Comparison of Immunohistochemical Expression of DNMT3B among Oral, Laryngeal and Skin SCC

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ABSTRACT

Background: Recently epigenetic alterations have received increased attention because of theirimportant role in the process of tumerigenesis. It has been found that more than half of genetic changes were epigenetic. Epigenetic alterations are catalyzed by DNMTs enzymes. Increased knowledge about this molecular event may achieve progress in the war against cancer. The aim of this study was to evaluate and compare the expression of DNMT3B among oral, laryngeal and skin SCC.

Materials and Methods: This study was performed on (120) formalin-fixed, paraffin-embedded blocks, histopathologically diagnosed as oral, laryngeal and skin SCC). Immunohistochemical staining of DNMT3B antibody was performed on each case of this study.

Results: The immunohistochemical analysis showed that the DNMT3b is over expressed in oral, laryngeal and skin squamous cell carcinoma as (77.5%), (77.5%) and (72.5%) respectively, withsignificant difference in expression among them (P=0.009), The expression in all the three types is correlated positively with the degree of differentiation (P<0.001), (P<0.001) and (P=0.015) respectively.

Conclusion: According to the results of this study, epigenetic alterations are believed to play a crucial role inthe development of oral, laryngeal and skin SCC.The results also, conclude that DNMT3B may act as a promising prognostic marker in cancer of epithelial in origin.

Keywords: Oral, laryngeal, skin, expression, Immunohistochemistry. **(J Bagh Coll Dentistry 2016; 28(4):61-67)**

INTRODUCTION

Epigenetics is the study of heritable alterations in activity of gene or its function that is not related to any alteration in DNA sequence itself [1]. DNA methylation is the most characteristic epigenetic mechanism which inherited without DNA sequences changes [2]. Methylation of DNA involving the transfer of methyl groups to the region 5 of cytosine residues that is located in the cytosine-guanine dinucleotides (CPG) via reactions catalyzed by protein called DNA methyltransferase (DNMTs). Any abnormal methylation may cause the development of many diseases, especially by direct action on process of tumerigenesis or silencing of tumor suppressor genes that contain CpG islands in promoter region [3].

DNA methylation in cancer has become the matter of intense study, compared to normal cells; the cancer cells show major disruptions in pattern of DNA methylation [4].Inappropriate silencing of gene resulting from abnormal DNA methylation largely contributes to malignant transformation, tumorigenesis, and progression of tumor [5].

DNMt3b enzyme is one of the three well known DNA methyltransferases with catalytic activity. It may play an important oncogenic role during the process of carcinogenesis, and its genetic variants had been recognized to be correlated with risk of various cancers [6]. It had been found that DNMt3b is over-expressed in many cancerous tissues [7], and its expression was found to be important for malignant cell survival [8]. DNMt3b has been reported to either contribute to DNA methylation or maintenance of aberrant patterns of DNA methylation in cancer [9].

This study was performed to find out the differences in expression of DNMT3B among oral, laryngeal and skin squamous cell carcinoma, and to identify the correlation with the histological grade.

MATERIALS AND METHODS

A total of one-hundred and twenty cases of Formalin-fixed, paraffin embedded tissue blocksthat histopathologically identified as oral, laryngeal andcutaneous squamous cell carcinoma (forty blocks for each type) were included in the study. Oral squamous cell carcinoma blocks were selected from the archives of Oral Pathology Department, College of Dentistry, University of Baghdad, while the laryngeal and cutaneous squamous cell carcinoma cases were obtained from histopathology laboratory of Ghazi al Hariry Hospital of Specialized Surgeries for the period from October 2014 till June 2015.

Immunohistochemical analysis was performed on the samples to study the expression of

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DNMT3b in tissue blocks. Five u thick tissue sections of the blocks were mounted on positively charged slides, dewaxed and rehydrated in xylene and serial dilutions of ethanol. Endogenous peroxidase activity and non-specific antibody binding were blocked with H2O2 and protein block respectively.

After blocking, the antigens were retrieved in a hot solution (100X Citrate Buffer pH 6.0) for 10 minutes.The sections were incubated with DNMt3b Rabbit polyclonal antibody ab71747 diluted into (1:50) for 6 hours. Subsequently, biotin free-HRP linked secondary antibodies were applied. Followed by application of diluted DAB (chromogenic solution) onto sections and counterstained with hematoxylin. Immunoreactivity was semi-quantitatively evaluated for positively stained cells as nuclear and/or cytoplasmic immunoreactivity in five representative microscopic fields, then calculating the percentage of positive cells.

The expression of DNMT3B in tissue sections was evaluated as 0 when no positive stained cells observed, score 1 (weak) for 11%- 50%, of positive tumor cells, score 2 for 51%-80% and score 3(strong) for more than 80% (81%-100%) positively stained tumor cells according to Chen et al [10]. Statistical analysis was performed using the SPSS version 21 computer software in association with Microsoft Excel. The statistical significance of variations in median was tested via Kruskal Wallis test, and assessed by Spearman Rank linear correlation coefficient.

RESULTS

Table1 shows that most cases of oral, laryngeal and skin squamous cell carcinoma were ranged from 50-69 years with (50%) for OSCC, (80.0%) for LSCC and (47%) for skin SCC. Also, this table showed that most of patients were males in oral, laryngeal and skin SCC, (52.5%), (72.5%) and (67.5%) respectively.

Table 2 shows that the most frequent degree of differentiation in OSCC is well differentiated 18 cases (45.0%), followed by moderately differentiated 15 cases (37.5%). Whereas in LSCC the predominant grade is moderately differentiated 17 cases (42.5%), followed by Well differentiated 12 (30.0%). In skin the well differentiated degree is so high 24 cases (60.0%) compared to moderately differentiated 11 (27.5%) and Poorly differentiated 5 cases (12.5%).

Table 3 shows that ninety one cases (75.8%) showed DNMT3B positive expression among the three types, whereas 29 cases (24.16%) were negative. As shown in table 3 in OSCC 31cases (77.5%) were positive. The commonest category of DNMT3B expression in OSCC was Score1 (18 cases) (45%), followed by score 2 (8 cases) (20%). Similarly, in LSCC (77.5%) were positive (31 cases). The commonest category was Score 2 (15cases) (37.5%), followed by score 3 (12 cases) (30%).In Skin SCC the positive cases were (29) (72.5%). As shown in table 3, Mann-Whitney statistical test revealed that the median DNMt3b score in oral SCC (score-1) was significantly lower than that in Laryngeal SCC(P= 0.024), while it was almost comparable to that of skin SCC(P= 0.59), but a significant difference was recorded between skin and laryngeal SCC (P=0.003).

As shown in table 4, the median DNMt3b score was significantly lower among those with well differentiated tumor (score 1) and increased with increasing tumor grade to reach its highest median score (score 3) among those with poorly differentiated tumor. This marker showed a statistically significant moderately strong positive linear correlation with tumor grade among Oral SCC cases (r=0.568, P<0.001).

As shown in table 5, the median DNMt3b score was significantly lower among those with well differentiated tumor (score 1) and increase with increasing tumor grade to reach its highest median score (score 3) among those with poorly differentiated tumor. This marker showed a statistically significant strong positive linear correlation with tumor grade among laryngeal SCC cases (r=0.668).

As shown in table 6, the median DNMt3b score was obviously lower among those with well differentiated tumor (score 1) and increased with increasing tumor grade to reach its highest median score (score 2) among those with poorly differentiated tumor. The observed differences, however failed to reach the level of statistical significance. This marker showed a statistically significant weak to moderately strong positive linear correlation with tumor grade among skin SCC cases (r=0.383). In this study the pattern of expression was nuclear and/or cytoplasmic. With more cytoplasmic expression .Only few exclusive cytoplasmic and no exclusive nuclear expression was observed as shown in figures (1, 2 ,3).

Table 1: Frequency distribution of the study groups by age and gender

	Study group					
	Oral SCC		Laryngeal SCC		Skin SCC	
	N	%	N	%	N	%
Age group (years)						
<50	11	27.5	3	7.5	13	32.5
50-69	20	50.0	32	80.0	19	47.5
70+	9	22.5	5	12.5	8	20.0
Total	40	100.0	40	100.0	40	100.0
Gender						
Female	19	47.5	11	27.5	13	32.5
male	21	52.5	29	72.5	27	67.5
Total	40	100.0	40	100.0	40	100.0

Table 2: Frequency distribution of the 3 study groups by tumor grade

	Study group					
	Oral SCC		Laryngeal SCC		Skin SCC	
	N	%	N	%	N	%
Tumor grade						
Well differentiated	18	45.0	12	30.0	24	60.0
Moderately differentiated	15	37.5	17	42.5	11	27.5
Poorly differentiated	7	17.5	11	27.5	5	12.5
Total	40	100	40	100	40	100

Table 3: The difference in median score category of DNMT3B between the 3 study groups.

	Study group						
	Oral SCC		Laryngeal SCC		Skin SCC		
	N	%	N	%	N	%	P
DNMt3b score							0.009
Negative (< 10%)	9	22.5	9	22.5	11	27.5	
Score-1 (11-50%)	18	45.0	4	10.0	16	40.0	
Score-2 (51-79%)	8	20.0	15	37.5	12	30.0	
Score-3 (80%+)	5	12.5	12	30.0	1	2.5	
Total	40	100.0	40	100.0	40	100.0	
Median	Score-1 (11-50%)		Score-2 (51-79%)		Score-1 (11-50%)		
Mean rank	56.1		73.6		51.8		
P (Mann-Whitney) for difference between:							
Laryngeal SCC X Oral SCC = 0.024							
Skin SCC X Oral SCC = 0.59[NS]							
Skin SCC X Laryngeal SCC = 0.003							

Table 4: The difference in median score category of DNMT3B among the three tumor grades among cases with oral SCC.

	Tumor grade						
	Well differentiated		Moderately differentiated		Poorly differentiated		
Oral SCC	**N**	**%**	**N**	**%**	**N**	**%**	**P**
DNMt3b score							<0.001
Negative (< 10%)	6	33.3	3	20.0	0	0.0	
Score-1 (11-50%)	10	55.6	8	53.3	0	0.0	
Score-2 (51-79%)	2	11.1	3	20.0	3	42.9	
Score-3 (80%+)	0	0.0	1	6.7	4	57.1	
Total	18	100.0	15	100.0	7	100.0	
Median	Score-1 (11-50%)		Score-1 (11-50%)		Score-3 (80%+)		
Mean rank	15.4		19.7		35.2		
r=0.568 P<0.001							

Table 5: The difference in median score category ofDNMT3B among the 3 tumor grades among cases with Laryngeal SCC.

	Tumor grade						
	Well differentiated		Moderately differentiated		Poorly differentiated		
Laryngeal SCC	**N**	**%**	**N**	**%**	**N**	**%**	**P**
DNMt3b score							<0.001
Negative (< 10%)	5	41.7	3	17.6	1	9.1	
Score-1 (11-50%)	4	33.3	0	0.0	0	0.0	
Score-2 (51-79%)	3	25.0	11	64.7	1	9.1	
Score-3 (80%+)	0	0.0	3	17.6	9	81.8	
Total	12	100.0	17	100.0	11	100.0	
Median	Score-1 (11-50%)		Score-2 (51-79%)		Score-3 (80%+)		
Mean rank	11.2		20.6		30.6		
r=0.668 P<0.001							

Table 6: The difference in median score category of DNMT3B among the 3 tumor grades among cases with Skin SCC.

	Tumor grade						
	Well differentiated		Moderately differentiated		Poorly differentiated		
Skin SCC	**N**	**%**	**N**	**%**	**N**	**%**	**P**
DNMt3b score							0.05[NS]
Negative (< 10%)	8	33.3	3	27.3	0	0.0	
Score-1 (11-50%)	12	50.0	2	18.2	2	40.0	
Score-2 (51-79%)	4	16.7	6	54.5	2	40.0	
Score-3 (80%+)	0	0.0	0	0.0	1	20.0	
Total	24	100.0	11	100.0	5	100.0	
Median	Score-1 (11-50%)		Score-2 (51-79%)		Score-2 (51-79%)		
Mean rank	17.3		23.5		29.2		
r=0.383 P=0.015							

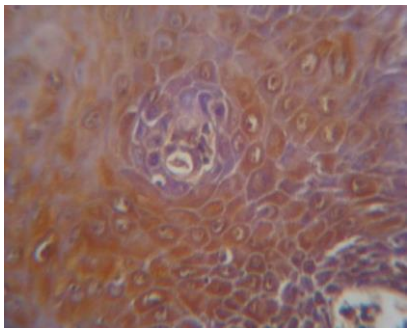

Figure 1: Nuclear and cytoplasmic expression of DNMT3B in OSCC (X40)

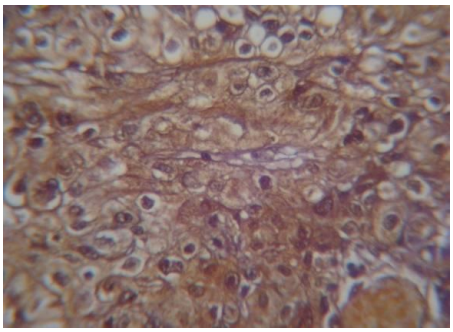

Figure 2: Nuclear and cytoplasmic expression of DNMT3B in LSCC (X40)

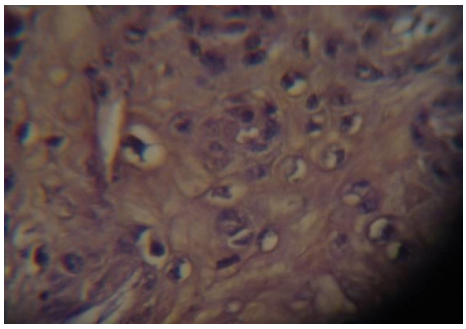

Figure 3: Nuclear and cytoplasmic expression of DNMT3B in Skin SCC

DISCUSSION

In the current study, of 120 oral SCC, LSCC and Skin SCC cases assayed for DNMT3B revealed high percentage expression of DNMT3B among oral, laryngeal and skin squamous cell carcinoma 91 (75.8%) and 29 (24.2%) were negatively stained. Ineach of OSCC and LSCC 31(77.5%) of the cases were positively immunoreacted to DNMT3b.DNMT3b immunoreactivity demonstrated no correlation with age/gender. while it exhibited strong association with tumor differentiation (P<0.001). This may be due to the fact that loss of p16 which is frequently inactivated in HNSCC by DNA hypermethylation is correlated with poorly differentiated HNSCC [11].

Also, TGF-β1 that enhances DNMTs expression is overexpressed in poorly differentiated SCC compared to well differentiated ones [12]. Although many previous studies have reported alteration in methylation pattern of malignant cells compared to non-malignant, there is limits in understanding the exact mechanism [13]. However, many studies illustrated the role of DNMT3B in initiation of cancer by inactivation of several tumor suppressor genes which lead to progression of HNSCC cancer [14]. Among the most important genes that inactivated by DNMT3B via hypermethylation and associated with poor prognosis in oral and Laryngeal SCC is P16 and death associated protein-kinase (DAPK) [15].

P16 is cyclin-dependent kinase inhibitor that regulates the Rb pathway, which inhibits progression of cell cycle [16]. DAPK that associated with apoptosis also is hypermethylated in oral and laryngeal SCC [17].Over expression of TGF-β1been found in most of Head and Neck SCC [18] .TGF-β1 up- regulates the expression of IL- 6 [19]. Also, TGF-β1 is considered a master regulator of DNA methylation via an increase expression of DNMTs in cancers [20]. IL 6 which is a cytokine with multifunction [21] and secreted by many cancerous cells [22] plays an important role in determining the biological behavior of oral squamous cell carcinoma [23] and Laryngeal squamous cell carcinoma [24]. IL-6 induced hypermethylation by mediating over expression of DNMT3B which may contribute to an aggressive behavior [25].

Also, IL-6 cause activation of Janus kinase (JAK) following binding to its receptor which mediate phosphorylation of signal transducer and then activation of the transcription factor Signal Transducer and Activator of Transcription (STAT). The activated STAT translocated to nucleus to activate targets like vascular endothelial growth factor (VEGF) that increase the invasion of tumor [19]. Activated STAT controls proliferation, invasion, migration of cells and angiogenesis [26]. DNA methylation may enhance progression of cancer [27]. It has been found that Activated IL 6 might promote tumerogensis via altering methylation of DNA by increase expression of DNMT3B and decrease E-cadherin [28] increase VEGF and MMP9, so the mechanism by which DNMT3b mediate an aggressive behavior in oral and laryngeal were epithelial mesenchymal transition (EMT) promotion and angiogensis [10].

So over expression of DNMT3B and activated TGF-β1/IL-6 pathways may be responsible for the aggressive behavior of oral and Laryngeal squamous cell carciniomas.With regard to skin SCC our data showed high percentage of DNMT3B expression (72.5%). This high percentage of DNMT3b expression goes withthe results of Nandakumar et al. [29] who had observed higher level of DNMT3B in skin SCC compared to normal skin. The statistical results showed a positive correlation tohistological grade (p=0.015). This is due to fact that DNA methylation in poorly or moderately differentiated tumors is more frequent than in the well differentiated ones [30].

The molecular mechanism for role of DNA methylation in skin SCC pathogenesis may be due to that activity of DNMTs in UVB- exposed epidermis is significantly higher than that in non-UVB exposed epidermis [29]. Less role of p16 hypermethylation established in Skin SCC [31]. Compared to silencing of p53 tumor suppressor gene by DNA hypermethylation that caused by UV radiation [32], in this study the pattern of expression was nuclear and/or cytoplasmic. With more cytoplasmic expression. Only few exclusive cytoplasmic and no exclusive nuclear expression was observed. Cytoplasmic imunoreactivity of this biomarker counted, because it has been found that over-expression of DNA methyltransferases switch toaccumulating DNA hypermethylation [10].

Where over expression of this enzyme in cytoplasm give us a concept that it would translocate to nucleus soon or later and cause DNA methylation. It has been found that intracellular localization of DNMT3B enzyme is dynamic during cell cycle, where it is diffusely distributed between nucleus and cytoplasm throughout most G1phase of cell cycle, during S phase in which DNA replication enhanced, localization correlate with subnuclear sites. While through G2 and M phases there were preferences of binding with pericentric heterochromatin [33].

In conclusion: Although the expression of DNMT3b was high among the three types , but data showed that epigenetic alterations in OSCC and LSCC were more than that in Skin SCC and this may be attributed to more silencing of tumor suppressor genes in HNSCC as a result of multiple etiological factors compared to that occurred in Skin SCC. And it's positive correlation with histopathological grades in the three types indicating that this biomarker could be used as prognostic marker in cancer of epithelial origin.

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