations, which predominantly result in loss of function.[109,112] However, there is currently a lack of information about the frequency of

mutations, including single heterozygous mutation, for DJ-1 in both familial and sporadic parkinsonism, especially in large population samples. Moreover, in a recent complete mutational analysis of DJ-1 coding sequence in a large cohort of familial and sporadic PD cases from 12 countries, none had causative mutation in DJ-1, suggesting its mutations are very rare in either familial or in sporadic parkinsonism.[113]

UCH-L1

The *UCH-L1* gene (PARK5) encodes the ubiquitin carboxy-terminal hydrolase L1, which is a component of LB and possesses both a hydrolase activity to generate the ubiquitin monomer and a ligase activity to link ubiquitin molecules to tag proteins for disposal.[114] The detection of an Ile93Met mutation in the UCH-L1 gene in a German family with autosomal dominant PD[115] suggested a role for an impaired ubiquitin-proteasomal activity in PD pathogenesis. In contrast, a Ser18Tyr polymorphism affecting mainly the ligase activity has been suggested to have a protective effect in PD in some association studies.[116] However, a subsequent large case-control study involving 3,044 PD cases and 3,252 controls, failed to replicate the association.[117]

Omi/HtrA2

The gene Omi/HtrA2 (PARK13) encodes a serine-protease with pro-apoptotic activity containing a mitochondrial targeting sequence at its N-terminal region.[118] Several lines of evidence in the literature support a role for Omi/HtrA2 in neurodegeneration.[119,120] The first pathogenic mutation (G399S) a risk variant (A141S) for PD were identified in a German cohort.[121] However, a later case-control study screening the whole coding region of Omi/HtrA2 revealed that neither of the two variants was overrepresented in the patients.[122] Although another mutation, R404W, was found in Belgian PD patients,[123] it is not clear whether it is associated with PD patients in other populations. Further, the most recent large-scale analysis of the five most informative SNPs spanning the Omi/HtrA2 gene in a cohort of 6,378 cases and 8,880 controls from 20 sites worldwide again confirmed the lacking of association of Omi/HtrA2 variants with PD.[124] Therefore, the genetic basis for the involvement of Omi/HtrA2 is still not conclusive at this point.

GIGYF2

Recently, it has been proposed that the GIGYF2 gene corresponds to the PARK11 locus causes a form of autosomal-recessive familial PD.[125,126] In two independent French and Italian familial PD populations, 10 changes in 16 unrelated PD patients were found in the shortest form of GIGYF2, yielding a mutation frequency of 6.4%.[127] However, no disease-causing mutations were found in other European populations[128] and in recent months, over 10 replication studies have provided conflicting data, casting considerable doubt on the causal role of GIGYF2.[129] In addition, a pooled analysis of over 4,500 PD and 5,500 controls revealed that the estimated frequency of GIGYF2 mutations in the entire replication cohort was only about 0.001%.[127] Furthermore, the presence of mutations in healthy population controls or within asymptomatic family members of PD patients argues against causality even if longitudinal data are not available. Thus, unless new information emerges to suggest otherwise, it is reasonable to conclude that GIGYF2 does not play a major role in PD.

VPS35

The most recently described cause of monogenic PD is the mutations of a gene encoding vacuolar protein sorting-associated protein 35 (VPS35), which were identified by the next-generation of sequencing technique.[130,131] Vilariño-Güell *et al.*[130] described the identification of the p.D620N mutation in VPS35 within affected members of a Swiss kindred and three other families with late-onset, autosomal dominant PD, and in one sporadic PD case. At the same time, Zimprich *et al.*[131] published the identification of the p.D620N mutation in a large multigenerational Austrian family with PD and in two additional families screened for VPS35 mutations. Both groups also identified additional mutations in VPS35; however, the pathogenicity of these additional variants remains unknown. Moreover, VPS35 mutations have been detected only in whites with PD. Studies in both Chinese and Japanese have excluded an association between VPS35 mutations and sporadic PD.[132-134]

PLA2G6

Mutations in phospholipase A2, group VI (PLA2G6)[135] usually cause an early-onset recessive degenerative disorder with spasticity, ataxia and dystonia; however, later adult onset forms of the disease can present with a dystonia predominant parkinsonism.[136] The patients with PLA2G6 homozygous mutations presented in their 20s with slowly progressive gait problems, clumsiness, imbalance, hand tremor, cognitive decline and dysarthria. Most patients with Parkinsonism are Levodopa-responsive at first, but this usually lasts only 1-2 years. PLA2G6 mutations have been screened for both early- and late-onset PD. Although SNPs have been identified in PD patients, none of these has been convincingly associated with the risk for PD.[137,138]

EIF4G1

Most recently, translation initiator mutations in EIF4G1 were genetically linked to autosomal dominant late-onset PD with brainstem Lewy body pathology.[139] EIF4G1 is a central component of the EIF4F complex

that regulates translation of mRNAs with highly structured 5'-sequences. The most popular mutations, p.Ala502Val and p.Arg1205His mutation were found to be with PD in some population. However, later replication studies in multiple ethnicities failed to confirm EIF4G1 mutations as a cause or a susceptible factor for familial or sporadic PD.[133,140,141]

BST1

Recently, GWAS in PD have provided association evidence at 16 loci, including a region encompassing a gene encoding bone marrow stromal cell antigen 1 (BST1) on 4p15.[142] Interestingly, all PD-associated single-nucleotide polymorphisms (SNPs) on the BST1 locus lie within linkage disequilibrium blocks containing only the BST1 gene.[142] However, by direct sequencing of the entire coding region of BST1, we did not reveal a variant associated with PD.[143]

CONCERNS ON THE TRANSLATION OF GENETIC INFORMATION INTO CLINICAL APPLICATIONS

Molecular diagnosis of Parkinson's disease: possibilities and concerns

As mutations in several genes are able to cause monogenic forms of PD, molecular diagnosis using these mutations for familial PD is possible. However, cautions must take before extensive applications of these mutations to genetic counseling, because most of our knowledge about the genetic basis of PD remains preliminary. According to the latest European Federation of Neurological Societies guidelines on the molecular diagnosis of PD,[144] for mutations that are detected in rare familial forms of PD, such as point mutation or multiplication of SNCA in familial PD, molecular diagnosis should be considered only for clearly familial cases. Even for the LRRK2 genes in which mutations are much more prevalent in Europeans, molecular testing is only recommended for cases with dominant inheritance of parkinsonian syndromes, and testing for the G2019S mutation is only recommended for familial and sporadic patients in the Ashkenazi Jews or North African Arabs. Similarly, testing for mutations in recessive PD-genes (parkin, PINK-1, DJ-1) is only recommended for families suggestive of recessive inheritance (affected sib pairs) or sporadic patients with very early onset (< 35 years). For most of the other mutations, using of them for genetic testing should wait until their causative role in the disease is convincingly established.

Use of genetic variation as predictive biomarkers for Parkinson's disease: is it possible now?

A biomarker is a substance used as an indicator of normal biologic and pathogenic processes, or responses to a therapeutic intervention. Biomarkers for PD may be directed at disease risk, disease progression, or both. A mutation or genetic variant can be considered a risk biomarker for PD if it is associated with the disease. The discovery of mutations that cause monogenetic forms of PD has allowed clinical investigators to determine the cause of the disease and to predict the risk for developing the disease. However, at least two factors have to be simultaneously considered before defining such mutations as biomarkers: the penetrance of the mutations and the variability of AAO of PD caused by the mutations. Mutations that confer high risk of developing a disease usually display a high penetrance (> 80%), and the variability of AAOs of patients carrying such mutations is usually low. In autosomal dominant form of PD, the most affirmatively causative mutations are those within the SNCA and LRRK2 genes. Point mutations, duplications, and triplications of SNCA cause PD with high penetrance. However, the AAO of each mutation type in this gene is associated with a fairly high variability among cases (ranging from mid- 30s to late 80s), making it difficult to use these mutations to predict the onset and course. On the other hand, although the causative role of the LRRK2 G2019S mutation is not in question, and the AAO is less variable (usually at 60s), it is clear that the penetrance of this gene is only 30-40%. Therefore, carrying this mutation does not unequivocally predict development of PD during a lifetime.[145] The situation for the risk variants associated with the onset and progression of sporadic PD are even more complicated and puzzling as it may involve multiple independent and interactive factors. Thus, the value of a genetic biomarker in predicting an individual's risk of developing the disease is questionable at the current stage.

Can genetic variations help in molecular classification of Parkinsonian disorders?

Parkinsonian disorders are a group of clinically and pathogenically diverse disorders. For diagnostic and therapeutic purposes, it has long been expected to classify this clinical complex further. Currently, the classification of these disorders is mainly based on pathological findings. According to autopsy findings, the histological characteristics in the patients' brain have been classified as α-synucleinopathies and non-α-synucleinopathies, the latter including tauopathies, TDP-43 proteinopathies and nonspecific degeneration in the pars compacta of the substantia nigra (SNPc).[146] However, this classification is made postpartum and, therefore, less useful for preclinical and clinical diagnosis. Interestingly, studies have demonstrated that similar pathologies might result from the influence of mutations in genes that are part of the same pathways.[11,147] For example, PD cases with mutations in SNCA, LRRK2 and GBA genes usually display a common α-synuclein pathology,

while those with mutations in the MAPT gene tend to possess both α-synuclein and tau pathologies. In contrast, except for nonspecific neuronal loss and gliosis, no histopathological hallmark was revealed in most of the AR-JP patients caused by mutations in the parkin gene.[146] Moreover, many variations in these genes are not only associated with increased risk for PD, but strongly correlate with certain profiles of the disease. Hence, it is reasonable to assume that genetically determined loci, especially when combined with pathological and clinical information, can help in establishing a classification for PD. In a recent study, we have investigated clinical profiles of PD related to LRRK2 (LRRK2-PD), GBA (GBA-PD) variant, or none of the variants (idiopathic PD, IPD).[148] As a result, LRRK2-PD is largely similar to IPD, while GBA-PD patients had an earlier onset and more frequent and severe nonmotor symptoms. These results favor the feasibility of genetic classification of PD. However, since much of our knowledge about the genetic-pathologic-clinical axis of parkinsonism is quite limited so far, there is still a long way ahead before a rational nosology for parkinsonian disorders linked to their genetic underpinnings is made and before the classification becomes a practice guideline.

CONCLUDING REMARKS AND FUTURE RESEARCH CONCERNS

The past decade has been an exciting time for investigators involved in genetic research in PD. The rapidly emerging evidences of the genetic contribution to PD have changed the way we think about the disease. However, we are still not able to see a complete genetic picture of the disease. Many concerns remain to be addressed. First, the highly genetic heterogeneity among populations reminds us that the genetic information of a gene or locus provided by current studies for certain populations is limiting and segmentary. For example, although the link of the LRKK2 G2019S mutation to PD in multiple populations has been well-established, it provides little information for Eastern Asians. The emerging evidences for the contribution of another variant, G2385R, residing in a different domain of the protein may suggest a yet-unknown, but sharply different story of LRRK2 from that of the G2019S mutation. Thus, before characterizing the roles played by G2385R or other potential significant variants, a complete genetic behavior of LRRK2 should not be described merely by the G2019S information, nor should it be applied extensively to clinical practice. Similarly, it is not reasonable to overestimate the genetic contribution of the H1 haplotype of MAPT gene because the homozygous H1 allele is dominant while the H2 haplotype lacks in Asians. These problems necessitate clinical and genetic studies surrounding the population-specific variants. Second, most of the current genetic studies are focused on sequence variations (i.e. point mutations or SNPs) and much less have been directed to copy-number variations (CNVs), whose pathogenic or predisposing effects sometimes are even more evident and important. Dosage mutations of the parkin gene, for instance, have been suggested to be more pathogenic than the sequence mutations for familial PD among Europeans. Therefore, large-scale and multi-central analysis of CNVs are urgently needed to improve the image of risk genetic variants for PD. Third, the work on genetic mechanisms underlying PD is far away from just identifying mutations or risk variants. We have to figure out how these variants act on the disease, e.g. how they interact with other genes and/or environmental factors, and how they are linked to pathophysiological pathways involved in PD. In addition, prospective studies of presymptomatic carriers of mutations or risk genetic variants of PD genes are necessary to confirm their genetic roles in the disease development and progression.

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Review Article

Current overview of myasthenia gravis and experience in China

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ABSTRACT

Myasthenia gravis (MG) is an acquired autoimmune disease affecting synaptic transmission via the neuromuscular junction mainly due to the presence of auto-antibodies targeting acetylcholine receptors. Ocular or generalized MG is clinically diagnosed when the extra-ocular muscles or other muscle groups beyond the extra-ocular muscles are involved. MG occurs in both sexes at any ages from all races but shows a wide variability in incidence and prevalence. Differences in clinical phenotypes of MG patients between West and East countries have been observed. Herein, we review the current concept on epidemiology, classification, and generalized progression in MG, mainly focusing on the differential features from mainland China.

Key words: Classification, epidemiology, generalization, myasthenia gravis

INTRODUCTION

Myasthenia gravis (MG) is known as an autoimmune disease mainly mediated by auto-antibodies against the acetylcholine receptors (AChR) between the synaptic space of the skeletal muscles, leading to an impairment of the neuromuscular transmission and corresponding clinical symptoms such as fluctuating muscle weakness and fatigability.[1] According to clinical symptoms, MG is divided into ocular MG and generalized MG. Secondary generalization of clinical symptoms is common in MG, resulting in a poor prognosis for patients and a tremendous burden for families and society.[2] Although epidemiological studies have shown that all the races worldwide can be affected, differences between Caucasian and Asian patients were found in relation to clinical phenotypes.[3-5] In this mini-review, we address the current concepts of MG, including epidemiology, classification of clinical subtypes, and secondary generalization. We also focus on the different clinical features of MG in China.

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EPIDEMIOLOGY

It is well known that MG occurs worldwide affecting both males and females at any ages as shown in an epidemiological study with a large sample size.[6] However, the incidence and prevalence of MG are characterized by marked variation, depending on the time and/or the location of studies. A national epidemiological study in Australia has shown that the annual crude incidence and prevalence rates of MG were 24.9 and 117.1/million, respectively.[7] Other two population-based studies have been conducted in Taiwan and Norway. The reported annual incidence and prevalence of MG were 21 and 84-140/million in Taiwan,[8] and 16 and 131/million in Norway.[9] Moreover, the estimated annual incidence rate of MG is 30/million in central London,[10] 24/million in Ferrara province of Italy,[11] and 21.3/1 million in Barcelona of Spain.[12] Unfortunately, no national population-based epidemiological studies of MG have been conducted in mainland China. To obtain pooled data from a larger sample, Carr *et al.*[6] have collated 55 studies performed between 1950 and 2007, representing 1.7 billion population-years. By utilizing the meta-analysis, they have estimated that the annual incidence and prevalence rates of MG were 5.3 (range: 1.7-21.3) and 77.7/million (15-179), respectively.

The onset of MG may be influenced by sex and age. Regardless of age, the crude incidences of females

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and males in Australia are 27.9 and 21.9/1 million, respectively, with a female predominance.[6] A similar tendency was shown in Taiwan where the incidence ratio of males to females is 0.68.[8] However, three studies with large sample sizes showed a nearly equal incidence for both sexes in mainland China.[13-15] Considering age and sex, the occurrence of MG exhibits a bimodal fashion. Below 40 years of age, the ratio of female to male is nearly 3:1; however, during puberty and between 40 and 50 years, the incidence rate is roughly equal. Over 50 years, MG is more common in males, with a ratio of 3:2.[16] Osserman and Genkins have observed two peaks of incidence in MG, with the first one at 20-40 years old and the second one at 40-60 years old,[17] but in another study, the second peak of incidence was determined at ages of 60-80 years.[18] Childhood MG (onset < 15 years) is not common in North America and Europe, comprising 10-15% of MG cases.[19] However, MG occurs during childhood in up to 50% of Chinese patients, mainly with pure ocular symptoms.[5,13]

CLASSIFICATION OF MYASTHENIA GRAVIS

Myasthenia gravis is a heterogeneous disorder with variable clinical symptoms because of the different location of involved neuromuscular junction. Up to now, the most widely accepted classification is the Myasthenia Gravis Foundation of America (MGFA) Clinical Classification,[20] a Task Force that was formed by the Medical Scientific Advisory Board of MGFA since 1997. It was designed to identify subtypes of MG patients with distinct clinical features or severity of disease indicating different prognosis or treatment response, but it is not used to evaluate the outcome. According to MGFA, MG can be divided into 5 main classes and several subclasses [Table 1].

Another classification of MG is based on clinical symptoms, age of onset, auto-antibody profile and thymic histology.[21-24] Briefly, MG patients are divided into six subtypes: ocular MG, early-onset MG, late-onset MG, thymoma-associated MG, muscle-specific tyrosine kinase (MuSK) antibody-associated MG and seronegative MG.[25,26] Early-onset patients have several clinical characteristics such as female predominance, generalized involvement, no evidence of thymoma and presence of anti-AChR antibodies. A predominance of thymic hyperplasia is observed in this subtype. However, late-onset MG patients are more common among males. These patients have generalized symptoms, and usually have normal or atrophic thymus.[27] The titer of anti-AChR antibodies is usually lower in late-onset subtype than that in the early-onset subtype, and antibodies against titin and ryanodine receptor are detected in about 50% of such patients.[23] Thymoma-associated MG involves MG patients with thymoma regardless of the extent of muscular involvement, accounting for about 10-15% of all MG patients. Male and female patients are equally common in this subtype, and MG occurs at any age with a peak onset age of 50 years.[28,29] In seronegative MG patients, anti-AChR and anti-MuSK antibodies are undetectable. Clinical features such as variable age of onset, lack of thymoma and variable extent and severity of muscular involvement are also found.[30] The detailed characteristics of all subtypes are listed in Table 2.

Table 1: MG foundation of America clinical classification

Type	Characteristics
Class I	Any ocular muscle weakness, possible ptosis, no evidence of muscle weakness elsewhere
Class II	Ocular muscle weakness of any severity, mild weakness of other muscles
Class IIa	Predominantly limb and/or axial muscles weakness, possible lesser involvement of bulbar muscles
Class IIb	Predominantly bulbar and/or respiratory muscles weakness, possible lesser or equal involvement of limb and/or axial muscles
Class III	Ocular muscle weakness of any severity, moderate weakness of other muscles
Class IIIa	Predominantly limb and/or axial muscles weakness, possible lesser involvement of bulbar muscles
Class IIIb	Predominantly bulbar and/or respiratory muscles weakness, possible lesser or equal involvement of limb and/or axial muscles
Class IV	Ocular muscle weakness of any severity, severe weakness of other muscles
Class IVa	Predominantly limb and/or axial muscles weakness, possible lesser involvement of bulbar muscles
Class IVb	Predominantly bulbar and/or respiratory muscles weakness, possible lesser or equal involvement of limb and/or axial muscles
Class V	Intubation with or without mechanical ventilation except when employed during routine postoperative management, the use of feeding tube without intubation places the patient in class IVb

MG: myasthenia gravis

Table 2: Clinical subtypes of MG

Subtypes	Characteristics
Ocular MG	Purely ocular symptoms, no evidence of thymoma, adult in America and Europe, childhood in Asia, anti-AChR antibody positive in 50%
Early-onset MG	Age of onset < 50 years, thymic hyperplasia, usually females, antibodies against AChR
Late-onset MG	Age of onset > 50 years, normal or atrophic thymus, mainly males, presence of antibodies against AChR, titin, RyR
Thymoma-associated MG	Age of onset between 40 and 60 years, thymic neoplasia, antibodies against AChR, titin, RyR and voltage-gated K^+channel subfamily A member 4 (KCNA4)
MuSK antibody-associated MG	Onset age < 40 years in most patients, normal thymus, antibodies against MuSK
Seronegative MG	Variable muscular involvement and severity, variable age of onset, thymic hyperplasia in some patients, no detectable antibodies against AChR and MuSK

MG: myasthenia gravis; MuSK: muscle-specific tyrosine kinase; AChR: acetylcholine receptors; RyR: ryanodine receptor

Modified osserman classification is also commonly used to distinguish subtype of MG patients and indicates the different prognosis and treatment response. This classification has been frequently recommended and widely used over the past several decades in China. Although the modified Osserman classification is based on clinical symptoms, impact on work and daily life, course of disease and treatment response, it is extremely challenging to take into account the prognosis and disability of patients. Moreover, this classification does not contain MG-associated auto-antibodies and low-frequency repetitive nerve stimulation (RNS) tests.

In 1997, Wang *et al*.[31] proposed a new clinical absolute and relative score system for MG in Chinese patients.The absolute scoring system consists of 8 items: ptosis, palpebra superior fatigability, disability of ocular motion, fatigability of the upper and lower extremity muscles, disability of facial muscles, chewing difficulties, dysphagia and disability of respiratory muscles, with a score of each item ranging from 0 (normal) to 4 (severe dysfunction). The relative scores are obtained by subtracting the pretreatment scores from the posttreatment scores and then dividing the results by the pretreatment scores. Several studies have proven that the clinical absolute and relative scoring system has good reliability and sensitivity to evaluate the disabilities in MG patients[31,32] and the clinical absolute and relative system is officially recommended by the Consensus of Chinese Experts in the Diagnosis and Treatment for Myasthenia Gravis.[33]

SECONDARY GENERALIZATION

Generalization of clinical symptoms is an important hallmark of MG patients. Ocular MG is termed when weakness is only limited to the extra-ocular muscles for > 2 years,[34,35] while generalized MG is defined as an extension of weakness beyond ocular muscles. The involvement of muscles is confirmed mainly by clinical presentations. Due to the different involvement of muscle groups, clinical presentation varies from fluctuating extra-ocular muscular weakness to respiratory failure. Secondary generalization mainly occurs during the first 2 years[16,36] and sometimes leads to the deterioration of prognosis including death.

It is well-known that ocular muscle weakness is the most common initial symptoms of MG, occurring in approximately 85% of patients. About 50% of these ocular MG patients may progress to generalized MG within 6 months after onset, 80% of patients within 1-year, and 90% of patients after 3 years. Only 10% of MG patients do not progress to secondary generalization throughout lifetime.[2] Another published study has reported that up to 65% of MG patients initially show ocular muscle involvements, and generalization of symptoms occur in only 44% of patients within 2 years.[37] In a follow-up study including 96 Thai patients with ocular MG, only 15 patients (15.6%) developed generalized symptoms within 2 years from the initial diagnosis.[38] It is to be noted that about 50% of Chinese MG patients present with pure ocular manifestations during their entire lifetime,[5] with a relatively lower rate of generalization. Recently, Jing *et al*.[39] have also reported that only 26% of Chinese patients with ocular MG develop into generalized MG during a 13-year follow-up period. These differences in the rate of secondary generalization might be attributed to the difference in race, severity of disease and early treatment with immunosuppressive drugs, especially corticosteroids.[38,40]

Given the poor prognosis of generalized MG, it is important to detect the risk factors of secondary generalization in those MG patients with initial ocular presentations. Previous studies have revealed that onset age > 15 years, presence of thymoma, early corticosteroids therapy and abnormal RNS results on stimulating proximal limb muscles are predictors for the development of generalized MG.[39,41-43] Our recent study has shown that disease onset during adulthood and RNS abnormality of the facial nerve predict the progression from ocular to generalized MG while course of the disease is inversely correlated with secondary generalization (unpublished data). In a senior population, the ocular MG patients with anti-AChR antibodies, antistriated muscle antibodies, abnormal RNS findings and abnormal single fiber electromyography tend to develop generalized MG.[44] However, other studies have demonstrated that none of these factors significantly predict development of generalized MG in younger populations.[2,45,46] Although similar results have been obtained in some studies, there are also some limitations such as the use of retrospective methodology, incomplete clinical data, small sample size and single hospital or center. Larger-sample, multi-center, prospective studies are needed to obtain more convincing risk factors for generalization of ocular MG.

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Original Article

Prognostic significance of neutrophil-to-lymphocyte ratio in glioblastoma

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ABSTRACT

Aim: The neutrophil-to-lymphocyte ratio (NLR) has prognostic value in patients with a variety of cancers. The purpose of this study was to investigate the prognostic value of NLR in patients with glioblastoma. **Methods:** A prospective study was conducted on patients receiving surgery for glioblastoma. Preoperative NLR was recorded and correlated with other prognostic factors and survival. **Results:** Fifty-one patients were included in the study. The mean NLR ratio was 6.7 ± 4.6. Using receiver operating characteristic curve analysis, an NLR cut-off value of 4.7 was determined to best predict survival. Patients with NLR ratios exceeding 4.7 differed significantly from those with NLR ratios ≤ 4.7 and were associated with reduced survival. Patients with gross total tumor excision had a median survival of 18 months, whereas the median survival time was 11 months in patients with subtotal tumor excision. No significant difference in survival was observed with respect to patient age, gender, Karnofsky performance status, or tumor location. Using multivariate analysis, NLR and extent of tumor resection were identified as factors with independent prognostic power. **Conclusion:** Neutrophil-to-lymphocyte ratio is an inexpensive, widely available biomarker of glioblastoma aggressiveness and should be used alongside current glioblastoma prognostic factors.

Key words: Glioblastoma, neutrophil-lymphocyte ratio, prognosis

INTRODUCTION

Glioblastoma is by far the most common type of primary brain tumor that occurs in adults. This devastating disease is usually incurable and, despite aggressive treatment, the median survival time remains in the range of 15 months.[1] Cancer-associated inflammation has been correlated with outcome in patients with cancer.[2-4] Among the various inflammation markers, the neutrophil-to-lymphocyte ratio (NLR) has been examined in a variety of cancers and has been found to be elevated in patients with more advanced or aggressive disease.[5-8] The exact mechanisms by which neutrophilia is induced by tumors is unclear.[9,10] The secretion of angiogenesis factors and cytokines has been implicated to play a role in neutrophilia induction.[11,12] In gliomas, lymphocyte infltration around the tumor has been associated with a better prognosis.[13] To the best of our knowledge, only one study has assessed the role of NLR in glioblastoma patients.[14] In this study, we aimed to assess the prognostic value of NLR and correlate it with other prognostic factors of glioblastoma.

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METHODS

Study population

We prospectively studied patients who received surgery for glioblastoma in our institute between March 2007 and September 2013. Patients were included if they had full blood count results at first presentation, before any treatment. The extent of resection was determined by comparing magnetic resonance imaging (MRI) scans obtained before surgery with those obtained within the 1st month after surgery. Clinical variables that were analyzed included age, sex, and preoperative Karnofsky performance status score (KPS). Radiological variables included tumor lateralization, location and volume. Tumor volumes were approximated from preoperative, postgadolinium T1-weighted MRI using a modified ellipsoid volume equation ($radius_x \times radius_y \times radius_z$)/2.[15] All patients received postoperative radiotherapy with temozolomide, followed by temozolomide chemotherapy

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for up to 1-year or until recurrence. Radiotherapy was administered as fractionated focal irradiation at a dose of 2 Gy/fraction given once a day for 5 days/week over a period of 6 weeks up to a total dose of 60 Gy. Follow-up MRIs were performed every 2 months. Recurrence was defined based on MRI and/or single photon emission tomography findings.[16] The study was approved by the Institutional Review Board.

Statistical analysis

Pearson's correlation coefficient was used to assess continuous variables. Progression-free survival (PFS) was defined as the time from the initial surgery to demonstration of tumor progression on follow-up MRI or to death. Survival time was defined as the time between the date of diagnosis and the date of death for deceased patients, or to the last follow-up for surviving patients. The overall survival time was estimated using Kaplan-Meier methods, and log-rank analysis was performed to compare survival curves between groups. Patients who were still alive at last contact were treated as censored events in the analysis. Multivariate Cox regression analysis of the data was used to analyze possible prognostic factors. The forward step-wise model selection procedure was used (*P* value of likelihood-ratio test < 0.05 as inclusion criteria; likelihood-ratio test > 0.10 as exclusion criteria) to define the final model. The following variables were entered: gender, age at diagnosis, KPS, NLR, and the extent of resection. With respect to NLR, receiving operating characteristics (ROC) curve analysis was performed in order to determine the cut-off value for predicting survival. A 2-sided $P < 0.05$ was considered as statistically significant.

RESULTS

Study population

Table 1 summarizes the patient data. Fifty-one patients (30 males, 21 females, mean age 59.2 ± 14.2) met the inclusion criteria for the study. The majority of glioblastomas were lateralized, with 28 (54.9%) on the left and 21 (41.1%) on the right side. The most common tumor site was the temporal lobe (37.2%), followed by the occipital lobe (27.5%). The mean tumor volume was 32.1 ± 27.3 cm^3. In 19 cases (37.2%), the tumor was located close to a ventricle. Thirty-four patients had a KPS over 80. In 32 cases, gross total excision was achieved, whereas in 19 cases there was subtotal tumor resection. One patient was lost to follow-up, and one patient died in the immediate postoperative period. After a mean follow-up period of 17 months (range: 3-39 months), 14 patients were alive.

Table 1: Patient data

Patient characteristic	*n* (%)	OS *P*
Gender		
Male	30 (58.8)	0.3
Female	21 (41.2)	
Age		
> 60	20 (39.2)	0.4
< 60	31 (60.8)	
KPS		
> 80	34 (66.7)	0.052
< 80	17 (33.3)	
NLR		
> 4.7	29 (56.8)	0.01
< 4.7	22 (43.2)	
Extent of resection		
GTR	32 (62.7)	0.036
STR	19 (37.2)	

KPS: Karnofsky performance status, GTR: gross total excision (> 95%), STR: subtotal excision (75-95%), OS: overall survival, NLR: neutrophil-to-lymphocyte ratio

Neutrophil-to-lymphocyte ratio

The mean NLR was 6.7 ± 4.6. Using ROC curve analysis, a cut-off NLR value of 4.7 was determined to best predict survival. Patients with an NLR exceeding 4.7 differed significantly from those with an NLR ≤ 4.7 and were associated with decreased survival time (11 *vs.* 18.7 months, $P = 0.01$) [Figure 1]. There was a significant increase in PFS for patients with an NLR lower than 4.73 ($P = 0.03$).

Extent of resection

Patients with gross total tumor excision had a median survival of 18 months, whereas in patients with subtotal tumor excision, the median survival time was 11 months. The difference was statistically significant ($P = 0.036$) [Figure 2].

Karnofsky performance status score

The median survival for patients with KPS over 80 was 17 months, whereas survival for patients with KPS under or equal to 80 was 11 months. However, the difference was marginally significant ($P = 0.052$). No significant difference in survival was observed with respect to patient age ($P = 0.4$) or gender ($P = 0.3$).

Tumor characteristics

In 19 cases, the tumor was located close to a ventricle. These patients were associated with reduced survival ($P = 0.052$). No prognostic significance was found for tumor location or laterality [Table 2]. No correlation was found between NLR and tumor volume.

Multivariate analysis

Using multivariate analysis, NLR ($P = 0.011$, 95% confidence intervals [CI]: 1.4-17.3) and extent of tumor resection ($P = 0.025$, 95% CI: 1.2-8.7) were identified as factors with independent prognostic power.

DISCUSSION

In the present study, we found that patients with an NLR over 4.7 were associated with reduced median overall survival. Patients with subtotal tumor excision

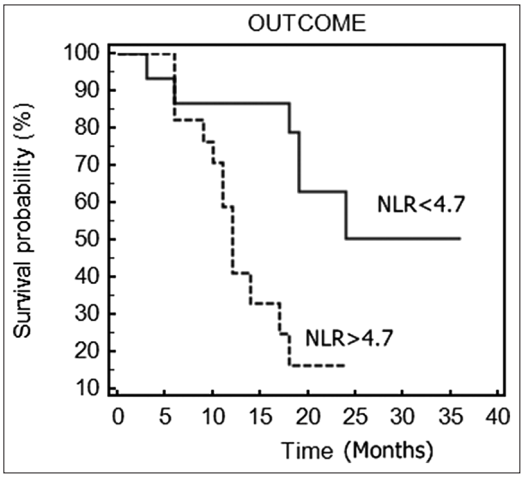

Figure 1: Relationship between neutrophil-to-lymphocyte ratio (NLR) index (cut-off = 4.7%) and survival in glioblastoma patients. NLR: neutrophil-to-lymphocyte ratio

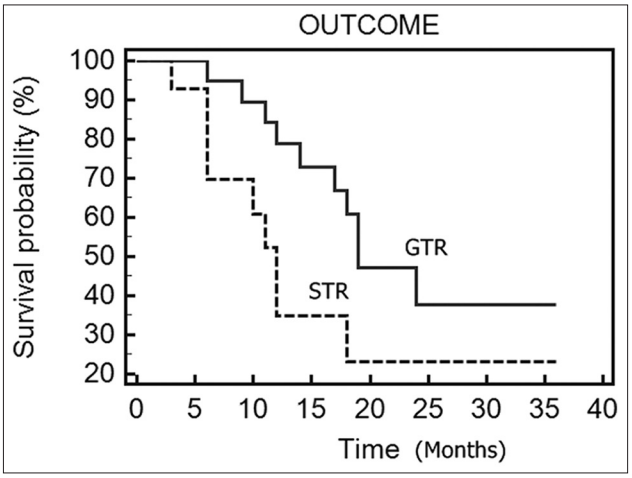

Figure 2: Representative Kaplan-Meier survival curve of patients grouped according to the extent of resection. GTR: gross total excision (> 95%); STR: subtotal excision (75-95%)

had reduced median overall survival compared with patients with gross total tumor excision. There was a trend toward increased survival for patients with KPS over 80 and tumors not related to the ventricular system.

In addition to genetic factors, systemic inflammatory response has also been implicated in carcinogenesis. Bambury *et al*. also studied the prognostic impact of the NLR in a cohort of patients with glioblastoma.[1] The authors studied 84 patients that had full blood count results available at first presentation with symptoms of glioblastoma, and the NLR was calculated. The results of this study showed that age over 65 years, gender, eastern cooperative oncology group performance status ≥ 2, frontal tumor, extent of surgical resection, completion of the adjuvant chemoradiation protocol, and NLR > 4 were significantly correlated with overall survival. The present study verified the above findings. Furthermore, we found no correlation between NLR and tumor volume. Patients with tumors not related to the ventricular system had a better prognosis.

In other cancer studies, a prognostic significance of NLR was found. A recent meta-analysis of 26 studies in primary liver cancer demonstrated that the high NLR can strongly predict poor survival in these patients, indicating the predictive value of the NLR as a new biomarker in primary liver cancer.[6] Furthermore, high NLR was associated with vascular invasion and correlated with alpha-fetoprotein levels. Proctor *et al*.[12] studied 12,118 patients who had been sampled within 2 years of their cancer diagnosis and found that NLR was independently associated with survival in all cancers studied.

Apart from NLR, other inflammatory markers have also been associated with patient prognosis. Steffens *et al*.[17]

Table 2: Tumor characteristics

Parameter	Proportion
Tumor location (%)	
Frontal	15.7
Temporal	37.2
Parietal	13.7
Occipital	27.5
Multifocal	5.8
Laterality (%)	
Right	41.1
Left	54.9
Midline	4
Located near ventricle (%)	37.2
Tumor volume (cm^3)	32.1 ± 27.3

have reported that a high preoperative serum C-reactive protein level is an independent predictor of poor survival in patients with renal cell carcinoma. In the present study, we verified the prognostic significance of gross total tumor excision. This is a well-established prognostic factor.[18] Karnofsky performance scale and age have been also associated with glioblastoma prognosis.[14,18] In the present study, there was a trend towards increased survival for patients with KPS over 80. Glioblastomas adjacent to the lateral ventricles have been suggested to harbor a dismal prognosis.[19,20] Neural and cancer stem cells have been found in the subventricular zone that lines the lateral ventricles.[20] Thus, tumors in this region may be more invasive with higher potential to recruit migratory progenitor cells.[20] In the present study, we verified the prognostic significance of tumors located adjacent to the ventricles.

In conclusion, our results are in agreement with Bambury *et al*., as well as other reports of the prognostic significance of NLR in a variety of cancers.[2,3] NLR is an inexpensive and widely available biomarker of glioblastoma aggressiveness, and thus should be used alongside current glioblastoma prognostic factors. Nevertheless, there is obviously a need for future studies

with larger numbers of patients in order to confirm our preliminary observations.

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Original Article

Tumor necrosis factor receptor superfamily member 9 is upregulated in the endothelium and tumor cells in melanoma brain metastasis

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ABSTRACT

Aim: The cytokine receptor tumor necrosis factor receptor superfamily member 9 (TNFRSF9) is mainly considered to be a co-stimulatory activation marker in hematopoietic cells. Several preclinical models have shown a dramatic beneficial effect of treatment approaches targeting TNFRSF9 with agonistic antibodies. However, preliminary clinical phase I/II studies were stopped after the occurrence of several severe deleterious side effects. In a previous study, it was demonstrated that TNFRSF9 was strongly expressed by reactive astrocytes in primary central nervous system (CNS) tumors, but was largely absent from tumor or inflammatory cells. The aim of the present study was to address the cellular source of TNFRSF9 expression in the setting of human melanoma brain metastasis, a highly immunogenic tumor with a prominent tropism to the CNS. **Methods:** Melanoma brain metastasis was analyzed in a cohort of 78 patients by immunohistochemistry for TNFRSF9 and its expression was correlated with clinicopathological parameters including sex, age, survival, tumor size, number of tumor spots, and BRAF V600E expression status. **Results:** Tumor necrosis factor receptor superfamily member 9 was frequently expressed independently on both melanoma and endothelial cells. In addition, TNFRSF9 was also present on smooth muscle cells of larger vessels and on a subset of lymphomonocytic tumor infiltrates. No association between TNFRSF9 expression and patient survival or other clinicopathological parameters was seen. Of note, several cases showed a gradual increase in TNFRSF9 expression on tumor cells with increasing distance from blood vessels, an observation that might be linked to hypoxia-driven TNFRSF9 expression in tumor cells. **Conclusion:** The findings indicate that the cellular source of TNFRSF9 in melanoma brain metastasis largely exceeds the lymphomonocytic pool, and therefore further careful (re-) assessment of potential TNFRSF9 functions in cell types other than hematopoietic cells is needed. Furthermore, the hypothesis of hypoxia-driven TNFRSF9 expression in brain metastasis melanoma cells requires further functional testing.

Key words: 4-1BB, brain metastasis, CD137, melanoma, tumor necrosis factor receptor superfamily member 9

INTRODUCTION

The cytokine receptor tumor necrosis factor receptor superfamily member 9 (TNFRSF9) (also known as CD137 or 4-1BB) is usually expressed upon cellular activation and acts as a co-stimulatory and the pro-survival molecule in different cellular subsets of the lymphoid and myeloid lineage.[1] Hematopoietic cells which have been activated via TNFRSF9 stimulation have shown an increased antitumor response in various preclinical models.[2,3] This effect was mainly attributed to increased numbers of CD8-positive cytotoxic T-cells as well as antigen-specific memory T-cells.

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Furthermore, tumor-infiltrating lymphocytes (TIL) which have been stimulated with agonistic TNFRSF9 antibodies show a stronger antitumor effect as well as prolonged survival.[4] Therefore, stimulating TNFRSF9 by the use of agonistic antibodies has been proposed as an additional immunotherapeutic approach in cancer treatment-especially for melanoma, which represents one of the most immunogenic tumors-and has already entered clinical trials.[5,6] An alternative approach uses genetically modified human T-cells to express higher TNFRSF9 levels.[7] TNFRSF9 stimulation has been suggested as a treatment for metastatic cancers.[8] However, to date there is only poor data about the distribution of TNFRSF9 in brain metastases, which still constitute one of the most deleterious clinical conditions in tumor patients.[9,10] This data is of importance since preliminary clinical studies targeting TNFRSF9 have already been stopped due to considerable side effects.[11] In a previous study, our group showed that TNFRSF9 was strongly upregulated by reactive astrocytes (so-called gemistocytes) in primary central nervous system (CNS) tumors, whereas both brain tumor cells and TIL were mainly TNFRSF9-negative.[12] Since most studies have only focused on TNFRSF9 expression on hematopoietic cells, there is an urgent need to decipher TNFRSF9 expression on other cell types and different microenvironmental conditions *in vivo* in more detail. Of note, a recent animal study discovered that TNFRSF9 is also expressed in neural stem cells, in which it induced cell death.[13] The expression of TNFRSF9 on cell types other than hematopoietic cells might be at least partly responsible for side effects in preliminary clinical trials targeting TNFRSF9. Therefore, the aim of our current study was to define the cellular source of TNFRSF9 expression in melanoma brain metastasis, in order to assess the suitability of an anti-TNFRSF9 treatment in this detrimental clinical condition.

METHODS

Patient data

The use of human tissue from cases of melanoma brain metastasis and the respective clinical data was approved by the ethical committee of the Eberhard Karls University of Tübingen and Tübingen University Hospital (project no. 408/2013BO2). Our cohort consisted of 78 patients suffering from melanoma brain metastases which underwent neurosurgical resection. A detailed overview of our patient cohort is provided in Table 1. Tissue microarrays were constructed from formalin-fixed and paraffin-embedded tumor samples for immunohistochemical analysis of TNFRSF9 expression. Brain magnetic resonance imaging data was analyzed for metastasis size (diameter) and number. In cases with > 10 metastases in one patient, the number of metastases was set to 10 for statistical analysis. Patient age at surgery and overall survival after surgery were registered.

Immunohistochemistry

For immunohistochemistry, a mouse monoclonal antihuman TNFRSF9 antibody (dilution 1:40; clone S16, Novocastra/Leica Microsystems, Germany) was used as previously published.[12] Tissue labeling was performed using the Discovery XT immunohistochemistry system (Ventana Medical Systems, France). A cell conditioning pretreatment was performed for 68 min followed by a 4 min blocking step with inhibitor D. The primary antibody was applied for 32 min, followed by secondary antibody (Discovery Universal Secondary Antibody) for 32 min. After washing steps, a blocking step with blocker D for 4 min and a 16 min incubation with one drop of SA-HRP D were performed. For diaminobenzidine (DAB) visualization, the sections were incubated with one drop of DAB D and one drop of DAB H_2O_2 D for 8 min, followed by a copper enhancer (Copper D, all Ventana Medical Systems, Tucson, AZ, USA) for 4 min. Specimens were washed, counterstained with hematoxylin and bluing reagent, and mounted. In addition, our cohort was immunohistochemically assessed for BRAF V600E mutations using mouse monoclonal IgG2a antihuman BRAF V600E (dilution 1:100; clone VE1, Spring Bioscience). Images were analyzed and recorded on an Olympus BX-50 microscope (Olympus, Germany).

Scoring

Tumor necrosis factor receptor superfamily member 9 expression was separately assessed in both tumor and endothelial cells by taking staining intensity and frequency into account, using a previously established protocol.[14,15] The semi-quantitative scores consist of a frequency score ranging from 0 to 4 (0 = 0-1%, 1 = 2-10%, 2 = 11-25%, 3 = 26-50%, and 4 ≥ 50% of all cells showing positive nuclear staining). Likewise, intensity was recorded in a similar semi-quantitative approach as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. The scores for staining intensity and frequency were multiplied together, so that the final expression cell score reflected both. The evaluation and photographic

Table 1: Patient data

Characteristic	Data
Patient age, median (range)	60 (20-83)
Sex, male/female	47/31
Number of brain metastases, median (range)	1 (1-10)
Tumor size in mm, median (range)	28 (5-61)
BRAF V600E mutation assessed by IHC, yes/no	38/40
Survival in days, median (range)	177 (17-4166)

IHC: Immunohistochemistry, BRAF: proto-oncogene B-Raf

documentation of immunohistochemical staining was performed using an Olympus BX50 light microscope.

Statistical analysis

The semi-quantitative TNFRSF9 scores were assigned as ordinal scaled response variables and analyzed together with nominal, ordinal, or continuous variables. Nominal and ordinal data was analyzed using a contingency table followed by likelihood ratio and Pearson tests. Survival analyses were performed using Kaplan-Meier and multivariate analyses. In order to compare survival curves, Wilcoxon and log-rank tests were used for censored data. TNFRSF9 expression levels were dichotomized at the median and referred to as low or high. A significance level of alpha = 0.05 was selected for all tests. Statistical analysis was performed using JMP 11.0.0 software (SAS Institute, Cary, NC, USA).

RESULTS

Tumor necrosis factor receptor superfamily member 9 is expressed on tumor and endothelial cells in melanoma brain metastasis

Immunohistochemical analyses of our melanoma brain metastasis cohort revealed that reactive astrocytes (gemistocytes) were strongly TNFRSF9-positive, especially at the border between melanoma metastasis and infiltrated CNS tissue, and similarly to our previous findings in a large cohort of primary brain tumors [Figure 1a].[12] Melanoma cells of brain metastasis showed a very heterogeneous TNFRSF9 staining pattern [Figure 1b]. Frequently, TNFRSF9 expression on melanoma cells became stronger with increasing distance from intra-tumoral blood vessels [Figure 1b], especially in perinecrotic areas. As previously shown, TNFRSF9 was also consistently expressed on smooth muscle cells of larger intra-tumoral blood vessels [Figure 1c]. Of note, TNFRSF9 was also upregulated on endothelial cells of smaller blood vessels within melanoma brain metastasis [Figure 1d]. In addition, a subset of lymphomonocytic infiltrates within melanoma tissue also displayed strong TNFRSF9 expression [Figure 1e]. TNFRSF9 expression on melanoma cells was mainly detected within the cytoplasm [Figure 1f], at the cellular membrane [Figure 1g and h], or both.

Tumor necrosis factor receptor superfamily member 9 expression in melanoma cells does not correlate with expression in endothelial cells within individual melanoma brain metastases

Next, we assessed if TNFRSF9 expression in melanoma brain metastasis was equally upregulated on both tumor and endothelial cells within individual tumors. However, the expression on melanoma cells (median expression score: 4; range: 1-12) was strongly varied in tumors with similar endothelial cell scores (median expression: 3; range: 0-12). No significant correlation between tumor and endothelial cell expression scores was found [Figure 2a]. These findings point to distinct regulatory mechanism of TNFRSF9 in melanoma and endothelial cells. Of note, in cases with an endothelial cell score of > 8, no melanomas with a tumor cell score < 4 were found.

Survival of melanoma brain metastasis patients is not associated with tumor necrosis factor receptor superfamily member 9 expression

To address the question of a potential clinicopathological

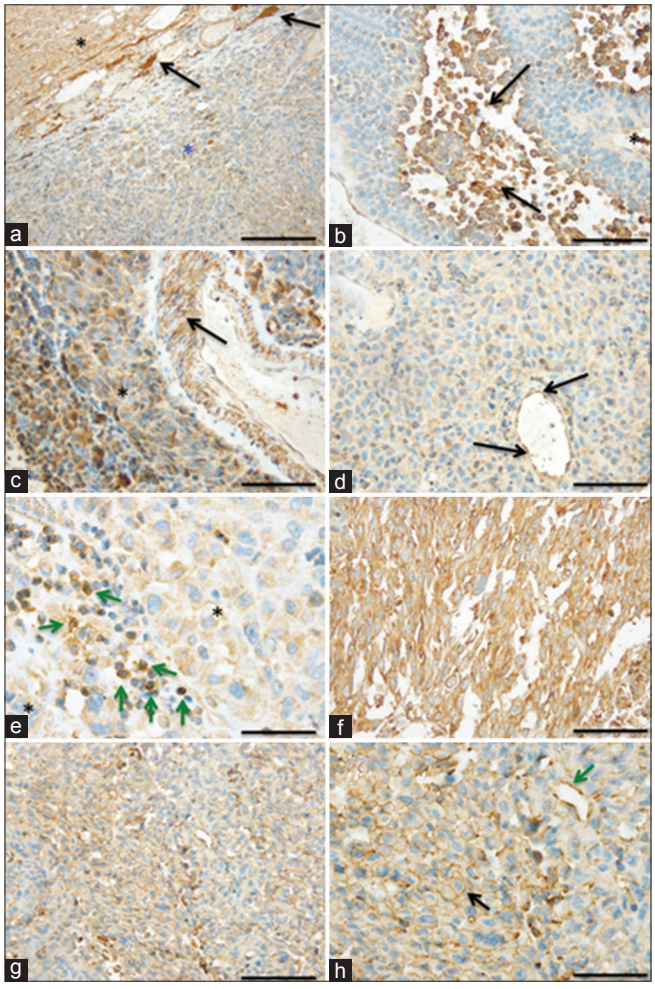

Figure 1: Tumor necrosis factor receptor superfamily member 9 (TNFRSF9) is upregulated on tumor and endothelial cells in melanoma brain metastases. Immunohistochemistry revealing (a) strongly TNFRSF9-positive reactive astrocytes (gemistocytes; arrows) at the border between central nervous system tissue (black asterisk) and melanoma brain metastasis (blue asterisk). (b) Frequently, TNFRSF9 expression on melanoma cells increases (black arrows) with the distance from blood vessels (asterisks). (c) Smooth muscle cells of larger vessels (arrow) within melanoma brain metastasis (asterisk) exhibit strong TNFRSF9-positivity. (d) Apart from melanoma cells, TNFRSF9 is also upregulated on endothelial cells (arrows) of small intra-tumoral blood vessels. (e) Intra-tumoral lymphocytic infiltrates (green arrows) in melanoma brain metastasis (asterisk) also display membranous TNFRSF9-positivity. While some melanoma brain metastases showed strong TNFRSF9 expression (f) both at the cell membrane and within the cytoplasm, (g) others displayed only weak to moderate TNFRSF9 staining at the cell membrane (h: higher magnification of g. Black arrow: melanoma cells; green arrow: blood vessel). (Scale bars: a: 200 μm; b, c, d, f, g: 100 μm; e: 50 μm; h: 50 μm)

relevance of TNFRSF9 expression in melanoma brain metastasis, we performed Kaplan-Meier survival analysis in our cohort of 78 patients. A survival analysis was performed separately for TNFRSF9 expression on melanoma [Figure 2b] and endothelial [Figure 2c] cells. No significant association of TNFRSF9 expression on melanoma (log-rank test: 0.23; Wilcoxon test: 0.31) or endothelial (log-rank test: 0.39; Wilcoxon test: 0.67) cells with patient survival was observed. However, although not showing statistically significant differences, there was a dichotomic tendency for high TNFRSF9 levels and patient survival in melanoma cells as compared to endothelial cells.

Tumor necrosis factor receptor superfamily member 9 levels in melanoma brain metastasis are independent of clinicopathological parameters

The finding of intra-individual differences in TNFRSF9 expression in melanoma brain metastasis [Figure 1b] led to the hypothesis that, in general, tumor size might be associated with increased TNFRSF9 levels due to nutritive changes with increasing tumor volume. However, no significant differences in tumor size were observed with respect to TNFRSF9 scores for melanoma cells [Figure 2d]. In fact, TNFRSF9 scores for melanoma [Figure 2d] and endothelial (data not shown) cells remained quite stable with increasing tumor size. Furthermore, no association of TNFRSF9 expression on melanoma or endothelial cells with patient age, sex, number of brain metastases, or BRAF V600E status was seen (data not shown).

DISCUSSION

The cellular source of TNFRSF9 expression in melanoma brain metastasis consists of a larger pool than the typically assessed tumor-infiltrating lymphomonocytic cells.[2,3] In our cohort of 78 melanoma brain metastasis patients, TNFRSF9 expression was frequently detected on both tumor and tumor-associated endothelial cells, but only to a moderate extent on tumor-infiltrating lymphomonocytic cells [Figure 1]. It has been previously shown that TIL that do not express TNFRSF9 display a significantly lower cytolytic antitumor activity.[16] Although melanomas are considered to be highly immunogenic tumors, they possess various strategies to escape from antitumor immune surveillance.[17] However, it is impossible to conclude from our data whether the low TNFRSF9 expression level on tumor-infiltrating immune cells is linked to a primary "underactivation" of the respective cells or an active counter-regulation exerted by melanoma cells. The fact that TNFRSF9 expression on melanoma cells was independent of expression on endothelial cells points to a cell lineage specific upregulation, rather than a general intra-individual regulatory mechanism. Our findings are in line with previous studies that described a selective upregulation of TNFRSF9 on tumor-associated endothelium, whereas endothelial cells from normal control cases remained negative.[18] In addition, TNFRSF9 expression has been discovered on endothelial cells of hypoxic or inflamed blood vessels.[19,20] Our observation that increased TNFRSF9 expression was especially seen in perinecrotic areas-and also with increasing distance from blood vessels-might be related to the fact that TNFRSF9 is also upregulated via hypoxia inducible factor-1 alpha (HIF-1α), indicating that hypoxia might also drive its expression on tumor cells.[21] Although it has been demonstrated that HIF1α-related TNFRSF9 upregulation is beneficial for the survival of hematopoietic cells, it can induce cellular apoptosis in other cell types such as liver or tumor cells.[22-24] This might at least partly explain why severe liver toxicity occurred in the first clinical studies targeting TNFRSF9 in humans.[25] Whether enhanced TNFRSF9 expression in perinecrotic areas in melanoma brain metastasis is beneficial or detrimental to the tumor remains an open question. However, a conclusion by analogy can be made, since these areas usually harbor an extremely elevated number of apoptotic cells. Thereby, one can speculate that the upregulation of TNFRSF9 in hypoxic areas is an indication of elevated cell death. Of note, TNFRSF9 expression on either tumor or endothelial cells was not associated with age, sex, patient survival, size or number of brain metastases, or BRAF V600E expression status. Therefore, TNFRSF9 does not serve as a prognostic marker on tumor or endothelial cells *per se*. Instead, differences in TNFRSF9 expression might reflect inter-individual tumor heterogeneity, including

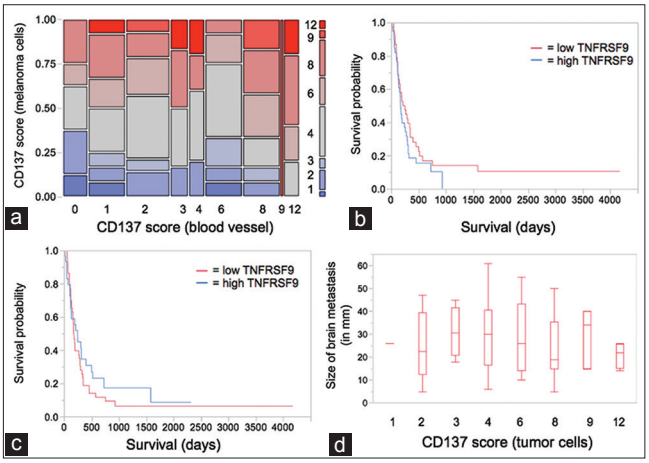

Figure 2: Tumor necrosis factor receptor superfamily member 9 (TNFRSF9) expression in melanoma brain metastasis is independent from clinicopathological data. (a) Contingency analysis of TNFRSF9 expression scores for melanoma and endothelial cells ($n = 78$). (b and c) Kaplan-Meier survival curves stratified by median split of TNFRSF9 expression scores for (b) melanoma and (c) endothelial cells. (d) Box-plot diagram of brain metastasis size (in mm) versus TNFRSF9 score for melanoma cells

alteration of oxygenation or nutrition related to vascularization, rather than a tumor-intrinsic phenomenon. Since we could not define the factors which are responsible for TNFRSF9 upregulation in melanoma brain metastasis, we can only speculate about the underlying reasons. Previous studies have shown that activating protein-1 (AP-1) and NF-kappaB in particular are involved in regulating TNFRSF9.[26] In contrast to AP-1, NF-kappaB DNA-binding is strongly upregulated in melanomas, indicating that NF-kappaB, but not AP-1, might be one candidate that could drive TNFRSF9 expression in melanomas.[27] However, the relevance of the NF-κB pathway in stimulating TNFRSF9 expressing in human melanoma brain metastasis definitely needs further investigation.

In summary, our results show that TNFRSF9 is frequently upregulated on both tumor and endothelial cells in melanoma brain metastasis, without being associated with patient survival or any of the clinicopathological parameters assessed in our study. In conclusion, further studies are needed to decipher the exact role of the TNFSF9-TNFRSF9 axis in tumor cells, as well as cells of the tumor micromilieu, in order to understand its link to observed severe side effects in clinical studies targeting TNFRSF9.

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Original Article

Assessment of cognitive function in patients with myasthenia gravis

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ABSTRACT

Aim: During the past decade, there has been an increasing interest in the evaluation of cognitive function in myasthenia gravis (MG), neuromuscular transmission disorder caused by acetylcholine receptor auto-antibodies. However, the results of previous studies on cognition and MG are inconsistent and controversial. This study aimed to evaluate cognition in patients with mild/moderate grades of MG. **Methods:** This study included 20 patients with MG with a mean age of 28.45 ± 8.89 years and duration of illness of 3.52 ± 1.15 years. Cognition was tested using a sensitive battery of psychometric testing (Mini-mental State Examination [MMSE], Stanford-Binet Intelligence Scale 4th edition [SBIS] and Wechsler Memory Scale-Revised [WMS-R]) and by recording P300 component of event-related potentials (ERPs), a neurophysiological analog for cognitive function. **Results:** Compared with healthy subjects (n = 20), patients had lower total scores of cognitive testing (MMSE, SBIS and WMS-R) (P = 0.001), higher Beck Depression Inventory 2nd edition scores (P = 0.0001) and prolonged latencies (P = 0.01) and reduced amplitudes (P = 0.001) of P300 component of ERPs. Correlations were identified between total scores of cognitive testing and age (r = -0.470, P = 0.010), duration of illness (r = -0.788, P = 0.001) and depression scores (r = -0.323, P = 0.045). Using linear regression analysis and after controlling for age and depression scores, a significant correlation was identified between total scores of cognitive testing and duration of illness (β = -0.305, P = 0.045). **Conclusion:** Patients with mild/moderate MG may have cognitive dysfunction. This is important to determine prognosis and managing patients.

Key words: Cognition, myasthenia gravis, nicotinic acetylcholine receptors

INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disease caused mainly by auto-antibodies against skeletal muscle nicotinic acetylcholine receptors (nAChRs) at the postsynaptic membrane resulting in depletion of ACh at the neuromuscular junction.[1] MG is uncommon with a prevalence of (25-125)/10^6. The disease tends to affect women more often than men (3:2) in their second and third decades.[2] The cardinal symptoms of MG are fatigue and weakness of skeletal muscles with repeated or sustained exertion in the course of the day but improved by rest. Ocular muscles are initially involved in about 2/3 of patients then spread to the bulbar and limb muscles. Approximately, 85% of patients develop generalized weakness. Many patients progress from mild to severe disease, and if weakness of respiratory muscles becomes severe enough to require mechanical ventilation, the patient is said to be in crisis.[3] Spontaneous remissions are very rare and last for varying periods that mostly occur during the first 3 years of the disease.[4] In adults, the thymus gland is abnormal in up to 90% of people with MG with approximately 70% of them have thymic hyperplasia while 10-20% have benign thymic tumors or thymoma.[5] The currently used treatment modalities for MG include acetyl choline esterase inhibitors (AChE-Is) (as pyridostigmine),[6] immunopharmacologic drugs (as prednisone,[7] azathioprine,[8] cyclosporine,[9] mycophenolate mofetil,[10] cyclophosphamide,[11] tacrolimus[12] and rituximab[13]) plasmapharesis,[14] intravenous immunoglobulins (IVIGs)[15] and thymectomy.[16]

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Subjective impairments of memory and other cognitive functions are very frequent in patients with MG, however, previous studies, which investigated cognitive

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function in such patients showed contradictory results. Some reported memory difficulties and other cognitive dysfunction[17-21] and electroencephalographic (EEG) abnormalities.[22-24] In contrast, others reported lack of neuropsychological impairments, normal intelligence, attention, memory and motor performance with MG.[25-27]

The exact mechanisms of the co-morbid cognitive dysfunction in patients with MG are unknown. The most likely suggested mechanism is central cholinergic deficiency due to the involvement of central neuronal nAChRs and other cholinergic nervous systems and pathways by the immune-mediated processes of MG.[20,28-30] However, controversial views suggest that the co-morbid nervous system manifestations with MG may result from nonspecific mechanisms as complications of MG, which include respiratory impairment, sleep apnea and hypoxia,[31-33] mental fatigue,[26,27,34] adverse effects from medications used for treatment of MG and mood disorder.[35,36]

This study aimed to investigate cognitive function in adults with mild/moderate MG. Cognitive functions were assessed using a battery of sensitive psychometric testing in addition to recording event-related potentials (ERPs), a neurophysiological analog of cognitive function.

METHODS

Subjects

This study included 20 patients (males = 6, females = 14) diagnosed clinically and electrophysiologically as MG. Their age ranged from 16 to 50 years, and duration of illness ranged from 1 to 4 years. Clinical grading of the patients was done based on the medical, scientific advisory board of MG Foundation of America classification.[37] Patients grading was based on their histories and diagnoses shown in their medical records. Patients reported histories of weakness of ocular muscles (ptosis) (class I), of mild and predominant weakness of the limb muscles (class II a) or oropharyngeal muscles (class II b), or with moderate and predominant weakness of the limb muscles (class III a). Before the presentation, all patients were treated with AChE-Is (pyridostigmine bromide or mestinon in a dose of 60 mg/4 h during the daytime and 60 mg at night time), immunotherapy with prednisolone and/or azathioprine (imuran) or plasmapharesis. Thymectomies were performed to the seven patients with thymoma. Table 1 shows the demographic and clinical characteristics of the studied group. Patients were recruited from the Out-patient Clinic of the Department of Neurology, Assiut University Hospital, Assiut, Egypt during their follow-up visits in which they were free of clinical manifestations (i.e. after resolution of active stage of the disease for at least 3 months) and were on maintenance treatment with low doses of AChE-Is and/or steroids. Twenty healthy subjects matched for age, sex and socioeconomic status were included in this study for statistical comparisons. Control subjects were recruited from the general population. This study was accepted by the regional Ethical Committee. Detailed information on the study was given to all patients, and control subjects, and all gave their written consent to attend the study.

Table 1: Demographic, clinical and laboratory characteristics of the studied groups

Demographic and clinical characteristics	Patients (*n* = 20) (%)	Control subjects (*n* = 20) (%)	*P*
Male/female	4/16	10/10	-
Age (years)	16-50	20-50	-
	28.45 ± 8.89	30.22 ± 5.76	0.380
Duration of illness (years)	1-4	-	-
	3.52 ± 1.15		
Clinical grade			
I	0	-	-
IIa/IIb	2/10	-	-
IIIa/IIIb	8/0	-	-
IVa/IVb	0	-	-
V	0	-	-
Thymic pathology			
Normal	5 (25)	-	-
Hyperplasia	8 (40)	-	-
Thymoma	7 (35)	-	-
Previous treatment (single or combination of the followings)			
Acetyl choline esterase inhibitors	20 (100)	-	-
Prednisolone	20 (100)	-	-
Azathioprine	8 (40)	-	-
Plasmapharesis	9 (45)	-	-
Thymectomy	7 (25)	-	-

Data are expressed as range, mean ± SD, *n* (%). SD: standard deviation

We excluded subjects (patients and controls) with: (1) respiratory involvement or in crisis (i.e. severe stages of the disease); (2) history of other primary neurological (e.g. transient ischemic attacks, cerebrovascular stroke or epilepsy), psychiatric (e.g. major depression) or medical (e.g. diabetes mellitus) diseases which are known to affect cognition; (3) previous serious head injury; (4) any sensory or motor disorder that would preclude psychological testing (as blindness or deafness); and (5) regular treatment with medications (other than those used for treatment of MG) which may alter cognitive testing (e.g. as benzodiazepines, beta-adrenoceptor antagonists, major tranquillizers and antidepressants).

Electroencephalographic recording

Electroencephalographic was done using the eight channels Nihon Kohden machine (4217), employing scalp electrodes placed according to the international 10-20 system with bipolar and referential montages.

Hyperventilation and photic stimulation were used as provocation tests.

Cognitive assessment

Cognitive functions were assessed independently for each participant by two experienced psychologists and under supervision of a psychiatrist, using a set of standardized Arabic translated neuropsychological tests that are sensitive for mild cognitive impairment and covering different cognitive domains. They included: Mini-Mental State Examination (MMSE),[38,39] Stanford-Binet Intelligence Scale 4th edition (SBIS)[40,41] and Wechsler Memory Scale-Revised (WMS-R).[42] From SBIS, we selected vocabulary and comprehension for assessment of verbal reasoning, pattern analysis for assessment of visual reasoning, quantitation for quantitative reasoning, and bead memory and memory for sentences for short-term memory. From WMS-R, we tested digit forward digit backward, mental control, associate learning, logical memory, and visual reproduction.

Event related potentials testing

Before examining ERPs, all participants underwent basic audiological testing (Amplaid Model 720, Milan, Italy). Testing for ERPs was done on a separate day after completion of neuropsychological testing (Neuropack S1 EMG/EP measuring system, MEB-9400 (Nihon Kohden, Japan). ERPs are series of scalp waves that are extracted from the EEG by time domain analysis and averaging of EEG activity following multiple stimulus repetitions. They were elicited with an auditory discrimination task paradigm by presenting a series of biaural 1000 Hz (standard) versus 2000 Hz (target) tones at 70 dB with a 10 ms rise/fall and 40 ms plateau time. P300, the late component of ERPs was obtained. Latencies and amplitudes (peak to peak) of P300 component of ERPs were measured. The P300 wave is a positive deflection in the human ERPs. It is most commonly elicited in an "oddball" paradigm when a subject detects an occasional "target" stimulus in a regular train of standard stimuli. The P300 wave only occurs if the subject is actively engaged in the task of detecting the targets. Its amplitude varies with the improbability of the targets. Its latency varies with the difficulty of discriminating the target stimulus from the standard stimuli. Typical peak latency is elicited when a young adult subject makes a simple discrimination in 300 ms. In patients with decreased cognitive ability, the P300 is smaller and later than in age-matched normal subjects. The P300 have multiple intra-cerebral generators, with the hippocampus and various association areas of the neocortex contribute to the development of this potential. The P300 component of ERPs represents the transfer of information to consciousness, a process that involves many different regions of the brain.[43]

Psychological evaluation

Standardized psychiatric interview was done by applying the Diagnostic and Statistical Manual of Mental Health Disorders, 4th edition (DSM-IV) criteria for the diagnosis of depression.[44] A differentiation between clinical depression and depressive symptoms was made throughout clinical interview of the patient. The Arabic version[45] of the Beck Depression Inventory, 2nd edition (BDI-II)[46] was used to assess the severity of depressive symptoms. BDI-II items are in alignment with DSM-IV criteria. BDI-II consists of 21 items each corresponds to a symptom of depression summed to give a single score for the BDI-II. According to that scale, the patient may have, not having or has minimal depressive symptoms if scoring 0-13, mild symptoms if scoring 14-19, moderate symptoms if scoring 20-28 and severe symptoms if scoring 29-63.

Statistical analysis

Calculations were done with the statistical package SPSS, version 12.0 (SPSS Inc. Chicago, IL, USA). Data were presented as mean ± standard deviation. Student's *t*-test was used for comparison of means. Correlations between score of cognitive testing and demographic and clinical characteristics and depression scores were assessed using Pearson's test. Linear regressions analyses were done using the total score of cognition testing as the dependent variable and age, duration of illness and depression scores as independent variables. For all tests, $P < 0.05$ was considered as significant.

RESULTS

This study included 20 patients with MG. They had a mean age of 28.45 ± 8.89 years and duration of illness of 3.52 ± 1.15 years. Patients reported normal EEG records. All patients had depressive symptoms of mild ($n = 15$, 75%) and moderate ($n = 5$, 25%) severities. Each patient had a different combination of abnormalities in various cognitive testing subsets particularly WMS-R ($n = 16$, 80%). Patients had significantly lower scores of MMSE, different subsets of SBIS, WMS-R and total scores of cognitive testing (MMSE, SBIS and WMS-R) ($P = 0.0001$) and higher scores of BDI-II ($P = 0.0001$) [Table 2]. The majority of patients had abnormalities in latency and/or amplitude of P300 component of ERPs ($n = 14$, 70%). Patients had significantly prolonged latencies ($P = 0.01$) and reduced amplitudes ($P = 0.001$) of P300 component of ERPs [Table 3]. Significant correlations were identified between total scores of cognitive testing and P300 latency, P300 amplitude, age, duration of illness and depression scores [Table 4] Using linear regression analysis and after controlling for age and depression scores, significant correlation was identified between

Table 2: Comparison between patients and controls in scores of cognitive functions and depression

Variable	Patients (n = 20)	Controls (n = 20)	P
MMSE	23.25 ± 2.35	27.56 ± 1.45	0.036
SBST			
Vocabulary	36.33 ± 5.45	50.45 ± 3.88	0.042
Comprehension	35.46 ± 9.07	49.76 ± 7.56	0.007
Total verbal reasoning score	75.32 ± 8.85	96.82 ± 16.25	0.0001
Visual reasoning	36.63 ± 4.64	48.68 ± 5.04	0.0001
Total visual reasoning score	68.43 ± 8.09	88.33 ± 14.70	0.0001
Quantitative test	36.57 ± 6.54	45.30 ± 5.43	0.0001
Total quantitative reasoning score	75.53 ± 8.67	96.65 ± 9.57	0.0001
Bead memory	45.30 ± 7.28	60.50 ± 10.08	0.0001
Memory for sentences	44.72 ± 6.34	65.56 ± 8.57	0.0001
Total score for short-term memory	85.65 ± 9.66	150.25 ± 25.26	0.0001
Total score of SBST	289.56 ± 55.48	360.34 ± 50.04	0.0001
IQ	78.53 ± 6.46	95.35 ± 8.73	0.0001
WMS-R			
Digit forward	4.56 ± 1.01	6.64 ± 0.88	0.035
Digit backward	2.23 ± 0.25	5.58 ± 0.45	0.010
Mental control	3.57 ± 1.45	5.89 ± 1.06	0.042
Logical memory	10.65 ± 1.30	14.83 ± 2.45	0.007
Associate learning	8.52 ± 2.04	12.06 ± 2.24	0.005
Total scores of cognitive testing (MMSE, SBST and WMS-R)	76.54 ± 8.35	96.54 ± 6.28	0.0001
Depression scores	20.64 ± 6.24	8.65 ± 3.55	0.0001

Data are expressed as mean ± SD. SBST: stanford Binet subtests testing, MMSE: mini-mental state examination, WMS-R: wechsler memory scale-revised, SD: standard deviation, IQ: intelligence quotient

Table 3: Comparison between patients and controls in event-related potentials

Variable	Patients (n = 20)	Controls (n = 20)	P
P_{300} latency (ms)			
Right sided	250.00-450.00	285.00-353.00	-
	350.80 ± 35.88	320.88 ± 25.75	0.010
Left sided	270.00-450.00	250.00-350.00	-
	355.60 ± 33.08	325.45 ± 20.45	0.010
P_{300} amplitude (mv)			
Right sided	2.20-20.25	6.88-20.54	-
	7.55 ± 2.45	12.45 ± 2.84	0.001
Left sided	2.55-18.09	6.80-22.25	-
	6.67 ± 3.23	12.63 ± 2.56	0.001

Data are expressed as range, mean ± SD. SD: standard deviation

Table 4: Pearson's correlation (r and P value) between total scores of cognitive testing and clinical variables, lab variables, depression scores and ERPs variables

Variables	Total scores of cognitive testing (MMSE, SBST and WMS-R)	
	r	P
P_{300} latency	-0.650	0.001
P_{300} amplitude	0.557	0.001
Age	-0.470	0.010
Duration of illness	-0.788	0.0001
Depression scores	-0.323	0.045

ERPs: event-related potentials, MMSE: mini-mental state examination, SBST: stanford Binet subtests testing, WMS-R: wechsler memory scale-revised

total scores of cognitive testing (MMSE, SBIS and WMS-R) and duration of illness (β = -0.305, P = 0.045).

DISCUSSION

The results of this study indicate that patients with mild/moderate MG may experience significant manifestations of cognitive impairment in the absence of disease activity and despite the short duration of illness. Patients with MG may experience poor performance in different cognitive tasks indicating central or brain involvement. These included deficits in verbal relations, comprehension, visual reasoning, pattern analysis, quantitation, bead memory, short-term memory and memory for sentences, digit forward, digit backward, mental control, logical memory, and associate learning. In the agreement with our findings, patients with MG commonly reported subjective cognitive complaints. In patients with MG, several previous studies reported memory difficulties[17,18] and impaired performance on varieties of cognitive tests as MMSE and memory tests,[19] the Boston Naming Test, the Logical Memory and Design Reproduction portions of the WMS, Rey Auditory Verbal Learning Test,[17] and measures of response fluency, information processing and verbal and visual learning.[20,21,47] In additions, the detected abnormalities in P300 component of ERPs also suggest the central or brain involvement in MG. In fact, abnormal evoked potential responses were noted in patients with MG.[48-50] In contrast, several studies reported normal IQ, memory, attention and motor performance and normal ERPs in MG.[25-27] We believe that such discrepancies could be explained by differences in methodologies, small sample size, different lists of inclusion and exclusion criteria and lack of control for potential confounding variables.

Several mechanisms have been hypothesized as etiologies of cognitive impairment in patients with MG. The central cholinergic deficiency due to the involvement of the central nAChRs and central cholinergic pathways by the disease process of MG have been suggested as the high likely mechanisms.[20,28-30] This hypothesis is based on the fact that there are structural identities between different muscle and neuronal nAChRs subunits with the possibility of cross-reactivity between different nAChRs antibodies.[51-53] The hippocampus, a cerebral structure highly involved in learning and memory, has abundant cholinergic innervation and enriched in nAChRs that modulate synaptic plasticity via mechanisms involved in long-term potentiation.[54] Few suggested that cognitive dysfunction co-morbidity may be due to the immune responses driven by muscle and neuronal nAChRs antibodies expressed by cancer (e.g. thymoma) (i.e. paraneoplastic syndrome).[55,56] Others suggested that it might be a nonspecific autoimmune response in presence or absence of tumor.[57] This it further supported by an association of MG with other nonnervous system medical

immune-mediated disorders as diabetes mellitus,[58] thyroiditis[59] and systemic lupus erythematosis.[60]

Despite the strength of our findings, this study had some limitations which include: (1) a relatively small sample size that is explained by the fact that we included only a homogenous group of adults with mild/moderate MG in their remission stages and on maintenance treatment; (2) due to the cross-sectional nature of the study, the temporal relation between the disease stage and occurrence of cognitive deficits is difficult to be determined. Future research should include longitudinal studies that prospectively assess the relation of the disease process to cognition over time; (3) CSF analysis for determination of immune markers of the disease is missing as our ethical committee did not allow CSF sampling for uncomplicated patients. It has been realized that CSF examination better detects immune involvement of CNS, and (4) our investigation did not include testing for mental fatigue or sleep pattern. These factors cannot be excluded as causes or potentials for cognitive dysfunction with MG.

In conclusion, adults with mild/moderate MG may experience prominent cognitive deficits in different domains regardless to the presence of depression. This is important to determine prognosis and managing patients.

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Original Article

Distinctive distribution of lymphocytes in unruptured and previously untreated brain arteriovenous malformation

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ABSTRACT

Aim: To test the hypothesis that lymphocyte infiltration in brain arteriovenous malformation (bAVM) is not associated with iron deposition (indicator of micro-hemorrhage). **Methods:** Sections of unruptured, previously untreated bAVM specimens ($n = 19$) were stained immunohistochemically for T-lymphocytes ($CD3^+$), B-lymphocytes ($CD20^+$), plasma cells ($CD138^+$) and macrophages ($CD68^+$). Iron deposition was assessed by hematoxylin and eosin and Prussian blue stains. Superficial temporal arteries (STA) were used as control. **Results:** Both T-lymphocytes and macrophages were present in unruptured, previously untreated bAVM specimens, whereas few B cells and plasma cells were detected. Iron deposition was detected in 8 specimens (42%; 95% confidence intervals = 20-67%). The samples with iron deposition tended to have more macrophages than those without (666 ± 313 *vs.* 478 ± 174 cells/mm^2; $P = 0.11$). T-cells were clustered on the luminal side of the endothelial surface, on the vessel-wall, and in the perivascular regions. There was no correlation between T-lymphocyte load and iron deposition ($P = 0.88$). No macrophages and lymphocytes were detected in STA controls. **Conclusion:** T-lymphocytes were present in bAVM specimens. Unlike macrophages, the load and location of T-lymphocytes were not associated with iron deposition, suggesting the possibility of an independent cell-mediated immunological mechanism in bAVM pathogenesis.

Key words: B-lymphocyte, human brain arteriovenous malformation, inflammatory cells, micro-hemorrhage, T-lymphocyte

INTRODUCTION

Human brain arteriovenous malformations (bAVMs) are tangles of abnormal vessels between arteries and veins and lack of capillary bed. Brain AVM is the most common cause of hemorrhagic stroke in young adults and children.[1-3] Commonly assumed to be congenital, postnatal formation may be more prevalent than previously thought,[4-6] and the etiology of bAVMs still remains unclear. Genetic factors,[7,8] aberrant vasculogenesis,[9-11] and inflammation may all play roles in the pathogenesis of bAVMs;[12] a confluence of these factors has been proposed in a "response-to-injury" paradigm.[5]

Evidence indicating the involvement of inflammation in bAVM pathogenesis includes neutrophil and macrophage infiltration, and increased expression of various inflammatory signals, such as matrix metalloproteinase-9, interleukin-6, myeloperoxidase and adhesion molecules.[13-18] About half of bAVMs cases present with an intracranial hemorrhage (ICH), which itself can induce inflammation. However, even in unruptured and untreated AVMs, substantial infiltration of inflammatory cells has been detected in the vascular wall and intervening stroma.[13] Magnetic resonance imaging has detected hemosiderin deposition in unruptured bAVMs,[19,20] consistent with episodes of clinically silent intralesional micro-hemorrhage.

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We recently described a strong association between imaging evidence of old silent hemorrhage and the risk of clinically symptomatic ICH.[21] Further, histological examination demonstrated that the degree of hemosiderin deposition is positively correlated with the number of macrophages in the lesion.[21] It is not clear, however, whether the macrophage response is specific or whether other inflammatory cells are also correlated with hemosiderin deposition and macrophage. Our previous studies demonstrated that both macrophage and neutrophil may play roles in bAVM pathogenesis.[13-15] Shi *et al*. described evidence of adaptive immunological responses in cavernous malformation.[22] Although bAVM tissue was used as control in Shi's study and while no oligoclonal response was observed, bAVM had a higher polyclonal response compared to normal brain tissue, suggesting that lymphocytes may also play a role in bAVM.

In this study, we analyzed lymphocytes in addition to macrophages, and tested the hypothesis that, unlike the innate immune cells (macrophages), adaptive immune cell (lymphocytes)-infiltration is not associated with micro-hemorrhage and iron deposition.

METHODS

All studies involving patients were approved by the Institutional Review Board of the University of California, San Francisco (UCSF), and patients gave informed consent.

Patients

Patients with AVMs evaluated at UCSF have been entered into an ongoing prospective registry since 2000.[23] We identified 24 unruptured brain AVMs from patients who did not undergo preoperative embolization or radiosurgery with frozen tissue available in our database; 19 samples were located and used in this study [Table 1]. Three superficial temporal arteries (STA), obtained from autopsies of patients who died from nonbrain-related diseases, were used as control.

Histology

Prussian blue staining was performed using Accustain Iron Stain kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol.

For immunohistochemistry, adjacent sections were used to stain different surface markers. CD68, CD3, CD20 and CD138-specific antibodies were purchased from Abcam (Abcam, Cambridge, MA). Brain AVM specimens were embedded in optimum cutting temperature, sectioned into 8 μm sections, and fixed with 4% paraformaldehyde. Endogenous peroxidase activity was quenched by incubating slides in 0.3% H_2O_2 in phosphate-buffered saline (PBS) for 15 min. After blocking with 10% normal donkey serum, sections were incubated at 4 °C overnight with primary antibodies diluted in PBS with 1% BSA in the following concentrations: mouse monoclonal antihuman CD68, 1:1000; rabbit monoclonal antihuman CD3, 1:400; rabbit monoclonal antihuman CD20, 1:200; mouse monoclonal antihuman CD138, 1:800. After washing in PBS, the sections were incubated with horseradish peroxidase labeled antimouse or antirabbit IgG (vector labs) for 1 h at room temperature. The positive staining was visualized using 3, 3-diaminobenzidene. Negative controls were performed by omitting the primary antibodies during immunostaining.

Table 1: Patient and lesion characteristics

Patients	Age (years)	Sex	Size (cm)	Presentation/ clinical details
1	38	Female	1	
2	24	Female	3	
3	37	Male	3	
4	44	Male	1	Incidental
5	54	Female	2	Seizure
6	49	Female	1	
7	63	Male	1	Seizure
8	54	Female	3	
9	47	Female	2	Seizure
10	39	Female	4	
11	53	Female	2	
12	20	Male	3	Focal deficit
13	53	Male	4	Incidental
14	30	Male	3	Headache
15	49	Female	1	Incidental
16	41	Male	3	Incidental
17	30	Male	4	
18	30	Female	3	
19	45	Female	2	Seizure

The criteria for identifying hemosiderin were birefringent or brownish particles seen in the vascular wall or interstitial tissue between vessels, and were confirmed by Prussian blue staining on adjacent sections. $CD68^+$, $CD3^+$, $CD20^+$, $CD138^+$ cells were quantified by counting the positively stained cells using stereological microscopy (Olympus, Japan).

Statistical analysis

All data are expressed as mean ± standard deviation the differences of means were analyzed using unpaired Student's *t*-test. Exact binomial 95% confidence intervals (CIs) for proportions are reported. $P < 0.05$ was considered as statistically significant.

RESULTS

Hemosiderin deposition was present in unruptured brain arteriovenous malformations

Consistent with our published data,[21] hemosiderin deposition was found in 8 out of 19 specimens

(42%; 95% CI: 20-67%) [Figure 1]. Hemosiderin positive cells were scattered mainly around the abnormal vessels [Figure 1a]. Prussian blue positive staining was detected in the areas that had hemosiderin deposition [Figure 1d], suggesting the presence of previous micro-hemorrhage.

T-lymphocytes and macrophages were detected in unruptured brain arteriovenous malformations

To analyze whether the lymphocytes were present in unruptured bAVM and whether their location was associated with macrophages and iron deposition, we analyzed T- and B-lymphocytes, plasma cells and macrophages. We found that T-lymphocyte was the predominant type of lymphocytes present in unruptured bAVM. Whereas the macrophages were scattered mostly in the vessel walls and intervening stromal regions [Figure 2], T-lymphocytes were clustered on the luminal side of the endothelial surface, in the vascular wall, and in the tissue between abnormal vessels [Figure 3]. Few B-lymphocytes were detected; they were mostly present in samples that had a large number of T-lymphocytes, and were co-localized with the T-lymphocytes [Figure 2]. In addition, a few plasma cells were identified in 5 samples, of which 4 had hemosiderin deposition (data not shown). No lymphocytes and macrophages were detected in STA [Figure 2].

Compared to the specimens that had no hemosiderin deposition, hemosiderin-positive specimens tended to have more macrophages (478 ± 174 *vs.* 666 ± 313 cells/mm^2; P = 0.11). The T-cell numbers were similar in hemosiderin-positive and hemosiderin-negative samples (147 ± 108 *vs.* 157 ± 139 cells/mm^2; P = 0.88) [Figure 4].

DISCUSSION

We found in this study that T-cells are the predominant lymphocytes in unruptured bAVMs. Few B-lymphocytes and plasma cells were detected. Unlike macrophages, the number and location of T-lymphocytes did not correlate with hemosiderin, suggesting an independent cell-mediated immunological mechanism in bAVM pathogenesis.

Previously, immune cells were mostly analyzed in ruptured[24] and irradiated[25] bAVMs. Our previous study showed that adaptive immune cells were rarely observed in unruptured bAVM.[13] We found in this study that many T-lymphocytes were present in unruptured, previously untreated bAVMs. The possible reason for the discrepancy is that we used a different immunohistochemical staining procedure in this study. Previously, we incubated sections in 0.3% H_2O_2 in methanol to quench the activity of endogenous peroxidase. However, lymphocyte surface markers have been shown to be sensitive to methanol/H_2O_2 treatment. Treating sections with 0.3% H_2O_2 in methanol can reduce our ability to detect membrane markers on frozen sections,[26] and thus, we used 0.3% H_2O_2 in PBS in this study. The case selection could also be responsible for the discrepancy.

Humoral immunity has been reported to play an important role in cerebral cavernous malformation, which might be due to chronic deposition of iron and blood degradation products.[22,27,28] Consistent with this view, we found that plasma cells were present mainly in specimens that had hemosiderin deposition. However, we cannot draw any conclusion regarding adaptive immune responses to the presence of iron from our small descriptive study.

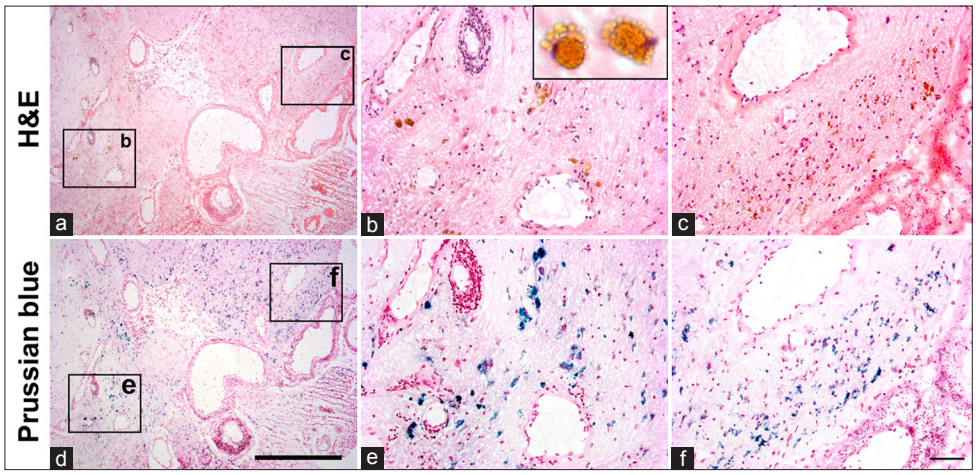

Figure 1: Hemosiderin deposition in unruptured brain arteriovenous malformations. H and E (a-c) and prussian blue staining (d-f) on the adjacent sections. (b) and (c) are enlarged pictures of the regions in squares (b) and (c) in (a) showing hemosiderin-positive areas. Insert in (b) shows two hemosiderin-laden macrophages. (d) Prussian blue staining of an adjacent section of (a). (e) and (f) are enlarged images of the regions in squares (e) and (f) in (d). Scale bars for a and d: 500 μm; for b, c, e and f: 50 μm

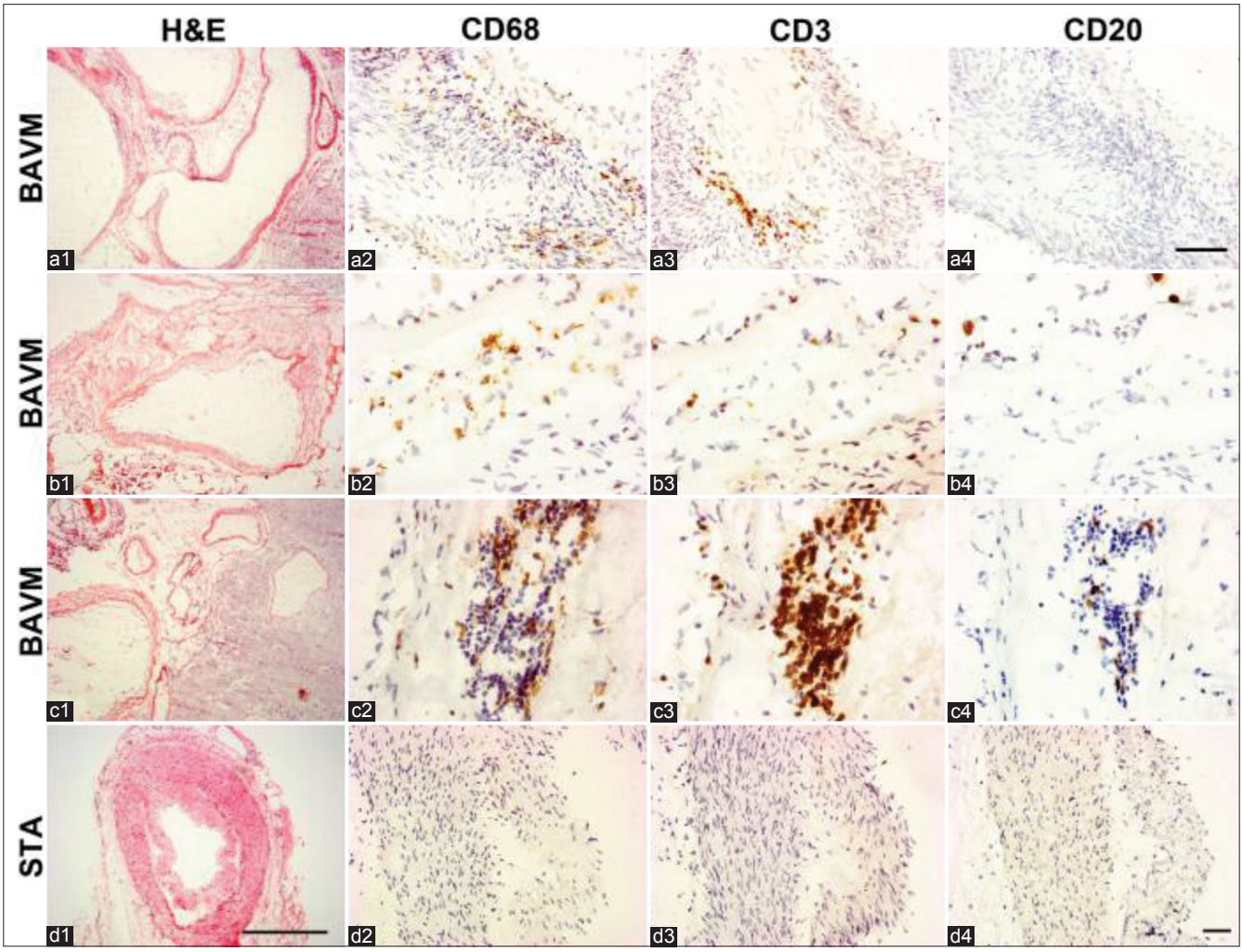

Figure 2: CD3⁺ T-lymphocytes and CD68⁺ macrophages. (a-c) Sections from 3 individual bAVM specimens. (c) Sections from an superficial temporal arteries (STA). Squares in H and E, images are enlarged to show CD68, CD3 and CD20 positive cells in the images next to them. T-lymphocytes and macrophages were detected on the vessel wall (a2 and a3) and between vessels (b2, c2, b3 and c3). Only a few CD20⁺ B-lymphocytes were detected in the lumen (b4) and between vessels (c4). No T- and B-lymphocyte, and macrophage were detected on the wall of STA. Scale bar for a1-d1: 500 μm; scale bar for a2-a4: 100 μm; scale bar for b2-b4, c2-c4, d2-d4: 20 μm

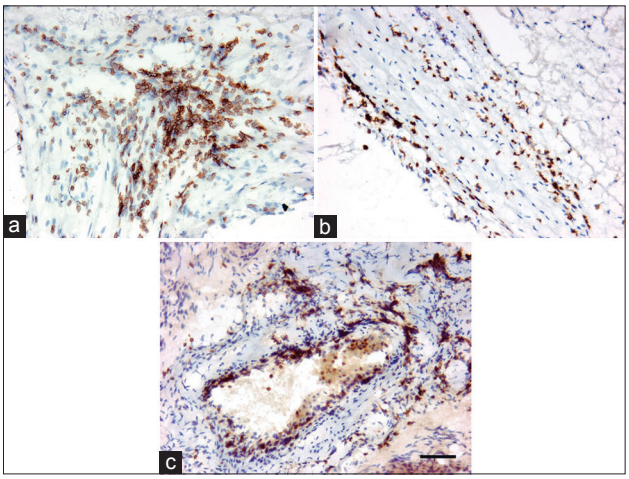

Figure 3: Location of CD3⁺ T-lymphocytes. T-lymphocytes were distributed in the perivascular region (a), in the vessel wall (b), and on the surface of the endothelial lining (c). Scale bar: 50 μm

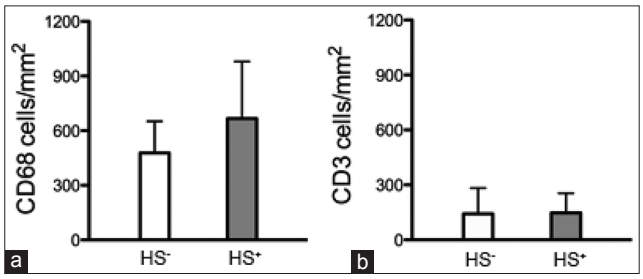

Figure 4: Quantification of inflammatory cells in brain arteriovenous malformation (bAVM). (a) Bar graph shows a trend towards more CD68⁺ cells in hemosiderin-positive (HS+) bAVMs than in hemosiderin-negative samples (HS-). (b) Bar graph shows that the numbers of CD3⁺ T-cells were similar in hemosiderin-positive (HS+) and negative (HS-) samples

Our study was underpowered to detect a difference in macrophage loads between hemosiderin-positive and negative specimens, although our data show a strong trend toward that hemosiderin-positive specimens having more macrophages ($P = 0.11$). The most important finding, however, was that macrophages were present even in the hemosiderin-negative specimens, suggesting that the presence of macrophages is not merely a response to hemorrhage and iron deposition. What remains to be determined is whether the baseline level of macrophage load is causally related to the formation of micro-hemorrhage (e.g. will bAVM with

high macrophage burden develop micro-hemorrhage?). This will be difficult to test in human studies, and would probably be best addressed in an animal model. An animal study has shown that in bAVM, vessel integrity is impaired.[11] Therefore, the macrophages in bAVM could also be a response to the extravasation of blood content.

One limitation of the study is that we only used one marker for each cell-type. Adding additional markers, including positive and negative controls, would make our data more convincing. However, the markers we used in this study are the most commonly used for macrophages, total lymphocytes, T- and B-lymphocytes, and plasma cells. A future study will employ more markers to confirm the cell-types we have identified here, and to define the subtypes of T- or B-lymphocytes or other inflammatory cells.

In summary, we found that the load and location of T-lymphocytes were not associated with hemosiderin and macrophages. Macrophages are present in unruptured and previously untreated bAVMs, and their load was greater when hemosiderin is present. However, the presence of macrophages is not uniquely driven by hemosiderin, because they were also found in hemosiderin-negative specimens. Future studies need to be conducted to determine (1) how macrophages and lymphocytes contribute to the pathogenesis and progression of the disease, and (2) whether the burden of these cell loads is causally related to the development of micro-hemorrhage, and ultimately, clinically symptomatic hemorrhage.

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Original Article

Increased circulating rather than spinal cytokines accompany chronic pain behaviors in experimental bone cancer and arthritis

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ABSTRACT

Aim: Peripheral cytokines contribute to arthritis and bone cancer pain through sensory nerve actions. However, increased spinal cytokine and glial filament expression, coined neuroinflammation, has also been proposed to play a part in chronic pain. Therefore, spinal cord, dorsal root ganglia and circulating cytokines were compared in murine arthritis and bone cancer models in relationship to behavioral signs of pain. **Methods:** Exploratory behaviors were studied after intra-articular complete Freund's adjuvant or bone intramedullary sarcoma cell injection. Nervous tissue and blood cytokine expression were determined by real-time polymerase chain reaction (PCR) and multiplex immunoassays, respectively. **Results:** PCR analysis did not reveal any hallmark of spinal neuroinflammation in spontaneously-behaving mice with cartilage or bone lesions. However, imposed paw stimulation during joint inflammation increased spinal interleukin-1β (IL-1β) expression. Spontaneous paw guarding during rearing was displayed by animals with joint inflammation and bone destruction and was accompanied by increased circulating IL-6 and monocyte chemoattractant protein-1, respectively. In addition, dorsal root ganglia were found to constitutively express receptors for this chemotactic cytokine. **Conclusion:** Our findings indicate that spinal neuroinflammation is not a necessary condition for chronic pain and suggest that circulating cytokine action in dorsal root ganglia may contribute to experimental joint inflammation and bone cancer pain.

Key words: Arthritis, bone cancer, CCL2, cytokines, dorsal root ganglia, pain, spinal cord

INTRODUCTION

Painful joint inflammation affects millions of people with osteoarthritis and rheumatoid arthritis, whereas bone pain occurs in hundreds of thousands of patients with metastasized cancer.[1-3] Arthritic and bone cancer pain are worsened by movement and thus reduce autonomy,[2,4-6] for instance by interfering with the capacity to prepare daily meals.

Local cytokine production is important in arthritis and bone cancer, but increased spinal cytokine and glial filament expression, coined neuroinflammation, may also contribute to pain.[7] Indeed, both peripheral or intrathecal administration of the pro-inflammatory cytokines interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) or of the chemotactic cytokine monocyte chemoattractant protein-1 (MCP-1/CCL2) increase experimental pain sensitivity.[8-14] Moreover, peripheral and intrathecal cytokine antagonists attenuate hyperalgesia in inflammatory and bone cancer pain models.[8,15-21] However, intrathecally-administered molecules readily spread to dorsal root ganglia (DRG),[22] where receptor proteins for some cytokines are expressed,[23,24] indicating that intrathecal cytokines or their antagonists may act centrally or peripherally.

Although some studies have reported increased spinal cytokine expression in experimental inflammatory and bone cancer pain,[19,25-28] most studies have addressed spinal glial responses and found these to be variable.[27-32]

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Interestingly, paw palpation similar to pain sensitivity testing, and direct sensory nerve stimulation induce spinal transcription factor and IL-1β expression, respectively.[32-34] In the present work, we therefore studied spinal and DRG expression as well as plasma concentrations of cytokines in murine models of arthritis and bone cancer in relationship to signs of spontaneous pain and paw palpation, rather than to pain sensitivity.

METHODS

Animals

One hundred male C57/Bl6 mice(CharlesRiver,Arbresle, France) weighing 25-30 g and 46 male C3H/HeN mice (Janvier Labs, Le Genest St-Isle, France) weighing 20-26 g were used. Four days before surgery, animals were housed individually in plastic transparent cages with unrestricted access to food and water in a room maintained at 21.5-22.5 °C. Lights were on from 3:00 a.m. to 15:00 p.m. All experimental procedures were approved by the local ethical committee (No. AP/2/11/2006).

Arthritis and bone cancer induction

C57/Bl6 mice were anesthetized with isoflurane and placed in a supine position to insert a 26-gauge needle into the knee joint as described by Gauldie *et al*.[35] Fifty µL of complete Freund adjuvant (CFA; Sigma-Aldrich, St. Louis, MO, USA) or mineral oil vehicle were injected on days 0 and 6 into the same joint [Figure 1]. C3H/HeN mice were anesthetized with isoflurane and injected with 10 µL of phosphate buffer saline (PBS) containing 10^5 NCTC-2472 cells (LGC Promochem, Molsheim, France) propagated *in vitro* or 10 µL of PBS into the intramedullary canal of the femur in accordance with a previous report by Schwei *et al*.[33]

Behavioral testing

Reduced food intake and exploration are signs of pain in rodents.[36] After surgery, food pellets and body weight were measured every day. To assess exploratory activity during the dark phase, animals were placed in a dimly-lit (10 Lux) open field device (40 cm × 40 cm) divided into 16 equal zones. Number of entries of the animal into a different zone and rearing with or without leaning against the wall were scored during 10 min.[37] To study hind paw guarding during rearing, animals were introduced into an inverted glass beaker of 20 cm diameter for 4 min during the light phase.[38]

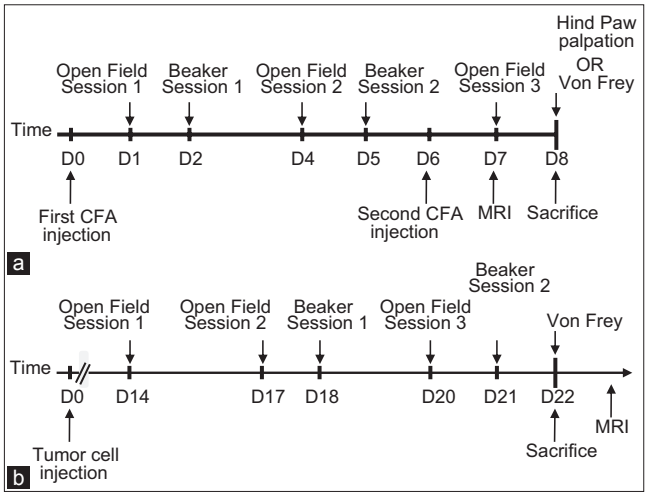

Figure 1: Timelines representing joint inflammation (a) and bone cancer (b) experiments. CFA: complete freund adjuvant; MRI: magnetic resonance imaging

As hallmarks of spinal neuroinflammation are variable between studies, in particular among those using CFA, we tested if mechanical non-noxious stimulation is one of the underlying factors. Therefore, half of the animals underwent hind paw palpation every second for 2 min and were sacrificed 90 min later [Figure 1]. To avoid any effect of mechanical allodynia testing on spinal gene expression, hind paw responses to von Frey filaments (0.16-2.4 g) applied to the plantar surface were studied a few minutes before sacrifice.

Articular inflammation and bone destruction

To assess inflammation, extracellular fluid was detected *in vivo* using T2-weighted magnetic resonance imaging (MRI) on a 4.7 T horizontal magnet (Bruker, Ettlingen, Germany). To determine bone destruction, 3D FLASH-based magnetic resonance microscopy of femurs was carried out *ex vivo* on a 9.4 T vertical magnet (Bruker Biospec 47/50, Ettlingen, Germany).

Tissue preparation

Two days after the second intra-articular injection, that is 8 days after the first injection, or 21 days after femur injection [Figure 1], animals were deeply anesthetized with sodium pentobarbital to allow for intracardiac puncture. Animals were rinsed with PBS after which animals assigned to immunohistochemical analysis were perfused with 4% paraformaldehyde in 0.1 mol/L PBS. L3-L5 spinal cords and DRGs of these animals were post-fixed for 4 h, cryoprotected in 30% sucrose, frozen on dry ice and stocked at -80 °C. For animals allocated to polymerase chain reaction (PCR) experiments, L3-L5 spinal cords and DRGs were removed within 3 min after rinsing with PBS and then frozen at -80 °C.

Spinal Fos expression

Immunohistochemical detection of c-Fos and FosB transcription factors in the spinal cord was performed using rabbit antisera (diluted 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as previously described.[39]

Circulating cytokines

Blood samples were collected in EDTA-coated vials, centrifuged for 15 min at 3000 *g* at 4 °C and the plasmas

were frozen at -80 °C. IL-1β, IL-6, IL-12, MCP-1/CCL2, TNF-α and interferon-gamma (IFN-γ) were measured using a 6-plex kit (BIORAD, Hercules, CA, USA). When estimated values were below the detection limit, animals were excluded from the analysis.

Cytokine and cytokine receptor expression in the spinal cord and dorsal root ganglias expression

RNA was extracted with Trizol (Invitrogen, Carlsblad, CA, USA) and concentrations were measured using a Nanodrop (Thermo scientific, Waltham, MA, USA). Quality check was performed with a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) before reverse transcription to cDNA. Primers were designed [Table 1] and the resulting amplicon was validated using melting curve analysis. Real-time SYBR green-based comparative PCR was performed (DyNamoTM SYBER Green qPCR Kit, Finnzymes Oy, Espoo, Finland). Animals were excluded from the analysis if melting curves did not show a single peak. Relative expression of mRNA expression of IL-1β, IL-1 receptor type 1 (IL-1R1), TNF-α, TNF receptor 1 and 2 (TNFR1 and 2), MCP-1/CCL2, cyclooxygenase-2 (COX-2), prostaglandin E synthase and glial fibrillary acidic protein (GFAP) to glyceraldehyde 3-phosphate dehydrogenase expression was calculated as described by Pfaffl *et al*.[40]

Although the constitutive expression of IL-6 receptor protein has convincingly been shown in DRG,[23,24] this is not necessarily the case for other cytokine receptors. Tyramide-amplified (PerkinElmer, Waltham, MA, USA) immunohistochemical detection of mouse CCR2 (rabbit antiserum diluted 1:25000, Avia Systems Biology, San Diego, CA, USA) was used on free-floating 20 µm DRG cryostat sections to study constitutive protein expression of the MCP-1/CCL2 receptor. Specificity of immunoreactivity was assessed in CCR2-C57/Bl6 knockout mice (Jackson Laboratory-JAX® Mice, Bar Harbor, USA). Double-labelling for transient receptor potential vanilloid 1 (TRPV1; guinea pig antiserum diluted 1:500; Neuromics, Edina, MN, USA) was performed to determine if CCR2 was present in nociceptors.

Data representation and statistical analysis

Data were expressed as mean ± standard error of mean or in case of PCR experiments as mean ± standard error. Weekly food intake, body weight changes and exploratory behaviors were analyzed with two-way repeated measures analysis of variances (ANOVAs). Mechanical allodynia and spinal Fos expression were analyzed with two-way ANOVAs. Plasma cytokine concentrations were analyzed using *t*-test. Nonparametric Mann-Whitney tests were performed when normality or equal variance test failed. Differences in spinal mRNA expression were analyzed with Pair-Wise fixed reallocation randomization tests.[40] In all cases, $P < 0.05$ was considered as a statistically significant difference.

RESULTS

Two C57/Bl6 mice died during the second anesthesia for intra-articular injection of CFA or mineral oil.

Magnetic resonance imaging

T2-weighted MRI indicated some stifle joint edema after mineral oil injection [Figure 2a], but revealed much more intense and widespread inflammatory edema after CFA administration [Figure 2b]. No signs of inflammation were observed in contralateral joints. FLASH-based MRI revealed intact bone and marrow after PBS injection into the femur intramedullary canal [Figure 2c], whereas NCTC tumor cell injection resulted in trabecular bone destruction and irregular bone surfaces [Figure 2d].

Food intake and body weight

Food intake [$Z = -2.520$; $P < 0.012$; Figure 3a] and body weight gain ($Z = -2.588$; $P < 0.010$) were significantly reduced during the week after CFA injection as compared to mineral oil. Weekly food intake [$Z = -2.588$; $P < 0.010$; Figure 3a] and body weight changes ($Z = -3.076$; $P < 0.003$) were significantly reduced during the 3rd week after tumor cell injection into the femur in comparison to PBS administration.

Table 1: List of forward and reverse primers used in this study (5'-3')

Gene	Forward primer	Reverse primer
IL-1β	GAAGAAGAGCCCATCCTCTG	TCATCTCGGAGCCTGTAGTG
IL-1R1	CCAGAAGTCTGTGGGAGTGA	TACGTTTTTGGGATGACAGG
TNF-α	GCCTCTTCTCATTCCTGCTT	TGGGAACTTCTCATCCCTTT
TNFR1	AAGAAATGTCCCAGGTGGAG	TCTCACTCAGGTAGCGTTGG
TNFR2	CCAATTGGTCTGATTGTTGG	AGGAGGGCTTCTTTTTCCTC
MCP-1	AGGTGTCCCAAAGAAGCTGT	ATGTCTGGACCCATTCCTTC
COX2	AATCCTTGCTGTTCCAATCC	AGAATCCAGTCCGGGTACAG
mPGES	TAGAATAGGGACGGGGTCTG	AGCATCCCAAAAGGCTAAGA
GFAP	TTTCTCAACCTCCAGATCC	CCGCATCTCCACAGTCTTTA
GAPGH	TCAAGAAGGTGGTGAAGCAG	TGGGAGTTGCTGTTGAAGTC

IL-1β: interleukin-1 beta; IL-1R1: interleukin-1 receptor type 1; TNF-α: tumor necrosis factor-alpha; TNFR1 and 2: tumor necrosis factor receptor 1 and 2; MCP-1: monocyte chemoattractant protein-1; COX2: cyclooxygenase-2; mPGES: microsomal prostaglandin E synthase; GFAP: glial fibrillary acidic protein; GAPGH: glyceraldehyde 3-phosphate dehydrogenase

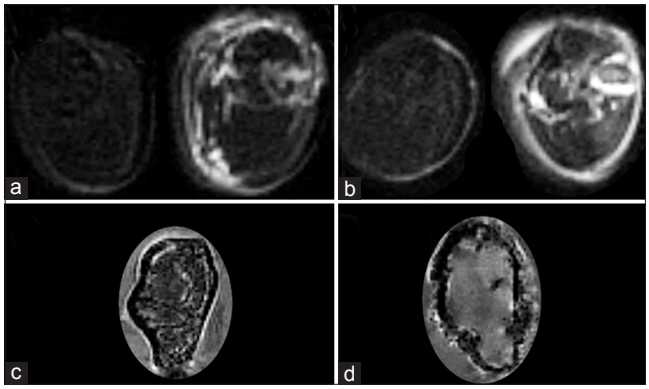

Figure 2: Joint inflammation and bone destruction. T2-weighted MRI indicating edema in contralateral (left) and ipsilateral (right) stifle joint one week after mineral oil (a) or CFA (b) injection. FLASH-based MRI of femurs after intramedullary injection of PBS (c) or NCTC cells (d) three weeks earlier. CFA: complete freund adjuvant; MRI: magnetic resonance imaging; PBS: phosphate buffer saline

Exploratory behavior

Dark phase exploratory activity in a dimly-lit open field device was significantly decreased on the 1st day after intra-articular CFA injection as compared to that of mineral oil [Z = -4.059; $P < 0.001$ and Z = -3.553; $P < 0.004$; Figure 3b]. No differences in activity were observed 14, 17 or 20 days after tumor cell or PBS injection into the femur intramedullary [Figure 3b].

During the light phase, animals injected with CFA into their stifle joint reared less under the inverted beaker glass on days 2 and 5 compared to animals that received mineral oil (Z = -4.860; $P < 0.001$ and Z = -2.198; $P < 0.0280$, respectively). They also reared less against the wall compared with control animals on day 2 (Z = -2.857; $P < 0.0043$). While rearing against the wall, animals injected with CFA displayed significantly more hind paw guarding than those administered mineral oil on days 2 (Z = 5.411; $P < 0.001$) and 5 [Z = -5.650; $P < 0.001$; Figure 3c] after injection. No differences in rearing were observed after NCTC tumor cell or PBS injection into the femur intramedullary canal, but while rearing the former showed significantly more hind paw guarding than the latter on days 18 (Z = 2.457; $P < 0.015$) and 21 [Z = 3.554; $P < 0.004$; Figure 3c].

Mechanical allodynia

Mice injected intra-articularly with CFA required significant lower forces to elicit paw withdrawal compared with those administered mineral oil [Z = -3.644; $P < 0.003$; Figure 3d]. Palpation of the hind paw had no effect on mechanical allodynia. Although bone tumor-bearing mice did not display active paw withdrawal, they allowed their paws to be lifted with the filament. This pressure-reducing behavior was significantly more frequent after femur NCTC tumor cell injection than after PBS administration [Z = -2.124; $P < 0.034$; Figure 3d].

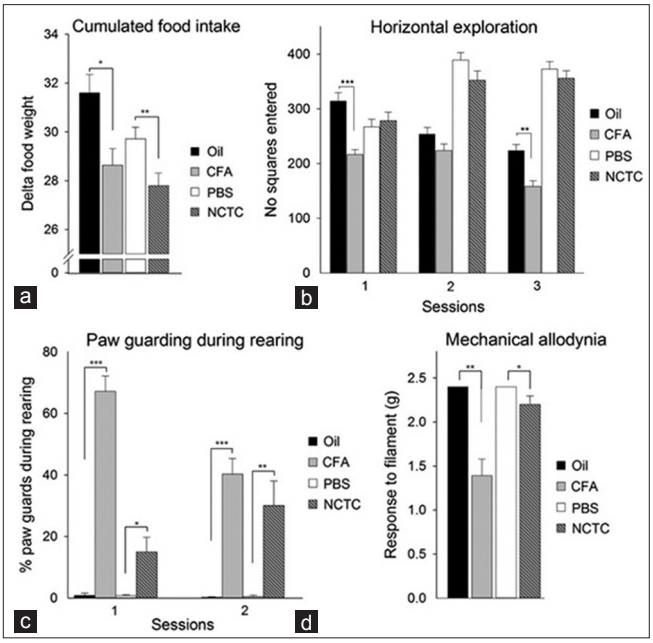

Figure 3: Behavioral effects of joint inflammation and bone cancer (n = 18-23, except for von Frey testing where n = 9-13). a: food intake during last week of experiment. b: horizontal exploration of open field. Sessions 1, 2 and 3 correspond to days 1, 4 and 7 after stifle joint injection and days 14, 17 and 20 after femur injection, respectively. c: percentage of paw guarding during rearing against wall. Sessions 1 and 2 correspond to days 2 and 5 after stifle joint injection and days 18 and 21 after femur injection, respectively. d: paw reaction to von Frey filament stimulation on the last day. Statistical differences: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. CFA: complete freund adjuvant; PBS: phosphate buffer saline

Spinal Fos expression

The number of c-Fos immunoreactive cells in L3-L5 spinal cord increased significantly after intra-articular CFA injection (F[1,27] =10.24; $P < 0.004$) and after non-noxious hind paw palpation (F[1,27] =17.85; $P < 0.001$), whereas the number of FosB-immunoreactive cells did not differ. No differences in the numbers of c-Fos-and FosB-immunoreactive cells were found between NCTC-bone tumor-bearing and PBS-injected control animals.

Plasma cytokine concentrations

Significantly higher IL-6 concentrations in plasma were found in mice that received intra-articular CFA as compared to mineral oil [Z = -2.237; $P < 0.019$; Figure 4] while significantly increased circulating MCP-1/CCL2 levels were observed in animals injected with NCTC tumor cells rather than with PBS into their femur [Z = 3.269; $P < 0.002$; Figure 4]. Circulating MCP-1/CCL2 was probably tumor-derived as NCTC bone tumors were highly MCP-1/CCL2-immunoreactive.

Spinal and dorsal root ganglia mRNA expression

L3-L5 spinal expression of COX-2 mRNA was significantly increased ($P < 0.004$) in animals that received intra-articular CFA compared with those receiving vehicle in the absence of paw palpation [Table 2]. Among animals that underwent paw palpation, CFA-injected mice showed significantly

increased IL-1β ($P < 0.009$), IL-1R1 ($P < 0.002$) and COX-2 ($P < 0.003$) mRNA expression compared with vehicle-treated animals [Table 2]. Spinal gene expression was not found to be different between femur injections, except for a decrease in TNFR1 expression in bone cancer-bearing mice compared to control animals ($P < 0.011$).

No changes in DRG mRNA expression were observed, except for a significant increase in COX-2 mRNA ($P < 0.027$) in animals that received intra-articular CFA compared to those administered vehicle in the absence of paw palpation [Table 2]. In animals that underwent paw palpation, this effect was absent.

Dorsal root ganglia CCR2 protein expression

Numerous CCR2-immunoreactive cells were observed in C3H/HeN DRGs, but their numbers were not statistically different between femur injections. Similar findings were obtained in C57/Bl6 wild-type mice [Figure 5a]. The signal was specific since no labeling was observed in CCR2-C57/Bl6 knockout mice, except for some interstitial staining [Figure 5b]. DRG CCR2-immunoreactivity occurred in nociceptors as it was found to be present in TRPV1-positive cells both by epifluorescence [Figure 5c-h] and confocal microscopy [Figure 5i-k].

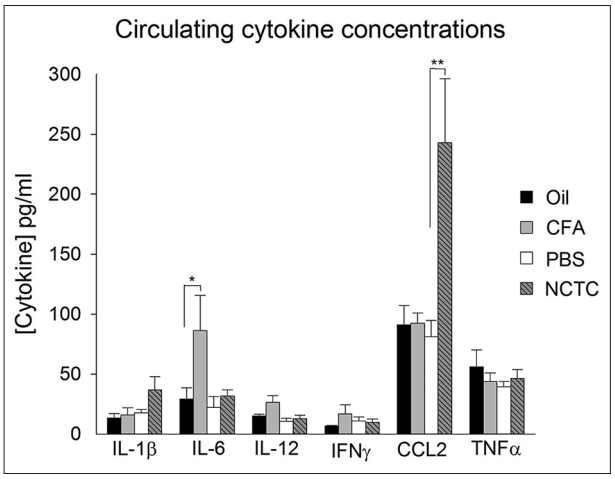

Figure 4: Plasma cytokines in joint inflammation and bone cancer (n = 9-11). Statistical differences: *P < 0.05 and **P < 0.01. CFA: complete freund adjuvant; PBS: phosphate buffer saline; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor

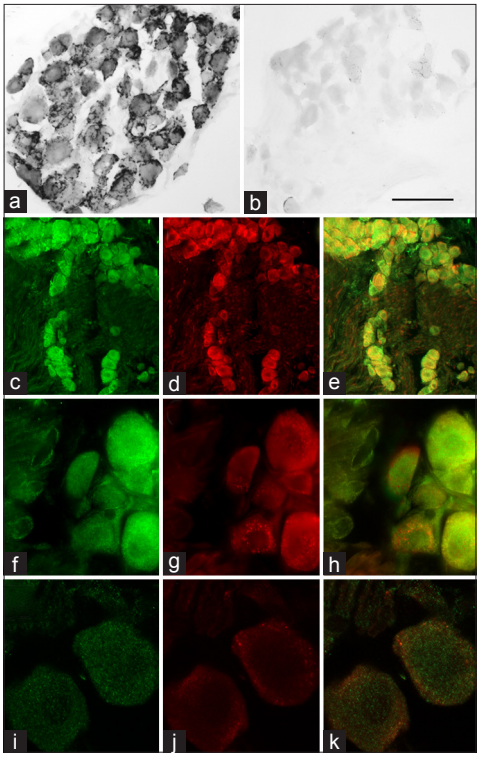

Figure 5: DRG CCR2-immunoreactivity. Presence of CCR2-immunoreactive DRG cells in wild-type (a) but not in CCR2 knockout (b) mice. CCR2-immunoreactivity (c; f; i) occurred largely in TRPV1-positive neurons (d, g, j) as illustrated by merged images from epifluorescence (e; h) or confocal microscopy (k). Scale bar indicates 100 μm for a and b. DRG: dorsal root ganglia

Table 2: Relative spinal cord and DRG expression of genes indicated

	CFA↔oil	CFA_p↔oil_p	CFA_p↔CFA	Oil_p↔oil	NCTC↔PBS
Spinal cord					
IL-1β	1.21 ± 0.35	1.80 ± 0.43**	1.27 ± 0.33	0.85 ± 0.23	0.96 ± 0.29
IL-1R1	1.76 ± 0.44	2.68 ± 0.55***	1.50 ± 0.36*	0.98 ± 0.92	0.90 ± 0.23
TNF-α	1.78 ± 0.54	0.99 ± 0.27	0.98 ± 0.33	1.75 ± 0.41	0.91 ± 0.23
TNFR1	1.38 ± 0.34	1.54 ± 0.45	1.31 ± 0.39	1.17 ± 0.28	0.73 ± 0.14**
TNFR2	0.90 ± 0.29	1.06 ± 0.22	1.15 ± 0.33	0.81 ± 0.27	0.73 ± 0.24
MCP-1	1.31 ± 0.68	0.98 ± 0.23	0.66 ± 0.19	0.90 ± 0.44	1.04 ± 0.32
COX2	3.43 ± 1.19*	4.38 ± 1.83**	1.24 ± 0.58	1.00 ± 0.26	1.11 ± 0.35
mPGES	0.79 ± 0.25	1.13 ± 0.22	1.56 ± 0.29	0.95 ± 0.29	0.86 ± 0.21
GFAP	1.24 ± 0.30	1.31 ± 0.24	1.11 ± 0.24	1.10 ± 0.22	0.87 ± 0.17
DRG					
IL-1β	1.47 ± 0.79	0.82 ± 0.29	0.55 ± 0.30	0.99 ± 0.34	1.22 ± 0.96
IL-1R1	0.97 ± 0.55	1.13 ± 0.44	1.32 ± 0.76	1.15 ± 0.44	0.59 ± 0.43
TNF-α	2.00 ± 1.47	0.89 ± 0.33	0.97 ± 0.67	2.17 ± 1.09	0.99 ± 0.72
TNFR1	1.10 ± 0.62	1.01 ± 0.34	0.97 ± 0.54	1.05 ± 0.35	0.71 ± 0.50
COX2	6.69 ± 6.08*	1.57 ± 0.92	0.35 ± 0.32	0.67 ± 0.40	ND
GFAP	1.29 ± 0.97	0.95 ± 0.56	1.16 ± 0.79	1.57 ± 1.05	0.58 ± 0.47

P indicates paw palpation. Significantly altered expression ratios between groups are represented by *P < 0.05, **P < 0.01, ***P < 0.001. DRG: dorsal root ganglia; CFA: complete freund adjuvant; CF: complete freund; PBS: phosphate buffer saline; ND: not determined; IL-1β: interleukin-1beta; IL-1R1: interleukin-1 receptor type 1; TNF-α: tumor necrosis factor-alpha; TNFR1 and 2: tumor necrosis factor receptor 1 and 2; MCP-1: monocyte chemoattractant protein-1; COX2: cyclooxygenase-2; mPGES: microsomal prostaglandin E synthase; GFAP: glial fibrillary acidic protein; GAPGH: glyceraldehyde 3-phosphate dehydrogenase; NCTC: National Collection of Type Cultures 2472 sarcoma

DISCUSSION

The present study shows that chronic pain behaviors during deep tissue damage occur in the absence of spinal neuroinflammation but in the presence of circulating cytokines. In addition, evidence is provided to indicate that the latter may act on DRG cytokine receptors and that mechanical stimulation increases spinal cytokine expression.

Our work extends a number of previous studies showing variable spinal GFAP responses across models of inflammatory and cancer pain.[27-33] It reports the absence of increased spinal cytokine expression in addition to the lack of GFAP up-regulation in models of moderate deep tissue pain. Indeed, the fact that we injected less biologically-active agents and induced less severe pain behavior and tissue damage, compared to previous reports,[27,29,38] may partly explain discrepancies concerning spinal neuroinflammation between studies.

However, studies employing concentrations and routes of administration of biologically-active agents comparable to those used currently have shown increased spinal GFAP and cytokine expression.[16,30,31] Interestingly, these studies, like those using higher concentrations of disease biologically-active agents, imposed mechanical stimulation or movement on animals. Non-noxious palpation of bone tumor-containing paws increases transcription factor expression in the spinal cord.[32,33] We show here that the palpation induced spinal c-Fos expression, although not to the same extent as CFA-provoked articular inflammation. Since sensory nerve stimulation can induce CNS IL-1β expression,[34,41] we tested the effect of paw palpation on spinal cytokine expression. Our observation that palpation increased spinal IL-1β and IL-1R1 expression in mice with joint inflammation indicates that afferent nerve stimulation during deep tissue injury can indeed induce hallmarks of neuroinflammation. Results obtained in models employing imposed mechanical stimulation to assess pain sensitivity should, therefore, be interpreted with caution.

As we hypothesized that imposed paw stimulation during deep tissue injury influences spinal gene expression, we assessed spontaneous behaviors indicating pain, such as decreased food intake and exploration as well as paw guarding,[36,42] and we studied allodynia only minutes before sacrifice. Although joint inflammation affected exploration more than bone cancer, both conditions reduced food intake and provoked hind paw guarding. The latter behavior is in accordance with earlier studies and has been shown to be reversed by morphine,[38,42] thus suggesting that paw guarding reflects spontaneous pain.

Although experimental joint inflammation and bone cancer gave rise to similar pain behaviors in the absence of spinal neuroinflammation, the underlying mechanisms differ. Subcutaneous CFA injections may result in blood-brain barrier breakdown and increase spinal COX-2 expression,[29,43] that mediates mechanical pain hypersensitivity.[44] Our work confirmed increased spinal COX-2 expression after less severe intra-articular CFA injections suggesting that it also mediates mechanical allodynia during local inflammation. However, in contrast to what has been reported after subcutaneous CFA injection,[27] we did not observe increased spinal cytokine expression in the absence of mechanical stimulation. Interestingly, in addition to IL-1β and TNF-α, peripheral IL-6 also increases central COX-2 expression and pain sensitivity during inflammation.[45] Since we observed increased circulating IL-6 concentrations, we propose that IL-6-induced spinal COX-2 upregulation underlies mechanical allodynia after intra-articular CFA injection. Alternatively, circulating IL-6 may have acted on IL-6 receptors in DRG,[23,24] accessible to circulating molecules,[22] to induce COX-2.

In the absence of increased spinal cytokine and COX-2 expression during bone cancer pain behavior, we considered nervous system actions of peripheral cytokines. We confirmed tumor MCP-1/CCL2 production and showed for the first time increased circulating MCP-1/CCL2 and constitutive CCR2 protein expression in murine DRG nociceptors. These findings are important given that circulating molecules can access DRGs and that MCP-1/CCL2 increases nociceptor excitability,[22,46] and suggest that circulating MCP-1/CCL2 action on DRGs contributes to bone cancer pain behavior.

In conclusion, our present work shows that in two different types of deep tissue lesions, inflammatory and neoplastic, signs of spontaneous, chronic pain are not correlated to spinal neuroinflammation, but rather to peripheral cytokines. In addition, we present evidence indicating that mechanical stimulation of the body segment containing the lesion, similar to what may occur during pain sensitivity testing, can induce spinal cytokine expression as a hallmark of neuroinflammation. As such, our work provides important new insights into the occurrence and role of spinal neuroinflammation in chronic pain. Finally, our findings suggest that circulating cytokine action in dorsal root ganglia may contribute to experimental joint inflammation and bone cancer pain.

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Case Report

Systemic non-albicans infections presented as meningitis in chronic hepatitis B patient: a case report

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ABSTRACT

Non-*albicans* candida meningitis is a relatively rare disease, with nonspecific clinical manifestation, which makes the misdiagnosis occur sometimes, especially in the early stage of the disease. Abuse of broad-spectrum antibiotics, corticosteroids, central vein cannulas, senility, big operation, malignancy, and total parenteral alimentation were all the susceptible factors of non-*albicans* candida infection. We present a case of this type of non-*albicans* infection in a 42-year-old woman who was early misdiagnosed as tuberculous meningitis and was treated with antibiotics and antituberculosis agents. The diagnosis of non-*albicans* infection was confirmed by fungus culture of the cerebrospinal fluid (CSF) with a low detectable rate. This case reminds us that the non-*albicans* candida meningitis had a nonspecific clinical presentations and laboratory data, and was difficult to differentiate from tuberculosis meningitis. Hence, we should highly suspect this disease if central nervous system infections with uncertain pathogens. Test cell counts; protein and fungus culture of CSF should be used to confirm the diagnosis. Once the diagnosis was established, the patients should receive antifungal treatment based on drug sensitivity tests as early as possible.

Key words: Central nervous system, fungi, non-*albicans* candida

INTRODUCTION

Central nervous system (CNS) infection caused by candida is a type of systemic candidiasis.[1] It is rare in clinical practice, with unnspecific clinical features and laboratory data, which makes this disorder prone to misdiagnosis. The morbidity of non-*albicans* candida infection rises in recent years as the abuse of broad-spectrum antibiotics and corticosteroids, human immunodeficiency virus (HIV) infections, and so on. Here, we present candidal meningitis case mimics tuberculous meningitis in a chronic hepatitis B patient.

CASE REPORT

A 42-year-old female, farmer was admitted in the Neurology Department of our hospital on November 24th, 2009. Two months earlier, she had a sharp, intermittent occipital headache and fever, the temperature fluctuated between 37.5 °C and 38.5 °C and up to 40 °C sometimes, it increased at dusk and night, accompanied with nonprojectile vomiting occasionally and anorexia with no visual blurring, diplopia, preceding trauma, or history of migraine. The fever and headache continued for 2 months. Painless intumescent lymph nodes showed up in the neck 2 months later, and the patient was diagnosed as tuberculous infection. And treated with antibiotics and antituberculosis drugs in local hospitals, but the symptoms were not relieved. So she came to our hospital for further diagnosis and treatments. Physical examination revealed an ill-looking woman with yellow skin, white conjunctiva, and enlarged cervical lymph nodes. The neurological examination showed neck stiffness, positive Kernig's sign, and negative Babinski sign on both sides.

She received lumber puncture in the local hospital, the pressure of cerebrospinal fluid (CSF) was 265 mmH_2O, with normal cell counts, glucose, chlorides, and protein. The white blood cell account was 10.6 × 10^9/L, the neutrophilic granulocyte percentage was 72.9%. The erythrocyte sedimentation rate was 76 mm/h, liver function tests showed total bilirubin 75 μmol/L, direct bilirubin 40.7 μmol/L, indirect bilirubin 34.3 μmol/L, aspartate aminotransferase 89 U/L, alanine aminotransferase 67 U/L, alkaline phosphatase 323 U/L, and γ-glutamyltransferase 400 U/L. Serology for

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HIV was negative, the hepatitis B surface antibody, hepatitis B core antibody, hepatitis B e antibody were all positive. Lung computed tomography (CT) scans showed nodules and fibrous stripes in the right middle lobe, left apex, and lingular lobe, with multiple enlarged lymph nodes in the mediastinum. We repeated a lumbar puncture on November 16th, 2009. The CSF tests showed: pressure 204 mmH_2O, white blood cell count 36 × 10^6/L, protein 0.48 g/L, glucose 2.70 mmol/L, and chloride 117 mmol/L. The CSF cytology showed that lymphocytes increased mainly. Both acid-fast staining and the antigen of the tubercle bacillus were negative. Brain magnetic resonance imaging (MRI) scans [Figure 1] presented multiple high signal lesions on T2-weighted imaging (T2-WI) and fluid attenuated inversion recovery (FLAIR) (T2-WI and FLAIR). Remarkable intensify leptomeninges and abnormal strengthening signal in the right side of the caudate nucleus were observed. It was necessary to rule out metastatic tumor or granulomatous according to the history. Hence, we tested the carcinoembryonie antigen, alpha fetal protein, and carbohydrate antigens (CA-199, CA-125) in the blood. Only CA-199 was moderately elevated (90.1 U/mL). B-mode ultrasound scans of the abdomen revealed multiple low signals in the hepatic hilar region and splenic hilum region. Ultrasound doctor considered them as enlarged lymph nodes. CT intensified scans of the upper abdomen showed an occupied lesion in the right hepatic lobe with intra-hepatic bile duct dilation. Radiologists thought the occupied lesion was cholangiocarcinoma, combining with the history we thought it was apt to inflammatory pseudotumor, but could not exclude malignant tumor, however, because systemic candidiasis is always seen in immunocompromised individuals.

Cervical lymph node biopsy [Figure 2] showed mycotic lymphadenitis. There were a lot of granulomatosis-like structures and mold in macrophages. Lymph node puncture fluid smears [Figure 3] showed that hyphae were visible. CSF smears showed no fungus. CSF and lymph node puncture fluid cultures [Figure 4] we saw blastoconidia with India ink staining, and not saw capsule in the culture. Non-*albicans* candida was

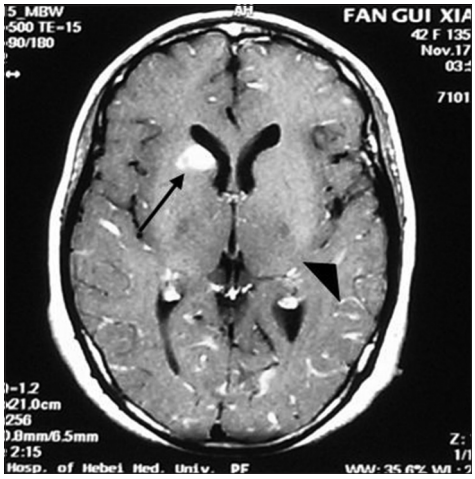

Figure 1: Brain magnetic resonance imaging scan presented multiple high-signal lesions on T2-weighted and fluid attenuated inversion recovery. Remarkable intensity of leptomeninges and abnormal strengthening in the right side of the caudate nucleus

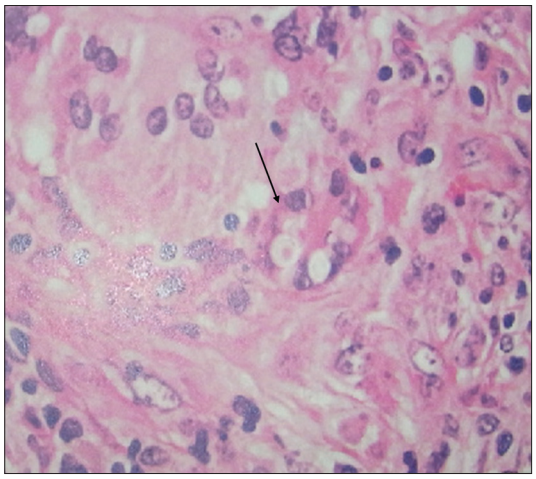

Figure 2: Cervical lymph node biopsy showed mycotic lymphadenitis (arrow). There were a lot of granulomatosis-like structures and mold in macrophages

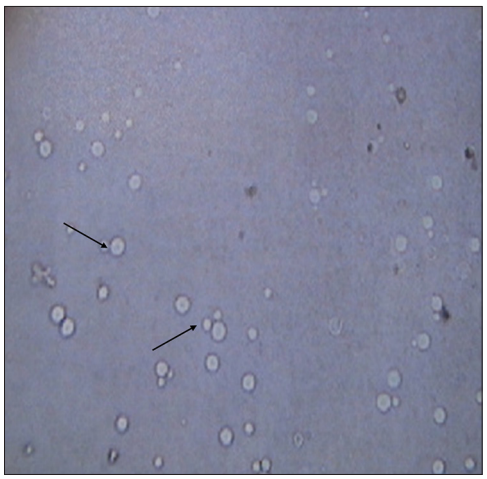

Figure 3: Cerebrospinal fluid and lymph node puncture fluid culture showed positive identification of blastoconidia (arrow) with India ink staining, but was not observed capsules

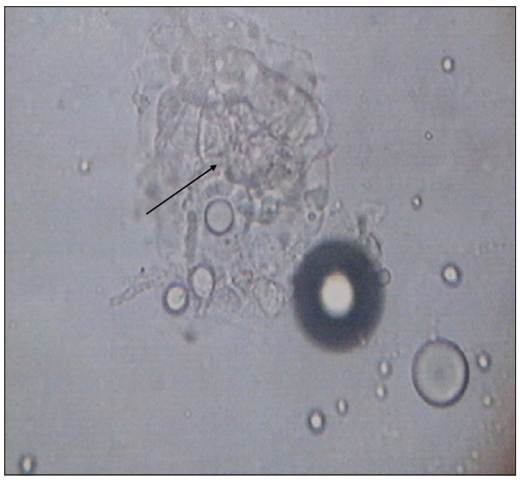

Figure 4: Lymph node puncture fluid smears showed that hyphae (arrow) was visiable

considered as a pathogen, and the diagnosis was clear as systemic non-*albicans* candida infections mainly presented as non-*albicans* candidal meningitis. She received amphotericin B 1 mg/day and fluconazole 400 mg/day intravenously as soon as diagnosis is clear. Unfortunately, the patient discharged from the hospital on the second day since administration of antifungal drugs because of economic reasons and hence we could not observe the therapeutic effect.

DISCUSSION

Many strains of fungal can cause CNS infection. Aspergillus, mucor, cryptococcosis, and yeast are the most common strains in China.[2] A foreign research showed that yeast, aspergillus, and cryptococcosis was the most common fungal pathogens for CNS infection in immune-competent hosts, while aspergillus, candida and nocardia were the most common pathogens in patients with impaired immunity.[3-5] Many factors contributed to the increasing incidence of systemic candidiasis, such as HIV transmission, the using of hormonal, immunosuppressive agents and broad-spectrum antibiotics, organ transplantation, the invasive intracranial examination, and so on. This patient has an infection of hepatitis B virus, and uncertained cholangio carcinoma. Her immune system was weak, such kind of patients should be considered of rare bacterial or fungus infection at the initial of treatment. Therefore, fungi, and bacterial culture could be done more actively.

Candida albicans was the most common pathogen of candidiasis in the past,[6] and the proportion of nonblastomyces albicans infection has tended to rise recently. However, meningitis caused by non-*albicans* candida is rare in the clinic and does not have specific manifestations, which make the misdiagnosis occur sometimes, especially in the early stage of the disease. This case reminds us that candidiasis should be considered if we found patients had unexplained fever responded poorly to antibiotics, thrush, esophagitis, vaginitis, atypical lung infiltrates, unexplained liver dysfunction, mental abnormality, endoophthalmitis, dry cough, rash, and tender muscle.[7] Candida is a kind of mold and belongs to *Saccharomyces*. It includes two types-yeast type and pseudohyphae type. Yeast type was round or oval and can produce blastoconidia. Monilia albicans, candida parapsilosis, candida tropicalis, candida glabrata, monilia guilliermondii, candida lusitaniae, and candida krusei are the most common candida albicans that can infect humans. In America and other European countries, candida parapsilosis and candida krusei are the major pathogens of non-*albicans* candida infection.[8] These fungus are resistant to the most common antifungal drugs, which makes them more likely to be opportunistic infections. The situation is more or less similar in China and Brazil.[9]

We summarize, the clinical features of this patient: (1) a 42-year-old woman, acute onset of fever, which is resistant to the antibiotics and antituberculosis agents; (2) liver dysfunction, intra-liver lesion, biliary ducts dilation; (3) hilar and portal hepatis lymph nodes enlargement; (4) used of broad-spectrum antibiotics in the early stage of the disease when the diagnosis was unclear; (5) with a low immune state, which was the basis of opportunistic infections. We considered the case was blastomycosis according to the lymph node biopsy results. Morphologically, giant blastoconidia presented a spectrum of forms such as blastoconidia with linear creases, with single broad-based buds resembling Blastomyces dermatididis, with multiple buds resembling *Paracoccidioides brasiliensis*. Its forms varied along with the environment and temperature. Such as, after growth on commercially prepared cultures in room temperature, we can see white fluffy colonmold to naked eyes and the characteristic thick-walled broad-based yeast in microscope with Periodic acid Schiff staining, as it shows mycelia-like type, while been cultured in 37 °C it presents on brown and frilly yeast-like colony, and in tissue it shows yeast-like type. Candida includes yeast type and pseudohyphae type and produces blastoconidium. In this case, we can see blastoconidia only with India ink staining in CSF and lymph node puncture fluid culture. Brain biopsy hadn't been done. Combing with the manifestation, brain MRI, CSF tests, and fungus culture results, we highly suspected systematic candidal albicans infection. This case reminds us that it is essential to do CSF culture for patients with unexplained CNS infection, not only the routine CSF cell counts and protein tests. If the diagnosis is established, patients should receive antifungal treatment based on drug sensitivity test as early as possible.

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Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
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Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. Proc Natl Acad Sci U S A. Forthcoming 2002.

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