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Analysis of methylation and expression profile of MOB1A in blood, saliva, and tissue of patients with oral squamous cell carcinoma (OSCC) and precancerous patients

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Background: Oral squamous cell carcinoma (OSCC) is considered the most common type of oral cavity malignancy worldwide. OSCC accounts for more than 90% of all oral neoplasms. This cancer affects the 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 that regulates tissue growth via balancing cell proliferation, death, and differentiation. MOB1A is a component of the hippo pathway that binds to Lats1/2 and may function as a tumor suppressor in human cancer cells. So, deregulation of the hippo pathway is the most common feature of solid tumors. Methods: In this research, 10 different cases of MOB1A promoter methylation status were compared with each other regarding 20 healthy controls (blood and saliva samples), 20 high-risk patients (blood and saliva samples), and OSCC patients (20 tissue, 12 blood, and saliva samples). Furthermore, the expression profile of MOB1A was investigated in the saliva cDNA of 12 healthy controls, and 12 high-risk patients 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, the only status that confirmed a significant difference was a comparison of MOB1A methylation status in high-risk group blood and OSCC group tissue (p-value: 0.025). Moreover, there was no significant difference in the expression profile of MOB1A in any of the studied conditions between the 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 the methylation status of the gene can be different in various samples. Moreover, it may be extracted from our findings that expression levels of MOB1A in the three aforementioned groups are not affected by various types of samples.

Introduction

Oral squamous cell carcinoma (OSCC) is considered as the most common type of oral cavity malignancy worldwide (Chen, et al., 2015). OSCC accounts for more than 90% of all oral neoplasms; therefore it is often used interchangeably with oral cancers (Choi et al.2008). The growth of OSCC is a multi-step process including a combination of genetic and environmental risk factors (Chien, et al., 2013).

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Precancerous lesions of the oral mucosa, known as potentially malignant disorders, include a group of diseases that should be diagnosed in the early stage (Yardimci, et al., 2014). 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 (Shirani, et al., 2014). Oral leukoplakia, a white lesion in the mucosa of the oral cavity, represents the most common precancer lesion of OSCC with a prevalence between 0.1-0.5% (Messadi2013).OLP is known as a chronic inflammatory disease affecting the oral mucosa. The most common features of the disease consist of dense sub-epithelial lymphocyte infiltration, increased number of intraepithelial lymphocytes, and the degeneration of basal keratinocytes (Tatulla, M., et al., 2015).

The hippo pathway is a serine/threonine kinase cascade that controls cell number and organ size by restricting cell proliferation and promoting apoptosis (Abylkassov, et al., 2016). 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(Kim et al.2019).MOB1A is a component of the hippo pathway that binds to Lats1/2 and may function as a tumor suppressor in human cancer cells(Chow et al.2010). Once activated, Lats1/2 phosphorylates YAP (Yes-associated protein). Phosphorylated YAP is sequestered in the cytoplasm via 14-3-3 protein, preventing it from entering the nucleus. When this kinase cascade is inactivated, YAP remains unphosphorylated, translocates into the nucleus, and activates transcription of target genes involved in Apoptosis, differentiation, and cell cycle control (Baron et al. 2014).

The aberrant DNA promoter methylation that influences gene expression is a common feature of many human cancers (Speel, E.J., 2017). Oncogenes and tumor suppressor genes are the two major groups that promote tumor progression whenever up-regulated or down-regulated, respectively (Kordi Tamandani, et al., 2016). The downregulation of tumor suppressor genes is preceded by various epigenetic modifications, mutations, loss of heterozygosities, and deletions (Perez-Sayans, et al.,2009). The present study aims to compare the methylation and expression profile of the MOB1A gene in blood, saliva, and tissue samples of healthy controls as well as high-risk (precancerous patients) and OSCC patients.

Materials and Methods

Subjects

Subjects investigated in this study include three groups: healthy controls, high-risk, and OSCC patients. In the control group, blood (3cc) and saliva (5ml) samples were collected from 20 persons who were free from any oral cavity (Mean age: 58.70±10.38). It should be mentioned that members of the control group were examined by the physician and their healthiness from any oral cavity disorders was approved. 20 patients with precancerous lesions of the oral cavity (15 oral lichen planus and 5 oral leukoplakia patients) composed of high-risk groups and blood as well as saliva samples were collected from them(Mean age:48.95±11.93). In the OSCC group, 20 paraffin-embedded tissues were collected from patients referred to the oral disease diagnosis department (Mean age: 60.55±15.37). Since 8 patients of the 20 investigated OSCC patients were dead, blood and saliva samples were collected only from 12 OSCC patients. Clinical-pathological data of the three investigated groups such as age, sex, and the clinical stage is shown in Table 1. The institutional review board approved this study and all participants confirmed the consent form.

Table1.Clinical-pathological and demographic characteristics of the investigated groups.

		Control (N=20)	Precancerous (N=20)	OSCC (N=20)
Gender	male	13	9	10
	female	7	11	10
Age Average		58.70± 10.38	48.95±11.93	60.55± 15.37
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

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DNA Extraction

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.

Methylation-specific PCR (MSP)

The process of bisulfite modification of DNA samples was performed using the Promega Wizard DNA clean-up system (Cat No.A7280, Promega) according to the manufacturer's instructions. Comparison of the methylation status of MOB1A promoter regions 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 Meth Prime online software (Table2). 5µl of bisulfite-modified DNA and 1 µl of each primer were added to each AccuPower® Hot Start PCR Premix tube which contained lyophilized PCR master mix; Taq DNA Polymerase, dNTP, reaction buffer, tracking dye, and patented stabilizer, then the reaction reached to a final volume of 20 µl using nuclease-free double-distilled water.MSP reactions were subjected to an initial incubation at 95°C for 10 min, followed by 40 cycles (95°C for 30s, the annealing temperature for MOB1A-methylated: 52°C and MOB1A-unmethylated: 55°C for 40s and extension at 72°C for 30s). Final incubation was completed at 72°C for 10 min. PCR products were loaded onto 3% agarose gel.

Table2.Methylation –Specific PCR primer sequences and annealing temperatures.

Genes	Sequences(5'→3')	Product size	Annealing Temp(°C)
MOB1A-methylated	F:GCGAACTAAAATTTCGCTACG R:GTTATTGTTTTTTTCGTAGGATCGT	153	52
MOB1A-unmethylated	F:TCTCACAAACTAAAATTTCACTACACC R:TTATTGTTTTTTTTGTAGGATTGT	156	55

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 a parstous total RNA extraction kit (Cat. No.A101231). The cDNA synthesis kit (vivantis, Cat.No.RTPL12) was used to reverse-transcribe 1µg of RNA in a final volume of 20 µl.

MOB1A Real-Time PCR reaction was performed using primers and annealing temperatures mentioned in Table 3 by SYBR green method. Cycle threshold (CT) at which the fluorescence for the reaction well crosses was recognized in all samples. Normalized CT (CT target gene/CT housekeeping gene) was used for the comparison of gene expression between samples and groups. The Real-time PCR data were normalized by 18sRNA.

Table3.Real-Time PCR primer sequences and annealing temperatures.

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

Statistical analysis

Statistical analyses were performed using SPSS version 16. The methylation status of MOB1A and the risk of OSCC development through promoter methylation were assessed using Logistic Regression. Analysis of relative gene expression between saliva samples of three groups and tissue samples of OSCC patients was done by Mann-Whitney test. The $p \leq 0.05$ was considered statistically significant.

Results

Promoter methylation of MOB1A

In this study, 10 different cases of MOB1A promoter methylation status were compared with each other in blood, saliva, and tissue samples of three investigated groups (tables 4-12).

The only status that showed a significant difference was a comparison of the MOB1A methylation profile in high-risk group blood and OSCC group tissue (p-value: 0.025, see Table 13).

Tables 4-12. Different comparisons of MOB1A promoter methylation status.

MOB1A	Control group blood	High Risk group blood	OR	95% CI	p-value
Unmethylated	14(70%)	14(70%)	_	_	Reference
Methylated	4(20%)	2(10%)	0.073	0.004 - 1.496	0.089
Methylated/ Unmethylated	2(10%)	4(20%)	0.167	0.015 - 1.872	0.147

MOB1A	Control group saliva	High risk group saliva	OR	95%CI	p-value
Unmethylated	7(35%)	9(45%)	_	_	Reference
Methylated	8(40%)	8(40%)	1.204	0.178-8.143	0.849
Methylated/ Unmethylated	5(25%)	3(15%)	1.882	0.319-11.305	0.485

MOB1A	Control group blood	OSCC group blood	OR	95%CI	p-value
Unmethylated	14(70%)	3(25%)	_	_	Reference
Methylated	4(20%)	7(55.33%)	1.007	0.045-22.304	0.996
Methylated/ Unmethylated	2(10%)	2(16.66%)	0.117	0.005-2.800	0.185

MOB1A	Control group saliva	OSCC group saliva	OR	95%CI	p-value
Unmethylated	7(35%)	1(8.33%)	_	_	Reference
Methylated	8(40%)	9(75%)	1.955	0.213-17.913	0.553
Methylated/ Unmethylated	5(25%)	2(16.66%)	0.304	0.012-7.804	0.472

MOB1A	High Risk group saliva	OSCC Group saliva	OR	95% CI	p-value
Unmethylated	9(45%)	1(8.33%)	_	_	Reference
Methylated	8(40%)	9(75%)	0.360	0.023-5.744	0.470
Methylated/ Unmethylated	3(15%)	2(16.66%)	0.078	0.003-1.955	0.121

MOB1A	Control group blood	OSCC group tissue	OR	95% CI	p-value
Unmethylated	14(70%)	5(25%)	_	_	Reference
Methylated	4(20%)	12(60%)	5.236	0.134-204.66	0.376
Methylated/ Unmethylated	2(10%)	3(15%)	0.404	0.012-13.703	0.614

MOB1A	Control Group saliva	OSCC group tissue	OR	95%CI	p-value
Unmethylated	7(35%)	5(25%)	_	_	Reference
Methylated	8(40%)	12(60%)	4.104	0.319-52.75	0.278
Methylated/ Unmethylated	5(25%)	3(15%)	3.461	0.215-55.77	0.381

MOB1A	High risk group saliva	OSCC group tissue	OR	95%CI	p-value
Unmethylated	9(45%)	5(25%)	_	_	Reference
Methylated	8(40%)	12(60%)	1.476	0.129-16.81	0.754
Methylated/ Unmethylated	3(15%)	3(15%)	1.058	0.080-14.00	0.966

Table13. Comparison of MOB1A methylation status between high-risk group blood and OSCC group tissue.

MOB1A	High risk group blood	OSCC group tissue	OR	95% CI	p-value
Unmethylated	14(70%)	5(25%)	_	_	Reference
Methylated	2(10%)	12(60%)	29.066	1.520-555.75	0.025
Methylated/ Unmethylated	4(20%)	3(15%)	0.843	0.093-7.653	0.879

OR: odds ratio, 95%CI: 95% confidence interval

Table14.Six different comparative assessments of MOB1A relative gene expression.

	MOB1A comparative expression assessments	Mean± Standard Deviation	p-value
1	Control group saliva	1.55±0.618	0.068
	High-risk group saliva	1.22±0.216	
2	Control group saliva	1.55±0.618	0.078
	OSCC group saliva	1.18±0.108	
3	High-risk group saliva	1.22±0.216	0.843
	OSCC group saliva	1.18±0.108	
4	Control group saliva	1.55±0.618	0.713
	OSCC group tissue	1.38±0.350	
5	High-risk group saliva	1.22±0.216	0.219
	OSCC group tissue	1.38±0.350	
6	OSCC group saliva	1.18±0.108	0.128
	OSCC group tissue	1.38±0.350	

MOB1A RNA levels

Assessment of MOB1A relative gene expression in saliva samples of three investigated groups as well as tissue samples of OSCC patients was done in 6 different comparative cases (table 14). According to the following results, No significant difference was observed in any of the studied cases.

Discussion

In this study, we compared 10 different statuses of MOB1A promoter methylation in blood, saliva, and tissue samples of control, high-risk, and OSCC groups. Among all of the investigated statuses, the amount of MOB1A promoter methylation between high-risk group blood and OSCC group tissue was significant. As we also compared the methylation status of blood samples of high-risk and OSCC groups as well as their saliva samples, simultaneously, we may be able to conclude that the methylation status of MOB1A can be different in various samples.

The hippo signaling pathway plays an important role in cell growth, apoptosis, and development. Among hippo pathway proteins, the MOB1 family controls the number of cells and size of organs. (Pinosa et al.2013). The two components MOB1A and MOB1B act as tumor suppressors by regulating downstream elements of the hippo-signaling pathway (Kordi Tamandani et al.2022).

The growing network of MOBs and MOB-interacting proteins suggests that modulation of MOB levels could provide a means of controlling the availability of specific regulatory nodes within a cell or tissue (Duhart et al.2020). Other studies have also highlighted the aberrant mutations of MOB1A in different cancers. Kosaka et al., (2007) have reported that MOB1A is mutated in melanoma and breast cancer cell lines and down regulated in human colorectal, nonsmall cell lung, and skin cancer (Kosaka et al.2007). According to Zhoud et al., (2009), impaired MOB1 phosphorylation occurs in 81% of human liver cancers (Zhou et al.2009). Methylation studies have also confirmed the role of MOB1A as a tumor suppressor gene in human cancers. Shen et al., (2015) indicated that treatment of human T-cell acute lymphoid leukemia (T-ALL) cells with 3-deazaneplanocin A (DZNep) will significantly reduce histone methylation of MOB1A promoter in T-ALL cells (Shen, et al.,2015). In the present study, we also tried to compare the expression levels of MOB1A in saliva and tissue samples of control, high-risk, and OSCC groups in six different comparative statuses. None of the studied comparisons clarified a significant difference in the mRNA levels of MOB1A. It may be extracted from our findings that expression levels of MOB1A in the three aforementioned groups are not affected by various types of samples. A study by Rong et al. showed overexpression of MOB1A in most colorectal cancers (Zhang, et al.,1997).

However, little is known about MOB1A methylation and expression profiles in oral cavity cancers. Our study has inspected methylation status and expression levels of MOB1A in a novel comparative way regarding different types of samples and various groups. It may be efficient to expand this study by investigating a high statistical population worldwide to discover new comparative aspects of MOB1A promoter methylation and mRNA levels as well as other genes of the hippo signaling pathway involved in oral cavity cancers regarding different types of samples. Moreover, it is suggested that other types of precancerous lesions of the oral cavity rather than those mentioned here be investigated for obtaining more meticulous results. Our suggestion for future willing researchers is to develop this kind of comparative study among other signaling pathways regarding precancerous and OSCC patients.

Conclusion

Results of the present study demonstrate that the methylation status of MOB1A can be different in various kinds of precancerous and OSCC samples (high-risk group blood and OSCC group tissue in this research) while the expression profile of this gene does not show any significant difference in diverse types of high risk and OSCC samples.

Compliance with ethical standards

Ethical approval

All procedures performed in this study involving human participants were following the ethical standards of the institutional and national research committee and informed consent was obtained from all individual participants included in the study.

Conflicts of interest

None to declare.

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