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Comparison of Methylation and Expression Profile of MOB-1A in Blood, Saliva and Tissue of Patients with Oral Squamous Cell Carcinoma (OSCC) and Precancerous Patients

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Abstract

Background: Oral Squamous Cell Carcinoma (OSCC) is the most common form of malignancy of head and neck zone and the sixth most common type of cancer in the world, leading to 8000 death per year. This cancer affects lips, palate and tongue and the average survival rate of patients is 5 years. The most important risk factors of OSCC are alcohol and tobacco and mostly middle aged males are affected. The hippo signaling pathway is a tumor suppressor pathway which regulates tissue growth *via* balancing cell proliferation, death and differentiation. So, dysregulation of this pathway is the most common feature of solid tumors.

Methods: In this research, methylation status of MOB1A gene promoter in blood and saliva DNA of 20 healthy controls and 20 patients with precancerous lesions of oral cavity as well as 20 tissue DNA and 12 blood and saliva DNA of OSCC patients were compared with each other by methylation-specific PCR technique. Furthermore, expression of MOB1A was conducted on saliva cDNA of 12 healthy controls, 12 patients with precancerous lesions as well as saliva and tissue cDNA of 12 patients with OSCC by real time PCR technique.

Results: Our results showed that, among all of the investigated conditions, there was only one significant difference in the methylation status of MOB1A in precancerous patients' blood and OSCC tissue (P-value: 0.025). Moreover, there was no significant difference in the expression profile of MOB1A in any of the investigated conditions between three groups.

Conclusion: From the significant difference observed in the methylation status of MOB1A in precancerous patients' blood and OSCC tissue, we may be able to conclude that type of the investigated sample (*i.e.* blood, saliva or tissue) can affect the methylation status of MOB1A gene. Moreover, treating patients with oral cavity disorders in the precancerous stage can help us to find biomarker genes involved in the progress of the disease and prevent these patients from moving toward OSCC.

Keywords: OSCC; Precancerous lesions; Hippo signaling pathway; Expression; Methylation; MOB1A

Introduction

Oral Squamous Cell Carcinoma (OSCC) is considered as the most common type of oral cavity malignancy and the sixth most common type of cancer all over the world [1]. OSCC leads to ~ 8000 death per year and the average 5 years survival for it is ~ 50% [2].

Precancerous lesions of oral mucosa, known as potentially malignant disorders, include a group of disease which should be diagnosed in the early stage [3]. Oral leukoplakia, Oral Lichen Planus (OLP), oral sub mucous fibrosis and oral erythroplakia are some of the common oral mucosal diseases with a very high malignancy rate [4]. Oral leukoplakia, a white lesion in the mucosa of the oral cavity, represents the most common precancer lesion of OSCC with prevalence between 0.1-0.5% [5]. OLP is known as a chronic inflammatory disease affecting the oral mucosa. Most common features of the disease consist of a dense sub-epithelial lymphocytes infiltration, increased number of intraepithelial lymphocytes and the degeneration of basal keratinocytes [6].

The hippo pathway is a serine/threonine kinase cascade that controls cell number and organ size by restricting cell proliferation and promoting apoptosis [7]. This signaling pathway has recently been recognized as a master regulator of the malignant progression of many cancers by regulating cell proliferation and stem/progenitor-cell expansion [8]. MOB1A is a component of hippo pathway that binds to LATS1/2 and may function as tumor suppressor in human cancer cells [9]. Once activated, LATS1/2 phosphorylates YAP. Phosphorylated YAP is sequestered in the cytoplasm *via* 14-3-3 protein, preventing it from entering the nucleus. When this kinase cascade is inactivated,

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YAP remains unphosphorylated, translocates into the nucleus and activates transcription of target genes [10].

The aberrant DNA promoter methylation that influences gene expression is a common feature of many human cancers [11]. So, the present study is trying to compare the methylation and expression profile of MOB1A gene in blood, saliva and tissue samples of healthy controls as well as high risk (precancerous patients) and OSCC patients.

Materials and Methods

Samples and DNA preparation

In this study, subjects included healthy controls, high risk and OSCC patients. In control group, blood (3 cc) and saliva (5 ml) samples were collected from 20 persons who were free from any oral cavity diseases after explanation of study purpose and signing of consent form (Mean age: 58.70 ± 10.38). 20 patients with precancerous lesions of oral cavity (15 oral lichen planus and 5 oral leukoplakia patients) composed high risk group and blood as well as saliva samples were collected from them (Mean age: 48.95 ± 11.93). In OSCC group, 20 paraffin-embedded tissues were collected from patients referred to oral disease diagnosis department (Mean age: 60.55 ± 15.37). Since 8 patients of 20 investigated OSCC patients were dead, blood and saliva samples were collected only from 12 OSCC patients. Clinico-pathological data of the three investigated groups such as age, sex and clinical stage are shown in Table 1. Genomic DNA was isolated from blood, tissue and saliva samples by salting out and phenol-chloroform methods and then its quality was estimated by spectrophotometer.

		Control (N=20)	Precancerous (N=20)	OSCC (N=20)	P-value
Gender	Male	13	9	10	
	Female	7	11	10	1
Age average		58.70 ± 10.38	48.95 ± 11.93	60.55 ± 15.37	0.177
Sample	Blood	20	20	12	-
	Saliva	20	20	12	-
	Tissue	-	-	20	-
Precancerous lesion	OLP	-	15	-	-
	Leukoplakia	-	5	-	-
Grade(OSCC)	One	-	-	12	-
	Two	-	-	6	-
	Three	-	-	2	-
Addiction	Yes	-	8	11	-
	No	20	12	9	-
Familial history	Yes		2	2	-
	No	20	18	18	-

Table 1: Clinic pathological and demographic characteristics of the investigated groups.

Methylation Specific PCR (MSP)

The process of bisulfit modification of DNA samples was performed using promega wizard DNA clean-up system (Cat No.A7280, promega) according to the manufacturer's instructions. Comparison of methylation status of the promoter regions of MOB-1A in blood, saliva and tissue samples of three investigated groups was determined by Methylation Specific PCR (MSP) using methylated specific and unmethylated specific primers designed at CPG sites of the promoter region using mat prime online software (Table 2). Accu power hot start PCR premix tubes were used for PCR reaction. The PCR reaction mixture included 5 µl of modified template DNA, 1 µl of each primer and 13 µl of nuclease free water in total volume of 20 µl. MSP amplification was performed as follows: 95°C for 10 min, and then 40 cycles consisting of (40 s at 95°C, 30s at 52°C for MOB1A (M), 55°C(U) and 30 s at 72°C), and a final extension at 72°C for 10 min. PCR products were loaded onto 3% agarose gel.

Genes	Sequences (5'→3')	Product size	Annealing temp (°C)
MOB1A-M	F: GCGAACTAAAATTTCGCTACG	153	52

	R: GTTATTGTTTTTTTCGTAGGATCGT		
MOB1A-U	F: TCTCACAAACTAAAATTTCACTACACC	156	55
	R: TTATTGTTTTTTTTGTAGGATTGT		

Table 2: Methylation-specific PCR primer sequences and annealing temperatures.

Gene expression analysis

Total RNA was extracted from saliva samples of control and high risk groups as well as saliva and tissue samples of OSCC patients using parstous total RNA extraction kit (Cat. No.A101231) according to the manufacturer's instructions. The cDNA synthesis kit (vivantis, Cat.No.RTPL12) was used to reverse-transcribe 1 µg of RNA in a final volume of 20 µl. As an internal standard, RNA18s was used. Real time PCR of MOB1A was performed using the primers and annealing temperatures according to Table 3. Cycle Threshold (CT) at which the fluorescence for the reaction well crosses was recognized in all samples and then, normalized CT (CT target gene/CT housekeeping gene) was used for comparison of gene expression between samples and groups.

Genes	Sequences (5'→3')	Product size	Annealing temp (°C)
MOB1A	F: CAGCAGCCGCTCTTCTAAAAC	134	58
	R: CCTCAGGCAACATAACAGCTTG		

Table 3: Real-time PCR primer sequences and annealing temperatures.

Statistical analysis

Data were analyzed using SPSS16 software. Analysis of relative gene expression between saliva samples of three groups and tissue samples of OSCC patients was done by Mann-Whitney test. The significance level was set at $p \leq 0.05$ for all tests.

Results

Promoter methylation of MOB-1A

In this study, 10 different situations of promoter methylation status of MOB1A were compared with each other in blood, saliva and tissue samples of three investigated groups: Comparison of methylation status in blood of high risk and control groups (P-value;0.089) comparison of methylation status in saliva of high risk and control groups (P-value;0.849) comparison of methylation status in blood of control and OSCC patients (P-value; 0.996) comparison of methylation status in saliva of OSCC and control groups (P-value; 0.553) comparison of methylation status in blood of OSCC and high risk patients (P-value; 0.085,) comparison of methylation status in saliva of OSCC and high risk patients (P-value; 0.470) comparison of methylation status in blood of control group and OSCC tissue (P-value; 0.376,) comparison of methylation status in saliva of control group and OSCC tissue (P-value; 0.278) comparison of methylation status in high risk saliva samples and OSCC tissue (P-value; 0.754). As it is clear from the results, there wasn't any significant difference between the comparisons of methylation status in any of the aforementioned situations. The only comparison that showed a significant difference was comparison of methylation status of MOB1A gene in blood of high risk patients and OSCC tissue (P-value: 0.025) (Table 4).

MOB1A	Precancer blood	OSCC tissue	P-value
Unmethyl	14 (70%)	5 (25%)	Refrence
Methyl	2 (10%)	12 (60%)	0.025
Methyl/Unmethyl	4 (20%)	3 (15%)	0.879

Table 4: Comparison of methylation status of MOB1A between blood of precancerous group and OSCC tissue.

MOB1A RNA levels

Assessment of relative gene expression of MOB1A in saliva samples of three investigated groups as well as tissue samples of OSCC patients was done by dividing target gene CT to housekeeping gene CT in 6 different comparative cases as follows: Comparison of gene expression in saliva samples of high risk and control groups (P-value; 0.068), comparison of gene expression in saliva samples of OSCC and control groups (P-value; 0.078), comparison of gene expression in saliva samples of OSCC and high risk patients (P-value; 0.84), comparison of gene expression in saliva samples of control group and OSCC tissue (P-value; 0.713), comparison of gene expression in saliva samples of high risk group and OSCC tissue (P-value; 0.219), comparison of gene expression in saliva and tissue samples of OSCC patients (P-value; 0.128). According to these results, there was not any significant difference in relative gene expression in any of the comparative cases.

Discussion

Epigenetic is the study of heritable variations that interfere with gene function without modifying the DNA sequences [12]. Realizing epigenetic mechanisms, including DNA methylation and chromatin remodeling, leads to rapid progress in diagnosis and treatment of various diseases [13]. The results of methylation status comparison in this study indicated statistically, significant difference in the amount of MOB1A promoter methylation between blood of high risk patients and OSCC tissue. As we also compared the methylation status of blood samples of these groups as well as their saliva samples, simultaneously, we are able to conclude that type of the investigated sample (*i.e.* blood, saliva or tissue) may affect the methylation status of MOB1A gene. Furthermore, the significant difference in MOB1A methylation status between high risk and OSCC patients shows that if we try to treat high risk patients in the early stages of the disease, we may be able to detect biomarker genes involved in the progress of the disease and prevent these patients from moving toward OSCC. The growing network of MOBs and MOB interacting proteins suggest that modulation of MOB levels could provide a means of controlling the availability of specific regulatory nodes within a cell or tissue [14]. Other studies have also highlighted the aberrant mutations of MOB1A in different cancers. Kosaka have reported that MOB1A is mutated in melanoma and breast cancer cell lines and downregulated in human colorectal, no small cell lung and skin cancer [15]. According to Zhoud (2009) impaired MOB1A phosphorylation occurs in 81% of human liver cancers [16]. Methylation studies have also confirmed the role of MOB1A as a tumor suppressor gene in human cancers. Shen indicated that treatment of human T-cell Acute Lymphoid Leukemia (T-ALL) cells with 3-deazaneplanocin A (DZNep) will significantly reduce histone methylation of MOB-1A promoter in T-ALL cells [17].

Conclusion

Different studies suggest that various factors such as genetic, epigenetic and environmental elements are involved in the progress of OSCC. OSCC is the most common type of malignancy of the oral cavity and is often diagnosed only in advanced stages. So, identification of genetic and epigenetic alterations, especially in the precancerous stages of the disease, can be effective in the early diagnosis and prevention of the disease development.

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