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Short Communication

Increased expression of DNA methyltransferase 1 and 3B correlates with tumor grade in laryngeal squamous cell carcinoma

Nooshin Mohammadzadeh^{1,2}, Fatemeh Mosafa^{3, 4}, Ehsan Khadivi⁵, Rosa Jahangiri⁶, Khadijeh Jamialahmadi^{3,7*}

¹School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

²School of Pharmacy, Pardis Campus, Azadi Square, Mashhad, Iran.

³Biotechnology Research Center, Institute of Pharmaceutical Technology, Mashhad University of Medical Sciences, Mashhad, Iran

⁴Department of Pharmaceutical Biotechnology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

⁵Department of Pharmaceutical Biotechnology, School of Pharmacy, Pardis Campus, Azadi Square, Mashhad, Iran.

⁶Sinus and Surgical Endoscopic Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

⁷Department of Medical Biotechnology and Nanotechnology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

*Corresponding author :Dr. Khadijeh Jamialahmadi,,Department of Medical Biotechnology and Nanotechnology, School of Medicine, Pardis Campus, Azadi Square, Mashhad, Iran, P.O. Box: 8594-917794, Mashhad, I.R. Iran. Orcid ID: [0000-0003-2173-003X](https://orcid.org/0000-0003-2173-003X)

Tel.: +98 513 8002293- 09155280860 Fax: +98 513 8002287 E-mail: jamialahmadikh@mums.ac.ir

Abstract

Background: DNA methyltransferase (DNMT) enzymes, encoded by DNMT1, DNMT3A and DNMT3B genes, play a major role in the development of cancers through aberrant promoter methylation. Due to little information about the biological and clinical significance of expression changes of these genes in Laryngeal Squamous Cell carcinoma (LSCC), the current study was designed to evaluate the contribution of DNMTs expression as potential diagnostic biomarkers in progression of LSCC.

Methods: DNMT1, DNMT3A and DNMT3B expressions in tumoral and normal tissues from thirty-three LSCC patients were evaluated by relative comparative real-time PCR, prior to any therapeutic intervention. Relationship between genes expression and clinicopathological features were also analyzed.

Results: We found the mRNA expression levels of all three DNMTs (DNMT1, DNMT3A and DNMT3B) were significantly elevated in LSCC tumor specimens compared to that of non-tumor tissues ($P < 0.0001$, $P = 0.011$ and $P < 0.0001$, respectively). The expression of DNMT1 and DNMT3B were strongly associated with histopathological tumor grade. Moreover, the mRNA expression levels of DNMT3A was significantly correlated with laryngopharyngeal reflux. No significant relationships existed with other clinicopathological parameters.

Conclusion: Our data showed that the expression levels of DNMT1, DNMT3A and DNMT3B markedly increased in LSCC tissues. DNMT1 and DNMT3B were mainly overexpressed in high grade LSCC tumors, therefore, they may have a role in LSCC progression. It seems that these genes may serve as diagnostic biomarkers in development of LSCC.

Keywords: Laryngeal squamous cell carcinoma; DNA methyltransferase; Expressional analysis; Histopathological grade; Biomarker

1. Introduction

Laryngeal squamous cell carcinoma (LSCC) is the second most prevalent type of malignancy in the head and neck squamous carcinoma.¹ In 2018, the GLOBOCAN database estimated 177,422 new cases of laryngeal cancer were diagnosed in the world and approximately 94,771 cases died from the laryngeal cancer.² In Iran, the incidence rate of laryngeal cancer is lower compared to other countries; the age-standardized rate (ASR) is about 2.62 per 100000 in men and 0.46 per 100,000 among Iranian women. Although the prevalence of LSCC in Iran is low, due to lifestyle changes and increased exposure to risk factors, there is a considerable growing trend in the incidence of this cancer in Iran.³

The most important risk factors affecting the development of LSCC are tobacco and alcohol consumption, human papilloma virus, chronic laryngeal inflammation, and radiation. In addition, esophageal reflux and occupational agents, such as asbestos and textile dust can play a role in the disease process.^{4,5}

Epigenetics is described as heritable and reversible modifications in gene expression without alteration of the DNA sequence,⁶ which plays an important role in the development and progress of cancer.⁷ Epigenetic modifications include DNA methylation, histone variants, chromatin remodeling processes and the epigenetic function of non-coding RNA. DNA methylation is the most important epigenetic mechanism which plays a key role in the modification of gene expression programs during development.⁸ DNA methylation, is mediated through DNA methyl transferases (DNMTs) enzyme family composed of DNMT1, DNMT3A and DNMT3B.⁹ DNMT1, the most abundant form of DNMT in mammalian cells, mainly preserves the paternal DNA methylation patterns, while DNMT3A and DNMT3B perform de novo DNA methylation.¹⁰ Nevertheless, methylation abnormality including global hypomethylation and local hypermethylation is implicated in various cancers. DNA hypomethylation can lead to disruption of gene expression but local hypermethylation of specific CpG islands in the promoter region, can cause tumor suppressor genes silencing which play an important role in the progression of cancer. Increased expression of DNMTs has been frequently reported in various types of human cancers.¹¹⁻²⁰ However, there are only a few studies in the literature that have investigated the DNMTs gene expression in LSCC patients. In the current study, for the first time in Iranian patients, we have investigated the expression of DNMT1, DNMT3A and DNMT3B in laryngeal squamous cell carcinoma patients to determine their impact on clinicopathological features in LSCC patients.

2. Methods

2.1. Patients and tissue samples

Thirty three fresh tumor and distant tumor-free tissue samples were collected from patients undergoing surgical resection for LSCC, over the period 2016-2018 at the Department of Head and Neck Surgery, Imam Reza Hospital, Mashhad University of Medical Sciences and Kasra Medical Clinic, Mashhad, Iran and transferred to RNA later solution (Qiagen, Hilden, Germany). All samples were stored at -80°C prior to mRNA extraction. Patients with diagnosis of LSCC, who had not received adjuvant chemotherapy or radiation prior to surgery were selected. Patients with other cancers or diseases were excluded from the current research. All fresh tissues were microscopically tested

by the pathologist to be certain about the originality of samples. The study protocol was acceded by the Medical Ethics Committee of the Mashhad University of Medical Science (Mashhad, Iran) and all patients signed informed consent letter after explaining the purpose of the study.

2.2. RNA extraction and cDNA synthesis

Total RNA of tumors and the adjacent normal tissues were extracted with RiboEx Total RNA extraction kit (GeneAll biotechnology, Korea) according to the manufacturer's instruction. The concentration and purity of all extracted RNAs were measured by NanoDrop 2000C Spectrophotometer (Thermo Scientific, USA). The integrity of the RNAs was confirmed by visualizing 28S, 18S, and 5S ribosomal RNA in an agarose gel. Extracted RNA samples were subjected to DNase I, RNase free enzyme (Thermo Scientific, Lithuania) treatments. The cDNA synthesis was conducted using random hexamer primers in RevertAid first-strand synthesis kit (Thermo Scientific, Lithuania), from 1 µg of total RNA in a final reaction volume of 20 µl.

2.3. Quantitative Polymerase Chain Reaction (qPCR)

Comparative relative real time PCR was performed on LightCycler® 96 real-time PCR system (Roche, Mannheim, Germany) using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus, Takara, Japan). The primer sequences (Table 1) were designed by Jahangiri et al. previously.²¹ qPCR reaction was performed in a total volume of 20 µl. Each reaction consisted of 10 µl of SYBR-Green master mix, 2µl cDNA, 0.8 µl of each primer (10 pmol), and 6.4 µl of DNase, RNase free water. Thermal profile was applied as initial denaturation step (30 s at 95 °C), followed by 40 cycles of denaturation at 95 °C for 5 s and annealing- extension at 60 °C for 30 s. Comparative (relative) Ct method was used to analysis DNMTs expression. β-actin was used as the housekeeping gene to normalize the data. Each evaluation was accomplished two times to verify the results, and the mean mRNA expression was used for the statistical analysis.

2.4. Statistical analysis

The statistical software SPSS version 22.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses. The DNMTs mRNA expression difference between tumor and normal tissues was evaluated by Paired sample t test or Wilcoxon signed-rank test. The correlations between DNMTs mRNA expression and clinical characteristics including age, T stage, N stage, M stage, cancer staging, histological grade, extracapsular nodal extension and laryngopharyngeal reflux were analyzed by the χ^2 or Fisher's exact tests. In addition, Spearman or Pearson's correlation coefficients analysis were used for the correlation of DNMT1, DNMT3a and DNMT3b expression level. P values < 0.05 were considered statistically significant.

3. Results

The median age of patients was 60 years (range 42-80), and all of them were men. There were 8 cases of low grade, 18 cases of intermediate grade and 7 cases of high grade. The other clinicopathological characteristics of patients are summarized in Table 2.

3.1. DNMT1, DNMT3A and DNMT3B mRNA expression in LSCC patients

The expression levels of DNMTs was analyzed in 33 fresh tumors and their adjacent normal margins by qRT-PCR . The results showed that DNMT1, DNMT3A and DNMT3B mRNA were expressed in all the tumor and corresponding non-cancerous tissues. Additionally, the mRNA expression level of DNMT1 was significantly increased in tumor tissue specimens in comparison to normal tissues ($P < 0.0001$). Similar findings were indicated for DNMT3A ($P = 0.011$) and DNMT3B mRNA expression levels ($P < 0.0001$).

3.2. Correlation between the expressions of DNMT1, DNMT3A and DNMT3B status in LSCC patients

Spearman correlation analysis showed that the expression of DNMT3A was positively correlated with the DNMT3B expression in laryngeal tumor tissues ($r: 0.570, P = 0.001$). In contrast, there were no significant correlation between the expression of (DNMT1 VS DNMT3B ($r: 0.201, P = 0.262$) and DNMT1 VS DNMT3A ($r: 0.234, P = 0.19$)).

3.3. Association between the expression of DNMTs and clinicopathological features

For every gene, the patients were divided in two groups, high and low expression, according to the median of mRNA expression. A significant correlation between DNMT1 and DNMT3B overexpression and the histologic grade ($P = 0.013$ and $P = 0.023$, respectively) of tumor cells was revealed (Fig. 1-2). Furthermore, DNMT3A mRNA expression was significantly associated with laryngopharyngeal reflux in patients ($P = 0.015$). There were no significant relationships with other clinicopathological parameters (Table 2).

4. Discussion

Studies have confirmed the role of epigenetic modifications especially DNA methylation in the development and occurrence of many cancers. DNA methylation is known to be abnormal in most cancers, while there are numerous instances of the over expression of DNMTs in different cancers.^{11,17,22} In mammals, the DNA methylation process is catalyzed by DNMTs.⁹ The expression of DNMT enzymes plays an important role in the dispersion of methylated regions in the promoter regions. These enzymes, themselves have CpG islands in their promoter and regulatory sequences, consequently, the genes of DNMTs can also be regulated by methylation.²³

Laryngeal squamous cell carcinoma is the most prevalent type of laryngeal cancers.¹ Genetic and environmental factors play a critical role in the occurrence of this cancer.²⁴ Although there are many published studies that reported the overexpression of DNMTs in various cancer tissues, but little is known about the role of DNMTs expressions in LSCC and the correlation between their expression and clinicopathological features during laryngeal carcinogenesis.

In the current study, for the first time in Iranian patients, we evaluated the significance of DNMT1, DNMT3A and DNMT3B mRNA expression and its association with clinicopathological parameters in laryngeal squamous cell carcinoma. We observed that mRNA expression of DNMT1, DNMT3A and DNMT3B were meaningfully elevated in LSCC tumor tissues compared with the corresponding normal tissues. These results were in line with other studies that exhibited the increased DNMTs expression in a variety of tumor types, such as hepatocellular carcinoma,²⁵ lip and oral squamous cell carcinoma,^{15,22} esophageal,¹³ pharyngeal,^{15,16} colon,²⁶ breast,^{17,21} lung cancers,²⁷ and ovarian tumors as well.²⁸ Recently the positive protein expression of DNMT1, DNMT3a and DNMT3b in LSCC tumor tissues were reported which was consistent with our results. Also, in the mentioned study, the expression of DNMT1 and DNMT3b were associated with age, tumor size and lymph node metastasis, while the expression of DNMT3a was markedly correlated with histological grade.²⁹ Chen et al. also have reported the DNMT3B protein levels was significantly increased in head and neck squamous cell carcinomas cell lines.¹²

Moreover, our analysis indicated that the expression levels between DNMT3A and DNMT3B were highly correlated ($r: 0.570, P = 0.001$). However, there was no significant correlations between the expression level of DNMT1 with the expressions of DNMT3A and DNMT3B. These results were similar to those observed in breast cancer,¹⁷ and hepatocellular carcinoma.³⁰ In contrast, in a recent study by Daniel et al. it was found that there was a correlation between the levels of nuclear X cytoplasmic immunoreactivity for DNMT 1 and DNMT3A in lip squamous cell carcinoma.²²

Furthermore, our findings represented a significantly higher expression of DNMT1 and DNMT3B in high grade LSCC in comparison with low grade ($p < 0.05$) which suggest that DNMT1 and DNMT3B might function as mediators of LSCC progression. It is agreeable to previous studies reporting a significant association between high DNMT1 expression and histological grading in oral SCC,¹⁴ gastric cancer,³¹ pancreatic ductal adeno carcinoma,²⁰ and clear cell renal cell carcinoma.³² Additionally, our previous study has demonstrated that in tamoxifen-resistance breast cancer patients, DNMTs mRNA expression was statistically correlated with high histologic grade.¹⁷ The exact mechanisms behind the roles of DNMTs in LSCC progression is not clear. According to several studies it seems that overexpression of DNMTs can regulate the expression of different genes which are important in cancer progression.

The expression of Fragile Histidine Triad Diadenosine Triphosphatase (FHIT), p16, Ras association domain family 1 isoform A (RASSF1A), Retinoic acid receptor beta (RAR β) and hRAB37 were changed following over expression of DNMTs in lung cancer. Additionally, the altered gene expression of p53 in ovarian cancer, Thrombospondin 1 (THBS-1) gen in gastric cancer were reported as the result of DNMTs overexpression.³³

5. Conclusion

In conclusion, results from this study show that DNMT1, DNMT3A and DNMT3B were highly expressed in LSCC tissues. In addition, DNMTs might be a significant biological markers of tumor progression for patients with

laryngeal cancer. However, more studies are required to confirm overexpression of DNMTs as a clinical biomarker of prognosis or response to therapy.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The study protocol was acceded by the Medical Ethics Committee of the Mashhad University of Medical Science (Mashhad, Iran)(IR.MUMS.fm.REC.1395.252) and all patients signed informed consent letter after explaining the purpose of the study.

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Table 1. Primer sequences for Quantitative PCR

Gene name	Primer sequences	Ampliqon size (bp)
DNMT1	F: 5'-GCAAACCACCATCACATCTCAT- 3' R: 5'-GTCTAGCAACTCGTTCTCTGGA- 3'	158
DNMT3A	F: 5'-ACCACGACCAGGAATTTGACC- 3' R: 5'-CAATGTAGCGGTCCACCTGAA- 3'	150
DNMT3B	F: 5'-TTGGAATAGGGGACCTCGTGTG- 3' R: 5'-AGAGACCTCGGAGAACTTGCCATC- 3'	152
β-actin	F: 5'-CAGGAGGAGCAATGATCTTGATCT- 3' R: 5'-TCATGAAGTGTGACGTGGACATC- 3'	156

Table 2. Clinicopathological features of LSCC patients and their correlation with DNMTs gene expression.

Variable		mRNA expression (Total N = 33)					
		DNMT1		DNMT3A		DNMT3B	
		Low	High	Low	High	Low	High
Age (\geqmedian)	<60	8(24.2%)	8(24.2%)	8(24.2%)	8(24.2%)	8(24.2%)	8(24.2%)
	\geq 60	6(18.1%)	11(33.3%)	5(15.1%)	12(36.3%)	7(21.2%)	10(30.3%)
	P*	NS		NS		NS	
Laryngopharyngeal reflux	Positive	9(27.2%)	13(39.3%)	11(33.3%)	8(24.2%)	9(27.2%)	13(39.3%)
	Negative	5(15.1%)	6(18.1%)	2(6%)	12(36.3%)	6(18.1%)	5(15.1%)
	P*	NS		<i>0.015</i>		NS	
T stage	T1	4(12.1%)	7(21.2%)	3(9.1%)	8(24.2%)	4(12.1%)	7(21.2%)
	T2	2(6.1%)	1(3%)	1(3%)	2(6.1%)	2(6.1%)	1(3%)
	T3	2(6.1%)	5(15.2%)	1(3%)	6(18.2%)	3(9.1%)	4(12.1%)
	T4	6(18.2%)	6(18.2%)	8(24.2%)	4(12.1%)	6(18.2%)	6(18.2%)
	P*	NS		NS		NS	
N stage	N0	11(33.3%)	15(45.5%)	10(30.3%)	16(48.5%)	13(39.4%)	13(39.4%)
	N2	2(6.1%)	4(12.1%)	2(6.1%)	4(12.1%)	1(3%)	5(15.2%)
	N3	1(3%)	0(0%)	1(3%)	0(0%)	1(3%)	0(0%)
	P*	NS		NS		NS	
M stage	M0	13(39.4%)	16(48.5%)	10(30.3%)	19(57.6%)	14(42.4%)	15(45.5%)
	M1	1(3%)	3(9.1%)	3(9.1%)	1(3%)	1(3%)	3(9.1%)
	P*	NS		NS		NS	
Cancer staging	Stage I	4(12.1%)	7(21.2%)	3(9.1%)	8(24.2%)	4(12.1%)	7(21.2%)
	Stage II	2(6.1%)	1(3%)	1(3%)	2(6.1%)	2(6.1%)	1(3%)
	Stage III	2(6.1%)	3(9.1%)	1(3%)	4(12.1%)	3(9.1%)	2(6.1%)
	Stage IV	6(18.2%)	8(24.2%)	8(24.2%)	6(18.2%)	6(18.2%)	8(24.2%)
	P*	NS		NS		NS	
Extracapsular nodal extension	Positive	1(3%)	3(9.1%)	2(6.1%)	2(6.1%)	0(0%)	4(12.1%)
	Negative	13(39.4%)	16(48.5%)	11(33.3%)	18(54.5%)	15(45.5%)	14(42.4%)
	P*	NS		NS		NS	
Histological grade	Grade I	6(18.2%)	2(6.1%)	4(12.1%)	4(12.1%)	5(15.2%)	3(9.1%)
	Grade II	8(24.2%)	10(30.3%)	8(24.2%)	10(30.3%)	10(30.3%)	8(24.2%)
	Grade III	0(0%)	7(21.2%)	1(3%)	6(18.2%)	0(0%)	7(21.2%)
	P*	<i>0.013</i>		NS		<i>0.023</i>	

NS non-significant*

Significant values ($P < 0.05$) are italicized.

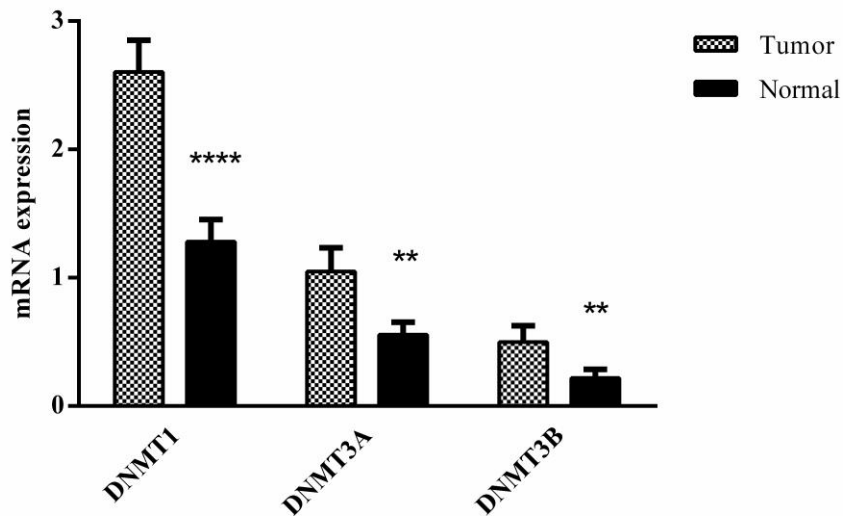


Fig.1. Expression profile of DNMT1, DNMT3A and DNMT3B in laryngeal tumor tissues and their normal adjacent tissues. DNMTs mRNA expression levels significantly reduced in laryngeal tumors, compared to normal tissues. The expression levels were calculated using $2^{-\Delta CT} \times 100$ and data are presented as mean \pm SEM. ** $P < 0.01$; **** $P < 0.0001$

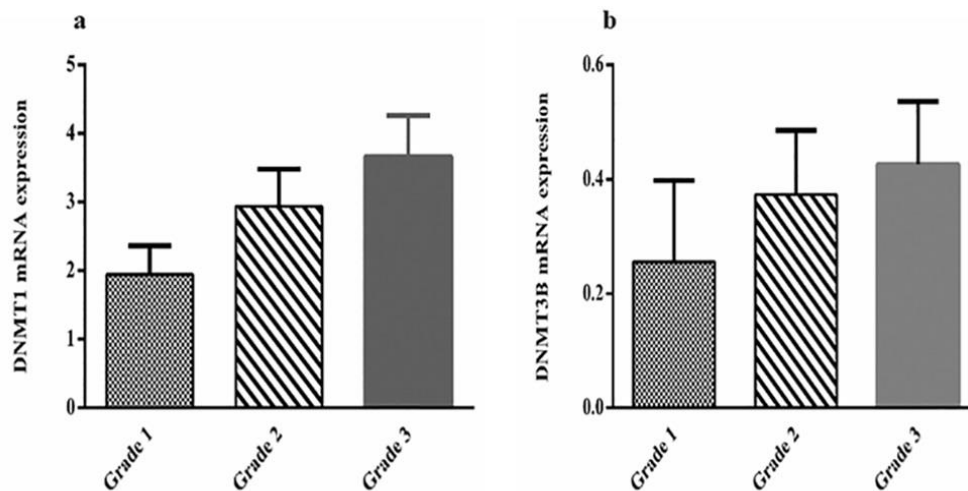


Fig.2. Plot showing DNMT1 and DNMT3B mRNA expression levels in relation to histologic grade. **a** DNMT1 mRNA expression. **b** DNMT3B mRNA expression. The expression levels were calculated using $2^{-\Delta CT} \times 100$ and Data are presented as mean \pm SEM.