ORIGINAL ARTICLES

N-cadherin and hyaluronan expression in head and neck squamous cell carcinoma, relation to patient outcomes

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ABSTRACT

Background: Epithelial-mesenchymal transition (EMT) is regarded as an essential step for tumor invasion and metastasis. In squamous cell carcinoma of head and neck (HNSCC), N-Cadherin expression and its involvement in tumor progression remains a controversial topic.
Aim of the study: The present study aimed to assess the expression of N-cadherin and HA in HNSCC and further study their relation to patients survival and outcomes.
Material and methods: Fifty-eight retrospective selected cases of head and neck squamous carcinomas (HNSCCs) with available paraffin blocks. Complete clinico-pathological and follow-up data were recorded. Immune staining for N-cadherin and hyaluronan were done, also, we study the correlation of the results with patients survival data.
Results: Squamous cell carcinoma islands demonstrated high N-cadherin expression in 55.2% and low expression in 44.8%. N-cadherin high expression was significantly ($p < .05$) associated with large tumor sizes, advanced TNM clinical stage, increased incidence of recurrence and patient's death. A significant correlation was recorded between the presence of neural invasion and N-cadherin expression ($p = .004$). Strong intensity of stromal HA was significantly ($p < .05$) associated with an oral site, nodal metastasis, and higher TNM stage. Patients with high N-cadherin expression, diffuse hyaluronan, and strong stromal hyaluronan reaction had significantly lower DFS rates ($p < .05$). High N-cadherin expression, diffuse hyaluronan immunoreactivity, and strong stromal hyaluronan reaction intensity had significantly lower OS rates ($p < .05$).
Conclusion: N-cadherin and hyaluronan could be important and promising biomarkers during surveillance of patients with HNSCC.

Key Words: N-cadherin, Hyaluronan, Squamous cell carcinoma, Head and neck, Survival

1. INTRODUCTION

N-cadherin (CDH2) is a calcium-dependent adhesion protein located on chromosome 18q11.2. Previous studies have shown that expression, re-expression also, up-regulation and down-regulation of N-cadherin in human tumors and cell lines.[1] In particular, breast, prostate, bladder, and thyroid tumors have shown de novo N-cadherin expression.[2] N-cadherin silencing inhibits tumor growth by upregulating

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E-cadherin, inhibiting regulators of epithelial-mesenchymal transition (EMT), and reversing the invasive mesenchymal phenotype to epithelial phenotype.[3] N-cadherin was shown to be expressed in the advanced and de-differentiated breast cancer cell lines, also, its expression in tumor cells associated with increased tumor cell motility, invasion, and metastasis. N-cadherin has a role in the systemic dissemination of tumor cells as it enables circulating tumor cells to bind with the stroma and the endothelium at distant sites.[4] In head and neck squamous cell carcinoma, role of N-cadherin expression in tumor progression remains a controversial topic.[5,6]

Previously, the extracellular matrix (ECM) was considered to be an inert complex of macromolecules, but recently, several valuable biological actions of matrix molecules have been described, such as the role of glycosaminoglycan (GAGs), in normal tissue development, homeostasis and pathological processes.[7] The GAGs are involved in cell proliferation, regulating angiogenesis, invasion, and metastasis of malignant cells.[8] Hyaluronan (HA) is one of the most important GAGs molecules; it has the main role in tissue homeostasis, and inflammatory conditions.[9] It has been suggested that HA production by tumors can stimulate proliferation, increase tumor cell invasion and epithelial-mesenchymal transition and hence, promote cancer progression.[10,11] High HA expression in various carcinomas significantly associated with poorly differentiated tumors and shorter patient's survival.[12–14]

Therefore, the present study aimed to evaluate the expression of N-cadherin and HA in patients with HNSCC and their correlation to patient's outcomes.

2. METHODS

2.1 Data retrieval

Fifty-eight retrospective selected cases of head and neck squamous cell carcinomas (HNSCC) with available paraffin blocks. Complete clinico-pathological, and follow-up data without distant metastasis at the initial diagnosis from January 2014 to December 2016. All the studied cases underwent surgical resection. Some cases underwent neck lymph node dissection. Patients with a close margin, multiple lymph node metastases, perineural invasion, high-grade lesions, extracapsular invasion, or advanced stage received postoperative radiotherapy (RT). None of the patients received preoperative RT. The study also included 5 paraffin blocks of normal oral mucosa obtained from the surgical removal of the operculum.

The patients' clinico-pathological data were obtained from archives of the pathology laboratory and oncology unit. These data included the patient's age, sex, primary tumor site, primary tumor size, lymph node status, distant metastasis status, TNM staging, the presence or absence of tumor tissue necrosis, lymph vascular invasion and perineural invasion. The clinico-pathological characteristics of patients are shown in Table 1. Patient follow-up was done every 3 to 6 months for the first 2 years then once a year. Overall survival (OS) and disease-free survival (DFS) data were retrieved from the archive of the Oncology Unit. Previous clinico-pathological and follow-up data were obtained after getting an approval from the institutional review board. This study was approved by the ethics committee.

2.2 Immuno-histochemical staining

All selected blocks were cut at 4 μm thickness. The slices were placed on coated slides. After xylene deparaffinization, the sections were rehydrated in descending grades of alcohol followed by water. Antigen retrieval was performed by using 0.01 M citric acid buffer (pH = 6.0) and heated for 10 minutes in the microwave. The sections were then incubated in a blocking medium (3% H_2O_2) for five minutes followed by washing with distilled water. Rabbit monoclonal anti-human antibody (clone; EPR 5111, 1:50, dilution, Abcam, 1 Kendall Square, Suite B2304, Cambridge, MA 02139-1517, USA) was used against N-cadherin and HA antibodies. Assessment of positivity of the used antibodies (N-Cadherin and HA) was performed by staining sections of colon and breast cancer (the positive control for the two antibodies respectively) at the same time and under the same conditions. Negative control slides obtained by replacement of the primary antibodies by plain phosphate buffer saline. Immuno-detection was executed using Power-stain TM1.0 poly HRP DAB kit for mouse + rabbit (Cat No 52-0017, Genemed Biotechnologies, Inc., 458 Carlton Ct., South San Francisco, CA 94080, USA). Immune staining was performed based on manufacturer's instructions. Immunoreaction was visualized by adding DAB. Counterstaining of slides was performed with the Mayer hematoxylin.

2.3 Immunostaining evaluation

Assessment of tissue immunoreactivity for N Cadherin was semi-quantitatively was evaluated using the method described by Afrem et al.[15] Each pot of tissue evaluated quantitatively as score (0) absence of reactivity, (+ 1) positive reaction in less than 10% tumor cells, (+ 2) positive reaction in 10%-75% of tumor cells and (+ 3) positive reaction in more than 75% of tumor cells. The intensity of reaction was subjectively evaluated as the score (1) for weak reaction, score (2) for moderate reaction and score (3) for intense or strong reaction. The final score was determined by multiplying the intensity scores with staining area scores (0, 1, 2, 3, 4, 6, 9). Finally, tumors were divided into tumors with

low expression (final score ≤ 4) and tumors with high expression (final score ≥ 4). Besides, reactivity was evaluated according to sub-cell localization as (N) nuclear, (C) cytoplasmic, (N + C) diffuse nuclear and cytoplasmic and (M) membranous staining. Also, the pattern of immunoreactivity was evaluated as (P) peripheral or (C) central of proliferated tumor islands.

Hyaluronan immunoreactivity was evaluated using the method described by Afify et al.[16] They defined the positive staining as a droplet to diffuse staining. The staining either intracytoplasmic or extracellular. The staining intensity was graded from + 1 to + 3. Also, the pattern of immunoreactivity was evaluated as (P) peripheral or (C) central of the proliferated tumor islands. The histopathological and immunohistochemical evaluation was done by two pathologists independently and blindly. The images were acquired utilizing a Nikon Eclipse microscope equipped with a 5-megapixel cooled CCD camera and the Image ProPlus AMS7 software.

2.4 Statistical analysis

Data analysis was conducted using SPSS program version 17 (Inc., Chicago, IL, USA). Descriptive data were presented in number and percentage format. Quantitative statistics were calculated in the form of mean $\pm$ standard deviation (SD).

The association between the different clinico-pathological parameters and N-cadherin and HA expression were tested using Chi-square (χ^2) and Fisher's exact probability test. The independent sample t-test (compare continuous variable in 2 groups) and one-way ANOVA (compare continuous variable in three groups) were applied to compare the duration of survival between factors. The construction of survival curves was conducted using the Kaplan-Meier method, and the significance was assessed with the log-rank test. Univariate and multivariate survival analyses were performed with the Cox proportional hazards model to detect an independent prognostic factor. A p-value less than .05 was considered statistically significant. DFS was estimated as the time interval from the time of surgery until the time of local or distant recurrence. OS was considered the time period from the time of initial diagnosis to the time of the last follow-up or the time of patient's death.

3. RESULTS

3.1 Clinico-pathological features of studied cases

As shown in Table 1, fifty-eight cases of HNSCC were studied; 41(70.68%) of cases were above the age of 60 years old, thirty-seven cases (63.79%) were females. Forty cases (68.9%) were located in the oral cavity, 14 (24.13%) were in the pharyngeal wall, only 4 (6.89%) located in the jaw. Regarding size, 43 (74.13%) were less than 4 cm. 20 (34.4%) were grade I, 16 (27.5%) were grade II, and 12 (20.68%) were grade III. 39 (67.2%) were of conventional type while other were of histologic variants mostly of basaloid type. Necrosis was detected in 36 (62.06%). Lymph vascular invasion was detected in 28 (48.27%), and neural invasion in 15 (25.58%). Lymph node involvement was noticed in 28 (48.27%) while distant metastasis was only in 16 (27.58%). Low TNM stage (I & II) was detected in 22 (37.93%) while advanced stage (III & IV) was in 36 (62.06%), recurrence of disease was observed in 25 (43.10%).

Table 1. Clinico-pathological data of the studied HNSCC cases

Characteristics		No.	%
Age	< 60 years	17	29.31
	≥ 60 years	41	70.68
Gender	Male	37	63.79
	Female	21	36.2
Site	Oral cavity	40	68.9
	Pharyngeal wall	14	24.13
	Jaw bone	4	6.89
Size	< 4 cm	43	74.13
	≥ 4 cm	15	25.86
Recurrence	Yes	25	43.10
	No	28	48.27
	unknown	5	8.6
Deaths	Yes	16	27.58
	No	37	63.79
	unknown	5	8.6
Histologic grade	I	20	34.4
	II	16	27.5
	III	12	20.68
Histologic type	Conventional	39	67.2
	Variant	19	32.7
Necrosis	Yes	36	62.06
	No	22	37.93
Lymph vascular invasion	Yes	28	48.27
	No	30	51.72
Neural invasion	Yes	15	25.86
	No	43	74.13
Nodal metastasis	Negative	28	48.27
	Positive	30	51.72
Distant metastasis	Yes	16	27.58
	No	42	72.41
TNM	I, II	22	37.93
	III, IV	36	62.06

Note. HNSCC: head and neck squamous cell carcinoma; TNM: tumor, node, metastasis.

3.2 N-cadherin immuno-histochemical expression in normal, dysplastic epithelium and in SCC tumor islands

N-cadherin showed weak expression in normal oral mucosa that limited only to basal and para-basal cell layers (see Figure 1A). On the other hand, Peritumoral dysplastic ep-

ithelium showed stronger expression of N-cadherin but still weaker than its expression in tumor islands. Squamous cell carcinoma islands demonstrated high expression (see Figure 1, D & E) (final score $\geq$ 6) in 55.2% (32 cases) and low expression in 44.8% (26 cases) (see Figure 1C). Thirty-five cases (60.3%) demonstrated only membranous expression. Meanwhile, mixed membranous and cytoplasmic expression was observed in 23 cases (39.7%). Regarding pattern of immunoreactivity; 36 cases (62.1%) demonstrated diffuse reaction at periphery and center of the proliferating tumor islands (see Figure 1E), 20 cases (34.5%) showed only peripheral reaction and 2 cases (3.4%) showed a central reaction.

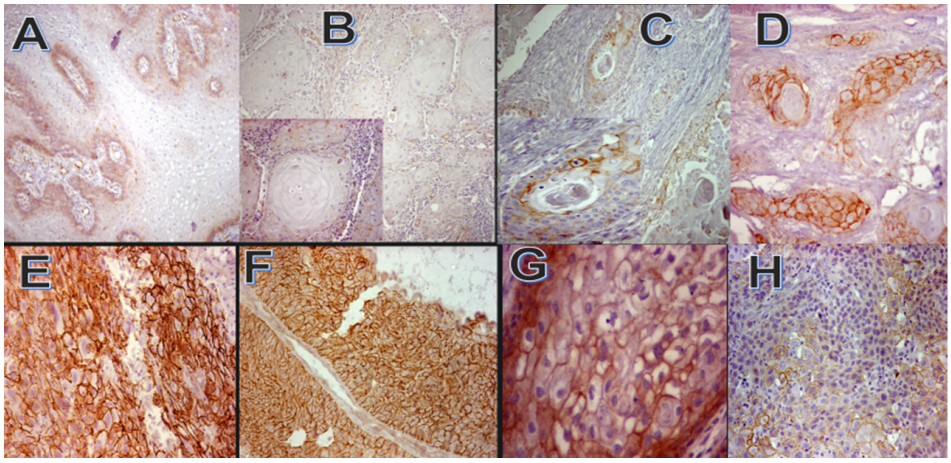

Figure 1. IHC staining for N-cadherin showed weak to moderate (low) expression in the adjacent dysplastic squamous epithelium (A: N-cadherin $\times$ 200), in HNSCC G1 showed mild weak membranous (low) expression (B: $\times$ 100), moderate focal membranous expression (low expression) (C: $\times$ 100). Moderate diffuse membranous staining score in GII HNSCC (high expression) (D: $\times$ 100), (D: CD3 immunostaining $\times$ 100), Strong diffuse membranous staining (high expression) in GIII HNSCC (E: $\times$ 200). Moderate diffuse membranous staining in basaloid SCC (F: $\times$ 100), moderate staining mainly peripheral (G: $\times$ 200), High expression of N-cadherin in SCC metastatic deposits in LN (H: CD20 immunostaining $\times$ 100)

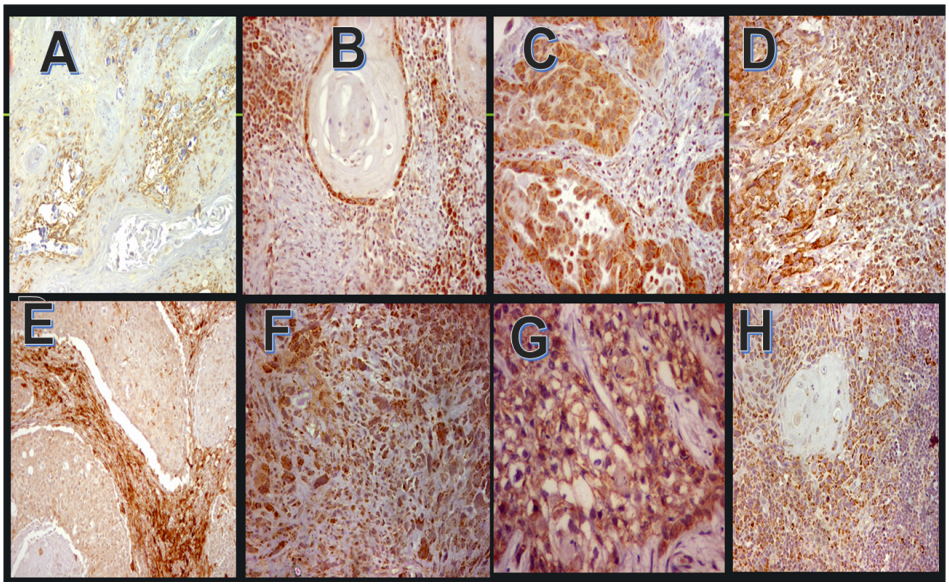

Figure 2. IHC staining for hyaluronan (HA) showed weak to moderate expression in adjacent dysplastic squamous epithelium (A: HA $\times$ 200), in HNSCC G1 showed cytoplasmic expression of HA in periphery of tumor islands and stroma cells (B: HA $\times$ 200), diffuse expression in tumor islands and stroma in GII (C: $\times$ 100). Strong diffuse cytoplasmic staining score in GIII HNSCC (D: HA $\times$ 200), Strong cytoplasmic staining for HA in stroma with weak staining in tumor islands (E: HA $\times$ 200), Strong diffuse nucleocytoplasmic staining in anaplastic variant of HNSCC (F: HA $\times$ 200), cytoplasmic staining in clear cell variant of SCC (G: $\times$ 200), Positive staining of lymph nodal tumor deposits for HA (H: HA $\times$ 100)

3.3 The association of N-cadherin immuno-histochemical expression with clinico-pathological characteristics

As shown in Table 2, N-cadherin high expression was significantly ($p < .05$) associated with large tumor sizes (T3 and T4, 93.3%), advanced TNM clinical stage (III and IV, 69.4%), increased incidence of recurrence (60% of cases with high expression showed recurrence) and increased incidence of patient's death. Patient's age, gender, tumor site, the status of nodal and distant metastasis were not significantly associated with N-cadherin expression ($p > .05$).

Table 2. Association of clinico-pathologic features to N-cadherin expression (final score)

Characteristics		Final score of N-cadherin		*p*
		High	Low	
Age	< 60 years	6 (35.3)	11 (64.7)	.34
	≥ 60 years	20 (48.8)	21 (51.2)	
Gender	Male	18 (48.6)	19 (51.4)	.4
	Female	8 (38.1)	13 (61.9)	
Tumor site	Oral cavity	18 (45)	22 (55)	.67
	Pharyngeal wall	7 (50)	7 (50)	
	Jaw bone	1 (25)	3 (75)	
Tumor size	< 4 cm	25 (58.1)	18 (41.9)	.001
	> 4 cm	1 (6.7)	14 (93.3)	
Nodal metastasis	Negative	16 (57.1)	12 (42.9)	.07
	Positive	10 (33.3)	20 (66.7)	
Distant metastasis	Yes	0	16 (100)	.34
	No	26 (60.9)	16 (38.1)	
TNM stage	I, II	15 (68.2)	7 (31.8)	.005
	III, IV	11 (30.6)	25 (69.4)	
Recurrence	Yes	7 (30.4%)	18 (60%)	.03
	No	16 (69.6%)	18 (60%)	
Grade	I	16 (80)	4 (20)	< .001
	II	7 (43.8)	9 (56.3)	
	II	0	12 (100)	
Type	Conventional	17 (43.6)	22 (56.4)	.78
	Variant	9 (47.4)	10 (52.6)	
Necrosis	Yes	14 (38.9)	22 (61.1)	.24
	No	12 (54.5)	10 (45.50	
Lymph vascular invasion	Yes	12 (54.5)	10 (45.50)	.18
	No	10 (35.7)	18 (64.3)	
Neural invasion	Yes	2 (13.3)	13 (86.7)	.004
	No	24 (55.8)	19 (44.2)	

The current study included 39 cases of conventional histological type and 19 cases of SCC variants. Chi-square test revealed no statistically significant differences between the two histologic types in relation to N-cadherin final score of expression and also, reaction pattern ($p > .05$). On the other hand, there was a highly significant difference ($p = .002$) between the two histologic types in relation N-cadherin sub-cell localization. Thirteen cases (68.4%) of SCC variants demonstrated mixed membranous and cytoplasmic reaction while the remaining 6 cases (31.6%) showed (only) membranous staining. Opposite to that finding, conventional histologic type demonstrated (only) membranous staining in 29 cases (74.4%) while the rest of cases (10 cases, 25.6%) showed mixed membranous and cytoplasmic reaction.

Expression of N-cadherin revealed high significant difference ($p < .001$) among the studied three histologic grades. Sixteen cases (80%) of grade I carcinomas demonstrated low expression, while all the examined cases of grade III carcinomas showed high expression. The higher percentage of grade II cases (9 cases, 56.3%) showed high expression meanwhile the remaining 43.8% (7 cases) was for low expression.

A significant association between neural invasion and high N-cadherin expression ($p = .004$) was detected. 86.7% (13 cases) of cases with neural invasion expressed N-cadherin highly (final > score 6). No statistically significant difference of final score of N-cadherin expression among the studied cases regarding lymph-vascular invasion and tumor tissue necrosis ($p > .05$).

3.4 Hyaluronan immuno-histochemical expression in normal, peritumoral dysplastic epithelium and in SCC tumor islands

Hyaluronan immuno-histochemical reaction mainly observed granular cytoplasmic reaction (52 cases, 89.7%) (see Figure 2), while mixed nuclear and cytoplasmic reaction was encountered in 6 cases (10.3%). Hyaluronan absent in normal oral mucosa. On the other hand, Peritumoral dysplastic epithelium showed a higher level of its expression but still weaker than that shown in tumor islands (see Figure 2A). The examined carcinomas demonstrated diffuse HA expression involving the proliferating tumor islands and the surrounding stroma (see Figure 2, B, C & D), but with different intensity of the reaction. HA intensity in the proliferating tumor islands was moderate in 25 (43.1%) and strong in 33 (56.9%) of the studied cases. The intensity of HA immunohistochemical reaction in stroma was moderate in 27 cases (46.55%) and strong in 31 cases (53.4%). Regarding pattern of HA immunoreactivity in the proliferating tumor islands, the higher percentage of cases (33 cases, 56.9%) showed diffuse reaction involving both tumor islands center and periphery. Meanwhile, tumor islands periphery were solely immune-stained in 43.1% (25 cases) (see Figure 2B).

3.5 The association of hyaluronan immuno-histochemical expression with clinico-pathological characteristics

3.5.1 *Hyaluronan reaction site and pattern of expression*

Only two cases expressed HA in tumor islands without stroma expression, the remaining cases expressed hyaluronan both in stroma and tumor with no significant difference related to clinical and histopathological parameters except gender, site, No. of death ($p = .05, .04, .028$ respectively). Pattern of reaction was significantly correlated to size of tu-

mor, and TNM staging, grade, and type (p = .001, .003, .001, .001 respectively).

3.5.2 *Stromal HA intensity of reaction*

As shown in Table 3, regarding clinical parameters, strong intensity of stromal HA expression was significantly ($p < .05$) associated with oral cavity carcinomas (21 cases, 67.7%), presence of nodal metastasis (24 cases, 77.4%) and higher TNM clinical stage (III and IV; 26 cases, 83.9%). Regarding incidence of recurrence, 17 out of 25 cases (58.6%) with a positive history of recurrence revealed strong HA stromal intensity but p-value was not significant (p = .07). From histopathological parameters, strong stromal expression of HA was significantly associated with grade and histologic type (p = .006, 0.01 respectively).

Table 3. Clinico-pathologic features of HN SCC in relation to Hyaluronan expression intensity in stroma

Characteristics		Hyaluronan expression		*p*
		Moderate	Strong	
Age	< 60	7 (25.9%)	10 (32.3%)	.597
	≥ 60	20 (74.1%)	21 (67.7%)	
Gender	Male	19 (70.4%)	18 (58.1%)	.331
	Female	8 (29.6%)	13 (41.9%)	
Tumor site	Oral cavity	19 (70.4%)	21 (67.7%)	.040*
	Pharyngeal wall	4 (14.8%)	10 (32.3%)	
	Intraosseous	4 (14.8%)	0	
Tumor size	T1 + T2	23 (85.2%)	20 (64.5%)	.073
	T3 + T4	4 (14.8%)	11 (35.5%)	
Nodal metastasis	N	21 (77.8%)	7 (22.6%)	< .001*
	P	6 (22.2%)	24 (77.4%)	
Distant metastasis	N	21 (77.8%)	21 (67.7%)	.394
	P	6 (22.2%)	10 (32.3%)	
TNM stage	Stage I & Stage II	17 (63.0%)	5 (16.1%)	< .001*
	Stage III & Stage IV	10 (37.0%)	26 (83.9%)	
Recurrence	Yes	16 (66.7%)	12 (41.4%)	.070
	No	8 (33.3%)	17 (58.6%)	
Mortality	Yes	18 (75.0%)	19 (65.5%)	.45
	No	6 (25.0%)	10 (34.5%)	
Grade	I	16 (59.3%)	4 (19.0%)	.01*
	II	9 (18.5)	7 (52.4%)	
	III	6 (22.2%)	6 (28.6%)	
Type	Conventional	23 (85.2%)	16 (51.6%)	.007
	Variant	4 (14.8%)	15 (48.4%)	
Necrosis	Yes	18 (66.7%)	18 (58.1%)	.5
	No	9 (33.3%)	13 (41.9%)	
Lymph-vascular invasion	Yes	11 (40.7%)	17 (54.8%)	.28
	No	16 (59.3%)	14 (45.2%)	
Neural invasion	Yes	6 (22.2%)	9 (29.0%)	.55
	No	21 (77.8%)	22 (71.0%)	

3.5.3 *HA intensity in tumor islands*

As shown in Table 3, strong HA intensity in tumor islands was significantly ($p < .05$) was associated with pharyngeal wall carcinomas (10 cases, 71.4%), large sized tumors (≥ 4 cm; 12 cases, 80%) , presence of nodal metastasis (25 cases, 83.3%) and higher TNM clinical stage (III and IV; 28 cases, 77.8%). Regarding incidence of recurrence, 18 out of 25 cases (58.6%) with a positive history of recurrence revealed strong HA intensity in tumor but p-value was not significant (.06).

With respect to pathological parameters, hyaluronan intensity of expression in tumor islands revealed significant differences among the studied different histological grades and types ($p < .05$). On the other hand, presence or absence of tumor tissue necrosis, lymph-vascular invasion, and neural invasion revealed no significant difference in the studied HNSCC cases ($p > .05$).

3.6 Follow-up and survival analysis of patients with HNSCC

3.6.1 *Recurrence*

As shown in Table 4, 43.10% out of the studied 58 HNSCC cases developed recurrence. Patients with large sized tumors (T3 + T4), positive nodal and distant metastatic tumors, high TNM clinical stage (stage III & IV), high histologic grade, high N-cadherin expression (final score ≥ 6) and strong HA expression in stroma are associated with high risk of recurrence.

Table 4. Recurrence COX regression

Characters		*p*
Age	≥ 60/< 60	.466
Gender	Female/Male	.945
Histologic grade		**.012**
Histologic type	Variant of SCC/Conventional	.874
Necrosis	Absent/Present	.255
Lymph-vascular invasion	Absent/Present	.459
Neural invasion	Absent/Present	.167
Tumor size	T3 +T4/T1 + T2	< .001
Nodal metastasis	P/N	.03
Distant metastasis	P/N	< .001
TNM stage	Stage III & Stage IV/ Stage I & Stage II	.062
N-cadherin final score	high expression/ low expression	**.007**
N cadherin reaction pattern	Periphery of tumor islands/ Center of tumor islands/ Periphery and center of tumor islands	.118
N cadherin sub cell localization	Membranous and cytoplasmic/ Membranous	.717
HA site of reaction tumor/stromal	Tumor + Stroma/ Tumor only	.132
HA reaction pattern	Diffuse/Periphery	**.010**
HA subcell localization	Nuclear and cytoplasmic/ Cytoplasmic	.067
HA intensity in tumor	Strong reaction/ Moderate reaction	0.510
HA intensity in stroma	Strong reaction/ Moderate reaction	**.010**

3.6.2 *Disease free survival*

As shown in Kaplan Meier curves in Figure 3, high and peripheral expression of N-cadherin, strong expression of Hyaluronan in stroma and tumor associated with decreased DFS rates ($p < .05$).

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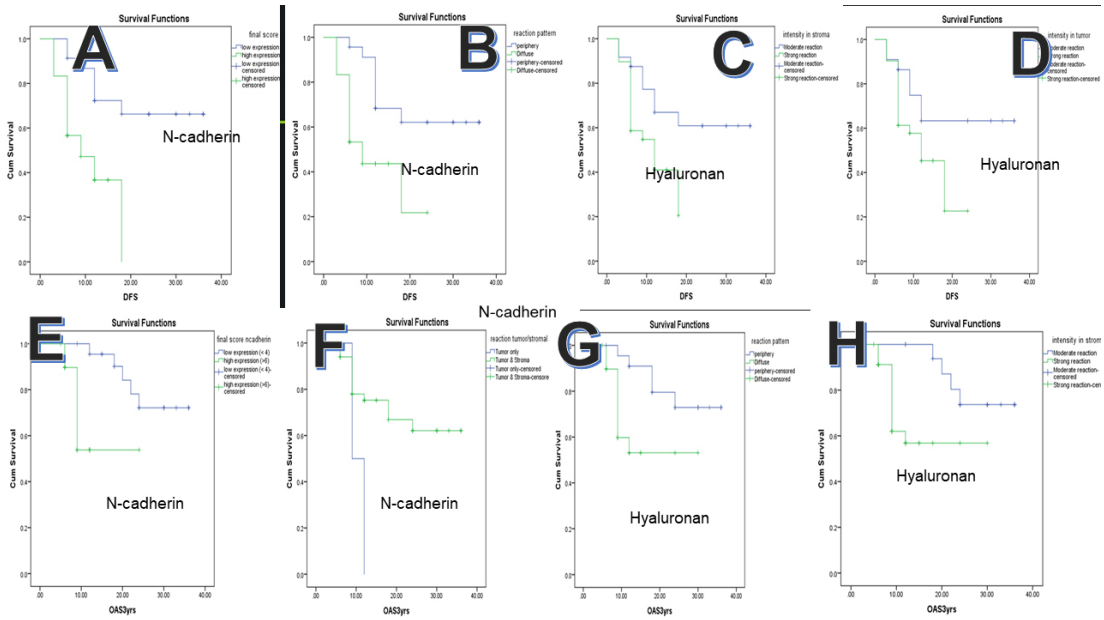

Figure 3. Kaplan-Meier disease-free survival curves; Stratified by low versus high expression in N-cadherin in HNSCC (A, B), Stratified by peripheral versus diffuse expression of hyaluronan (B), also, stratified by moderate versus strong stromal expression of HA (C), and moderate versus strong tumor expression of HA (D). Kaplan-Meier overall survival curves; Stratified by low versus high expression in N-cadherin in HNSCC (E), peripheral versus diffuse expression of N -cadherin (F), moderate versus strong stromal expression of HA (G), and moderate versus strong tumor expression (H).

As shown in Table 5, The univariate Cox proportional hazards model analysis of various prognostic factors in HNSCC in relation to DFS showed that numerous predictors for DFS rate however multivariate analysis revealed that final score of N-cadherin expression (p = .008), distant metastasis (p = .002) and histologic grade (p = .006) remained as independent prognostic factors for DFS .

Table 5. The univariate Cox proportional hazards model analysis of various prognostic factors in HNSCC in relation to DFS

Clinicopathological characters		*p*	95.0% CI for Exp (B)	
			Lower	Upper
Histologic grade		**.006**	1.698	23.844
Tumor size	T3 + T4/T1 + T2	.478	0.423	6.272
Nodal metastasis	Positive/Negative	.931	0.154	5.555
Distant metastasis	Positive/Negative	**.002**	3.633	365.379
TNM stage	Stage III&IV/I&II	.934	0	6.880E+137
Final score n-cadherin	**High expression /Low expression**	**.008**	0.003	0.415
Reaction pattern (Hyaluronan)	Diffuse /Periphery	.390	0.299	1.602
HA Intensity in stroma	Strong/Moderate	.050	0.999	5.668

3.6.3 *Overall survival*

As shown in Kaplan Meier plots are shown in Figure 4. We also found that 16 (27.58%) out of the studied 58 HNSCC cases died during their follow up. Patients with large sized tumors (T3 + T4), positive nodal and distant metastatic tumors, high N-cadherin expression (final score $\geq$ 6), diffuse hyaluronan immunoreactivity involving both periphery and center of the proliferating tumor islands and strong stromal hyaluronan intensity of reaction had significantly lower OS rates (p < .05).

As shown in Table 6, the univariate Cox proportional hazards model analysis of various prognostic factors in HNSCC in relation to OS revealed that revealed that tumor size (p < .001), nodal metastasis(p = .03), distant metastasis (p < .001), final score of N-cadherin (p = .007), reaction pattern of hyaluronan (p = .017), and HA Intensity in stroma (p = .020). However, by multivariate analysis, only final score of N-cadherin expression (p = .037) and distant metastasis (p = .000) remained as independent prognostic factors for OS.

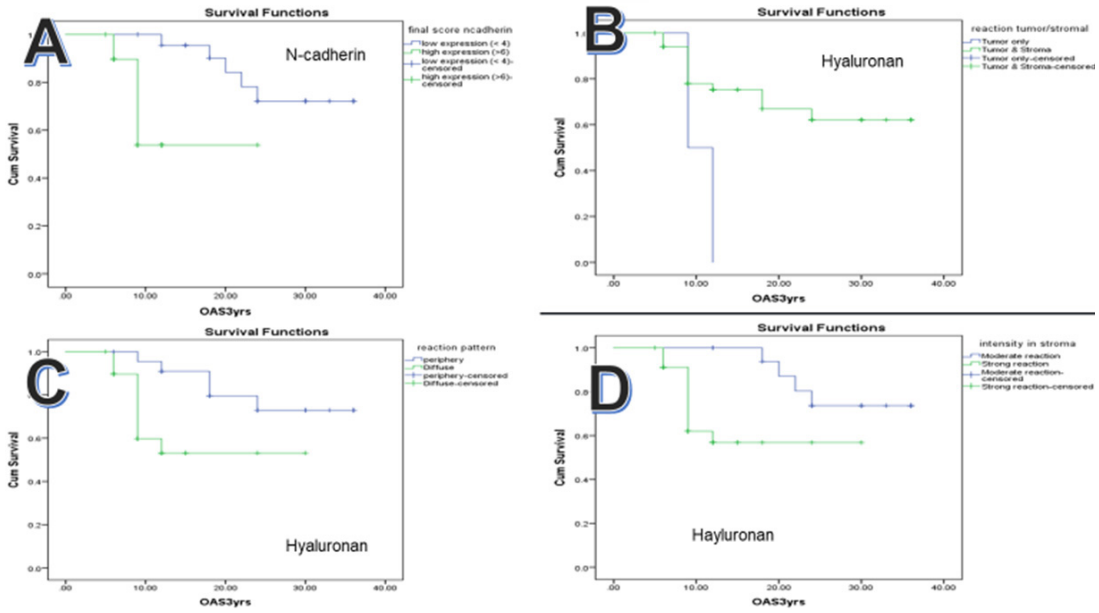

Figure 4. Kaplan-Meier overall survival curves; Stratified by low versus high expression in N-cadherin in HNSCC (A), Stratified by expression of HA in tumor only, stroma only or stromal and tumor (B), also, stratified by moderate versus strong, stromal expression of HA (C), and moderate versus strong tumor expression of HA (D)

Table 6. The univariate Cox proportional hazards model analysis of various prognostic factors in HNSCC in relation to OS

Clinicopathological characters		*p*	95.0% CI for Exp (B)	
			Lower	Upper
Histologic grade		.06	0.8	7.516
Tumor size	T3 + T4/T1 + T2	< .001	2.538	20.481
Nodal metastasis	Positive/Negative	0.03*	1.151	14.679
Distant metastasis	Positive/Negative	< .001	5.276	109.163
TNM stage	Stage III&IV/I&II	.062	0.820	3,353.993
Final score n-cadherin	High expression /Low expression	.007	1.593	19.194
Reaction pattern (Hyaluronan)	Diffuse /Periphery	.017	1.133	3.605
HA Intensity in stroma	Strong/Moderate	.020	1.261	1.261

4. Discussion

Cancer of the head and neck is the sixth most common malignancy worldwide. The most common malignancy is squamous cell carcinoma (SCC) and its variants. More than 90% of tumors in the head and neck are squamous carcinomas. Patients with early-stage disease can be treated effectively with local surgery or radiotherapy. Advanced disease is incurable or requires an aggressive treatment.[17]

The biological behavior of oral SCC is still not fully predictable based on clinico-pathological predictors. To date, no valuable immuno-histochemical biomarkers predicting the clinical outcomes of head-and-neck SCC have been described.[18] This led researchers to study factors that could predict the prognosis. These factors may help personalized therapy.[19]

Recently, studies focused on the role of EMT in tumor invasion. Previous studies suggested that EMT mediates HNSCC progression. Cadherin's are important biomarkers that are involved in EMT.[5,6,20] N-cadherin is one of the cadherin families. It is expressed mainly in neural tissue and striated muscle but not expressed in epithelial cells. "Cadherin switch described as loss of E-cadherin expression and expression of N-cadherin expression increases the mobility of neoplastic epithelial cells and their ability to invade locally".[21,22]

Also, it was described that the key step in gaining the invasive phenotype is the functional elimination of E-Cadherin and up-regulation of N-Cadherin. Previous studies demonstrated that expression of N-Cadherin was associated with invasive

and de-differentiated breast cancer cell lines, and also linked to motility, invasion, and metastasis.[4]

The prognostic value of E-cadherin expression in several tumors as in breast cancer has been studied,[21] bladder carcinoma.[22] Few studies were carried on biological and prognostic role of N-cadherin in HNSCCs, so further studies are needed for confirmation and more clarification of this role.[5,21,22]

This study assessed the expression of N-cadherin in HNSCCs and adjacent normal and dysplastic epithelium; it revealed that N-cadherin showed low expression in dysplastic basal and para basal cells of covering epithelium with higher expression in the malignant squamous epithelium. These findings were consistent with Domenico et al.[5] Moreover, we noticed that increased expression of N-cadherin was positively correlated with large sized tumors, advanced TNM clinical stage, increased tumor recurrence, positive neural invasion and higher histologic grade.

Numerous studies have been carried on E cadherin, its relation with cancer metastasis and poor prognosis.[23–26] However, N-cadherin has scarcely been studied in HNSCC.

In the present study, immuno-histochemical expression of N-cadherin was markedly increased in the HNSCC tissues when compared with the noncancerous tissues from oral mucosa and peritumor dysplastic surface epithelium. Consistent with our finding, results of previous studies investigated N-cadherin expression in other cancers including gastric, prostate , oral carcinomas,[23,24,27] nasopharyngeal carcinoma,[28,29] breast cancer, lung cancer reported similar findings of increased N-cadherin expression in tumor in comparison to adjacent non tumor tissue.[5,27,30] On the other hand, Some tumors showed decreased expression of N-cadherin, such as osteosarcoma,[31] ovarian carcinoma,[32] glioblastoma[33] and renal carcinoma.[34] These opposing expression profiles of N-cadherin in different types of cancer suggest that N-cadherin expression pattern based on the diverse background of cancer, and need further study.

The different sub-cell pattern of N expression was noted in carcinomatous cells. HNSCCs revealed a significant difference (p = .002) of N-cadherin expression between the studied two histologic types, whereas SCC variants mainly demonstrated mixed membranous and cytoplasmic staining and conventional histologic type mainly showed only membranous staining. In agreement with our finding Kehagias et al.[35] reported similar finding in malignant salivary gland tumors. They observed N-cadherin membranous staining in all of the studied malignant salivary gland tumors and mixed membranous and cytoplasmic reaction was observed in a case of adenocarcinoma not otherwise specified.

Contradictory to our finding, Domenico et al.[5] reported different sub-cell localization of N-cadherin in OSCCs. They demonstrated N-cadherin cytoplasmic expression in all the studied OSCC cases with exception of two cases; one exhibited droplet invasion pattern revealed nuclear and cytoplasmic expression, and another case with single cell pattern of invasion exhibited intense membranous-cytoplasmic N-cadherin staining. Maryam Rezaei[36] observed membranous and nuclear expression in the studied breast cancer tumors. Krisanaprakornkit et al.[37] reported predominant cytoplasmic N-cadherin immunoreactivity at the periphery of the proliferative islands of poorly differentiated tongue squamous cell carcinomas. This difference could be explained by differences in immuno-histochemical procedures.[47]

In the present study, we also found a significant (p = .04) association between high N-cadherin expression and neural invasion as 86.7% of cases that showed neural invasion exhibited high expression of N-cadherin. Kehagias et al. also reported similar results in their series of salivary gland tumors as N-cadherin was not expressed in benign neoplasms, but was expressed in half of the malignant ones.[35] This pattern of expression, together with our results suggests a role for N-cadherin as an indicator of aggressive biologic behavior and invasion potentiality of HNSCC.

Moreover, we found a correlation between N-Cadherin expression and histologic grade was statistically significant (1 vs. 2 and 3, $p < .001$). Eighty percent of the examined well differentiated SCCs revealed low expression, while all poorly differentiated carcinomas had high expression. Regarding moderately differentiated SCC we observed high (9 cases, 56.3%) and low expression (7 cases, 43.8%). Many previous studies on OSCCs, ovarian cancer, tongue SCCs[4,37,39] reported similar finding.

Contradictory to our finding, Hashimoto et al.[40] suggested that expression of N-cadherin was limited in OSCCs and lack a correlation with clinico-pathological parameters.[49] Sirin et al. had observed similar finding in ovarian carcinoma as N-cadherin had no significant relationship with tumor grade.[39]

N-cadherin expression was significantly related to tumor stage ($p < .05$) as 69.4% of HNSCCs with advanced TNM stage demonstrated high expression of N-cadherin. Similar to our finding was observed by Sirin et al.[39] in ovarian cancer, Krisanaprakornkit et al.[37] in tongue SCCs, Domenico et al. in OSCCs.[5]

Increased incidence of recurrence and death also demonstrated significant correlation with N -cadherin expression

as 60% of cases with high expression showed recurrence. Many studies reported similar findings where N-cadherin expression showed significant correlations with the grade of differentiation, the clinical stage of disease, prognosis, survival rates and tendency to relapse.[4, 20, 29, 37]

High expression of N-cadherin was observed in 66.7% of the studied HNSCC cases that had positive nodal metastatic deposits but the p-value was not significant ($p = .007$) that may be due to the limited number of cases in our study. On the other hand, many studied revealed a significant association of N-cadherin to nodal metastasis.[4, 20, 29]

Tumor progression is accomplished by several mechanisms; extracellular matrix (ECM) modification is one of the theses mechanisms. HA is a major component of ECM s and a member of the glycosaminoglycan family of polysaccharides. HA is synthesized at the cell surface in healing wounds and in tumors. HA is overexpressed in various types of solid tumors and this was suggested to be associated with worse prognosis.[41–43]

Previous studies have demonstrated that HA is involved in tumor cell migration and invasion in vitro, also, tumor growth and progression in vivo.[44, 45] In Cell culture studies of invasive breast cancer cells revealed that the cancer cell accumulates larger amounts of HA than normal tissue.[46] HA acting through promoting cell adhesion and motility, proliferation, and differentiation.[47] Growth factors and chemokines produced in tumor microenvironment can induce HA production.[48]

In the current study, HA was expressed in the proliferating tumor islands and in the surrounding stroma with variable intensity of the reaction. Peritumoral dysplastic epithelium showed a higher level of its expression but still weaker than that shown in tumor islands. In agreement with our finding, previous studies investigated HA content in breast carcinoma and found that HA was more expressed in stromal tissue in breast cancer than HA expression in normal breast or benign lesions.[49–51]

Afify et al. noticed that stromal HA expression in breast carcinoma stroma was progressively increased from in situ to infiltrating carcinomas. Also, HA was exclusively confined to the stroma in all cases of breast cancer and was not detected in epithelial cells.[16] However, in our study, HA was expressed in a tumor and stroma. Consistent with our finding, the expression of HA is correlated with malignancy in many cancers such as breast, lung and ovarian cancer.[46, 52, 53] Alexandra reported a high level of HA expression in desmoid tumors in comparison with the normal controls.[54] Also, previous few studies reported high levels of in HNSCC.[55, 56]

In contrast, the HA expression is low in melanoma, and its absence is significantly correlated with the metastatic potential.[57, 58] Accumulation of HA can decrease tumorigenic potential and confer resistance to cancer.[59–62] HA oligosaccharides increase the metastatic potential through the affection of the interactions between the high-molecular-weight HA and its receptors on tumor cells and thus inhibiting tumor growth in vitro and in vivo.[63–65]

Regarding pattern of HA immunoreactivity in the proliferating tumor islands, the higher percentage of our cases (33 cases, 56.9%) showed diffuse reaction involving both tumor islands center and periphery. Meanwhile, tumor islands periphery were solely immuno-stained in 43.1% (25 cases). Contradictory to our finding, Afify et al. found HA tends to be expressed at the periphery of invasive tumor islands than the center, these findings supporting the argument that tumors release many soluble factors that increase HA.[16, 66]

In the present study, hyaluronan intensity of expression in tumor islands and stroma revealed significant differences among the studied different histological grades and types ($p < .05$). A Higher number of the examined SCC variant cases revealed strong HA expression in comparison with conventional SCC type. In more words, moderately and poorly differentiated SCCs frequently showed strong HA expression in contrast with well-differentiated carcinomas agreement with our finding, many studies indicated high HA levels were detected mostly in poorly differentiation in various types of carcinomas.[12–14, 46] Increased HA production by tumors has a role in increasing tumor proliferation, promoting cell invasion and epithelial-mesenchymal transition, and stimulate angiogenesis.[67–69] Previous studies reported significant correlation to poor differentiation of the tumors.[16, 70, 71]

The current study indicated a strong intensity of stromal and tumoral HA expression was significantly ($p < .05$) associated with the presence of nodal metastasis. A similar finding was reported by Wernicke et al., Suwiwat et al., Afify et al.[16, 56, 70–72]

Regarding incidence of recurrence, 58.6% of cases with a positive history of recurrence revealed strong tumor and stromal HA expression but was not statistically significant by chi-square test (p values .06 and .07). Kaplan Meier curves demonstrated a statistically significant difference of stromal HA intensity of expression in relation to DFS ($p = .050$). On the other hand, tumoral HA intensity of expression revealed no significant difference in relation to DFS but p-value approach significance ($p = .078$). This results don't cope with that obtained by Johrens et al., who reported no significant differences for hyaluronan positive versus negative cases. This could be due to short follow up period.

The studied HNSCC cases that showed strong stromal hyaluronan intensity of reaction had significantly lower OS rates ($p < .05$) with Kaplan Meier curves. In agreement with our finding, many studies indicated high HA levels associated with poor prognosis and shorter patient survival.[16, 46, 56, 70–73]

5. Conclusion

In summary, EMT is regarded as the first necessary step for invasion and metastasis. Previous studies have suggested that EMT facilitates the dissemination of tumor cells and thus promotes distant metastasis. Recently, it has been demonstrated that the loss of the 'invasion suppressor' E-cadherin and upregulation of N-cadherin occurred in invasive tumor cell lines. High expression of N-cadherin and hyaluronan were significantly associated with adverse prognosis in patients with HNSCC. The present study demonstrated high expression of N-cadherin, and Hyaluronan was significantly associated with large sized tumors (T3 + T4), advanced TNM clinical stages (III & IV), increased the incidence of tumor recurrence and patient's death. Also, different histologic grades and types.

Follow up data and survival analysis of the studied cases of HNSCC using Kaplan Meier curves revealed significant lower rates of DFS and OS in patients with high expression of N-cadherin, diffuse immunoreactivity of hyaluronan, strong stromal hyaluronan intensity of expression. Absent expression of hyaluronan and the weak expression of N-cadherin in normal oral mucosa contrasting their expression in peritumor dysplastic epithelium and in the main bulk of tumor indicates that N -cadherin and hyaluronan could be promising biomarkers for patients with HNSCC.

Conflicts of Interest Disclosure

The authors declare that there is no conflict of interest statement.

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