

Roles of dysregulated Notch pathway and small DNA tumor viruses in cancer initiation and progression

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ABSTRACT

Notch pathway is a major determinant of cell fate, and research within the last 30 years has shown dysfunctions within this pathway in the majority of solid tumors and leukemias. The molecular mechanisms causing aberrant expression of Notch in cancer are still partially known. Mesotheliomas, breast, and cervical cancers are among the cancer types for which the dysregulation of Notch has been reported together with the association of simian virus 40 (SV40) or human papilloma virus (HPV) infections. In mesotheliomas and cervical cancer, there is clear evidence that these viruses cause and rely on dysregulation of the Notch pathway to promote and sustain cell transformation. The existence of a relationship in tumors between DNA viruses and Notch could have an impact on cancer therapy by implementing Notch inhibition to interfere with the growth of SV40- and HPV-positive cancers. In addition, since Notch links innate and acquired immunity and plays a key role in the regulation of the anti-viral response, targeting Notch in the presence of oncogenic viruses infections may help prevent the onset and progression of cancers associated with the exposure to these viruses.

Key words: Cancer; human papilloma virus; Notch; pathway; simian virus 40

INTRODUCTION

Notch has been identified as a critical pathway aberrantly expressed in many types of solid tumors and leukemias. Dysregulation of Notch signaling is a result of many factors including interactions with viral proteins. In this short review, we took in consideration significant articles dealing with the dysregulation of the Notch pathway and/or presence of oncogenic viruses, mainly simian virus 40 (SV40) and human papilloma viruses (HPVs), in cancer. Indeed, the proteins encoded by Notch pathway genes and the viral oncoproteins of SV40 and HPV were found in some models of study, interconnected in the cell transformation *in vitro* and tumor initiation and progression *in vivo*.

BASICS OF NOTCH SIGNALING

Beginning in the early 20th century, the discovery of a new

Type 1 transmembrane receptor came after the identification of a specific mutation in *Drosophila melanogaster*, which formed a Notch on the wing of the fly. This discovery led to the naming of “Notch” to the mutated gene.^[1] In *Drosophila*, the Notch receptor was found to encode a 300 kDa single-pass transmembrane receptor. Later, Notch-like molecules were identified from *Caenorhabditis elegans* (LIN-12) to humans, which are highly conserved and play pivotal roles in development, stem cell renewal, and differentiation in postnatal tissues.^[2] In mammals, there are four Notch Type I transmembrane receptors (Notch 1, 2, 3, and 4) and five known ligands (delta-like 1, 3, and 4 and Jagged 1, 2). Notch signaling relies on cell-cell contact to initiate its eventual signaling activation.^[3] To be primed for mature Notch signaling activation, the protein is processed first in the trans-Golgi apparatus by furin-like convertase creating a heterodimer, which is shuttled to the cellular membrane and held

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together by Ca^{2+} cations. The mature receptor is available to interact with its ligand, which subsequently stimulates through conformational changes a second proteolytic cleavage by tumor necrosis factor- α converting enzyme or a disintegrin and metalloprotease (10/17).^[4] This in turn results in shedding of the extra-cellular portion of Notch, which through receptor-mediated endocytosis, propagates signaling events in neighboring cells. The final cleavage occurs within the membrane through an associated aspartyl protease known as the γ -secretase complex composed of presenilin, nicastrin, APH 1 and PEN2.^[5] Intra-cellular Notch cleaved protein translocates to the nucleus where it binds with the transcription factor recombining binding protein-Jk or C-promoter-binding factor 1/suppressor of hairless/Lag1 (CSL)^[6] and, after displacing co-repressors and recruiting co-activators such as p300, histone acetyl transferases, and mastermind-like protein 1 (MAML1), it activates downstream pathways [Figure 1].^[7] The “canonical” Notch signaling is known to activate genes coding for transcriptional factors such as those belonging to the hairy/enhancer of split (Hes1-5), the hairy-related (Hrt), and the Hes1-5-Hrt with YRPW motif (Hey) families involved in inhibiting neuronal differentiation.^[8] The “canonical” Notch pathway is a major determinant of cell proliferation and survival through the activation of genes controlling cell cycle progression such as cyclin D1^[9] and genes belonging to the anti-apoptotic pathway nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B).^[10,11] Notch activation can also be attained in a “non-canonical” fashion initiated by a

non-canonical ligand or may not require cleavage of the Notch receptor.^[1] Among suggested mechanism of “non-canonical” Notch signaling are interactions of Notch with non-CSL transcription factors, such as β -catenin,^[12] hypoxia-inducible factor-1 α , NF- κ B,^[13] and estrogen receptor α (ER α).^[14] Anti-apoptotic activity independent of canonical functions has been associated with active Notch1, which signals via the kinase AKT to prevent the loss of mitochondrial function and consequent nuclear damage and requires mitochondrial remodeling proteins mitofusins-1 and 2.^[15] Notch activity is finely regulated by interactions with other key proteins and pathways, among them p53,^[16] ER α ,^[17,18] the epidermal growth factor B2 (ErbB-2)^[19] and the vascular endothelial growth factor receptors (VEGFRs),^[20] the Wingless (Wnt)^[21,22] and Hedgehog^[23] signaling pathways. Recent genome-scale studies in *D. melanogaster* have revealed an even more complex network of genes that can affect Notch activity^[24] consistent with decades of work showing that the highly conserved Notch pathway is extremely complex, and the output of its activation or its inhibition will result in differentiation, proliferation or increased survival based on the existing cellular context.

NOTCH SIGNALING PATHWAY IN TUMORS

Many reports have been published on the role of the Notch pathway in the development of the cardiovascular system,^[25,26] in regulation of stem cells functions such as survival of cardiac progenitor cells,^[27] the differentiation

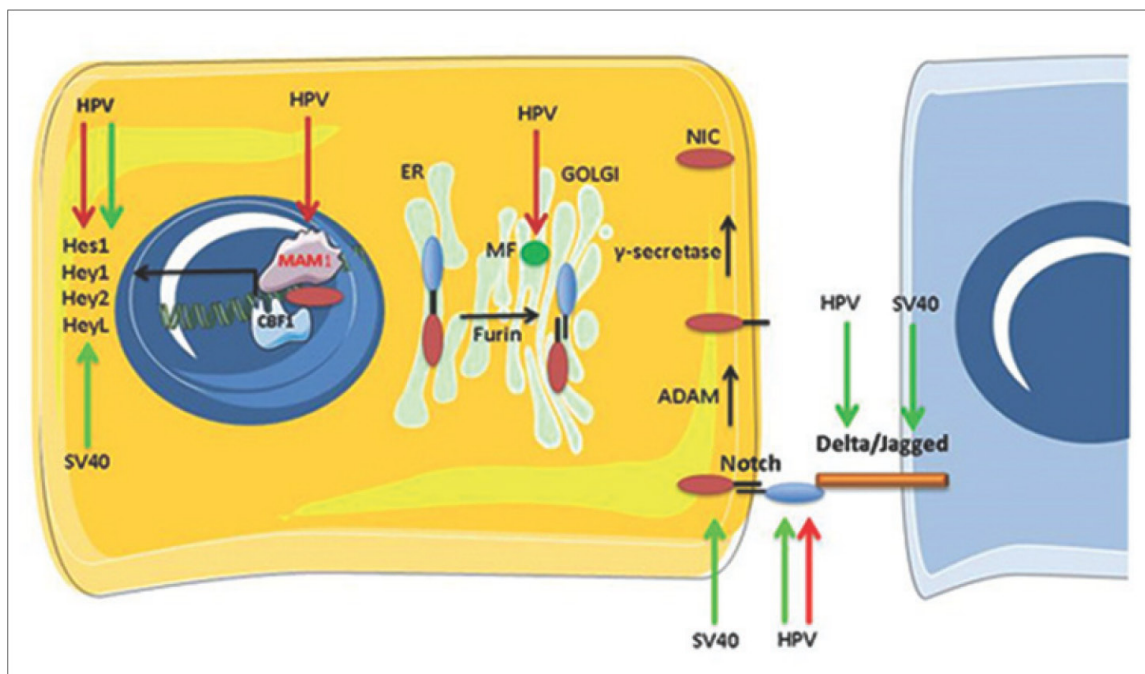


Figure 1: Schematic representation of the events leading to Notch signaling activation and the steps of this process affected by the oncogenic viruses simian virus 40 and human papillomavirus. Notch precursor is cleaved in the Golgi apparatus by a furin-like convertase and then exposed on the cell membrane. Notch ligands Delta/Jagged bind Notch extra-cellular subunit. This causes a disintegrin and metalloprotease to clip the extra-cellular portion of Notch transmembrane generating an intermediate, cleaved by γ -secretase which releases active Notch. Active Notch enters the nucleus, where it causes the dissociation of silencing mediator of retinoic acid and thyroid hormone receptor corepressor complex from C-promoter-binding factor 1/suppressor of hairless/Lag1, and recruits mastermind-like 1 coactivator complex, resulting in transcription of target genes. Simian virus 40 induces upregulation of the Notch pathway, whereas conflicting reports exist on the modulation of Notch by human papillomavirus (green arrow indicates up-regulation, red arrow indicates down-regulation or inhibitory binding)

of insulin-secreting pancreatic cells^[28] of inner ear hair cells,^[29] and intestinal crypt and goblet cells.^[30] Accordingly, the important role of the Notch pathway for normal tissues development has been proven by the identification of Notch mutations in human inherited diseases. Indeed, Notch alterations have been detected in: (1) Cerebral autosomal dominant arteriopathy, with subcortical infarcts and leukoencephalopathy (a heritable arteriopathy that leads to damaged small blood vessels and irreversible dementia); (2) spondylocostal dysostosis (characterized by abnormal development of bones in the spine and ribs); (3) Alagille syndrome affecting the liver, heart, kidney, and other systems of the body;^[31] (4) congenital heart diseases.^[32] Similarly, in the last decade, it has been demonstrated that the Notch signaling pathway contributes to the regulation of the immune system by playing a role in multiple lineage decisions of developing lymphoid and myeloid cells.^[33] Recent work has shown that Notch, through macrophage-dependent delta-like ligand 1 and 4 signaling, is critical in providing an antiviral response by linking innate and acquired immunity during influenza^[34] and dengue^[35] viral infections.

Notch has emerged as a potent oncogene when it was first shown that a subset of T-cell acute lymphoblastic leukemias (T-ALL) contained a chromosomal translocation, t(7;9), leading to abnormal expression of the Notch1 intracellular domain,^[36] which was later shown to be able to cause T-cell neoplasm in mice.^[37] Later studies confirmed the existence of Notch1 mutation in 60% of human T-ALL.^[38] In T-cell neoplasms, Notch1 represses p53,^[38] induces c-myc,^[39] and inhibits phosphatase and tensin homolog, a downregulator of the phosphatidylinositol 3 kinase (PI3K)-AKT pathway involved in promoting cancer cell survival.^[40] Recent work has shed light on the role of Notch in T-ALL showing that in these tumors aberrant Notch activity counteracts the tumor suppression function of the transcription factor IKZF1 (IKAROS).^[41]

The major role played by Notch in breast cancer is also well established. Reports of an involvement of Notch in mammary gland development and neoplasia came from the observation of the Notch4/int3 gene as a common provirus integration site in mammary tumors of mice infected with mouse mammary tumor virus (MMTV),^[42] followed by the report that transgenic female mice carrying Notch1 and 3 activating mutations (caused by the insertion of the MMTV) developed mammary gland tumors.^[43] Notch has been found activated in ER α positive-, negative-, triple negative-breast cancer cell lines and breast cancer cell lines overexpressing the oncogene *Her2/neu*.^[18,19,44] Dysregulation of Notch has been shown in human breast cancer biopsies^[45-47] in which overexpression of Notch1 and one of its ligands, Jagged1 has been linked to poor prognosis and overall diminished survival.^[48,49] Of interest, Notch2 overexpression was instead associated with increased survival in breast cancer patients,^[49]

suggesting a role for Notch2 as a tumor suppressor gene in these cancers. In agreement with this observation, active Notch2 induces reduction in tumor take and increased apoptosis in human MDA-MB-231 (ER α , Her2 negative cell line) xenograft tumor growth.^[50] The Notch pathway is a major determinant of breast cancer stem cells survival, and Notch activation in these cells has been linked to resistance to tamoxifen.^[51,52] Consistently, Notch activation plays a role in tamoxifen resistance observed in protein kinase C- α overexpressing estrogen-responsive breast cancers^[53] and in ErbB-2-positive breast tumors.^[54]

Dysregulated expression of Notch proteins, ligands, and targets has been described in a multitude of solid tumors, including cervical, head and neck, endometrial, renal, lung, pancreatic, ovarian, prostate, esophageal, oral, hepatocellular and gastric carcinomas, osteosarcoma, mesothelioma melanoma, gliomas, medulloblastomas, and rhabdomyosarcoma.^[8] Dysregulation of Notch signaling has been reported in some hematological malignancies, other than T-ALL, including Hodgkin lymphomas, anaplastic large-cell non-Hodgkin lymphomas, acute myeloid leukemias, and B-cell chronic lymphoid leukemias multiple myeloma (for the original articles on the subject the reader is referred to).^[8]

Tumor angiogenesis is crucial for cancer growth and progression.^[55] The Notch pathway promotes cancer growth not only by enhancing the survival of cancer cells and their progenitors but also by controlling tumor vascularization. Dll4/Notch1-mediated signaling modulates VEGF-A-driven angiogenesis by affecting the number of sprouts (new branches) on endothelial cells. This interplay between Dll4/Notch1/VEGFR determines the balance between the number of tip cells (leading and guiding the blood vessel sprout) and stalk cells (proliferating cells forming the vascular lumen).^[24,56] Interference with tumor angiogenesis by inhibition of Dll4-mediated signaling has been effective in blocking cancer growth in animal models.^[57] Recently, high levels of Jagged1 have also been shown to promote tumor angiogenesis by destabilizing the tip and stalk cell fates^[58] and by regulating levels of VEGFR1, 2^[59] and activate Notch3/Hey1 in tumor cells thus promoting proliferation, survival, and epithelial to mesenchymal transition.^[59] Consistently, inhibition of experimental tumors growth has been obtained by blocking Jagged1-dependent Notch signaling.^[60]

Notch inhibitors are currently under clinical investigation, in combination with existing therapies for the treatment of several types of cancers.^[61] Considering the role of Notch in maintaining intestinal homeostasis, patients treated with Notch inhibitors require clinical monitoring of the gastrointestinal tract.^[62] Furthermore, due to the effect of Notch in promoting angiogenesis and survival of cardiac progenitor cells, cancer patients with preexisting ischemic diseases should also be monitored for possible

cardiotoxicity linked to the use of Notch inhibitors.^[63]

The mutations causing the activation of Notch signaling have been identified for T-ALLs;^[38] however, little is known about the molecular mechanism involved in dysregulating Notch in other malignancies. Few activating mutations of the Notch pathway have been found in solid tumor patients, with most being observed in non-small cells lung^[64] and head and neck cancers.^[65] In breast and lung cancers, inactivation of Numb, a protein involved in Notch1 downregulation, has also been identified.^[64,66] Rearrangements of the Notch gene families have been found in breast cancer.^[67]

THE ROLE OF SMALL DNA TUMOR VIRUSES IN THE PATHOGENESIS OF CANCER

SV40

SV40^[68] is a monkey virus, which was accidentally administered to humans, in the years 1955-1963, through contaminated poliovirus vaccines.^[69,70] However, a more recent study indicates that some oral poliovirus vaccines were contaminated with infectious SV40 in sub-sequent years.^[71] Early experiments both *in vitro* and *in vivo* classified SV40 as a transforming and oncogenic viral agent. These activities are due to SV40 large tumor antigen (Tag) and small tumor antigen (tag), which act as activated viral oncogenes.^[69,70] These studies addressed a new wave of investigations into the potential of SV40 to induce cancer in humans. To date, hundreds of molecular and epidemiologic studies aimed at investigating whether SV40 infects humans, its potential mode of transmission and its putative role in human tumors have been carried out.^[72-74]

SV40 was assigned to the family of Papovaviridae, an acronym proposed by Melnick^[75] obtained by fusing the names of the 3 representative viruses papilloma, polyoma, and vacuolating agent. However, this nomenclature at present is considered obsolete. More recently, SV40 has been enclosed among polyomaviruses, together with the human polyomaviruses (HPyV), BK Polyomavirus (BKPyV), and JC polyomavirus (JCPyV). The virion is about 45 nm, an icosahedral particle, with a density of 1.34-1.35 g/cm³. The viral genome is a circular, double-stranded DNA molecule. SV40 encodes for six main viral proteins: Two early non-structural polypeptides, Tag and tag, an agnoprotein, probably involved in the assembly of viral particles and processing of late messenger RNA (mRNA) and 3 capsid proteins, VP1, VP2 and VP3.^[76-78] The early and late genes are transcribed on different DNA strands in a way that the transcription proceeds divergently from the regulatory region. This region contains the origin of DNA replication and binding sites for the transcription factors that control viral gene expression and terminates within DNA sequences containing the polyadenylation signals. Recently, a predicted late polarity pre-microRNA to the untranslated region 3' of the polyadenylation cleavage site

in the late pre-mRNA has also been detected.^[79,80] SV40 is phylogenetically, closely related to HPyV. There is evidence of similarity with respect to size (about 5.2 Kb), genome organization, and DNA sequence. The tags of SV40, BKPyV, and JCPyV strongly cross-react with the same antisera^[81,82] while a less, strong cross-reactivity is observed in most structural antigenic determinants of the viral proteins, named VP1, 2 and 3. A genus-specific capsid antigen, located on viral peptide VP1, has been identified.^[83] The DNA sequences of SV40 share 70% homology with BKPyV,^[84] and 69% with JCPyV.^[85] The greatest homology is found in the early region coding for the Tags and tags, whereas a lower homology is detected in the regulatory region.

Transformation of rodent and human cells by SV40 is induced by the 2 oncoproteins, Tag and tag, which display multiple functions. The main activity of Tag for cell transformation^[69] and tumorigenesis is to target key cellular proteins,^[86-88] such as the tumor suppressor p53^[89-91] and retinoblastoma protein (pRB) family proteins, inactivating their functions.^[92-94] SV40 Tag may also lead to transformation by inducing mutations to the cellular genome^[95] or numerical and structural alterations of chromosomes,^[96,97] such as gaps, breaks, dicentric and ring chromosomes, chromatid exchanges, deletions, duplications, and translocations.^[98] The principal role of the tag in transformation is to bind the catalytic (36 kDa) and regulatory (63 kDa) sub-units of protein phosphatase 2A (PP2A),^[69,86] inactivating their function. Moreover, tag interacts with the centrosome and blocks mitosis in human cells,^[99] suggesting that it may disrupt cell cycle progression. Recently, it has been shown that in human mammary epithelial cells tag activates PI3K^[100] an enzyme involved in pathways crucial for cell proliferation, and transformation through phosphorylation of the hydroxyl moiety present on the phosphatidylinositol inositol ring. Aberrant regulation of EGFR upstream from PI3K through mutations in EGFR can lead to cancer promotion in glioblastoma.^[101,102] In addition, SV40 tag can enhance transcription from E2F-activated promoters of early growth response genes.^[103,104] The process of rodent cell transformation induced by SV40 typically depends on the integration of the viral DNA into the host genome where it produces a high level of expression of the major viral oncogenic proteins, Tag, and tag. However, human cells experimentally transformed by SV40 harbor viral genomes in an episomal state in addition to integrated viral DNA. SV40 immortalized^[105] and transformed human cells^[106-108] can induce tumors when implanted subcutaneously in autologous hosts.^[107] An SV40 Tag needs cooperation of the catalytic sub-unit of telomerase and the activated c-HRas oncogene, for the complete transformation of human cells, as shown in cotransfection experiment.^[109] SV40 is highly oncogenic in rodents and when inoculated subcutaneously, intra-cerebrally, or intra-venously in newborn hamsters induces soft

tissue sarcomas, osteosarcomas, ependymomas and choroid plexus papillomas, and neoplasms of the hematopoietic system, such as lymphocytic leukemia, histiocytic lymphomas and rarely, and B-cell lymphomas, respectively.^[87,110-112] Direct inoculation of SV40 into the pleural space induces malignant mesothelioma in 100% of the injected hamsters.^[111] The oncogenic potential of SV40 is confirmed by the generation of transgenic mice in which polyomavirus large Tag expression is regulated by the native viral early promoter enhancer.^[113] Furthermore, SV40-transgenic mice develop ependymomas and choroid plexus papillomas, as well as other neoplasms.^[87,114-116] Many reports were published on SV40 sequences detected, at high prevalence in human cancers of the same histotypes induced by this small DNA tumor virus in experimental animals, that is, lymphoproliferative disorders, mesothelioma, and bone and brain tumors.^[72,117,118] SV40 sequences were also detected at low prevalence in healthy subjects.^[119-121]

Most of these studies were obtained by polymerase chain reaction techniques. More recently, investigations reported the detection at high prevalence of specific antibodies in serum samples from patients affected by malignant pleural mesothelioma,^[122] glioblastoma multiforme,^[123] osteosarcoma,^[124] ocular melanoma,^[125] and non-Hodgkin lymphoma,^[126] suggesting an association of SV40 with these human cancers. Indeed, in serum samples from normal individuals^[127-129] or patients affected by tumors, and^[130,131]/other pathologies^[132,133] unrelated to SV40, the prevalence of antibodies against SV40 is lower than that detected in human cancers found to be associated with SV40. It is worth noting that taken at all, the prevalence of SV40 sequences and the prevalence of specific antibodies against SV40 in these human tumors/normal tissues and sera, respectively, are very similar. This result indicates that SV40 is also a human virus, which infection occurs at low prevalence in normal individuals. Altogether, these data suggest that this small DNA tumor virus of monkey origin seems to be associated at high prevalence with specific human cancers. It is also possible that the immunologic data are due to the cross-reactivity with a new, still undetected, human polyomavirus closely related to SV40.

HPV

HPV infection is considered to be the main oncogenic agent for the onset of female genital tumors.^[134] HPVs are non-enveloped small DNA tumor viruses, with a double-stranded genome of approximately 8.2 kb. HPVs are sub-divided into 2 classes such as low-risk, which are detected in mainly genital warts, and high-risk (HR), which are associated with invasive cervical cancer. HR HPV includes 15 types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), whereas low-risk HPV includes 12 types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and 108).^[135] However, the oncogenic potential of HPV

is mediated by the expression of the viral oncoproteins identified as E6 and E7. The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis is mainly due to their interaction with the cellular tumor suppressor p53 and members of the pRB family, respectively.^[136-138] The mechanisms of action of HPV cause genetic instability and cell transformation resulting in cell cycle regulated escape and inhibition of apoptosis-hallmarks of cancer initiation and progression.^[139] Studies on the association between HPV and cervical neoplasia have indicated a strong link between these oncogenic virus types.^[140]

Research demonstrates that only a fraction of HPV-positive women develops genital tumors.^[141] Indeed, the majority of patients who are infected with HPV can clear these viral agents naturally within 1 year.^[142] Persistent infection with HR HPV at a high viral load in cervical mucosa is considered the main cause of the initiation and progression of genital tumors^[143] as it is a well-established cause of cervical cancer. In addition to E6 and E7 transformations, HR HPV oncogenic types 16, 18, 31, 33, 35, 45, 52, 58, and 66 are associated closely with > 95% of cases of squamous cell carcinoma of the cervix.^[144] Moreover, only genotype HPV 16 accounts for > 55% of diagnosed tumors.^[145] Although infection with HR HPV is the major risk factor associated with cervical cancer, some studies have reported a possible tumor-initiating and promoting role in cervical cancer for other DNA tumor viruses. Taken together, this interaction may synergize with HPV in a normal cell to initiate and progress a tumorigenic cell.^[146]

ONCOGENIC DNA VIRUSES AND MODULATION OF THE NOTCH PATHWAY

As previously discussed, the Notch signaling pathway influences cell fate decisions, proliferation versus differentiation, and cell survival. Similarly, viruses in infected cells promote cell survival, promote or block cell cycling and employ a variety of mechanisms to evade innate cellular anti-viral responses to ensure their own survival and multiplication. In light of these similarities, it is not surprising that several viruses hijack the Notch pathway to ensure the completion of their own life cycles.^[147]

The first report of an interaction between a virus and the Notch pathway came from studies showing that binding of Epstein-Barr virus (EBV) nuclear antigen 2 (the transcriptional activator essential for EBV-driven B-cell immortalization) to responsive promoters requires the interaction with the nuclear effector of Notch signaling CSL.^[148] More recently, also, the Kaposi's sarcoma (KS)-associated herpes virus replication and transcription activator protein (involved in controlling the switch from latency to lytic replication) has been found to activate lysis-related gene by binding to CSL.^[149] Studies using γ -secretase

inhibitors (GSI-I Z-Leu-Leu-Nle-CHO and LY-411, 575), small molecules which block Notch activation, resulted in apoptosis in KS cells and established KS cell tumors in mice, demonstrating the requirement for an active Notch signaling in KS.^[150] Notch pathway interactions have also been shown with adenoviral oncoprotein 13S E1A, which binds to CSL, displaces associated corepressor complexes, and activates CSL-dependent gene expression.^[151]

In agreement with reports of an association between SV40 infection and human mesothelioma,^[152] SV40 infection upregulates the expression of Notch1 in mesothelial cells.^[153] SV40-mediated Notch1 induction is achieved at the transcriptional level; it requires both SV40 Tag and tag and tag-induced activation of the mitogen-activated protein kinase-extracellular-signal-regulated kinase pathway. Notch activation is necessary for the growth of SV40-transformed mesothelial cells, as treatment of these cells with a Notch inhibitor leads to G2/M cell cycle arrest.^[153] Consistently, upregulation of Notch1 and ligands Jagged1 and 2 is maintained in SV40-transformed human mesothelial clones and SV40-positive mesotheliomas and derived cell lines.^[153]

Other than in mesothelial cells, Notch1 expression and signaling has been linked to SV40-mediated transformation of primary astrocytes.^[154] In both mesothelial cells and astrocytes, SV40-mediated activation of Notch signaling determines the survival of cells grown in suspension. Of interest, the archetypal (1 copy of enhancer sequence in the regulatory region) and the non-archetypal (2 copies of enhancer sequences in the regulatory region) SV40 strains are both able to transform astrocytes whether only the non-archetypal strain can transform mesothelial cells. Differences in expression levels of Notch1 and its downstream effectors (c-Myc, Hey1, Hes1 and HeyL) appear to explain these differences in SV40-mediated transformation of primary astrocytes and mesothelial cells.^[154]

SV40 tag, which forms a complex and inhibits PP2A activity, plays a critical role in the malignant transformation of human cells. Microarray analyses on human embryonic kidney cell lines overexpressing SV40 tag have identified induction of Dll1 and Jagged1 suggesting a role for SV40 tag in the activation of the Notch pathway.^[155] Of interest, in these cells, Notch signaling was found to be upregulated in association with Hedgehog and Wnt pathways but inhibition of Hedgehog and not of Notch interfered with cell survival suggesting that Notch signaling is not essential for survival in cells expressing SV40 tag.^[155] A link between SV40 tag and Notch has been observed also in human bronchial epithelial cells. Specifically, Wang *et al.* have shown that miR-27a is upregulated in SV40 tag-transformed human bronchial epithelial cells (HBERST) following the interaction between tag and PP2A. In these cells, miR-27a promotes cell cycle progression by downregulating Fbxw7, a regulator of ubiquitin-dependent proteolysis of a set of protein involved in cell cycle progression, including

Notch1. Suppression of miR-27a expression in HBERST cells leads to cell cycle arrest in the G0-G1 phase.^[156]

Both SV40 Tag and tag have been shown to induce the immortalization of mammary gland epithelial cells.^[157,158] SV40 tag expression inhibits mammary gland differentiation during mid-pregnancy and about 10% of multiparous tag transgenic animals develop breast tumors with latencies ranging from 10 to 17 months, whereas expression of N-terminal truncated Tag molecules harboring the intact p53 and pRB binding region does not have this effect.^[158] Expression of SV40 Tag in the epithelium of the mammary glands results in cancers which resemble the human disease and do not require hormone supplementation or pregnancy for insurgence.^[157] Breast cancer has been associated to SV40 infection^[159] and a specific gene signature in transgenic models of breast cancer intrinsic to the functions of the SV40 T/t-antigens has been identified which is associated with poor prognosis.^[160] It is not known whether SV40 is involved in the dysregulation of Notch signaling observed in breast cancer.^[19,152] Of interest, the Notch target gene cyclin D1 is overexpressed in the SV40 tag-positive mammary gland epithelial cells and in the breast tumor cells from SV40 tag-expressing mice.^[157]

HPV is the most significant causative agent in the development of cervical cancer. Despite its presence in almost all cervical cancers, it is widely recognized that HPV by itself is unable to transform a normal cell to a cancerous one, and additional cellular mutations are required to supplement the HPV oncoproteins E6 and E7. The activation of the Notch signaling pathway induced by HPV infection has been proposed as one of the cellular changes that cooperate with the E6 and E7 proteins to cause cervical cancers.^[161] This proposition is based on several studies showing overexpression of Notch signaling in HPV-cervical cancer or cell lines. Specifically, active Notch1 expression has been shown in high-grade cervical lesions and cancers^[162,163] and progressively increasing up-regulation of Notch3 expression with severity of disease as compared to normal cervix tissue has been reported in a set of 168 tissue biopsy samples comprising of tumor specimens, precancer, and non-neoplastic cervical tissues.^[164] Noteworthy, in the same specimens, Notch1 was found to be downregulated thus suggesting the existence of a complex interplay between Notch signaling and HPV in the context of the development of cervical carcinogenesis.^[164] Upregulation of both Jagged1 and Hes1 and downregulation of Manic Fringe, a negative regulator of Jagged1-Notch1 signaling, have been shown in squamous cell carcinoma of cervix compared to high-grade lesions and in late-passage, but not early-passage, HPV type 16-positive human cervical low-grade lesion-derived cell line W12.^[165] Overexpression of all Notch receptors, Hes1, and MAML1, the transcriptional co-activator originally identified by its role in Notch signaling, has been found in HeLa, SiHa, and CaSki, three other cell lines derived from

HPV-positive human cervical cancer.^[166] Evidence in favor of an oncogenic role for Notch in cervical cancer comes from the observation that activated Notch1 synergizes with HPV16 E6 and E7 proteins in conferring apoptosis protection through the activation of the prosurvival PI3K-protein kinase B/AKT (PI3K-PKB/AKT) pathway and in the transformation of the immortalized human keratinocytes HaCaT cell line.^[167] Furthermore, in HaCaT cells active Notch1, through the PI3K-PKB/AKT-dependent pathway, inhibits p53-induced apoptosis and sustains transformation by HPV 16 E6 and E7.^[168] Consistently with the findings of high level of Jagged1 in cervical cancer, Jagged1 but not Dll1 expression correlates with the rapid induction of PI3K-mediated epithelial-mesenchymal transition both in HaCaT cells and in a human cervical tumor-derived cell line.^[169] Microarray studies by the same authors show that Notch-PI3K oncogenic functions can be independent of CSL activation and rely instead on Deltex 1, an alternative Notch effector.^[169] The anti-apoptotic role played by Notch in cervical cancer progression has also been revealed by immunohistochemistry conducted in cervical cancer specimens in which high levels of Jagged1, Hes1, and Cdk9 were paralleled by nuclear translocation of both NF- κ B p50 and p65 and NF- κ B target genes expression (I κ B- α , B-cell lymphoma 2 and cyclin D1).^[170] An active Notch pathway is necessary for the survival and the maintenance of the neoplastic phenotype of HPV-positive cervical cancer cell lines as demonstrated by experiments in which Notch signaling was inhibited by anti-sense Notch1 oligo,^[46,171] by upregulation of Manic Fringe,^[165] by small interfering RNA against Jagged1^[165] or by inhibition of γ -secretase in combination with dominant negative MAML1, a regulator of crosstalk between the Notch and NF- κ B pathways.^[166]

Experimental evidence shows that as with SV40, HPV proteins have a direct effect on the activation of Notch signaling. Weijzen *et al.* have reported that transfection of mouse primary embryonic cells and human primary fibroblasts with HPV16 E6 and E7 upregulates Notch1 not only transcriptionally but also post-translationally by upregulating presenilin-1, a protein involved in Notch processing.^[46] Microarray analyses have revealed enhanced expression of Notch1 mRNA in HPV16 E6-expressing keratinocytes when NFX1-123 (a protein involved, together with E6, in binding and stabilization of mRNA coding for human telomerase reverse transcriptase, the catalytic subunit of telomerase) was overexpressed. A moderate increase in Notch1 mRNA was seen with overexpression of NFX1-123 alone, but with 16E6 coexpression the increase in Notch1 was enhanced.^[172] A recent study by the same group has shown that the Notch canonical pathway genes Hes1 and Hes5 were increased with overexpression of NFX1-123 in 16E6 - expressing keratinocytes, and their expression was directly linked to the activation or blockade of the Notch1 receptor. Of interest, keratin 1 and keratin 10 were also increased in this model, but in contrast to Notch target genes, their upregulation was only indirectly associated

with Notch1 receptor stimulation, and it did not lead to growth arrest, increased p21 (Waf1/CIP1), or decreased proliferative factor Ki67.^[173]

Notch signaling pathway is a key determinant of keratinocyte growth arrest and differentiation.^[174] and it has been recently shown that it promotes expression of differentiation markers acting together with the TAp63 β isoform of the p63 transcription factor.^[175] This evidence supports a role for Notch as putative tumor suppressor in HPV-associated tumorigenesis rather than an oncogene, as discussed so far. It is well established that Notch activity regulates tumor biology in a context-dependent manner and may act as an oncogene or a tumor-suppressor gene within the same tumor type. In human, esophageal keratinocytes overexpression of Notch1 induces senescence (induction of G0/G1 cell-cycle arrest, Rb dephosphorylation, flat and enlarged cell morphology, and senescence-associated beta-galactosidase activity) requiring both canonical CSL-dependent transcriptional activity and the p16INK4A-Rb pathway. Loss of p16INK4A or the presence of HPV E6/E7 oncogene products (which inactivate both the p53 and pRB) in these cells have been shown not only to prevent intracellular Notch1 (N1IC) from inducing senescence, but also to facilitate N1IC-mediated anchorage-independent colony formation and xenograft tumor growth with increased cell proliferation and reduced squamous-cell differentiation.^[176] These observations provide a possible molecular mechanism to explain and support the hypothesis of the oncogenic role on Notch in HPV-positive cervical cancer.

In agreement with a protective role of Notch against HPV-induced transformation, Talora *et al.* have reported that the expression of the endogenous Notch1 gene is markedly reduced in a panel of cervical carcinoma cells, whereas expression of Notch2 remains elevated, and Notch1 expression is reduced or absent in invasive cervical cancers.^[177] The authors show that increased Notch1 signaling, but not Notch2, causes a dramatic downmodulation of HPV-driven transcription of the E6/E7 viral genes, through suppression of AP-1 activity by upregulation of the Fra-1 family member and decreased c-Fos expression. According to the authors, the downmodulation of Notch1 expression would play an important role in late stages of HPV-induced carcinogenesis.^[177] In agreement with these observations, E6 protein from cutaneous HPVs of the β -genus, such as bovine papillomavirus Type 1 and β -HPV5 and 8, induces a repression of Notch transcriptional activation, which is dependent on an interaction with MAML1^[178-180] and it has been shown to inhibit keratinocyte differentiation.^[181]

Technical approaches (type of anti-body used) for Notch detection have been invoked to explain the differences in expression levels of Notch in HPV-positive cervical tumors linked to the different roles of Notch as an oncogene or tumor suppressor gene.^[182] As previously discussed, the opposite

roles on Notch in the context of HPV-cervical cancer have also been attributed to the cellular context. Extremely high levels of Notch1 seem to adversely affect HPV E6 and E7 expression and cellular proliferation whereas moderate levels of Notch1 and PI3K exhibit oncogenic properties that transform primary cells containing HPV16 E6 and E7 proteins.^[161] More recently, in SiHa cervical cancer cells, it was shown that moderate Notch activation contributed to increased viability and anchorage independent growth, whereas high-level Notch activation decreased anchorage independent growth. The shift in phenotypical outcome was correlated to altered AP-1 activity and complex composition.^[183]

Interactions between the Notch pathway and HPV may play a role also in the progression of head and neck squamous cell carcinoma. Exome sequencing of head and neck squamous cell carcinoma have revealed inactivating mutations in Notch1^[184] and recent work by Seiwert *et al.* has shown an enrichment in the frequency of Notch1 mutations in HPV-positive compared to HPV-negative head and neck squamous cell carcinomas.^[185]

CONCLUSION

Many reports indicate that dysregulated Notch pathway and oncogenic viruses may act together in the initiation and progression of different human tumors. More investigations are necessary to acquire new knowledge on the molecular mechanisms involved in the oncogenic process, which are regulated by oncogenic viruses-mediated Notch dysregulation [Figure 1]. These studies could lead to the identification of biomarkers or the development of targeted therapeutic approaches specific for Notch-associated malignancies characterized by the presence of the oncogenic viruses. Furthermore, considering the role of Notch in the regulation of the host immune response against viral infections, a deeper understanding of the interactions between oncogenic viruses and the Notch pathway could lead to the targeting of Notch to prevent or reduce oncogenic virus infections and, possibly, onset of cancers associated with exposure to these viruses.

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Conflicts of interest

There are no conflicts of interest.

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