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Nibrin expression in oral squamous cell carcinoma: association with clinicopathological parameters

Jigna H. Dave, Hemangini H. Vora, Trupti I. Trivedi, Nandita R. Ghosh

Division of Molecular Endocrinology, Department of Cancer Biology, the Gujarat Cancer and Research Institute, Asarwa, Ahmedabad 380016, India.

Correspondence to: Dr. Nandita R. Ghosh, Division of Molecular Endocrinology, Department of Cancer Biology, the Gujarat Cancer and Research Institute, Asarwa, Ahmedabad 380016, India. E-mail: nandita.ghosh@gcriindia.org

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ABSTRACT

Aim: The present study sought to discover the role of Nibrin protein in 100 patients with oral squamous cell carcinoma (OSCC) and its potential relationship with clinicopathological parameters. **Methods:** Nibrin expression was evaluated immunohistochemically using the modified H-score method. **Results:** The present study included 20% of patients with stage I disease, 22% of patients with stage II disease, 18% of patients with stage III disease, and 40% of patients with stage IV disease. Nibrin showed a significant positive correlation with moderately/poorly differentiated tumor tissues ($P = 0.028$), while significant inverse correlation of Nibrin expression was observed with tumor size ($P = 0.018$) and tumor stage ($P = 0.039$). Further, using univariate survival analysis it was observed that strong Nibrin expression was significantly associated with disease relapse in early stage OSCC patients ($P = 0.049$). **Conclusion:** Thus, the present study revealed that Nibrin could be used as a prognostic marker in patients with early stage OSCC.

INTRODUCTION

Carcinomas of the oral cavity, including cancer originating from the buccal mucosa and tongue are of 10 most common cancers in the world with an increasing trend of incidence.[1,2] Squamous cell carcinoma (SCC) is the most common type of oral cancer which accounts for more than 90% of oral malignancies which is characterized by an aggressive growth pattern, high-degree of local invasiveness, and cervical lymph node spread.[1,3] In India, oral squamous cell carcinoma (OSCC) is the leading cause of death which stands for 35-40% of all malignancies which is owed to the increased prevalence of lifestyle habits like chewing areca-nut/betel nut quid/tobacco and smoking with heavy alcohol consumption serving as a potent cofactor.[4-6] The survival of patients with oral cancer has remained unchanged even with the improved therapeutic modalities, over the last 3 decades.[4] The resultant poor prognosis is owed to a late stage diagnosis, low response rate to current therapeutic strategies, high risk of primary site recurrence and

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aggressive metastases to loco-regional lymph nodes, strongly suggestive of an urge to improve the treatment efficacy and diagnostic capabilities. Over the last decade, scientific research related to the specific pathways which are relevant to the development and progression of this disease has been performed to investigate biological, diagnostic and prognostic parameters.[7-10]

DNA damage is one of the underlying causes for mutations which is very numerous and appears to be a fundamental problem for life leading to cancer. In human cells, the estimated average number of DNA damages occurring per hour is about 800 which reach to 19,200 per day.[11] If, such DNA damages are not repaired in dividing cells, cause errors during DNA synthesis leading to mutations which can give rise to cancer. Thus individuals are often at increased risk of cancer with an inherited damage in DNA repair capability.[12]

Nijmegen breakage syndrome (NBS) is a chromosomal-instability syndrome associated with cancer predisposition, growth retardation, microcephaly, radiosensitivity and immunodeficiency.[13-15] The NBS1/Nibrin/p95 is a member of the DNA double-strand break (DSB) repair complex (hMre11 complex) which is a product of the defective gene in NBS (the *NBS* gene) located on human chromosome 8q21.[14-16] The Nibrin containing protein complex [Mre11-Rad50-Nbs1(MRN) complex] binds to the edges of the DNA double stranded break and remains attached to this site until the break gets repaired.[17] Nibrin is also involved in various signaling cascades other than DSBs induced by irradiation such as mitotic V(D)J rearrangements in T and B lymphocytes, maintenance of telomere function and meiotic recombination.[18,19] Once ataxia-telangiectasia mutated protein phosphorylates NBS1, it then carries out its checkpoint functions following ionizing radiation.[20-22] However, in certain types of human cancer rare or no mutations of NBS1 have been studied.[23-25] In addition, during the process of carcinogenesis NBS1 is expressed in highly proliferating tissues.[26] On the basis of this information, the aim of this study was to assess whether the Nibrin expression would relate to clinicopathological variables and if it could predict survival or recurrence in OSCC.

METHODS

Study population

A total of 100 untreated patients with histolpathologically confirmed OSCC of tongue and buccal mucosa evaluated between 2011 and 2013 at our institute were included in this study. Formalin fixed and paraffin embedded primary tumor tissue blocks (buccal mucosa: n = 39, tongue: n = 61) and histologically confirmed adjacent normal tissue blocks were collected from the histopathology department of our institute. The detail clinical history of the patients [age, gender, tobacco habit, site of disease, tumor-node-metastasis (TNM) stage, histopathological findings, treatment given, *etc*.] was obtained from the case files maintained at our institute. In patients with OSCC the disease was staged according to the criteria of the American Joint Committee on Cancer pTNM classification. Thus, the present study included 20 patients with stage I disease, 22 patients with stage II disease, 18 patients with stage III disease and 40 patients with stage IV disease. This study was approved by our institutional review committee for dissertation/thesis/publications/conference presentations and institutional ethics committee.

Immunohistochemistry

Immunohistochemistry of Nibrin was performed using the avidine-biotin complex technique in which formalin fixed paraffin embedded tissue sections (4 µm) were mounted on 3-aminopropyletriethoxy silane coated glass slides. The sections were first deparafinized using xylene and then rehydrated using graded alcohol. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide prepared in methanol for 15 min. Antigenicity of the processed tissue sections was retrieved by cooking the sections in 10 mmol/L tri-sodium citrate buffer (pH 6.0) solution with 0.05% tween-20 for 20 min in a pressure cooker. Sections were then allowed to cool at room temperature. For Nibrin immunostainings, a commercial mouse monoclonal antibody (clone 1D7, Santa Cruz Biotechnology, Santa Cruz, USA) at dilution of 1:100 prepared in tris buffered saline was applied to the sections and were then incubated overnight at 4 ˚C. For immunohistochemical detection of the antibody reaction, we used Novolink polymer detection kit from Novocastra. Sections were then dehydrated, cleared in xylene and mounted in dibutylphthalate xylene. As positive controls, formalin-fixed paraffin-embedded tissue sections with intense staining for a given marker were included with each staining procedure.

Assessment of Nibrin expression

All sections were scored independently by two independent researchers in a blinded fashion. The staining intensities and the percentage of positive cells were separately assessed in primary tumor tissues (n = 100) and their corresponding adjacent normal squamous epithelium (n = 100). As the Nibrin expression was not uniform in different parts of the epithelium or cancerous tissue, we used modified

histoscore (H-score) method to combine the staining intensity and percentage of Nibrin expressing cells. More specifically, the staining intensity was assessed with a four-point scale from negative (0); weak (1); moderate (2); and strong intensity (3). The extent of the staining was expressed as percentage of positive cells (0-100%) by 10% intervals. The Nibrin histoscore was counted by multiplying the intensity level by percentage of positive cells resulting in a value between 0 and 300. Data were divided into groups by histoscore levels. Accordingly the cancer and their corresponding adjacent normal specimens were grouped by Nibrin expression score based on the median score value of cancerous and adjacent normal tissues respectively into "weak expression" (Tumor: scores 0-209 and Normal: scores 0-144) and "strong expression" (Tumor: scores 210-300 and Normal: scores 145-300).

Follow-up and disease status of OSCC patients

Out of total 100 OSCC patients, for overall survival analysis, only 90 patients could be followed for a period of 24 months or until death within that period. On the other hand, for relapse-free survival study, 78 of 100 patients with or without recurrence within that period were considered. The remaining 12 patients could not be included for relapse-free survival study due to presence of persistent disease.

Statistical analysis

The data were analyzed statistically using SPSS software version 17.0 (Chicago, IL, USA). The two tailed chi-square test was used to assess associations between two parameters. Correlations between two parameters were calculated using spearman's correlation coefficient (*r*). To compare the Nibrin expression in cancerous and adjacent normal tissues, paired sample *t*-test was used. Univariate survival analysis was performed using Kaplan-Meier survival function and differences in survival were tested for statistical significance using the log-rank statistics. *P* values ≤ 0.05 were considered significant.

RESULTS

Nibrin expression in OSCC

Of the tongue and buccal mucosa cancer tissue, Nibrin protein expression was evaluated with nuclear location of the immunoreactions, Nibrin was expressed in 99% of tumors and 92% of the adjacent normal squamous epithelium [Figure 1]. H-score varied from 0 to 300 in both OSCC and adjacent normal tissues. Median H-score for tumor tissues was 210 while that for the adjacent normal tissue was 145. The tissues expressing Nibrin below the median H-score was consider as a weak expression and tissue expressing Nibrin above the median H-score was considered as a strong expression.

Relation of Nibrin expression with clinical and histopathological parameters

Two tailed chi-square test and spearman's correlation coefficient (*r*) were used to assess correlation between the Nibrin protein expression and clinicopathological parameters in tumor tissues. The relations of Nibrin immunoreactivity with clinical and histopathological parameters are depicted in Table 1, respectively. In tumor tissues an inverse correlation of Nibrin expression was found with tumor size (χ^2 = 5.622, *r* = -0.237, *P* = 0.018) and tumor stage (χ^2 = 6.600, *r* = -0.194, *P* = 0.039) while, significant positive correlation was found with strong Nibrin expression and moderately/poorly differentiated tumor tissues (χ^2 = 4.857, *r* = +0.220, *P* = 0.028). No significant association was detected with Nibrin expression and other clinicopathological parameters.

Univariate survival analysis

According to Kaplan-Meier survival analysis similar incidence of death observed in total patients with strong (42%, 20/48, log-rank = 0.112, df = 1, *P* = 0.737)

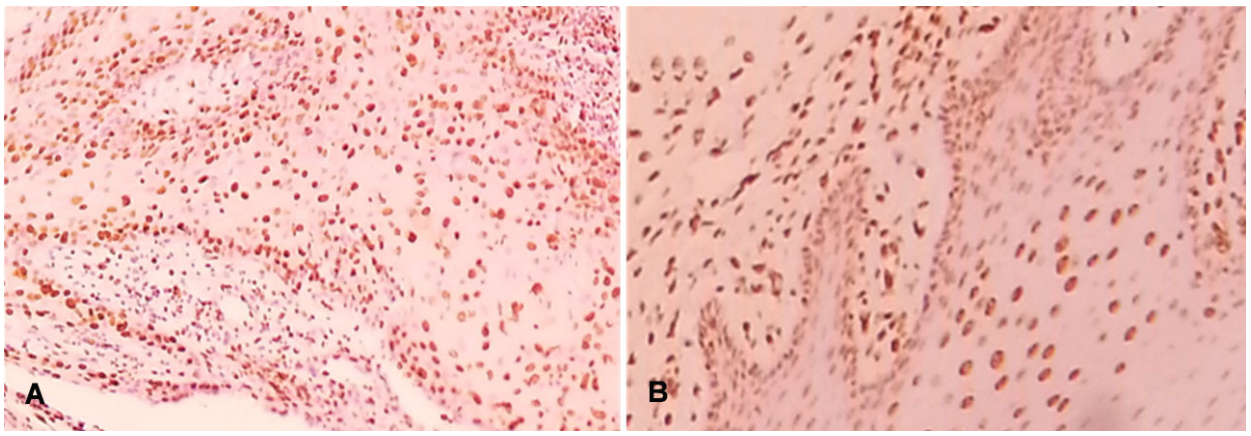

Figure 1: Nibrin protein expression (IHC, ×10). (A) Nuclear protein expression of Nibrin in primary tumor of OSCC; (B) nuclear protein expression of Nibrin in adjacent normal tissue of primary OSCC tumor tissue. OSCC: oral squamous cell carcinoma

Table 1: Relation of Nibrin immunoreactivity with clinical and pathological parameters in OSCC tissue

	Nibrin expression in tumor (Median: 210)				
Variables	***n***	**Weak, *n* (%)**	**Strong, *n* (%)**	**Correlation (*r*)**	***P***
Age (year): median 45					
< 45	47	22 (47)	25 (53)	-0.004	0.972
≥ 45	53	25 (47)	28 (53)		
Gender					
Male	75	33 (44)	42 (56)	+0.104	0.303
Female	25	14 (56)	11 (44)		
Anatomic site					
Tongue	61	30 (49)	31 (51)	+0.055	0.589
Buccal mucosa	39	17 (44)	22 (56)		
Tobacco habit					
Absent	14	9 (64)	5 (36)	+0.140	0.166
Present	86	38 (44)	48 (56)		
Disease status (*n* = 78)					
No recurrence	46	23 (50)	23 (50)	+0.031	0.789
Recurrence	32	15 (47)	17 (53)		
Disease outcome (*n* = 90)					
Alive	51	23 (45)	28 (55)	-0.036	0.737
Dead	39	19 (49)	20 (51)		
Tumor size					
T1 - T2	71	28 (39)	43 (61)	-0.237	0.018
T3 - T4	29	19 (66)	10 (34)		
Tumor stage					
I	20	8 (40)	12 (60)	-0.194	0.039
II	22	8 (36)	14 (64)		
III	18	6 (33)	12 (67)		
IV	40	25 (62)	15 (38)		
Nodal status					
Negative	59	25 (42)	34 (58)	-0.111	0.271
Positive	41	22 (54)	19 (46)		
Tumor differentiation					
Well	50	29 (58)	21 (42)	+0.220	0.028
Moderately/poorly	50	18 (36)	32 (64)		
Keratin					
Absent	79	34 (43)	45 (57)	-0.154	0.126
Present	21	13 (62)	8 (38)		
Lymphatic permeation					
Absent	91	43 (47)	48 (53)	+0.095	0.874
Present	9	4 (44)	5 (56)		
Vascular permeation					
Absent	99	47 (47)	52 (53)	+0.095	0.349
Present	1	0 (0)	1 (100)		
Perineural invasion					
Absent	82	40 (49)	42 (51)	+0.076	0.451
Present	18	7 (39)	11 (61)		
Lymphocytic stromal response					
Absent	46	21 (46)	25 (54)	-0.025	0.806
Present	54	26 (48)	28 (52)		

OSCC: oral squamous cell carcinoma

and weak Nibrin expression (45%, 19/42). In relation to relapse free survival also we were unable to find any significant incidence of disease relapse in patients with strong (42%, 17/40, log-rank = 0.006, df = 1, *P* = 0.937) and weak Nibrin expression (39%, 15/38). Although we were unable to obtain any significant findings in total patients, we further sub grouped patients into early and advanced stage disease and surprisingly, we observed that in patients with early stage disease, a significant high incidence of disease relapse was observed in patients with strong Nibrin expression (43%, 10/23, log-rank = 3.884, df = 1, *P* = 0.049) as compared to patients with weak Nibrin expression (8%, 1/12) [Table 2 and Figure 2]. No such significant difference was noted for overall survival in this subgroup of patients. On the other hand in patients with advanced disease, Nibrin expression failed to discriminate such high and low risk sub group patients for survival.

DISCUSSION

Nibrin (p95, NBN, NBS1, NBS) is a 754-amino acid polypeptide which is involved in the recognition and the repair of DSBs.[15,27-29] It interacts with Mre11 and RAD50 to form the MRN complex and is required for translocation of this complex to sites of DSBs.[29] Although, in advanced head and neck SCC the prognostic significance of over expression of Nibrin by immunohistochemistry has been identified.[30] However, data on the correlation of Nibrin with the clinicopathological prognosticators are limited. So, the current study evaluated correlation between Nibrin expression with clinicopathological parameters in total 100 patients with SCC of tongue and buccal mucosa.

In the present study, we observed nuclear expression of Nibrin in OSCC tissues and its corresponding adjacent normal tissues. However, there was no significant

Table 2: Univariate survival analysis (Kaplan-Meier survival function) of Nibrin expression

Variable	*n*	Patients relapsed or died, *n* (%)	Log-rank	df	*P*
Relapse free survival					
Nibrin (total patients, *n* = 78)					
Weak	38	15 (39)*	0.006	1	0.937
Strong	40	17 (42)*			
Nibrin (early stage patients, *n* = 35)					
Weak	12	1 (8)*	3.884	1	0.049
Strong	23	10 (43)*			
Nibrin (advanced stage patients, *n* = 43)					
Weak	26	14 (54)*	0.593	1	0.441
Strong	17	7 (41)*			
Overall survival					
Nibrin (total patients, *n* = 90)					
Weak	42	19 (45)#	0.112	1	0.737
Strong	48	20 (42)#			
Nibrin (early stage patients, *n* = 38)					
Weak	13	2 (15)#	0.659	1	0.417
Strong	25	7 (28)#			
Nibrin (advanced stage patients, *n* = 52)					
Weak	29	17 (59)#	0.010	1	0.920
Strong	23	13 (56)#			

*: patients relapsed; #: patients died

difference in Nibrin expression between OSCC tissues and their corresponding adjacent normal tissues (*t* = -0.455, df = 99, *P* = 0.657). Along with that in OSCC tissues, Nibrin expression was significantly positively correlated with tumor differentiation and significantly inversely correlated with tumor size and tumor stage, suggesting that up-regulation of Nibrin may be an early event in OSCC development. In accordance with our results, Ali-Fehmi *et al*.[31] also showed that NBS1 does not show markedly higher expression in all ovarian cancer patients compared to women with serous cyst adenoma and those with normal ovaries.

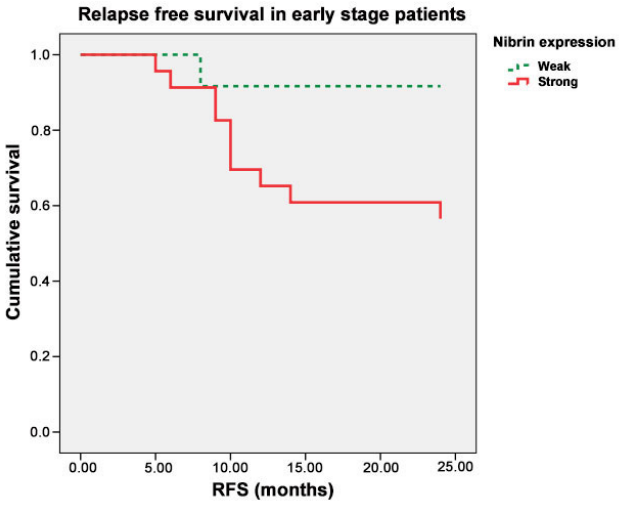

Figure 2: Kaplan-Meier univariate survival analysis of patients with early stage disease indicating significant high incidence of disease relapse in patients with strong Nibrin expression (*P* = 0.049). RFS: relapse-free survival

Plisiecka-Halasa *et al*.[25] also showed that in human ovarian tumor tissues Nibrin expression was marked as strong nuclear staining which was present in both tumors and normal tissues. Further, Nibrin expression is up-regulated in adjacent normal tissues of OSCC tissue which is compatible with the hypothesis that Nibrin is a tumor suppressor gene.[32] In contrast with our findings, Hsu *et al*.[30] showed that Nibrin over expression was significantly correlated with high tumor size and metastatic dieses in OSCC patients which may be because of the inclusion of more number of patients with locally advanced diseases. Ehlers *et al*.[33] also showed that Nibrin was associated with strong tumor severity and metastatic death marker in uveal melanoma. However, similar expression of NBS1 in class 1 tumors and normal uveal melanocytes suggests that up-regulation of NBS1 may be a late event in melanoma progression.

Kaplan-Meier univariate survival analysis showed that in patients with early stage disease high number of patients relapsed with strong Nibrin expression. However, our findings not only observed increased expression pattern of Nibrin in early stage patients but also found a strong correlation between increased Nibrin expressions in the onset of the disease with higher probability of recurrence. This could be attributed to the fact that since Nibrin acts as a sensor molecule of MRN complex which further activates the other DNA repair molecules, it might have a plausible role in constitutively activating these downstream molecules eventually leading to disease relapse in patients. While Hsu *et al*.[30] found that in OSCC

patients strong Nibrin expression was associated significantly with shorter overall survival compared with weak expression. Ehlers *et al*.[33] have also found that in uveal melanoma, the 6-year survival was 100% for the low NBS1 group and 22% for the high NBS1 group (P = 0.01). In the breast carcinoma, patients with NBS1-aberrant tumors seemed to have poorer survival than the patients with NBS1 normal tumors. This indicates that the NBS1 deficiency predicts poor survival of the breast carcinoma patients.[34]

In conclusion, our study discovered that a Nibrin protein expression is significant in lower tumor size and early stage disease in OSCC indicating its role in early event of disease progression. Further, high incidence of disease relapse was found to be present in early stage patients with strong Nibrin expression. Thus, it could be used as a favorable prognostic factor in developing disease recurrence in patients with early stage disease. Further, among various cancers, the different patterns of the Nibrin expression have observed which indicates that the expression of Nibrin is important in cancer development and progression with cancer cell type specificity, although the mechanism behind it is unclear.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained from the patients.

Ethics approval

Ethics approval was obtained from the Gujarat Cancer and Research Institute.

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