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STUDY OF THE PROMOTER METHYLATION STATUS OF RASSF1A GENE IN ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Oral cancer is one of the major global health problems. Methylation is one of the epigenetic modifications that play an important role in the transcriptional inactivation of tumor suppressor genes in human cancer. **Aim:** The present study was directed to assess the Ras-association domain family 1A (RASSF1 A) gene promoter methylation status in oral squamous cell carcinoma (OSCC) and its correlation to patient's clinicopathological data. **Material and methods:** Formalin-fixed, paraffin-embedded tissue blocks of fifty patients with histopathologically confirmed OSCC in addition to 10 normal oral mucosa blocks were included in this study. Hematoxylin and Eosin (H&E) stain was used to confirm the diagnosis of cases as 16 well differentiated, 19 moderately differentiated and 15 poorly differentiated OSCC. Sections of each block were deparaffinized and prepared by DNA extraction for detection of RASSF1A gene methylation status by methylation-specific polymerase chain reaction (MSP) technique. **Results:** RASSF1A promoter methylation was not detected in normal cases, but it was found in 30% (15/50) of OSCC cases. The increase of methylation in OSCC was significant (p-value=0.045). The increase of methylation in poorly differentiated OSCC (6/15, 40%) was more than that.0 in moderately differentiated OSCC (7/19 36.8%) but both were significante in relation to normal mucosa (p-values= 0.021, 0.027 respectively). Well-differentiated OSCC was the lowest in methylation (2/16, 12.5%), while its increase than normal was not significant (p-value= 0.244). There was direct correlation between methylation and the lymph nodes metastasis. **Conclusion:** RASSF1A promoter methylation significantly associated with OSCC, the decrease of cell differentiation in histopathological stages and lymph node metastasis. Hence, RASSF1A promoter methylation could be used as a tumor marker in addition to histopathological stage and lymph nodes metastasis predictor in OSCC.

Keywords: oral squamous cell carcinoma, promoter methylation, RASSF1A gene.

INTRODUCTION

Oral cancer is one of the major global health problems with an annual estimated incidence of 300,000 newly diagnosed cases[1]. In Egypt, about 4.500 cases are diagnosed as oral cancer every year[2]. Oral squamous cell carcinoma (OSCC) is the commonest single entity in oral cancer and accounting alone for about 90% of all malignancies of the oral cavity[3]. Despite the development of diagnostic screening equipment, operation techniques, and postoperative care, there are a high number of patients manifesting oral cancer advanced-stage as well as high mortality. This five-year survival rate remains approximately 50 %, which is not satisfactory despite new treatment modalities[4]. Because of this unfortunate situation, which persists until now, there have been continuous studies and reports on the deciding factors that would improve the survival rate of oral cancer patients[5].

Besides genetic changes, epigenetic alterations may lead to changes in gene expression as well. Epigenetic alterations include modifications of the genome without changes in the underlying DNA sequence[6]. DNA methylation is a major

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epigenetic modification that is strongly involved in the physiological control of genome expression. Aberrant DNA methylation may lead to silencing of important genes, such as tumor suppressor genes, affecting their related transcriptional pathways and ultimately leading to development of cancer[7]. One of the greatest challenges facing modern oncology is the development of adequate biomarkers that could improve both diagnosis and treatment[8]. DNA methylation can be used as a marker for early cancer detection[9]. In contrast to genetic events, DNA methylation in cells is reversible. Therefore, it could serve as an attractive target for new therapeutic strategies[10].

Although hypermethylation and consequent silencing of more than forty tumor suppressor genes have been identified in OSCC, a clear correlation between the epigenetic-driven deregulation of gene expression and oral cancer progression is at present not fully demonstrated[11]. The Ras signaling pathway is frequently activated in human tumors [12]. Moreover, previous studies indicate that, in addition to Ras mutation, inactivation of negative effectors of Ras may play a significant role in the development of tumors. Among these negative effectors, the Ras association domain family (RASSF) is a group of proteins that contain Ras association (RA) domains and suppress Ras activity. RASSF1, for example, was identified as a potential tumor suppressor that can induce apoptosis in tumor cells [13, 14]. Moreover, RASSF1A is one of eight isoforms of RASSF1 that is involved in microtubule stabilization, cellular motility, invasion, apoptosis and cell cycle control[9].

To date, a number of studies have investigated the association between head and neck squamous cell carcinomas(HNSCC) and aberrant methylation of RASSF1A gene [15]. RASSF1A promoter methylation studies which conducted only on OSCC still limited and focused on cases with specific clinicopathologic or histopathologic characteristics. The promoter methylation percentages which resulted from these studies ranged from 2% to 93% [16-18]. Because of these inconsistent and inconclusive results, it was advantageous in the present study to assess the RASSF1A promoter methylation status, its role in carcinogenesis of OSCC and its correlation to patient's clinicopathological data.

MATERIAL AND METHODS

Cases collection

Formalin-fixed, paraffin-embedded tissue blocks of fifty patients with histopathologically confirmed OSCC in addition to ten normal oral mucosa tissue blocks, adjacent to non-neoplastic lesions obtained from operculectomy, were included in this study. The OSCC cases were diagnosed as: 16 well differentiated 19 moderately differentiated and 15 poorly differentiated OSCC cases. These blocks were collected including their clinicopathological data from the archival stored blocks in Pathology Departments of Nasr City Health Insurance Hospital and National Cancer Institute, Cairo University. Consecutive sections from paraffin-embedded tissue blocks were cut at 5-μm thickness and stained by H&E stain for confirmation of histopathological diagnosis.

Methylation specific PCR detection of methylated RASSF1A gene

Three main steps were followed in order to detect hypermethylated RASSF1A gene by methylation specific PCR technique: DNA extraction, bisulfite treatment of extracted DNA and PCR detection of methylated RASSF1A genes.

DNA extraction

It was done by using the QIAamp DNA FFPE Tissue Kit (56404, QIAGEN, Germany), the extracted DNA concentration and purity were evaluated by Beckman DU 7400 spectrophotometers.

Bisulfite treatment of extracted DNA

This step was performed by EZ DNA methylation direct kit (D5020, Zymo Research Corp., USA). In this reaction, all unmethylated cytosines were converted in to uracil but methylated cytosines are resistant to this modification. Therefore, the methylated cytosine remained intact while the unmethylated cytosines were completely converted into uracil following bisulfite treatment and detected as thymine after PCR amplification.

PCR detection of methylated RASSF1A genes

The methylation of DNA to inactivate any gene, it occurs at the promoter regions of this gene. The promoter DNA sequences which modified by bisulfite treatment could be detected by PCR using unmethylated inner primers and the sequences which resisted the modification could be detected by PCR using methylated inner primers. To increase the sensitivity and specificity we firstly amplified the promoter regions of the bisulfite treated DNA by using the outer primers. Secondly, we performed PCR by applying the inner unmethylated primer to each DNA sample resulted from the first step, and lastly we performed PCR by applying the inner methylated primer to every DNA sample resulted from the first step. The primer was purchased from metabion international AG Lena-Christ Strasse /Martinsried, Germany. Three types of primers used as follow:

Primer	Sequence
RASSF1A-outer F	(5'-GAG GAG GGG ATG AAG GAG G-3', upstream)
RASSF1A-outer R	(5'-CTC CAA CCA AAT ACA ACC CT-3' downstream)
RASSF1A-MSP-F	(5'-GGG TTT TGC GAG AGC GCG-3', upstream)
RASSF1A-MSP-R	(5'-GCT AAC AAA GCG GAA CCG-3' downstream)
RASSF1A-UM-SP-F	(5'-GGT TTT GTG AGA GTG TGT TTA G-3', upstream)
RASSF1A-UM-SP-R	(5'-CAC TAA CAA ACA CAA ACC AAA C-3' downstream)

The PCR conditions for outer primers were 95˚C for 5 min; 40 cycles at 95˚C for 30 sec, 53˚C for 45 sec, and 72˚C for 45 sec; and a final extension at 72˚C for 10 min. The PCR conditions for inner primers were 94˚C for 15 min; 40 cycles at 94˚C for 30 sec, 62˚C for 30 sec, 72˚C for 30 sec; and a final extension at 72˚C for 10 min. The PCR products were detected by agarose gel electrophoresis after staining by ethidioum promide and analyzed by gel documentation system. Distilled water was used as negative control (DW). Bisulfite-treated normal lymphocyte DNA (NL) from healthy volunteers served as a positive control for unmethylated DNA primers. This DNA was methylated by the use of SssI methyltransferase and used after bisulfite modification as a positive control for methylated DNA primers (Fig 1).

Statistical Analysis

Data were collected, revised, coded and entered to the statistical package for social science (IBM SPSS) version 20. The comparison between two

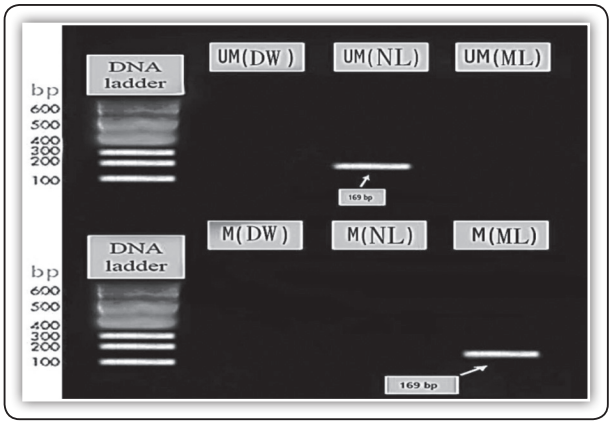

Fig. 1: Printed photograph of gel documentation system revealed agarose gel electrophoresis of methylation-specific PCR products showing DNA ladder with known base pair sizes. Other lanes showing DNA product (169 bp) of RASSF1A gene, in samples of distilled water (DW), normal lymphocytes (NL), and lymphocytes DNA methylated by SssI methyltransferase (ML), Letters UM denoted PCR product with primers specific for unmethylated sequences and M denoted PCR product with primers specific for methylated sequences.

groups with qualitative data was done by using Chi-square test and/or Fisher exact test was used instead of Chi-square test when the expected count in any cell was found less than 5. The comparison between two independent groups with quantitative data and parametric distribution was done by using Independent t-test. The comparison between more than two independent groups with quantitative data and parametric distribution was done by using one-way analysis of variance (ANOVA) followed by post hoc analysis using least significance difference (LSD) test. The p-value was considered significant if < 0.05 and highly significant if < 0.01.

RESULTS

Clinicopathological findings

Table 1 summarizes the clinicopathological features of all cases examined in the present study. The average age of the 50 patients with OSCC (male, 41; female, 9) was (67.56 ± 8.65) years. According to the site of the tumors, the most common site was gingiva and alveolar mucosa followed by tongue, palate, buccal mucosa, floor of the mouth and lips. The average size of the tumor was (7.38 ± 10.25). Histopathological examination showed that the presence of metastatic tumor cells in the regional lymph nodes was found in only 10 out of 50 cases (20%). There was no statistical significant difference between OSCC subgroups concerning to clinicopathological features except between well differentiated and poorly differentiated OSCC concerning to gender distribution and tumors size (P-value 0.046 and 0.038, respectively) (Table 2).

TABLE (1) Description of clinicopathological results of all the studied OSCC cases.

Clinicopathological / No.		No.= 50
Age	Mean ± SD	67.56 ± 8.65
	Range	50 – 85
Gender	Female	9 (18%)
	Male	41 (82%)
Site	Gingiva and alveolar mucosa	19 (38%)
	Tongue	9 (18%)
	Palate	8 (16%)
	Cheek	6 (12%)
	Floor of the mouth	3 (6%)
	Lip	3 (6%)
	lymph nodes	2 (4%)
Size cm3	Mean ± SD	7.38 ± 10.25
	Range	0.25 – 30
Lymph nodes metastasis	Negative	40
	Positive	10
	Cases with 1 +ve lymph node	1
	Cases with 2 +ve lymph nodes	3
	Cases with 3 +ve lymph nodes	3
	Cases with 4 +ve lymph nodes	1
	Cases with 7 +ve lymph nodes	2

Methylation specific PCR (MSP) results:

The results showed that methylation of the promoter region of RASSF1A was not detected in normal cases but unmethylated bands was detected in 10 of 10 (100%) of these normal cases. In OSCC cases promoter methylation bands were detected in 15 of 50 cases (30%) and unmethylated bands were detected in 49 of 50 (98%) of OSCC cases. Statistically, there was significant increase of methylation in OSCC group in relation to normal group (P= 0.045) (Table 3). Furthermore, there was no significant statistical difference between positive and negative methylated cases in age (P = 0.2), distribution of gender (P = 0.8) and mean of tumor size (P =

TABLE 2: Comparison between OSCC subgroups regarding age, gender and tumors size in all studied cases.

Age, gend. size / OSCC subgroups		Well	Moderate	Poor	One Way ANOVA		Post hoc analysis		
		No.= 16	No.= 19	No.= 15	F/x^{2*}	P-value	P1	P2	P3
Age	Mean ± SD	70.69 ± 10.20	65.53 ± 7.17	66.80 ± 8.18	1.674	0.199	0.081	0.211	0.668
	Range	53 – 85	54 – 79	50 – 82					
Gender	Female	1 (6.2%)	3 (15.8%)	5 (33.3%)	3.949*	0.139	0.377	0.046**	0.231
	Male	15 (93.8%)	16 (84.2%)	10 (66.7%)					
Size cm3	Mean ± SD	3.93 ± 2.69	6.97 ± 11.66	11.5 ± 12.33	2.306*	0.111	0.374	0.038**	0.187
	Range	0.5 – 9	0.25 – 30	0.8 – 30					

**: Chi-square test, **: P < 0.05: Significant, P1: well vs. moderate, P2: well vs. poor, P3: moderate vs. poor.*

0.54). However, there was high significant correlation between positive cases for methylation regarding to lymph nodes positivity and number of positive lymph nodes involvement (p-value 0.00 and 0.002 respectively) (Table 4).

Fourteen OSCC cases (n=14) showed both methylated and unmethylated bands, while one case showed only methylated band. In primary tumors, the presence of the normal stromal cells and inflammatory cells may contribute to the presence of unmethylated bands in most cases. All normal cases showed only unmethylated bands. Printed

TABLE (3): Comparison between OSCC group and control group regarding methylation and unmethylation.

Study groups / Methylation		OSCC group		Control group		Chi-square test	
		No.	%	No.	%	X^2	P-value
Methylation	Negative	35	70%	10	100%	4.000	**0.045***
	Positive	15	30%	0	0%		
Unmethylation	Negative	1	2%	0	0%	0.203	0.652
	Positive	49	98%	10	100%		

*: P < 0.05: Significant

TABLE (4) Comparison between positive and negative cases to methylation regarding lymph node metastasis and number of lymph nodes involvement.

Methylation / L.N.		-ve to methylation		+ve to methylation		Chi-square test	
		No.	%	No.	%	X^2	P-value
Lymph nodes	Negative	33	94.3%	7	46.7%	14.881	0.000*
	Positive	2	5.7%	8	53.3%		
No. of lymph Nodes	0	33	94.3%	7	46.7%	19.325	0.002*
	1	0	0.0%	1	6.7%		
	2	2	5.7%	1	6.7%		
	3	0	0.0%	3	20.0%		
	4	0	0.0%	1	6.7%		
	7	0	0.0%	2	13.3%		

TABLE (5) Comparison between OSCC subgroups and control group regarding methylation and unmethylation

Study groups / Methylation		Control group		Well		Moderate		Poor		Chi-square test		
		No.	%	No.	%	No.	%	No.	%	P1	P2	P3
Methylation	-ve	10	100%	14	87.50%	12	63.20%	9	60.00%	0.244	0.027*	0.021*
	+ve	0	0%	2	12.50%	7	36.80%	6	40.00%			
Unmethylation	-ve	0	0%	0	0.00%	1	5.30%	0	0.00 %	NA	0.460	NA
	+ve	10	100%	16	100%	18	94.70%	15	100%			

*P1: Control vs well, P2: Control vs moderate, P3 control vs poor. *: P < 0.05: Significant*

photograph of gel documentation system revealed agarose gel electrophoresis of methylation-specific PCR products of oral squamous cell carcinoma samples were shown in (Fig 2).

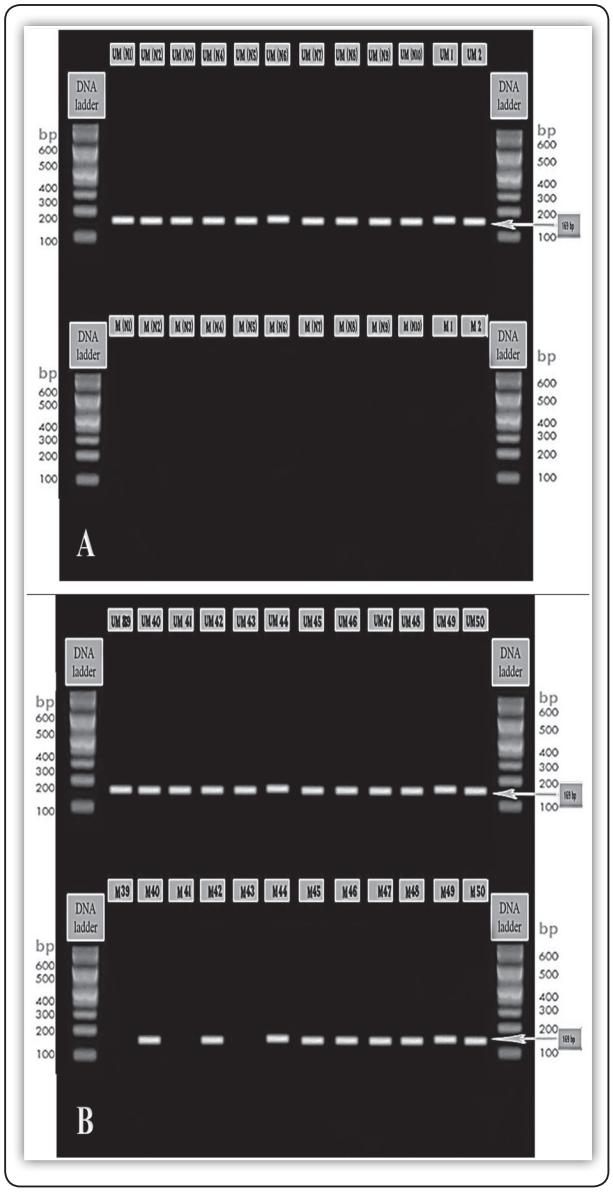

Fig. (2) Agarose gel electrophoresis of methylation-specific PCR products showing DNA ladder with known base pair sizes. Other lanes showing DNA products (169 bp) resulted from MSP of RASSF1A gene in 10 normal mucosa samples (N1-N10) and 2 cases of OSCC (Fig A) and cases of OSCC (39-50). Letters UM and M denoted PCR product with primers specific for unmethylated and methylated sequences, respectively.

The percentage of methylation in poorly differentiated OSCC (40%) was more than that in moderately differentiated OSCC (36.8%) and well-differentiated OSCC was the lowest in methylation (12.5%). There was a significant difference in the increase of methylation when moderately and poorly differentiated OSCC compared to normal group, but well-differentiated OSCC didn`t show significance (P= 0.027, P=0.021, and P=0.244 respectively) (table 5).

DISCUSSION

In the present study, a series of 50 blocks of OSCC tissue with their clinicopathological data used in order to assess the IHC expression of RASSF1A protein and its correlation to clinicopathological data compared to normal oral mucosa tissues as a control. Studied cases ranged from 50 to 85 years old with mean of 67.56 years. Being in the old age, the mean of OSCC patients age was nearly in line with that was reported in Egypt (56.85)[19]. The age range of studied OSCC patients differed from age range that reported in some Arabic countries population which may be due to the difference in habits and environment[20, 21]. In the present study, males were affected more than females that is in agreement with most OSCC studies. However, the present male to female ratio was 4.6:1 that is more than that reported in Egypt (1.4-1) and in Arabic countries (1-1 to 1.5-1)[1,20,21]. This difference may be attributed to the small number of cases in the current study. In the present study, the most affected site was the gingiva followed by the tongue. Contrarily, the anterior two thirds of the tongue was the most common site in recent published epidemiological studies of Egyptian, Arabic countries and worldwide population [3,20,21]. This difference may be also attributed to the small number of cases in the current study. The mean of tumors size of the presented cases were 7.38 cm^3. In addition, the sizes of 23 out of 50 (46%) OSCC cases were less than 4 cm that is in-between the results of previous studies which reported 59%, 39%, 40%,

respectively[17, 22, 23]. In the current study, 10 out of 50 (20%) OSCC cases showed positive lymph node metastasis with variable number of involved nodes. This percentage was nearly similar to that reported by Noorlag et al[18], who found lymph node involvement in 49 out of 215 (23%) and was less than that reported by other studies which reported 37%, 78% and 30% lymph node involvement respectively[17,22,23]. The mean of tumor size and percentage of positive lymph node metastasis were variable in previous studies due to deference in patient cohorts, environmental factor and methods of detection [3, 24].

In the present study, promoter methylated MSP bands were not detected in normal oral mucosa cases. The absence of methylation in control is congruent with previous studies[25-27] which found methylation in none of their control cases. On the contrary, another studies[19, 28, 29] investigated the RASSF1A-promoter methylation and the methylation has been reported to be present in (9/41), (4/18) and (11/48) of control cases, respectively. These results are in disagreement with the current study result. This divergence in methylation percentage of control cases can be explained by the difference in control type, either autologous or heterogeneous. Control cases in disagreement studies were autologous but in agreement studies were mostly heterogeneous resembling the current study.

The previous interpretation may be supported by the results of Su et al[30] who found methylation in none of 12 heterogeneous normal mucosa cases compared to (12/32) of autologous adjacent non-neoplastic mucosa. Moreover, the epigenetic changes occur firstly before the appearance of the subsequent histopathological changes or may occur in haploid pattern especially in normal tissues adjacent to tumor.

In the current study, RASSF1A promoter methylated MSP bands were detected in 30% (15/50) of OSCC cases which in agreement with previous HNSCC studies[28,31]. Similarly, Su et al [30] detected promoter methylation in 29% (9/31) of OSCC. However, promoter methylation of RASSF1A in OSCC were reported to be present in 2%, 22.4% and 95% of cases by Noorlag et al[18], Huang et al[17] and Tran et al [16] respectively, which appear to be in disagreement with the results of the current study. The divergence between the present study results and those of Noorlag et al[18] could be interpreted by that their study was restricted to histopathologically confirmed early SCC, which was reported to be less in methylation than more advanced and higher grades of HNSCC [32-34]. Interestingly, the promoter methylation was noticed only in (2/16) of well-differentiated OSCC in the current study.

The little difference between the present study results and those of Huang et al[17] in promoter methylation of OSCC can be attributed to the differences in methylation testing methodology, the variations in sample processing, the differences in composition of patient cohorts and the lack of *Human Papilloma Virus* determination. All these causes had been previously proved to be somewhat influential in methylation results[15, 16, 18, 35, 36].

Methylation results which reported by Tran et al[16] was not only in disagreement with the present study, but also it was higher than all previous HNSCC studies. They conducted their study in population with betel-associated OSCC. Therefore, the above-mentioned divergence in methylation can be elucidated by that the genetic pathway responsible for betel-associated OSCC without concomitant effects of ethanol and smoking may be different from the genetic pathway of OSCC of Egyptians, in the present study, which are mostly not betel-associated. Furthermore, Tran et al[16] study was restricted to non-smoking non-drinking Vietnamese female population. As methylation depends largely on environmental factors even during fetal life[37], ethnic and gender differences had been reported to influence methylation results as well[15, 38].

In the current study, unmethylated MSP bands were detected in 49 out of 50 (98%) of OSCC cases. The detection of unmethylated MSP bands in nearly all cases of SCC is in coordination with the results obtained by other studies[16, 39]. The unmethylated bands may have been detected due to the presence of normal cells, inflammatory cell infiltrates and nonmethylated alleles of cells in tumor tissue blocks. In the present study, it was noted that 1 out of 50 cases showed negative unmethylated band and positive methylated band. This observation may be attributed to using paraffin block containing large proportion from cancer tissue that is contain too little stromal cells to be detected by MSP technique.

In the present study, it was also noted that 14 out of these 49 cases showed both methylated and unmethylated bands. This finding is in coordination with results of other studies[16,39]. The methylated and unmethylated bands may have been detected in the same case because of the presence of methylated gene in cancer cells in addition to unmethylated gene in normal cells, inflammatory cell infiltrates and nonmethylated alleles of cancer cells in the same tumor tissue block.

Statistically in the present study, aberrant RASSF1A promoter methylation in OSCC was found to be significantly increased in relation to normal mucosa (p-value 0.045<0.05). This observation suggested that RASSF1A promoter methylation seems to play an important role in OSCC pathogenesis. This is in coordination with many previous studies [15,16,19]. Rarely, other studies reported that RASSF1A plays an important role in the development of cancer but is less important in HNSCC[18, 25]. The contradiction between these studies may reflect the known differences in the disease or genes other than RASSF1A may be of greater importance in disagreement studies.

In this study, RASSF1A promoter methylation was correlated to OSCC histopathological subgroups. It was found that the promoter methylation of RASSF1A in well, moderately and poorly differentiated OSCC present in 2 out of 16 (12.5%), 7 out of 19 (36.8%) and 6 out of 15 (40%) of their cases respectively while none of normal oral mucosa cases showed methylation. Statistically, the increase of RASSF1A promoter methylation in well-differentiated OSCC in relation to normal mucosa was not significant (p-value 0.244>0.05).

On the other hand, moderately and poorly differentiated OSCC showed significant increase of RASSF1A promoter methylation in relation to normal mucosa (p-value 0.021 and 0.027, respectively). These observations indicated the presence of significant increase of RASSF1A promoter methylation with the decrease of cell differentiation in histopathological grades especially the poorly differentiated OSCC that is, according to available knowledge in English literatures, not discovered before in studies restricted to OSCC. However, previous HNSCC studies reported similar observations[22, 40].

In the present study, 20% of the OSCC cases (10/50) showed positive lymph node metastasis and eight out of these 10 cases showed RASSF1A promoter methylation. Furthermore, 6 of the above mentioned 8 cases contain higher number of involved lymph nodes which mean that there was direct correlation not only between methylation and the lymph nodes metastasis but also with number of involved lymph nodes. Statistically RASSF1A promoter methylation in OSCC was found to be significantly correlated to lymph nodes metastasis and number of involved lymph nodes (p-value 0.00 and 0.002 respectively). These results are in consistence with previous study [32]. The relation between RASSF1A promoter methylation and lymph nodes metastasis could be explained by previous study results which reported that knocking down of RASSF1A results in reduced cell-cell adhesion [41] and RASSF1A inactivation by methylation may be involved in cell motility and migration control[42]. Accordingly, RASSF1A methylation may play a role in metastasis of OSCC.

In the present study, it was found that 11 out of 15 (73.3 %) of positive methylated cases located in age between 61 and 70 years old. This was in agreement with Zhou et al[38] who found that the prevalence of RASSF1A promoter methylation of ESCC patients over 50 years old were higher than those younger than 50 years old. This relation can be attributed to longer exposure time to environmental risk factors and indicated the impact of age on RASSF1A promoter methylation in OSCC. In summary, RASSF1A gene methylation showed highly significant association with OSCC and lymph nodes metastasis. It showed also highly significant association with advancement in OSCC histopathological sub groups. Therefore, it was indicated that RASSF1A gene methylation could be important in OSCC carcinogenesis, metastasis, diagnostic biomarkers and prediction of grade. Being reversible, RASSF1A promoter methylation could be a promising in discovering therapeutic alternatives for OSCC.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M et al: Cancer incidence and mortality worldwide: sources, methods and major patterns in globocan 2012. Int J Cancer 2015; 136(5):359-86.
2. El-Mofty S: Early detection of oral cancer. Egypt J Oral Maxillofac Surg 2010; 1:25-31.
3. Feller L, Lemmer J: Oral squamous cell carcinoma: epidemiology, clinical presentation and treatment. J Cancer Ther 2012; 3:263-68.
4. Taghavi N, Yazdi I: Prognostic factors of survival rate in oral squamous cell carcinoma: clinical, histologic, genetic and molecular concepts. Arch Iran Med 2015; 18(5):314-19.
5. Geum D, Roh Y, Yoon S, Kim H, Lee J, Song J et al: The impact factors on 5-year survival rate in patients operated with oral cancer. J Korean Assoc Oral Maxillofac Surg 2013; 39(5):207-16.
6. Tsai H, Baylin S: Cancer epigenetics: linking basic biology to clinical medicine. Cell Res 2011; 21(3):502-17.
7. Alokail M, Alenad A: DNA Methylation. In: Gunduz M: A concise review of molecular pathology of breast cancer. InTech. 2015; ch2:27-56.
8. Grawenda A, Oneill E: Clinical utility of RASSF1A methylation in human malignancies. Br J Cancer 2015; 113(3):372-81.
9. Shi H, Wang M, Caldwell C: CpG islands: their potential as biomarkers for cancer. Expert Rev Mol Diagn 2007; 7(5):519-31.
10. Gasche J, Goel A: Epigenetic mechanisms in oral carcinogenesis. Future Oncol 2012; 8(11):1407-25.
11. Mascolo M, Siano M, Ilardi G, Russo D, Merolla F, De Rosa G et al: Epigenetic disregulation in oral cancer. Int J Mol Sci 2012; 13(2):2331-53.
12. Schubbert S, Shannon K, Bollag G: Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer 2007; 7(4):295-308.
13. Dammann R, Li C, Yoon J, Chin P, Bates S, Pfeifer G: Epigenetic inactivation of a RAS association domain family protein from the lung tumor suppressor locus 3p21.3. Nat Genet 2000; 25(3):315-19.
14. Akino K, Toyota M, Suzuki H, Mita H, Sasaki Y, Ohe-Toyota M et al: The Ras effector RASSF2 is a novel tumor-suppressor gene in human colorectal cancer. Gastroenterology 2005; 129(1): 156-69.
15. Meng R, Li Y, Chen X, Huang Y, Shi H, Du D et al: Aberrant methylation of RASSF1A closely associated with HNSCC, a meta-analysis. Sci Rep 2016; 6:256-64.
16. Tran T, Liu Y, Takagi M, Yamaguchi A, Fujii H: Frequent promoter hypermethylation of RASSF1A and p16INK4a and infrequent allelic loss other than 9p21 in betel-associated oral carcinoma in a Vietnamese non-smoking/non-drinking female population. J Oral Pathol Med 2005; 34(3):150-56.
17. Huang K, Huang S, Chen I, Liao C, Wang H, Hsieh L: Methylation of RASSF1A, RASSF2A, and HIN-1 is associated with poor outcome after radiotherapy, but not surgery, in oral squamous cell carcinoma. Clin Cancer Res 2009; 15(12):4174-80.
18. Noorlag R, van Kempen P, Moelans C, de Jong R, Blok L, Koole R et al: Promoter hypermethylation using 24-gene array in early head and neck cancer: better outcome in oral than in oropharyngeal cancer. Epigenetics 2014; 9(9):1220-27.

19. Xu C, Ten B, LI C, Jin C: Promoter methylation of RASSF1A gene in laryngeal squamous cell carcinoma and protein expression. J Jilin University 2006; 32(2):326-29.

20. Labib N, El-Raghi H, Shoman T, Othman F: Epidemiology of oral and pharyngeal cancer at the National Cancer Institute, Cairo. Med J Cairo Univ 2012; 80(1):85-91.

21. Al-Jaber A, Al-Nasser L, El-Metwally A: Epidemiology of oral cancer in Arab countries. Saudi Med J 2016; 37(3):249-55.

22. Guo W, Cui L, Wang C, Guo Y, Shen S, Kuang G et al: Decreased expression of RASSF1A and up-regulation of RASSF1C is associated with esophageal squamous cell carcinoma. Clin Exp Metastasis 2014; 31(5):521-33.

23. Lo P, Xie D, Chan K, Xu F, Kuzmin I, Lerman M et al: Reduced expression of RASSF1A in esophageal and nasopharyngeal carcinomas significantly correlates with tumor stage. Cancer Lett 2007; 257(2):199-205.

24. Mirza S, Hadi N, Akram S, Noor-ul-Wahab A, Akram Z: Histopathological predictors of nodal metastases in oral squamous cell carcinoma. Pak J Mid Dent 2016; 5(3):12-16.

25. Koutsimpelas D, Pongsapich W, Heinrich U, Mann S, Mann W, Brieger J: Promoter methylation of MGMT, MLH1 and RASSF1A tumor suppressor genes in head and neck squamous cell carcinoma: pharmacological genome demethylation reduces proliferation of head and neck squamous carcinoma cells. Oncol Rep 2012; 27(4):1135-41.

26. Steinmann K, Sandner A, Schagdarsurengin U, Dammann R: Frequent promoter hypermethylation of tumor-related genes in head and neck squamous cell carcinoma. Oncol Rep 2009; 22(6):1519-26.

27. Righini C, Fraipont F, Timsit J, Faure C, Brambilla E, Reyt E et al: Tumor-specific methylation in saliva: a promising biomarker for early detection of head and neck cancer recurrence. Clin Cancer Res 2007; 13(4):1179-85.

28. Paluszczak J, Misiak P, Wierzbicka M, Wozniak A, Baer-Dubowska W: Frequent hypermethylation of DAPK, RARbeta, MGMT, RASSF1A and FHIT in laryngeal squamous cell carcinomas and adjacent normal mucosa. Oral Oncol 2011; 47(2):104-07.

29. Laytragoon-Lewin N, Chen F, Castro J, Elmberger G, Rutqvist L, Lewin F et al: DNA content and methylation of p16, DAPK and RASSF1A gene in tumor and distant, normal mucosal tissue of head and neck squamous cell carcinoma patients. Anticancer Res 2010; 30(11):4643-48.

30. Su P, Huang W, Wu H, Wu C, Liu T, Kao S: p16(INK4A) promoter hypermethylation is associated with invasiveness and prognosis of oral squamous cell carcinoma in an age dependent manner. Oral Oncol 2010; 46(10):734-39.

31. Wong M, Tao Q, Fu L, Wong K, Qiu G, Law F et al: Aberrant promoter hypermethylation and silencing of the critical 3p21 tumour suppressor gene, RASSF1A, in Chinese oesophageal squamous cell carcinoma. Int J Oncol 2006; 28(3):767-73.

32. Fendri A, Masmoudi A, Khabir A, Sellami-Boudawara T, Daoud J, Frikha M et al: Inactivation of RASSF1A, RARβ2 and DAP-kinase by promoter methylation correlates with lymph node metastasis in nasopharyngeal carcinoma. Cancer Biol Ther 2009; 8(5):444-51.

33. Yang X, Dai W, Kwong D, Szeto C, Wong E, Ng W et al: Epigenetic markers for noninvasive early detection of nasopharyngeal carcinoma by methylation-sensitive high resolution melting. Int J Cancer 2015; 136(4):127-35.

34. Zhang C, Zhao Y, Xia R, Han J, Wang B, Tian Z et al: RASSF1A promoter hypermethylation is a strong biomarker of poor survival in patients with salivary adenoid cystic carcinoma in a Chinese population. PLoS One 2014; 9(10):1-9.

35. Van der Weyden L, Arends M, Dovey O, Harrison H, Lefebvre G, Conte N et al: Loss of RASSF1A cooperates with Apc (Min) to accelerate intestinal tumourigenesis. Oncogene 2008; 27(32):4503-08.

36. Degli D, Sklias A, Lima S, Beghelli S, Cahais V, Fernandez N et al: Unique DNA methylation signature in HPV-positive head and neck squamous cell carcinomas. Genome Med 2017; 9(1):33-44.

37. Canovas S, Ivanova E, Romar R, Garcia-Martinez S, Soriano-Ubeda C, Garcia-Vazquez F at al: DNA methylation and gene expression changes derived from assisted reproductive technologies can be decreased by reproductive fluids. E life 2017; 6:1-24.

38. Zhou S, Cui J, Fan Z, Li X, Li J, Liu B et al: Polymorphism of A133S and promoter hypermethylation in Ras association domain family 1A gene (RASSF1A) is associated with risk of esophageal and gastric cardia cancers in Chinese population from high incidence area in northern China. BMC Cancer 2013; 13:259-69.

39. Kuroki T, Trapasso F, Yendamuri S, Matsuyama A, Alder H, Mori M et al: Promoter hypermethylation of RASSF1A in esophageal squamous cell carcinoma. Clin Cancer Res 2003; 9(4):1441-45.

40. Hogg R, Honorio S, Martinez A, Agathanggelou A, Dallol A, Fullwood P et al: Frequent 3p allele loss and epigenetic inactivation of the RASSF1A tumour suppressor gene from region 3p21.3 in head and neck squamous cell carcinoma. Eur J Cancer 2002; 38(12):1585-92.

41. Dallol A, Agathanggelou A, Tommasi S, Pfeifer G, Maher E, Latif F: Involvement of the RASSF1A tumor suppressor gene in controlling cell migration. Cancer Res 2005; 65(17):7653-59.

42. Donninger H, Vos M, Clark G: The RASSF1A tumor suppressor. J Cell Sci 2007; 120(18):3163-72.