

Semaphorin 4D (Sema4D) and HoxB9 Expression Levels in Head and Neck Squamous Cell Carcinomas (HNSCCs) and their Clinicopathological Values; an Immunohistochemical Study

Enas M Fouad^{1*}, Marwa M Ahmed², Ahmed Mohamed El Teliti³ and Ahmed Mohamed Yehia³

¹Department of Pathology, Faculty of Medicine, Zagazig University, Egypt

²Department of Pathology, Faculty of Medicine, Beni-Suef University, Egypt

³Department of General Surgery, Faculty of Medicine, Zagazig University, Egypt.

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ABSTRACT

Background: Semaphorin 4D is a glycoprotein which was found to be highly expressed in many cancers, but its detailed pathological and prognostic role in HNSCC is still controversial. Homeobox (HOX) genes which code for many transcription factors which were aberrantly expressed in HNSCC tissues, like HOXB9 but, there is minimal research on the relationships between HOXB9 and HNSCC and there is a lack of clarity about its importance in HNSCC carcinogenesis.

Therefore we aimed in the current study to explore the expression of Semaphorin 4D and HoxB9 in malignant tissues which were retrieved from HNSCC patients then to correlate our findings with clinical data and pathological criteria of patients.

Methods: Semaphorin 4D and HoxB9 expression was evaluated in malignant tissues which were retrieved from HNSCC patients using immunohistochemistry then correlate their expression with clinical data and pathological criteria of patients.

Results: High Semaphorin 4D and HoxB9 expression was correlated with higher grade ($p=0.043$ and 0.039 respectively), presence of lymph node metastases ($p=0.006$ and 0.023 , respectively), advanced stage of the tumor ($p=0.002$ and 0.015 , respectively), distant metastases ($p=0.025$ and 0.030 respectively). There is a significant positive association between Semaphorin 4D and HoxB9 expression Phi: phi coefficient= $+0.905$ ($p<0.001$).

Conclusion: Semaphorin 4D and HoxB9 overexpression are markers of poor prognosis in HNSCC patients.

Keywords: Semaphorin 4D, HoxB9, HNSCC, Immunohistochemistry, Prognosis

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCCs) are carcinomas which originate from the oral cavity, pharynx, larynx, nose and para-nasal sinuses [1]. They are considered the 6th commonest malignancy worldwide [2]. The nearby vital structures limits the radicalism of excision of such malignancies, moreover, patients could suffer from multiple primaries or have cancer recurrence with a 6 months average survival rate for advanced or recurrent cases [3,4]. Hence, there is a real need to find novel therapeutic intervention targets and new prognostic biomarkers in HNSCC [5]. Semaphorins are members of a large family of proteins which were discovered in the nervous system and they are involved in the maintenance of a correct neuronal network [6]. Semaphorin 4D is a glycoprotein which is a member of group IV semaphorin family and it could be present in a membrane bound or a secreted form [7]. It has a role in many physiological and pathological processes like angiogenesis and bone [8]. Semaphorin 4D was found to be highly expressed in many cancers [1,9], but its detailed

pathological and prognostic role in HNSCC is still controversial.

There are many transcription factors which are found to be deregulated in HNSCC; Homeobox (HOX) genes which code for many transcription factors that have important roles in embryogenesis and normal organogenesis [10]. There are about 18 HOX genes which were aberrantly expressed in HNSCC tissues, among which; HOXB9 which was aberrantly expressed in many cancers [5]. But, there is

Corresponding author: Enas M Fouad, Department of Pathology, Faculty of Medicine, Zagazig University, Egypt, E-mail: olaharb2015@gmail.com

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a lack of clarity about its importance in HNSCC carcinogenesis.

Therefore we aimed in the current study to explore the expression of Semaphorin 4D and HoxB9 in malignant tissues which were retrieved from HNSCC patients then to correlate our findings with clinical data and pathological criteria of patients.

MATERIALS AND METHODS

This is a retrospective cohort study; we have included sections from fifty paraffin blocks which are retrieved from fifty HNSCC cases that were retrospectively examined. All cases were surgically managed at the Department of General Surgery, Department, Faculty of Medicine, Zagazig University Hospital in the period from March 2014 and March 2018. All patients' data were collected from patients' files and the diagnosis was pathologically confirmed to be HNSCC in pathology department, Faculty of Medicine Zagazig University and reviewed in Pathology Department Faculty of Medicine, Beni Suif University. No patient had received chemotherapy or radiotherapy before surgery. The study was approved by the ethics committee of Faculty of Medicine, Zagazig University.

Immunohistochemistry

Sections from the fifty paraffin blocks were deparaffinized then autoclaved in 0.2% citrate buffer for fifteen minutes for antigen retrieval. All sections were incubated with 3% hydrogen peroxide for half an hour to block endogenous peroxidase activity. Sections were incubated with primary rabbit polyclonal anti-human Semaphorin 4D (1:200, Abcam, Cambridge, MA, USA) anti-HOXB9 (1:400, Sigma Aldrich, Poole, UK) overnight at 4°C. The specimens were incubated with the primary antibodies overnight at 4°C, followed by 3 washes with buffered saline, and then treated with a streptavidin-biotin complex for 60 min at a dilution of 1:100. The immunoreaction was assessed using a 3,3'-diaminobenzidine (DAB) substrate-chromogen solution. Finally, the sections were immersed in both ethanol and

xylene bath and then mounted for examination. Sections from colon cancer and submandibular salivary gland were used as positive controls for Semaphorin 4D and HoxB9, respectively.

Evaluation of the Immunohistochemical staining

Cytoplasmic immunoreactivity for Semaphorin 4D and nuclear immunoreactivity for HOXB9 were evaluated according to both the extent and intensity of staining; the extent of positive tumor cells were scored as follows: 0: none; 1: <10%; 2: 10-50%; and 3: >50%, while the staining intensity were scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. We multiplied both to give final stain score from 0-9. Tumor cells with a score of ≥ 5 of staining were classified as high expression and tumor cells with a score of less than 5 were classified as low-expression group [1,9].

STATISTICAL ANALYSIS

Statistics were performed using SPSS 22.0 for windows (Chicago, IL, USA) and MedCalc windows (Ostend, Belgium). Percent of categorical variables were compared by using Chi-square test or Fisher's exact test when was appropriate. Continuous variables were expressed as the mean $\pm$ SD and median (range), and the categorical variables were expressed as a number (percentage). Strength of relationship between Semaphorin 4D and HoxB9 expression were determined by computing phi coefficient with (+) sign was indicator for direct relationship and (-) sign was indicator for inverse relationship. A p-value<0.05 was considered significant.

RESULTS

Patient characteristics

Fifty samples retrieved from HNSCC patients. The demographic data of the 50 patients were summarized in **Table 1**. We have included 38 (76%) males and 12 (24%) females.

Table 1. Demographic data of patients with head and neck squamous cell carcinomas (HNSCCs).

Characteristics	All patients (N=50)	
	No.	%
Age (years)		
Mean $\pm$ SD	54.60 $\pm$ 9.73	
Median (Range)	56 (33-69)	
$\leq$ 60 years	29	58%
>60 years	21	42%
Sex		
Male	38	76%
Female	12	24%
History of smoking		
Non-smoker	15	30%
Smoker	35	70%
Grade		
Grade I	18	36%
Grade II	22	44%
Grade III	10	20%
CIS		
Absent	28	56%
Present	22	44%
LVI		
Absent	19	38%
Present	31	62%
PNI		
Absent	28	56%
Present	22	44%
p16		
Negative	18	36%
Positive	32	64%
T stage		
T1	12	24%
T2	12	24%
T3	14	27%
T4a	6	12.5%
T4b	6	12.5%
N stage		
N0	18	36%
N1	6	12.5%
N2	12	25%
N3	14	26.5%
M stage		
M0	40	80%
M1	10	20%
AJCC 2017 stage group		
Stage I	8	16%
Stage II	18	36%
Stage III	14	28%
Stage IVA	5	10%
Stage IVB	4	8%
Stage IVC	1	2%

Categorical variables were expressed as number (percentage). Continuous variables were expressed as mean $\pm$ SD and median (range)

Immunohistochemical results

Semaphorin 4D immuno-expression and its correlation clinicopathological features of HNSCC patients: High Semaphorin 4D expression was found in 35(70%) of cases of HNSCC and its expression was significantly positively correlated with higher grade ($p=0.043$), presence of lymph

node metastases ($p=0.006$), advanced stage of the tumor ($p=0.002$), distant metastases ($p=0.025$), presence of adjacent foci of CIS ($p=0.26$), PNI ($p=0.023$). No significant correlation was found between Semaphorin 4D expression with primary site of the tumor, age or sex of patients or previous history of smoking (**Table 2 and Figure 1**).

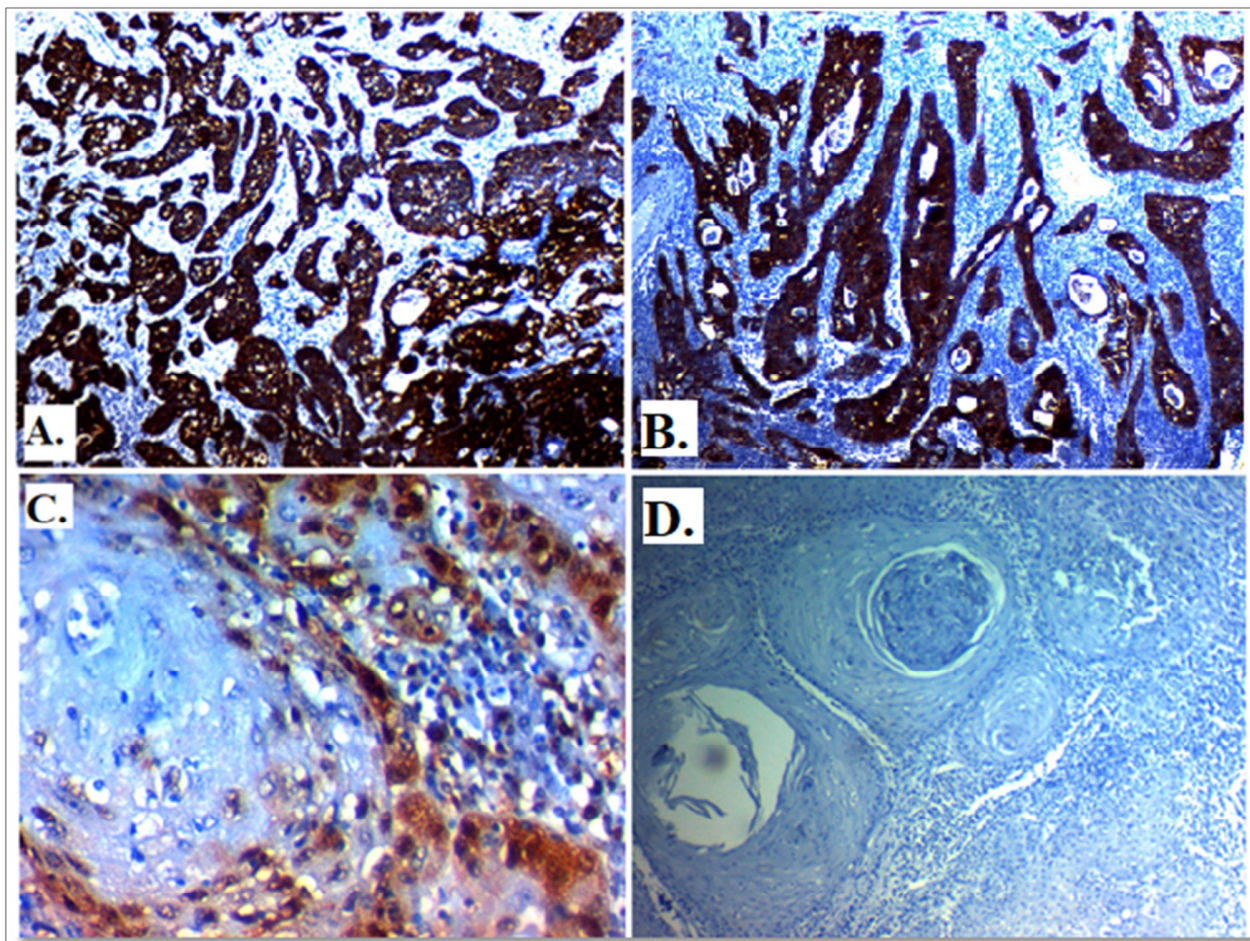


Figure 1. Semaphorin 4D expression in head and neck squamous cell carcinoma (HNSCC): (A) High cytoplasmic expression of Semaphorin 4D in poorly differentiated HNSCC stage IVx100. (B) High cytoplasmic expression of Semaphorin 4D in poorly differentiated HNSCC, stage IVx100. (C) Low cytoplasmic expression of Semaphorin 4D in moderately differentiated HNSCC stage IIIx400. (D) Negative cytoplasmic expression of Semaphorin 4D in well differentiated HNSCC; stage Ix200.

Table 2. Correlation between clinicopathological features and Semaphorin 4D expression in in head and neck squamous cell carcinomas (HNSCCs).

Characteristics	All		Semaphorin 4D				p-value
	(N=50)		Low (N=15)		High (N=35)		
	No.	(%)	No.	(%)	No.	(%)	
Age							
≤ 60 years	29	58%	12	(51.7%)	17	(48.3%)	0.385‡
>60 years	21	42%	3	(36.4%)	18	(63.6%)	
Sex							
Male	38	76%	11	(48.6%)	27	(51.4%)	1.000‡
Female	12	24%	4	(40%)	8	(60%)	
History of smoking							
Non-smoker	15	30%	5	(50%)	10	(50%)	1.000‡
Smoker	35	70%	10	(46.7%)	25	(53.3%)	
Grade							
Grade I	18	36%	7	(58.3%)	11	(41.7%)	0.043§
Grade II	22	44%	5	(52.6%)	17	(47.4%)	
Grade III	10	20%	3	(22.2%)	7	(77.8%)	
CIS							
Absent	28	56%	10	(50%)	18	(50%)	0.026‡
Present	22	44%	5	(44.4%)	17	(55.6%)	
LVI							
Absent	19	38%	8	(68.8%)	11	(31.2%)	0.045‡
Present	31	62%	7	(33.3%)	24	(66.7%)	
PNI							
Absent	28	56%	10	(61.5%)	18	(38.5%)	0.023‡
Present	22	44%	5	(21.4%)	17	(78.6%)	
p16							
Negative	18	36%	13	(72.2%)	5	(27.8%)	0.025‡
Positive	32	64%	2	(27.3%)	30	(72.7%)	
T stage							
T1	12	24%	8	(90%)	4	(10%)	0.034§
T2	12	24%	4	(40%)	8	(60%)	
T3	14	27%	2	(33.3%)	12	(66.7%)	
T4a	6	12.5%	1	(50%)	5	(50%)	
T4b	6	12.5%	0	(0%)	6	(100%)	
N stage							
N0	18	36%	11	(72.2%)	7	(27.8%)	0.006§
N1	6	12.5%	0	(0%)	6	(100%)	
N2	12	25%	2	(44.4%)	10	(55.6%)	
N3	14	26.5%	2	(18.2%)	12	(81.8%)	
M stage							
M0	40	80%	14	(54.5%)	26	(45.5%)	0.025‡
M1	10	20%	1	(14.3%)	9	(85.7%)	
AJCC 2017 stage group							
Stage I	8	16%	6	(87.5%)	2	(12.5%)	0.002§
Stage II	18	36%	4	(54.5%)	14	(45.5%)	
Stage III	14	28%	4	(35.7%)	10	(64.3%)	
Stage IVA	5	10%	1	(33.3%)	4	(66.7%)	
Stage IVB	4	8%	0	(0%)	4	(100%)	
Stage IVC	1	2%	0	(0%)	1	(100%)	

Categorical variables were expressed as number (percentage)

‡ Chi-square test; § Chi-square test for trend; p<0.05 is significant

HOXB9 immuno-expression and its correlation clinicopathological features of HNSCC patients: High HOXB9 was found in 30 (60%) of cases of HNSCC and its expression was significantly positively correlated with higher grade ($p=0.039$), distant metastases ($p=0.030$), advanced stage of the tumor ($p=0.015$), presence of lymph

node ($p=0.023$), LVI ($p=0.033$) and higher incidence of P16 positivity ($p=0.024$). No significant association was found between HOXB9 expression with primary location of the tumor, age or sex of the patients, presence of adjacent foci of CIS or history of smoking (**Table 3 and Figure 2**).

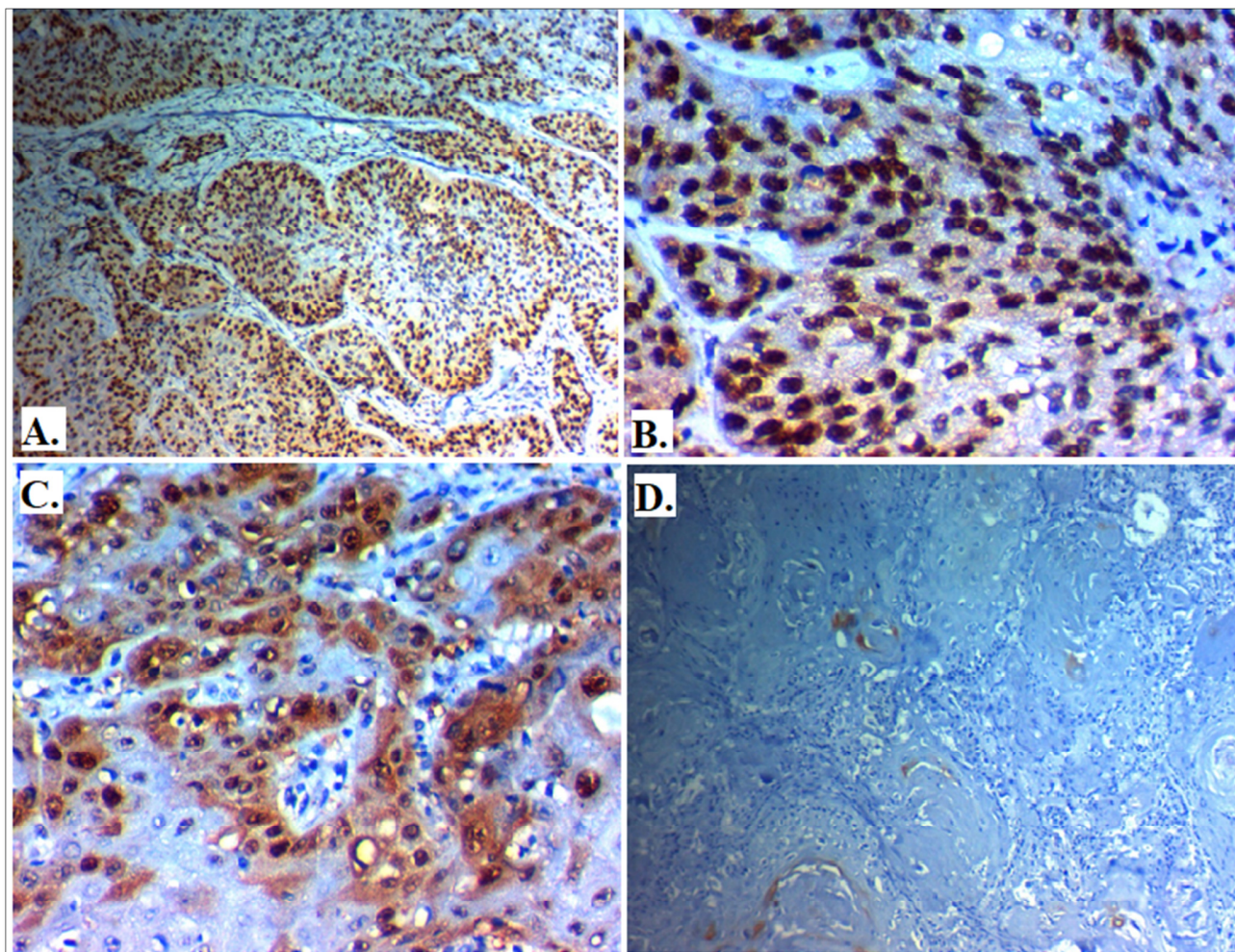


Figure 2. HoxB9 expression in head and neck squamous cell carcinoma (HNSCC): (A) High nuclear expression of HoxB9 in poorly differentiated HNSCC stage IVx100. (B) High nuclear expression of HoxB9 in poorly differentiated HNSCC, stage IVx400. (C) Low nuclear expression of HoxB9 in moderately differentiated HNSCC stage IIx400. (D) Negative nuclear expression of HoxB9 in well differentiated HNSCC; stage IIx100.

Table 3. Correlation between clinicopathological features and HoxB9 expression in head and neck squamous cell carcinomas (HNSCCs).

Characteristics	All		HoxB9				p-value
			Low		High		
	(N=50)		(N=20)		(N=30)		
	No.	(%)	No.	(%)	No.	(%)	
Age							
≤ 60 years	29	(72.5%)	14	(51.7%)	15	(48.3%)	0.873‡
>60 years	21	(27.5%)	6	(54.5%)	15	(45.5%)	
Sex							
Male	38	(87.5%)	16	(54.3%)	22	(45.7%)	0.654‡
Female	12	(12.5%)	4	(40%)	8	(60%)	
History of smoking							
Non-smoker	15	(25%)	5	(50%)	10	(50%)	1.000‡
Smoker	35	(75%)	15	(53.3%)	20	(46.7%)	
Grade							
Grade I	18	(30%)	5	(58.3%)	13	(41.7%)	0.039§
Grade II	22	(47.5%)	12	(57.9%)	10	(42.1%)	
Grade III	10	(22.5%)	3	(33.3%)	7	(66.7%)	
CIS							
Absent	28	(55%)	11	(54.5%)	17	(45.5%)	0.775‡
Present	22	(45%)	9	(50%)	11	(50%)	
LVI							
Absent	19	(40%)	14	(68.8%)	5	(31.2%)	0.033‡
Present	31	(60%)	6	(41.7%)	25	(58.3%)	
PNI							
Absent	28	(65%)	16	(65.4%)	12	(34.6%)	0.046‡
Present	22	(35%)	4	(28.6%)	18	(71.4%)	
p16							
Negative	18	(45%)	12	(72.2%)	6	(27.8%)	0.024‡
Positive	32	(55%)	8	(36.4%)	24	(63.6%)	
T stage							
T1	12	(25%)	7	(90%)	5	(10%)	0.013§
T2	12	(25%)	5	(40%)	7	(60%)	
T3	14	(30%)	6	(50%)	8	(50%)	
T4a	6	(10%)	2	(50%)	4	(50%)	
T4b	6	(10%)	0	(0%)	6	(100%)	
N stage							
N0	18	(45%)	13	(72.2%)	5	(27.8%)	0.023§
N1	6	(5%)	0	(0%)	6	(100%)	
N2	12	(22.5%)	4	(55.6%)	8	(44.4%)	
N3	14	(27.5%)	3	(27.3%)	11	(72.7%)	
M stage							
M0	40	(82.5%)	19	(60.6%)	11	(39.4%)	0.030‡
M1	10	(17.5%)	1	(14.3%)	9	(85.7%)	
AJCC 2017 stage group							
Stage I	8	(20%)	7	(87.5%)	1	(12.5%)	0.015§
Stage II	18	(27.5%)	6	(54.5%)	12	(45.5%)	
Stage III	14	(35%)	6	(50%)	8	(50%)	
Stage IVA	5	(7.5%)	1	(33.3%)	4	(66.7%)	
Stage IVB	4	(7.5%)	0	(0%)	4	(100%)	
Stage IVC	1	(2.5%)	0	(0%)	1	(100%)	

Categorical variables were expressed as number (percentage)

‡ Chi-square test; § Chi-square test for trend; $p < 0.05$ is significant

There is a significant positive association between Semaphorin 4D and HoxB9 expression. Phi: phi coefficient=+0.905 ($p<0.001$).

DISCUSSION

The molecular pathogenesis of HNSCC is still poorly understood and despite recent advances in the management of such cancer it still has dismal outcome. As the progression of HNSCC is complex process which involves interactions between plethoras of factors, so identification of novel prognostic variables could help in adequate management of such type of cancer to improve the prognosis.

In the current study we found that Semaphorin 4D was overexpressed in 35 (70%) of cases of HNSCC and its expression was correlated with poor clinicopathological criteria like higher grade, advanced stage, presence of lymph node and distant metastases, presence of adjacent foci of CIS, LVI and PNI.

and our results were similar to results of Takada et al. [1] and Derakhshandeh et al. [9] who proved similar results in SCC and Liu et al. [6] who proved that Semaphorin 4D expression is related to poor clinical outcome in cervical cancer patients.

Similarly, Semaphorin 4D was found to be highly expressed in many cancers and its expression was related to poor pathological parameters [1,9] and it was proved to enhance tumor angiogenesis through binding to its receptor Plexin-B1 on endothelial cells which promotes their proliferation which subsequently lead to malignant progression [11,12]. Previous research indicated that Semaphorin 4D has been shown to act directly cancer cells of different organs inducing invasion and metastasis [13,14].

Moreover, Takada et al. [1] demonstrated that Semaphorin 4D increased HNSCC cell proliferation, migration and invasion, which explain our results regarding the association between such marker and progression of HNSCC.

Capparuccia et al. [15] showed that Semaphorin 4D is related to cytoskeleton reorganization and focal adhesion formation which modulate cellular adhesion and motility. Moreover, Semaphorin 4D causes the AKT and MAPK phosphorylation [16,17], the signaling which suppresses apoptosis and promotes malignant cells multiplication, respectively. Taken together, these findings suggest that Semaphorin 4D plays a direct role in progression of HNSCC through the previously mentioned signaling pathways. Takada et al. [1], demonstrate that Semaphorin 4D high expression is present in HNSCCs with bone invasion compared to those without bone invasion. Derakhshandeh et al. [9] study presents a novel role for Semaphorin 4D in modulating the tumor microenvironment by upregulation of ECM collagen formation by fibroblasts, which accompanies tumor progression and facilitates invasion

The dense fibrosis which is rich in collagen type I has been linked with aggressive fatal malignancies and dismal outcome [18-20]. The limitation of the Derakhshandeh et al. [9] study is that it included stage IV malignancy with loco-regional metastases and did not include stage IV with distant metastases, so they did not assessed Semaphorin 4D expression in other stages. In addition, they provided no information regarding smoking history or HPV status was available, leaving their findings more descriptive rather than conclusive. But our study includes different grades, stages and clinical parameters which strengthen our results more. Additionally we assessed the expression of another transcription factor which was incriminated in HNSCC progression; HOXB9.

HOXB9 is a HOX family member, which functions as a transcription factor and it was incriminated in cancer progression. The role of HOXB9 in cancer progression is complicated [21]. HOXB9 was found to be overexpressed in lung and breast cancer, while its expression was decreased in colon, pancreatic and gastric carcinoma [22-24]. In the present study we proved that HoxB9 was overexpressed in 30 (60%) of cases of HNSCC and its expression was correlated with poor clinicopathological criteria like higher grade, advanced stage, presence of lymph node and distant metastases, presence of adjacent foci of CIS, LVI and PNI. which was similar to results of Sun et al. [25] in laryngeal SCC (LSCC) tissues. Xue et al. [5] in oral squamous cell carcinoma (OSCC) tissues and Wan et al. [21] in endometrial carcinoma. Wan et al. [21] found the expression of HOXB9 in endometrial carcinoma is higher than proliferative endometrium and endometrial hyperplasia. In addition, HOXB9 expression in cancer was correlated with higher histological grade and presence of lymph node metastasis which was similar to our results in HNSCC. Sun et al. [25] report that HOXB9 was up regulated in LSCC tissues when compared with the adjacent noncancerous tissues. Moreover they found that a high expression level of HOXB9 was found to be associated with high histological grade and poor prognosis in LSCC. Sun et al. [25] findings provide the first data regarding the association between HOXB9 expression and LSCC progression which is a subtype of HNSCC. Similarly, HOX gene expression is dysregulated in several cancers [25]. But, the expression of different subtypes varies among different types of cancer.

Xue et al. [5] observed that HoxB9 is associated with cancer progression in OSCC and that high levels of HoxB9 are associated with shorter overall survival in OSCC patients. Subsequently, Xue et al. [5] found that knockdown of HoxB9 in OSCC cells decreases cancer cell migration and invasion. This pointed to, the possibility of considering such factor as a targeted therapy for SCC. Effects of HoxB9 in OSCC progression might be due to EMT stimulation through activating the TGF- β 1/Smad2/Slug signaling pathway which increased malignant cells invasion and metastases. Although some HOX genes were found to act as

oncogenes in solid tumors, others showed down-regulation in other types of cancer [26] and acted as tumor-suppressor genes [27]. The discrepancy between such results may be caused by different roles of HOX genes in different cancer types and the different methods of preparing samples and assessment. Sun et al. [25] have concluded that HOXB9 is a novel biomarker which is responsible for progression of LSCC development and which simulate results other studies that that HOXB9 was frequently overexpressed in many tumors [24,28]. And its high expression was correlated with high tumor grade and poor prognosis [29]. By contrast, decreased HOXB9 expression was found to be related to poor survival in gastric cancer [30].

These findings suggest that protein expression HOXB9 varied according to tumor subtype. However, the underlying mechanism for such discrepancy remains elusive. In a recent study similar to ours in HNSCC, HOXB9 was highly expressed [10]. Several mechanisms could explain the association between high HOXB9 and poor prognosis in cancer one of them is activation of Epithelial-mesenchymal transition (EMT) which comprises a set of changes in the cellular phenotype in which epithelial cells experience a molecular switch from the epithelial phenotype to a highly mobile, mesenchymal phenotype [31]. EMT is mainly observed at the invasive front of tumors and is associated with occurrence of metastasis in tumor progression [32,33]. HoxB9 caused dysregulation of adhesion proteins including E-cadherin, Claudin-1 and Occludin in colon cancer [34]. Additionally, HoxB9 was found to induce EMT in breast cancer by activating the Wnt signaling pathway [28]. These results suggested that over expression of HoxB9 was associated with occurrence of EMT process and cancer progression through activation of TGF- β 1/Smad2/Slug signaling pathway [5]. Wan et al. [21] demonstrated that HOXB9 expression is increased in EC patients, and HOXB9 promotes EC cell migration by regulating E2F3 expression. Thus, HOXB9 may represent a prognostic marker and a potential therapeutic target for EC.

CONCLUSION

We found a positive association between Semaphorin 4D and HoxB9 expression in HNSCC and both are associated with progression of the tumor, poor clinicopathological criteria of the patients, that points to the possibility of considering both of them novel therapeutic targets for better management of such serious cancer.

In conclusion, this study confirmed that Semaphorin 4D and HoxB9 expression in HNSCC is increased. This finding supports the possibility of Semaphorin 4D and HoxB9 and/or their associated molecules as targets for anti-metastatic therapy against HNSCC.

LIMITATIONS

The limitations of our study are; small number of samples, lack of follow-up, the retrospective nature of the study and

assessment of the markers using single method of evaluation that is the immunohistochemical evaluation of markers so we have recommended performing a prospective study on a large number of patients using genetic methods of evaluation to prove our results and improve the findings.

CONFLICTS OF INTEREST

Authors declared no conflicts of interest.

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