Quantitative Expression of Cell-Free Salivary Transcriptomes (IL8, IL1β, And H3F3A) in Oral Squamous Cell Carcinoma and Oral Erosive Lichen Planus

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Abstract

Objective: Diagnosis of oral SCC at its earliest stage is crucial for increasing survival rate. Salivary transcriptome meets demands for noninvasive, accessible, and highly efficient diagnostic medium. This study designed to investigate whether quantification of cell-free salivary mRNA of certain genes (IL8, IL1β, H3F3A) is an informative diagnostic tool for early detection of oral SCC and malignant transformation in erosive oral lichen planus.

Methods: The mRNA of salivary transcriptomes of IL8, IL1B, and H3F3A genes were examined in 18 individuals (six oral SCC, six oral lichen planus, and six healthy people). Up to 5 ml of unstimulated saliva was collected from each person. The total RNA was extracted from the cell-free saliva samples and converted to cDNA. The mRNA expression level of the indicated genes was quantified by a qRT-PCR technique using specific primers and SyberGreen reagent. The data obtained from the qRT-PCR reaction was analyzed using the comparative CT (2-ΔΔCT) method. Independent t-test was performed to find the relation between variables. P-value < 0.05 was considered significant.

Results: The results show that the mRNA expression of all the three indicated genes (IL8, IL1B, and H3F3A) was up-regulated in all OSCC and erosive oral lichen planus cases in comparison to normal cases.

Conclusions: This work demonstrates a significant correlation between cell-free salivary mRNA of certain genes and cases of OSCC and oral lichen planus. These findings suggest that detection of salivary mRNA of certain related genes hold promises as future biomarkers for accurate and early diagnosis of oral malignancies.

Keywords: *Salivary transcriptomes, IL8, IL1B, H3F3A, Lichen planus.*

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Introduction

Oral SCC usually arises from a pre-existing potentially malignant disorder[1]. It includes a variety of lesions and conditions like leukoplakia erythroplakia, erosive oral lichen planus, and oral submucous fibrosis[2]. Early detection of OSCC can not only significantly reduce mortality and morbidity but also enable effective intervention and therapy.

Saliva sampling is a noninvasive, simple and low-cost method which does not bother the patient. Besides, saliva is an exceedingly desirable body fluid for clinical applications. It contains numerous biomarkers used for detecting and monitoring of oral and systemic health[3,4] and determining disease stages[5] such as IL-6 and IL-8. Previous studies show that the expression level of IL-6 protein is correlated with the severity of dysplastic changes in leukoplakia[5] while IL-8 protein proved to be an analyte for early OSCC detection[6].

At the molecular level, the quantity of transcriptome (the full range of mRNA) change in relation to health and disease[7-9]. There are about 3,000 mRNAs in cell-free saliva, and only 180 of them are common among healthy subjects[10]. It is worthy to mention that salivary transcriptomic profile reflects those originated in distant diseased tissues as well as transcripts that originated in salivary glands[3]. In a pioneer study that concern the associated of salivary RNAs and OSCC, authors registered that IL-8, SAT, IL-1β and OAZ1 as potential biomarkers for OSCC detection[11]. Later on, another two studies pre-validated these biomarkers and confirmed their feasibility in the discrimination of OSCC patients from control subjects[12,13]. Thus quantitative expression of different salivary transcriptomes makes saliva be considered as a sensitive novel resource that could readily be used in clinical diagnosis.

The present study aims to evaluate the diagnostic value of cell-free salivary transcriptomes of certain genes including IL1β, IL-8 and H3 histone family 3A (H3F3A) as a pre-diagnostic tool of malignant oral disorders. This work is set to be performed via extraction and quantification of the total mRNA of the indicated genes in cell-free saliva using qRT-PCR analysis.

Patients and methods

The study included a study group of 12 non-treated patients; six primary small sized (T1) OSCC and six erosive lichen planus (OLP) admitted to the maxillofacial surgery clinic of Sulaimani Teaching Hospital. Another six healthy volunteers served as a control group. The study obtained ethical approval from the Medical Ethics Committee of the medical college before it started. Each participant signed an informed consent then the demographic, clinical and histopathologic data were recorded.

Saliva mRNA extraction and cDNA synthesis: Unstimulated whole saliva samples were collected as described by Navazesh[14]. Samples were spun down for 15 min at 3500 rpm to pellet any particles including the cells. The total RNA was then extracted from the cell-free saliva using DNA/RNA prep K-2-9 kit (Sacace, Italy) following the manufacturer's instructions. The resultant RNA was eluted in 50 µl of nuclease-free water and tested with a nanodrop machine (FoodALYT photometer, single beam UV/VIS spectrophotometer) to check the purity and concentration of the RNA. The RNA samples were stored at -80°C until use. For cDNA synthesis, a maximum of 100 ng of total RNA was reverse transcribed to cDNA using a GoScript Reverse Transcription System (Promega, USA, # A5001) following the manufacturer's guidelines. The cDNA samples were either directly used for qRT-PCR analysis or stored at -20°C until use.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Specific primers for IL-1B, IL-8, and H3B3A were designed using the primer 3 web-based primer design tool. The specificity of the primers was checked by blasting the primer sequences against the NCBI human transcript database (Table 2). The specificity of the primers was further confirmed by running a small portion of the PCR product on 2% agarose gel to obtain the correct expected bands as shown in Figure 1.

All reactions were performed in triplicate using 96 well plates supplied by Applied Biosystem/USA (MicroAMP Optical 96-well reaction plate 96, #4306737). Each reaction consisted of 12.5 µl of 2X

SYBR Green I Master Mix (Promega, # 04707516001), two µl of each of the forward and reverse primer (10 µM), and five µl of undiluted cDNA template. Reaction volumes were made up to 25 µl with PCR graded water provided by Promega. For each set of reaction, a housekeeping gene such as glyceraldehyde-phosphate dehydrogenase (GAPDH) was used as a loading control. Samples without cDNA templates were also used as negative controls (NTC). Plates were sealed with Applied Biosystem MicroAMP optical adhesive film (#4311971) and run using Applied Biosystems® 7500 Real-Time PCR machine following the standard protocol provided by the manufacturer.

The data obtained from the above reaction was analyzed using the comparative CT (2-ΔΔCT) method as described previously[15,16].

Table 1: PCR primers used to amplify mRNA of *GAPDH, IL8, IL1B, H3F3A* genes by qRT-PCR.

Target genes	Primer sequences
GAPDH	**Forward (**5'- 3'): GTCAAGGCTGAGAACGGGAA **Reverse (**3'-5') : AAATGAGCCCCAGCCTTCTC
IL8	**Forward (**5'- 3'): GGTGCAGTTTTGCCAAGGAG **Reverse (**3'-5'): TTCCTTGGGGTCCAGACAGA
IL1B	**Forward (**5'- 3'): CCACCTCCAGGGACAGGATA **Reverse (**3'-5'): AACACGCAGGACAGGTACAG
H3F3A	**Forward (**5'- 3'): CCAGGAAGCAACTGGCTACA **Reverse (**3'-5'): CAGACGCTGGAAGGGAAGTT

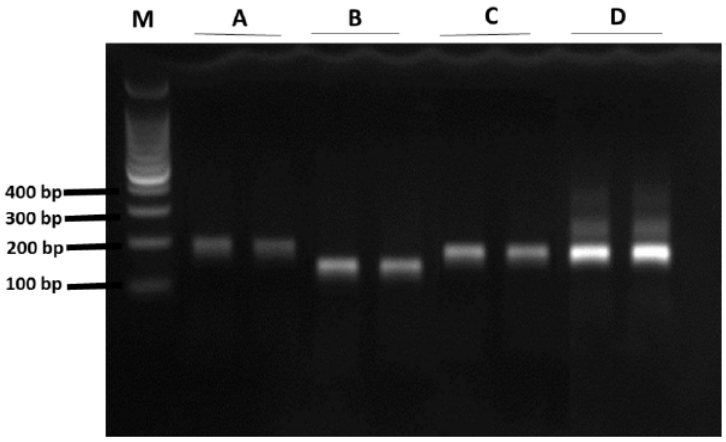

Figure 1: Agarose gel electrophoresis. A small amount (10µl) of RT-PCR product of each gene was run on 2% agarose gel in duplicate. Letter M denotes 100 bp DNA ladder (Promega, # G2101). Letters A-D corresponds to IL8, IL1β, H3B3A and GAPDH genes respectively.

Results

The age ranged between 25 to 61 years for healthy group, 48 to 64 years of OLP patients, and 61 to 79 years for OSCC patients. The data indicate that age in OSCC group 100% of cases were over 60 years old while only 50% of OPMDs cases occurred in people >60 years old (Table 2).

The qRT-PCR analysis revealed that saliva samples derived from erosive OLPs and OSCC contain a significant increase in the mRNA level expression fold of IL8 (both 0.61) and IL1B (1.12 and 0.75 respectively) in comparison to the normal group (0.15 for IL8 and 0.04 for IL1B). Notably, the expression level of both IL8 and IL1B were statistically not differed between both diseased groups. Although, the expression fold of H3B3A also increased in both diseased groups (0.96 for OSCC and 0.65 for OLP) as compared to the control (0.33) it does not reach a significant level (Figure 2).

Table 2: Frequency distribution of the three studied groups (healthy, PMD and OSCC) according to sex and age.

		Normal		OLP		OSCC	
		No.	%	No.	%	No.	%
Sex	Female	4	66.67	5	83.33	5	83.33
	Male	2	33.33	1	16.67	1	16.67
Age (years)	<50	3	50	1	16.67		
	50-60	2	33.33	3	50		
	>60	1	16.68	2	33.33	6	100
Range		25-61		48-64		61-79	

OLP= oral lichen planus, OSCC = oral squamous cell carcinoma, No = number, %= percentage

Discussion

Several studies have shown that genetic alterations can be identified in most body fluids that drain from organs affected by the tumor, whether it is near or away from the affected fluid through exosomes communication[3,17].

Quantification of different salivary transcriptome biomarkers from cell-free unstimulated saliva samples is a relatively new method worldwide. In this study, we applied this method for the diagnosis of OSCC at its earliest stage (when the lesion still small) or in a potentially malignant condition (erosive OLP) before a real malignant transformation. To our knowledge, this is the first record of using this approach in Iraq.

To make sure that primers are located within the coding region of the genes of interest, we used several bioinformatics resources and NCBI database to design specific primers, double check and match them with the corresponding genes. This step is crucial and critical to get robust results which can be easily achieved with the help of the primer designing program (primer3 web-based primer design tool). In this way, we made sure that the primer sequences for all the three mRNA are surely located in the coding region of the genes of interest. Thus, it gives more trusting and perfect blasting results.

IL-8 is significantly regulating tumor micro-environment[18]. Its expression correlates with the angiogenesis, tumorigenicity and metastasis[11, 13].

On the other hand, IL-1β is a chemical mediator of cell proliferation, differentiation and apoptosis and plays a significant role in the pathogenesis of cancers[19]. Our results showed that IL8 and IL1β mRNA expression significantly increased (0.61 fold) in saliva samples of both OLP and OSCC groups in comparison to the healthy group. The increase in salivary IL8 mRNA fold expression nearly equal to that reported by Brinkmann et al.[20], but less than that reported by Li et al., [11] (24.3 fold increase). Furthermore, the significant increase in IL1β mRNA was in line with Li et al.[11], (5.48 fold increase) and Brinkmann[20] (0.35 fold increase) despite the great difference in the reported value of fold expression. In contrast, Cheng et al.[21] did not find such differences in IL8 and IL1β. These differences may be due to various types of materials, sample size, criteria for evaluating the result, and site variation.

Histones are basic nuclear proteins, responsible for the compaction of DNA into nucleosomes, involved in the regulation of chromatin activity during DNA replication. It is considered as a proliferative marker[22]. Although the results of our study showed an increased mRNA level of H3F3A, it does not reach a significant level in both study groups as had been reported by Li et al.[11] and Elashoff et al.[13]. Nevertheless, the present finding supports Cheng et al.[22] study which showed no significant increase in the expression of H3F3A in OSCC group.

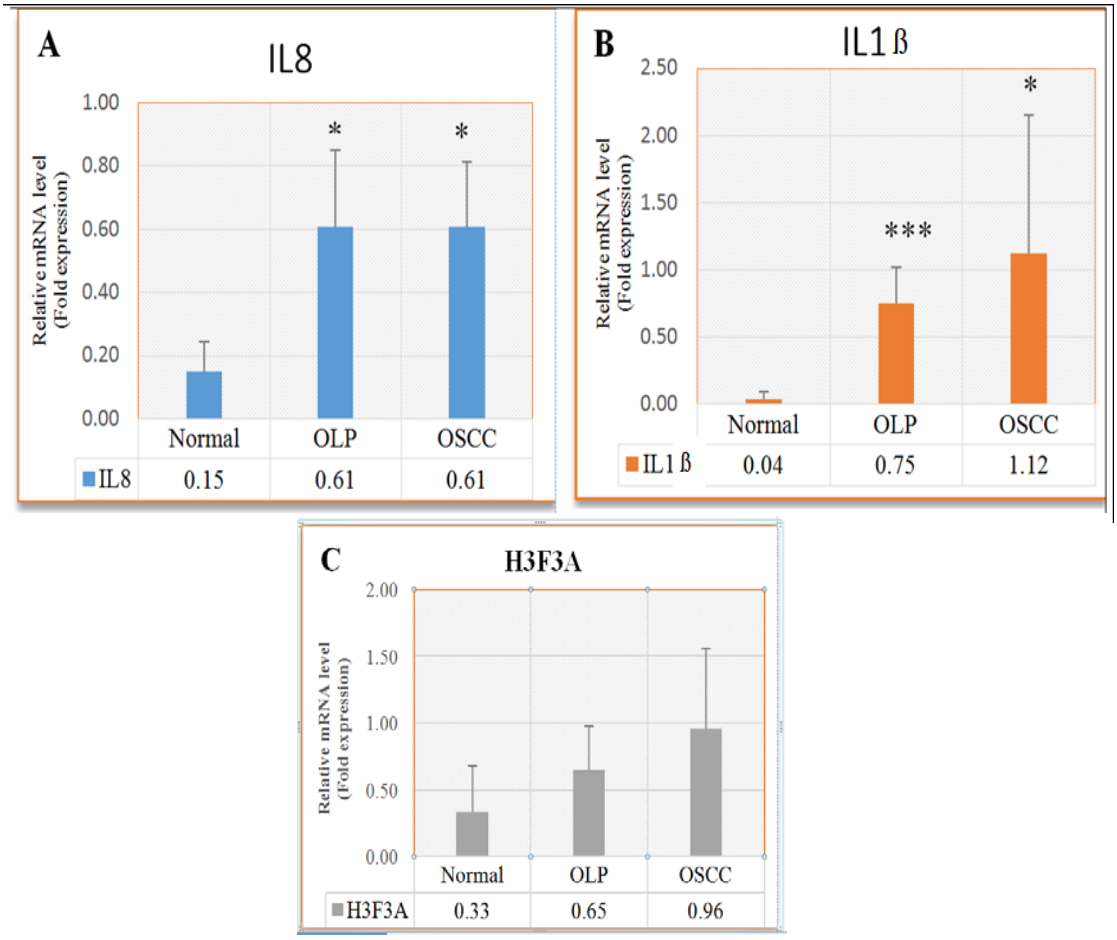

Figure 2: Gene expression and quantification. The mRNA expression level of IL8 (A), IL1ß (B), and H3F3A (C) in erosive OLP and OSCC in relative comparison to the control group. Data represent relative fold of expression from mean ± sd. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Concerning cell-free salivary mRNAs in OLP, the present work also supports the finding of Cheng and his co-workers[22] that show a significant increase in IL8 and IL1β mRNAs (1.76 and 1.39 folds increase respectively). However, there was no significant difference in the level of H3F3A (0.83 fold increases) when compared with the healthy subject.

The exact explanation of all the above findings is not clear yet. Such biomarkers have a great role in inflammation process and angiogenesis which both are signs of dysplastic and transforming changes associated with OSCC and OLP. The results obtained from this study support the new direction of research toward salivary transcriptome diagnostics to be an available aid for the development of noninvasive diagnostic, prognostic and follow-up procedure for OSCC and OLP.

Conclusions

Within the limitation of this study, one can conclude that the mRNA levels of IL8, IL1B, and H3F3A were increased in OSCC and OLP when compared to their expression in the normal healthy group. This indicates a successful usage of salivary transcriptomic analysis for diagnosis of OSCC at an early stage, and also to identify patients that are at high risk of developing OSCC.

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