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Salivary mRNA markers having the potential to detect oral squamous cell carcinoma segregated from oral leukoplakia with dysplasia

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ABSTRACT

Background: In the current study the presence of extracellular IL-1B, IL-8, OAZ and SAT mRNAs in the saliva was evaluated as a tool in the early detection of oral squamous cell carcinoma.
Methods: 34 patients with primary oral squamous cell carcinoma stage $T_1N_0M_0/T_2N_0M_0$, 20 patients with oral leukoplakia and dysplasia (15 patients with mild dysplasia and 5 with severe dysplasia/*in situ* carcinoma) and 31 matched healthy-control subjects were included in the study. The presence of IL-1B, IL-8, OAZ and SAT mRNA was evaluated in extracellular RNA isolated from saliva samples using sequence-specific primers and real-time RT-PCR. ROC curve analysis was used to estimate the ability of the biomarkers to detect oral squamous cell carcinoma patients.
Results: The data reveal that the combination of these four biomarkers provides a good predictive probability of up to 80% (AUC = 0.799, p = 0.002) for patients with oral squamous cell carcinoma but not patients suffering from oral leukoplakia with dysplasia. Moreover, the combination of only the two biomarkers (SAT and IL-8) also raises a high predictive ability of 75.5% (AUC = 0.755, p = 0.007) approximately equal to the four biomarkers suggesting the use of the two biomarkers only in the prediction model for oral squamous cell carcinoma patients limiting the economic and health cost in half.
Conclusion: SAT and IL-8 mRNAs are present in the saliva in high quality and quantity, with a good discriminatory ability for oral squamous cell carcinoma patients only but not for patients with oral leukoplakia and dysplasia an oral potentially malignant disorder.

1. Introduction

In 2015, over 450,000 new individuals are going to be diagnosed worldwide with oral cancer [1] and suffer from it. Despite the fact that oral cancer is really easily accessible because it is in the oral cavity, its five year survival rate (about 50–62%) still remains low [2,3], mainly because of its late diagnosis due to being fully asymptomatic in its early stages. The World Health Organisation WHO particularly stresses that early detection of oral cancer is the key to its management and successful therapeutic approach (www.who.int/cancer/detection/en) [4].

There are several types of oral cancer, but approximately 90% are oral squamous cell carcinomas [5,6]. Until now, there have been several attempts to develop biomarkers in order to detect oral squamous cell carcinomas early in their progression, the latest of which use saliva as a source [7–10]. Saliva is a valuable body fluid that has been used long ago for drug testing [11,12], therapeutic monitoring [13], and disease diagnosis [14–17]. It seems to be preferable than peripheral blood for disease detection and diagnosis [18] due to the non-invasive nature of its collection.

Various biomarkers have been proposed for oral squamous cell carcinoma detection and use for screening purposes [19,20] but all the biomarkers for OSCC detection that have been reported in articles until now have not been studied extensively or in a larger scale. This study focuses on the analysis of four mRNA markers in

Abbreviations: OSCC, Oral squamous cell carcinoma; OAZ, Ornithine decarboxylase antizyme 1; SAT, Spermidine/spermine N1-acetyltransferase 1; IL-8, (interleukin 8); IL-1B, interleukin 1B; ROC curve, receiver operating curve; AUC, Area under the curve.

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the saliva (OAZ, SAT, IL-8 and IL-1B mRNAs) which were previously reported as significantly elevated in patients diagnosed with oral squamous cell carcinoma in at least four studies one of which multicenter and subjected to a phase II validation by the NCI-EDRN (early detection Research Network Biomarker Reference Laboratory) in the USA [21]. They have not however yet been tested in patients with oral potentially malignant disorders.

Such mRNA molecules were first detected in the saliva back in 2004 [22], but their presence in the saliva was called into question [23]. Since then, several research papers overbid the use of mRNAs in the saliva for forensic human body fluids and tissue identification [24–26], in the diagnosis of periodontal disease [27,28] Sjögren's syndrome [29–31] and the detection of oral squamous cell carcinoma [21,32].

Some researchers however draw the attention towards some serious aspects in the process of biomarker development [33,34] in oral squamous cell carcinoma [20,35]. Among those issues are oral potentially malignant disorders which must not be overlooked in the research towards embracing candidate biomarkers for oral squamous cell carcinoma screening. The most common form of an oral potentially malignant disorder is oral leukoplakia with no, mild, moderate or severe dysplasia [36,37]. The term leukoplakia should be used to recognize white plaques of questionable risk having excluded other diseases or disorders that carry no increased risk for cancer [38] criteria proposed by the Workshop coordinated by the WHO Collaborating Centre for Oral Cancer and Precancer held in the UK, London, May 2005.

Since, to our knowledge, there are no other studies testing OAZ, SAT, IL-8 and IL-1B mRNAs as potential biomarkers in the early detection of oral squamous cell carcinoma and oral leukoplakia with dysplasia concurrently. The rationale of this study is to show that saliva could serve as a tool in the early detection and diagnosis of oral squamous cell carcinoma and to identify the appropriate biomarkers in the saliva in order to develop a good screening test for oral squamous cell carcinoma.

We thus performed the present study measuring the levels of the above biomarkers in order to investigate whether saliva can serve as a tool in tracking down oral squamous cell carcinoma in early stages and segregate it from oral leukoplakia with dysplasia.

2. Patients and methods

2.1. Patients

The patients were recruited at 1. the outpatient clinic of Oral Medicine and Oral Pathology in the School of Dentistry, Aristotle University of Thessaloniki, Greece, and two hospitals 2. The anticancer Hospital, Theageneion in Thessaloniki, Greece, and the 3. Department of Oral and Maxillofacial Surgery, Papanikolaou Hospital in Thessaloniki from 2009 until 2014.

All patients were informed about the research and had signed a written patient informed consent form. This research study was approved by the School of Dentistry Ethical Committee.

The patients selection criteria included: Newly diagnosed patients that had not received any remedy for the suspicious lesion, did not suffer from any virus disease or immunodeficiency and had not undergone any biopsy of the lesion.

Their squamous cell carcinomas were staged as $T_1N_0M_0$ or $T_2N_0M_0$, according to the TNM staging system. Their oral leukoplakias had various degrees of dysplasia. The diagnosis was based on clinical examination and histopathologic analysis of the tissue specimens. The diagnosis of epithelial dysplasia was based on the WHO criteria, according to which carcinoma *in situ* is also categorized in epithelial precursor lesions [39].

The clinical and histopathological data that were recorded were: Patients' age and gender, site, stage and histopathologic differentiation of the lesion.

2.2. Methods

2.2.1. Saliva collection and processing

Whole unstimulated saliva from patients and controls was collected before any biopsy performed always in the morning. Patients and healthy donors were asked to refrain from eating, drinking and any oral hygiene overnight and spit in 5-ml plastic vials used for biochemical examinations for 5 min. During the whole procedure and until centrifugation the vials were kept in ice.

The samples were centrifuged at 2600*g* for 15 min in 4 °C to remove any solid particles. Then the supernatant, that is the cell-free portion of the saliva, was separated from the debris. Five 5 ml of SUPERase•In™ that is an RNAase Inhibitor (20 U/mL) (Ambion Inc., Austin, Texas, USA). The RNA stabilizing reagent was added, in order to protect RNA from the RNases that exist in the saliva. The supernatant was then stored in 2 ml collection tubes at −80 °C.

2.2.2. Extraction of total saliva RNA

Extraction of total saliva RNA was performed using the QIAmp Viral RNA Mini Kit Cat. No. 52906 (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The supernatant (560–700 ml) was passed through the silica membrane of the mini spin columns provided in the kit in 140 ml aliquots. Total saliva RNA was eluted in 80 ml of elution buffer. The purity and quantification of the extracted RNA was determined by spectrophotometric analysis using a NanoDrop spectrophotometer (A_{260}/A_{280} ratio). Samples with a good amount and purity of RNA were further processed. All saliva RNA samples used in this study were treated with DNase I (Invitrogen), according to the manufactures' instructions in order to avoid any genomic contamination. Extracted saliva RNA samples were stored in aliquots in −80 °C.

2.2.3. cDNA synthesis

cDNA synthesis was performed following the manufacturer instructions (Invitrogen). Briefly, 1 ml primer oligodT (50 mM), the respective quantity of RNA (2 mg), 0.4 ml dNTPs (25 mM) and ddH_2O were added in an eppendorf tube to the final volume of 12 ml. The samples were then heated to 65 °C for 5 min and then quickly placed again in ice.

A master mix was synthesized using: 4 ml of 5X First synthesis buffer, 2 ml DDT (0.1 M), 1 ml of RNAse OUT an RNAse inhibitor and

Table 1
Gene symbols, accession numbers and primers' assay ID.

Gene symbol	Full name	Accession number	Assay ID
ACTB	Actin, beta	NM_001101.3	Hs99999903_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046.3	Hs02758991_g1
IL-1B	Interleukin 1, beta	NM_000576.2	Hs01555410_m1
IL-8	Interleukin 8	NM_000584.3	Hs00174103_m1
OAZ1	Ornithine decarboxylaseantizyme 1	NM_004152.2	Hs00427923_m1
SAT1	Spermidine/spermine N1-acetyltransferase 1	NM_002970.2	Hs00161511_m1