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CLINICAL RESEARCH:

Immunohistochemical and Clinicopathologic Correlation of DNA Methyltransferase 3A and (C-X-C motif) Ligand 1 in Oral Squamous Cell Carcinoma

Correlación inmunohistoquímica y clínico-patológica de la ADN metiltransferasa 3A y el ligando 1 (motivo C-X-C) en el carcinoma oral de células escamosas

Basma Abdelrahman Ahmed PhD[1] https://orcid.org/0000-0001-9216-3146
Mai Hafez Mohamed PhD[2] https://orcid.org/0000-0003-2639-1380

1. Lecturer of Oral Pathology, Faculty of Dentistry, Ain Shams University, Cairo, Egypt.
2. Lecturer of Oral Pathology, Faculty of Dentistry, British University in Egypt, Cairo, Egypt.

Correspondence to: Basma Abdelrahman Ahmed - basmaabdelrahman@dent.asu.edu.eg

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ABSTRACT: DNA methyl transferase 3A (DNMT3A) is an enzyme acting by adding a new methyl group to DNA favoring DNA silencing and carcinogenesis. Cytokines were said to assist epigenetic switch and enhance the activation of methyltransferases in many cancer types. The role of chemokine (C-X-C motif) ligand 1 (CXCL1) in cancer development was proved in many reports. In this study, we suggested that CXCL1 might induce activation of DNMT3A, affecting carcinogenesis of oral squamous cell carcinoma (OSCC). Immunohistochemical (IHC) scoring was calculated and statistical correlation was performed to evaluate the expression of epithelial DNMT3A in addition to epithelial and mesenchymal CXCL1 in OSCC and normal mucosal samples. DNMT3A, epithelial, and mesenchymal CXCL1 revealed a statistically significant increase in immune scoring from normal mucosa and between different tumor grades, besides a significant relation of the expressions with tumor size, stage, and lymph node involvement. Pearson's correlation detected a statistically significant correlation of DNMT3A with epithelial and mesenchymal CXCL1. Thus, CXCL1 overexpression may be associated with DNMT3A upregulation. DNMT3A, epithelial, and mesenchymal CXCL1 were associated with histological grades and advanced tumor characters suggesting them as reliable prognostic biomarkers in patients of OSCC.

KEYWORDS: Oral squamous cell carcinoma; DNA methyltransferase 3A; CXCL1; Immunohistochemistry; Tumor grading; Clinical characteristics.

RESUMEN: La ADN metil transferasa 3A (DNMT3A) es una enzima que actúa añadiendo un nuevo grupo metilo al ADN favoreciendo el silenciamiento del ADN y la carcinogénesis. Se decía que las citoquinas ayudaban al cambio epigenético y mejoraban la activación de las metiltransferasas en muchos tipos de cáncer. El papel del ligando 1 de quimiocina (motivo C-X-C) (CXCL1) en el desarrollo del cáncer quedó demostrado en muchos informes. En este estudio, sugerimos que CXCL1 podría inducir la activación de DNMT3A, afectando la carcinogénesis del carcinoma oral de células escamosas (OSCC). Se calculó la puntuación inmunohistoquímica (IHC) y se realizó una correlación estadística para evaluar la expresión de DNMT3A epitelial además de CXCL1 epitelial y mesenquimal en OSCC y muestras de mucosa normal. DNMT3A, CXCL1 epitelial y mesenquimatoso reveló un aumento estadísticamente significativo en la puntuación inmune de la mucosa normal y entre diferentes grados tumorales, además de una relación significativa de las expresiones con el tamaño, el estadio y la afectación de los ganglios linfáticos del tumor. La correlación de Pearson detectó una correlación estadísticamente significativa de DNMT3A con CXCL1 epitelial y mesenquimal. Por tanto, la sobreexpresión de CXCL1 puede estar asociada con la regulación positiva de DNMT3A. DNMT3A, CXCL1 epitelial y mesenquimatoso se asociaron con grados histológicos y caracteres tumorales avanzados, lo que los sugiere como biomarcadores de pronóstico confiables en pacientes con OSCC.

PALABRAS CLAVE: Carcinoma oral de células escamosas; ADN metiltransferasa 3A; CXCL1; Inmunohistoquímica; Clasificación de tumores; Características clínicas.

INTRODUCTION

Squamous cell carcinoma is the most globally occurring neoplasm in the oral cavity. The genetic and epigenetic regulations of oral squamous cell carcinoma (OSCC) have been widely studied in the last few years (1). Epigenetics is the term describing the reversible changes in gene expression without any difference in DNA sequences, a process achieved through DNA methylation, histone modification, chromatin remodeling, and post-transcriptional modifications (2, 3).

Transferring a methyl group to the C5 position of the cytosine during DNA methylation regulates gene expression either by activating proteins engaged in repressing gene expression or by weakening the binding of the transcription factors to DNA. This process occurs, in normal development and cancer, under the control of a group of enzymes called DNA methyltransferases (2, 4).

DNA methyl transferase 3A (DNMT3A) is an enzyme responsible for de novo methylation which means the addition of methyl group to unmodified DNA. DNMT3A increased expression was proved to be related to progression, poor prognosis, aggressiveness, and recurrence in hepatocellular carcinoma, breast cancer, vulvar cancer, and OSCC (5-8). In their study, Daniel *et al*. (7) declared that DNMT3A has an early significant role in the carcinogenesis of OSCC when compared to the other studied methyltransferases.

The effect of inflammatory mediators on carcinogenesis has been widely investigated (9, 10). Cytokines like Interleukin- 1, Interleukin-6 (IL-6), transforming growth factor beta (TGF β),

Interferon-γ, and many others were reported to have high expression in skin SCC and OSCC (11-13).

The chemokine (C-X-C motif) ligand 1 (CXCL1) is one of the CXC family (14). This agent is either secreted by the tumor cells (15, 16) or by cells of the tumor microenvironment (17,18). It is considered a promoter of cancer cell proliferation, angiogenesis, and metastasis in numerous cancer types including OSCC (15, 18-21). Previous studies on different epithelial malignancies attempted to clarify the interaction between different chemokines and epigenetic regulation of cancer cells whether on the expression of methyltransferase enzymes or specific gene silencing (22-24). Therefore, we aimed to detect the immunohistochemical correlation of the potent epigenetic marker DNMT3A with epithelial and mesenchymal CXCL1, as well as detect their association with different histological grades and clinical parameters in OSCC.

MATERIALS AND METHODS

SPECIMEN COLLECTION

Hundred and twenty specimens of formalin-fixed paraffin-embedded (FFPE) blocks were included in this study. The blocks were obtained from the archives of the Oral Pathology Department, Faculty of Dentistry, Ain Shams University, and General Pathology Department Faculty of Medicine Cairo University, Cairo, Egypt. The blocks used were present for diagnostic purposes from years (2015-2020). The work was done according to the Declaration of Helsinki. The ethics committee of the Faculty of Dentistry, Ain Shams University considered the study with exemption from consent (FDASU-Rec ER072201), as all the blocks were coded, the patients were unidentified and their information was anonymous.

The cases were divided into 30 normal control samples from blocks of gingival tissue obtained during implant surgeries. The other 90 were OSCC cases of primary neoplasms. The cases were divided into 30 each well differentiated, moderately differentiated, and poorly differentiated according to the WHO grading system (25). Two pathologists not familiar with clinical data of patients re-diagnosed the cases.

IMMUNOHISTOCHEMISTRY

FFPE tissues of 4-µm thick sections were mounted on positively charged glass slides. Sections were deparaffinized, rehydrated, and antigen retrieved using Trilogy (Cell Marque, Millipore, Sigma-Aldrich, Germany). The slides were stained automatically and counterstained by Hematoxyllin according to the manufacturer's protocol (Ventana-Benchmark GX, Tucson, AZ, USA). The primary rabbit polyclonal antibodies; anti-DNMT3A (HPA026588) and anti-CXCL1 (SAB4301833) were obtained from (Sigma-Aldrich, Germany) and diluted to (1:50) and (1:100) respectively.

At least 4 fields from each slide at magnification 400X were captured using (Canon EOS 650D) digital camera mounted on a light microscope (BX60, Olympus, Japan). We assessed DNMT3A expression in the nucleus of epithelial cells only (7), while CXCL1 immunoreactivity was assessed in either the cytoplasm or the nucleus of epithelial and mesenchymal cells.

The Immunohistochemical (IHC) scoring was the used semiquantitative system for immunohistochemical expression detection. The percentage and intensity of positively stained epithelial cells to the total number of epithelial cells were determined in both DNMT3A and CXCL1 sections, while those of the mesenchymal CXCL1 stained cells, were detected in relation to the total number of mesenchymal cells. Then, the IHC scoring formula was applied as follows: 3 × percentage of strongly stained cells (brownish black) + 2 × percentage of moderately stained cells (brown) + 1 ×

percentage of weakly stained cells (light brown) + 0 × percentage of negatively stained cells (26). Two independent observers counted the cells and evaluated the intensity.

The IHC cutoff value was chosen based on the mean IHC score for each marker (27). The IHC score was defined as high when the DNMT3A score was higher than 174 while the high IHC score for CXCL1 was defined as higher than 125.

STATISTICAL ANALYSIS

Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). Data was summarized using mean and standard deviation for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Data was double-checked for normality using normality plots and Shapiro Wilk test and proved not to deviate from normal distribution. Comparisons of IHC scores between normal mucosa and different tumor grades were done using analysis of variance (ANOVA) with multiple comparisons (Tukey) post hoc test. Correlations between the pathologic variables and DNMT3A, epithelial, and mesenchymal CXCL1 were performed using the Chi-square test. Correlations between DNMT3A and epithelial CXCL1 besides DNMT3A and mesenchymal CXCL1 were done using Pearson's correlation coefficient. P-values less than 0.05 were considered statistically significant.

RESULTS

Eighty-three male and 37 female FFPE tissue samples with an age range from (31-92) years were used in this study. Of them, 65 males and 25 females were OSCC patients with an age range of (40-92).

DNMT3A AND CXCL1 IMMUNOHISTOCHEMICAL EXPRESSION IN DIFFERENT HISTOLOGICAL GRADES

The epithelial tissues of all groups showed positive nuclear immunoreactivity of DNMT3A (Figure 1.a-d). A statistically significant increase in the IHC score of DNMT3A protein was observed in each OSCC grade in comparison to the normal mucosa. Besides a significant increase between well differentiated when compared to moderately and poorly differentiated and between moderately differentiated in comparison to poorly differentiated groups ($p<0.001$) (Table 1).

CXCL1 immunostaining in epithelial cells revealed cytoplasmic positive expression in the normal group and all OSCC groups, while nuclear immunostaining appeared in some tumor cells of the poorly differentiated group (Figure 1.e-h). The peripheral cells in cell nests showed higher staining intensity than central cells (Figure 1.g). Mesenchymal cells showed immunoreactivity of CXCL1 in all study groups (Figure 1.e-h). A significant rise in the mean IHC score of both epithelial and mesenchymal CXCL1 was observed in each grade when compared to normal mucosa and between one grade and the other ($p<0.001$) (Table 1).

CORRELATION OF DNMT3A AND CXCL1 WITH CLINICAL PARAMETERS

Next, we examined the correlation of DNMT3A, epithelial, and mesenchymal CXCL1 with different clinical parameters. Correlation with age was adjusted to cut off value of 60 years which was the mean age of the OSCC cases used in the study.

Chi-square test analysis of the relation of clinical data with the IHC expressions of DNMT3A, epithelial, and mesenchymal CXCL1 did not reveal any association of any of the markers with age,

sex, or tumor site. However, all three markers showed a correlation with lymph node metastasis (p=0.010, 0.018, and 0.019 respectively). Moreover, all DNMT3A, epithelial, and mesenchymal CXCL1were seen to be related to the change in tumor size from T1/T2 to T3/T4 (p=0.020, 0.040, and 0.040 respectively) and tumor stage from I/ II to III/ IV (p=0.008, 0.009 and 0.010 respectively) (Table 2).

CORRELATION OF DNMT3A EXPRESSION WITH EPITHELIAL AND MESENCHYMAL CXCL1 EXPRESSIONS

Pearson's correlation test revealed a statistically significant strong correlation between DNMT3A IHC score and that of epithelial and mesenchymal CXCL1 (r=0.937, p<0.001 and r=0.917, p<0.001 respectively) (Table 3).

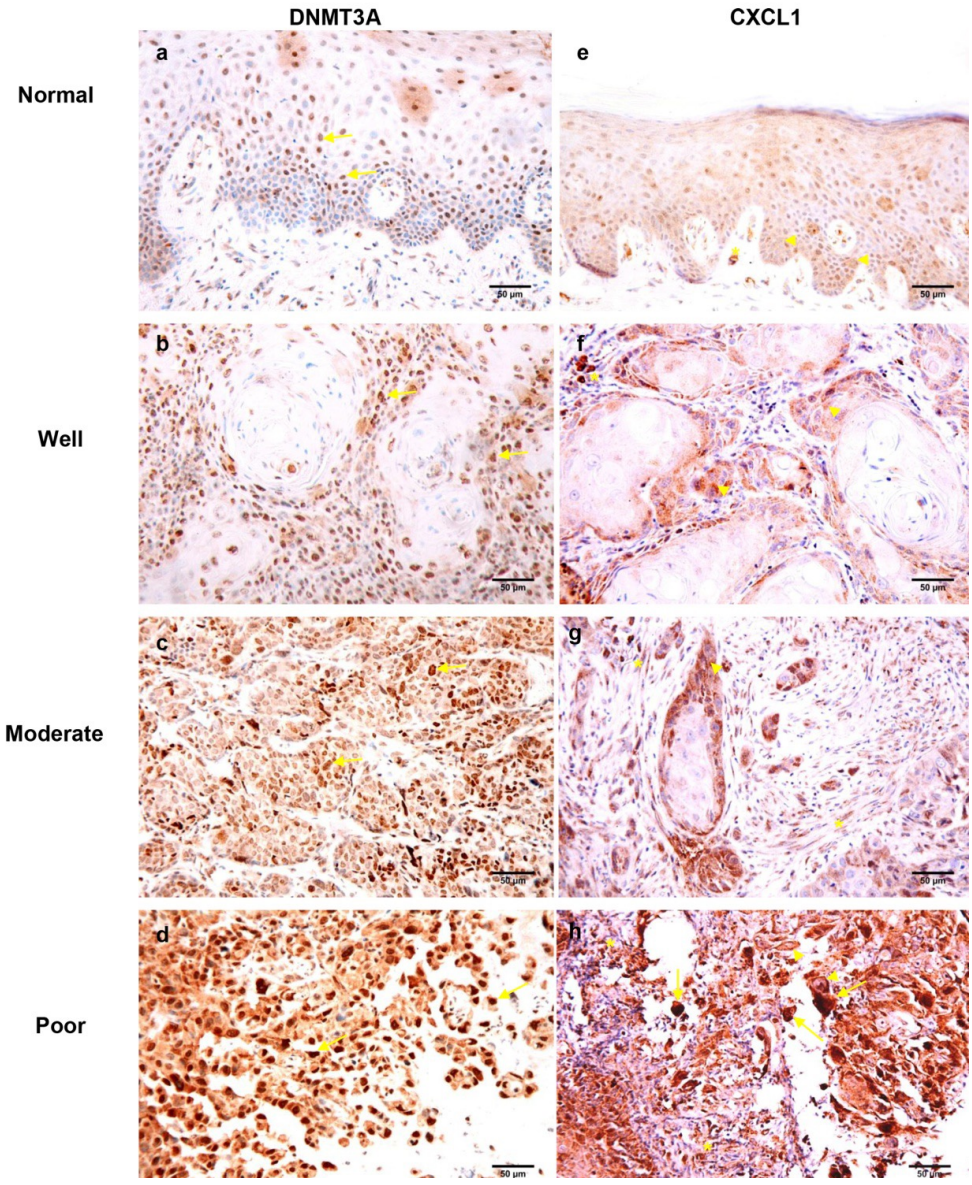

Figure 1. Immunohistochemical expression of DNMT3A and CXCL1 in normal mucosa and different grades of OSCC (400X). (a-d) nuclear expression of DNMT3A (yellow arrows), (e-h) cytoplasmic expression of epithelial CXCL1 (yellow arrowheads), nuclear expression of epithelial CXCL1 in poorly differentiated OSCC (yellow arrows), mesenchymal expression of CXCL1 (yellow astricts).

Table 1. Comparison between IHC scores in different histological grades and normal mucosa of DNMT3A, epithelial, and mesenchymal CXCL1 (Anova and Tukey).

		Normal	Well	Moderate	Poor	P value
DNMT3A	Mean±SD	34.82[a]±7.04	153.63[b]±12.85	229.13[c] ±8.71	277.36[d]±6.76	<0.001
Epithelial CXCL1		64.80[a]±4.23	111.33[b]±14.94	161.27[c] ±15.16	231.80[d]±9.14	<0.001
Mesenchymal CXCL1		56.06[a]±3.64	88.27[b]±12.78	121.97[c] ±8.55	164.91[d]±17.57	<0.001

Means among the same row with different superscript letters are statistically significant.
P value<0.05 is statistically significant.

Table 2. Correlation between IHC expressions of DNMT3A, epithelial, and mesenchymal CXCL1 with clinical features in OSCC patients (Chi square test).

Items	N	DNMT3A expression			CXCL1 expression in Epithelial Cells			CXCL1 expression in Mesenchymal Cells		
		High	Low	P value	High	Low	P value	High	Low	P value
No. of Patients	90	60	30		66	24		43	47	
Age				0.697			0.660			0.380
<60	37	25	12		29	10		20	19	
≥60	53	35	18		37	14		22	29	
Sex				0.683			0.551			0.051
Male	65	43	22		47	18		30	35	
Female	25	17	8		19	6		13	12	
Tumor Site				0.747			0.786			0.798
Lip	10	5	5		6	4		2	8	
Tongue	38	29	9		31	7		22	16	
Buccal mucosa	9	4	5		6	3		4	5	
Floor of mouth	10	8	2		9	1		5	5	
Gingiva	14	7	7		8	6		5	9	
Palate	9	6	3		6	3		4	5	
Lymph node metastasis				0.010			0.018			0.019
No	46	16	30		22	24		9	37	
Yes	44	44	0		44	0		34	10	
Size				0.020			0.040			0.040
T1/T2	51	23	28		28	23		14	37	
T3/T4	39	37	2		37	2		28	11	
Stage				0.008			0.009			0.010
I/ II	36	10	28		12	24		4	32	
III/ IV	54	2	50		53	1		39	15	

P value <0.05 is statistically significant.

Table 3. Correlation of DNMT3A with epithelial, and mesenchymal CXCL1 IHC scores (Pearson's Correlation)

		DNMT3A
CXCL1 in Epithelial cells	Pearson Correlation (r)	0.937
	P value	<0.001
CXCL1 in Mesenchymal Cells	Pearson Correlation (r)	0.917
	P value	<0.001

P value <0.05 is statistically significant.

DISCUSSION

DNMT3A is an epigenetic regulator, acting by silencing tumor suppressor genes favoring proliferation and carcinogenesis (2, 28). Understanding the effect of cytokines on epigenetic regulation paves the way for more treatment strategies for OSCC. CXCL1 whether produced from tumor or stromal cells was reported as a predictive factor for cancer progression (15, 18, 29). Although some studies shed light on the cross-talk between cytokines and epigenetic management (22, 23), the complete picture in OSCC remains unclear.

Our study revealed an increase in the IHC score of DNMT3A in different OSCC grades from normal mucosa and between tumor grades. Previously, the expression of the methyltransferase in OSCC was detected to be statistically significant with the non-neoplastic tissue (7, 8, 30). It was declared that the high expression of de novo DNMT3A renders it responsible at least partly for the silencing of tumor suppressor genes during oral carcinogenesis (7). In addition, DNMT3A immunoreactivity was found to increase from nonneoplastic tissues through high-grade nodules to advanced carcinoma in liver cancer (31). It should be noted here that only the nuclear expression was regarded in scoring, as the enzyme is considered active just when transported to the nucleus (32).

The association of DNMT3A with tumor size, stage, and lymph node metastasis was found to be statistically significant, a finding indicating a correlation between the expression and poor prognosis. Yang J. *et al*. (33) proposed similar results in gastric cancer. They detected an association between DNMT3A immunoscoring, III /IV TNM stage, and lymph node involvement. Moreover, DNMT3A overexpression was found to be associated with high recurrence in vulvar carcinoma (6). Similar to our results, Daniel *et al*., did not detect any association between DNMT3A expression and age or sex in OSCC (7).

The expression of CXCL1 revealed a statistically significant increase in epithelial and mesenchymal immunoscoring between normal mucosa and OSCC and by the transition from one grade to another. Following our results, a study on bladder cancer detected higher IHC scores in high-grade than low-grade tumors (34). The mechanisms of CXCL1-induced tumorigenesis have been well-established (18, 21, 30,35). Lee *et al*. (21) indicated that CXCL1 was involved in the interleukin 1 β (IL-1 β)-mediated phosphorylation of epidermal growth factor receptors promoting proliferation of oral premalignant cells. It was shown that lung cancer growth was induced by tumor cell-secreted CXCL1 via recruitment of neutrophils into the tumor (35). Also, gastric cancer cells induced

apoptosis, and decreased proliferation and migration were reached by CXCL1 silencing (36).

The nature of CXCL1-positive mesenchymal cells in different cancer types was verified by dual immunostaining and cell line studies to be cancer-associated fibroblasts (CAF) and tumor-associated macrophages (TAM) (18,29,37). Wei *et al*. (19) reported that besides the high expression of CXCL1 in OSCC cells, IL-1β expressed in tumor cells significantly increased CXCL1 production by CAF, which in turn promoted oral malignant cell invasion and migration. CXCL1 produced by TAMs stimulated breast cancer metastasis via Nuclear factor-κB/SOX4 induction (37). TAMs/CAFs-secreted CXCL1 enhanced tumor growth of subcutaneous bladder cancer tumors in nude mice and was responsible for repeated recurrence and disease progression through its ability to enhance invasion (29).

Comparing the CXCL1 IHC score in tumor and mesenchymal cells with clinical variants revealed a significant increase with advanced tumor size and stage, and lymph node metastasis, rendering CXCL1 a prognostic indicator of OSCC. Similarly, Wan *et al*. (15) and Yu *et al*. (16) detected high marker expression, in large tumors, high stages, and tumors with nodal metastasis in cervical and lung cancer respectively. Moreover, the expression positively correlated with tumor stage and nodal infiltration in breast cancer (37).

In our study, the correlation of DNMT3A was statistically significant with epithelial and mesenchymal CXCL1. The results were supported by the postulate of cytokine-derived epigenetic reprogramming. The modulation of DNMTs under the effect of pro-inflammatory cytokines during cancer initiation and progression was previously determined in literature (38). IL-6 upregulated DNMT-induced gene methylation, and tumor suppressor gene silencing (11). TGF β was acknowledged to be involved in activating DNA methyltransferases including DNMT3A in ovarian and liver cancers (23, 39). In their study on breast cancer cell lines, Mathot *et al*. (40) claimed that stromal cells-secreted factors were linked to the activation of genes characterized by DNA methylation patterns. They also reported that the change in methylation could be related to stromal-cancer cell interaction.

The aforementioned studies clarified the role of stromal elements in the epigenetic modification of cancer cells; nevertheless, the autocrine effect of cytokines produced by tumor cells could not be detected earlier. It is recommended to follow this immunohistochemical study with further cell line studies for the detection of the exact causative relation between CXCL1 and DNMT3A in the initiation and progression of OSCC.

CONCLUSION

Our data showed that DNMT3A positively correlated with epithelial and mesenchymal CXCL1 in OSCC. Each of DNMT3A, epithelial, and mesenchymal CXCL1 were well associated with tumor histological grades, advanced tumor features, and lymph node metastasis, rendering them potential prognostic markers for OSCC.

AUTHOR CONTRIBUTION STATEMENT

Conceptualization and design: B.A.A.
Interpretation of data: B.A.A., M.H.M.
Writing and revising the work: B.A.A., M.H.M.

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