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Nanomaterial-based biosensors for detection of oral cancer biomarkers

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Abstract
Biosensors are a diverse category of sensors and can be differentiated not only by their mechanical components but by the biological sensing unit they utilize and several other specialization mechanisms, most orally administered drug that are soluble in water and capable of penetrating biological membrane during the passage of gastrointestinal tract will eventually become bioavailable in the body, due to low solubility in the drug. Comprehensive knowledge of the pathogenesis of vascular aging may lead to the identification of new biomarkers and therapeutic targets, providing new insights toward future vascular aging treatment. The advancement in nanotechnology has resulted in an amazing revolution in the diagnosis and treatment of vascular aging-related diseases.

Keywords: Biosensing, optical fibre, oral squamous carcinoma cell, Saliva, Serum, Diagnosis

INTRODUCTION

Oral squamous carcinoma (OSCC),which accounts for more than 40% of head and neck malignancies, ranks the sixth most common cancer worldwide.

A large number of article have demonstrated that serum may contain reliable biomarkers for detecting OSCC, such as Porphyromonas gingivalis IgG, Interleukin 6, Cripto-1 and Endothelin-1 [11-13]. However, the usefulness of serum biomarkers is unsatisfactory due to their low diagnostic sensitivity and specificity in the ear5ly stage of OSCC.Therefore a more effective, reliable and convenient diagnostic method is urgently needed in early diagnosis of OSCC.

Threefactors govern the speed and degree of absorption of orally administered drugs: (i) dissolution rate,(ii) solubility and (iii) intestinal permeability, which are grouped according to the biopharmaceutical classification system in the categories

Nanomaterial – based optical fiber biosensors

Advances in nanotechnology have allowed us to build structures or devices in the nanoregime, such as nanoparticles, nanotubes, nanorods and nanowires, which can directly probe and interact with the biomolecules we intend to detect using biosensors. Nanobiosensors have advanced ability to identify specific analytes and obtain detailed information regarding biomolecules profiles of various diseases.

COST EFFECTIVE FIBER OPTIC SOLUTIONS FOR BIOSENSING

In the case of optical biosensors, the signal generated can be coded in wavelength, phase, and signal intensity, among other optical features [5]. Optical absorbance, fluorescence, bioluminescence, interferometry, ellipsometry, reflectometric interference spectroscopy, and surface-enhanced Raman scattering are also examples of highly applied optical techniques to biosensing [6].

The optical fibres the are usually applied in cost effective biosensing approaches, different

kind of fibres can be used, from regular silica single -mode fibres (SMFs) [12] to multimode fibres (MMFs) such as plastic- clad silica (PCS) [13] and plastic optical fibres (POFs)

FUNDAMENTAL OF OPTICAL FIBRE SENSORS

2.1. Theoretical Background and Main Architectures

An optical fibre is a waveguide that transmits light of different wavelengths. The propagation mechanism can be analysed by Maxwell equations as well as by ray theory because the signal wavelength is smaller than the physical dimensions of the waveguide. A standard optical fibre is made of silica and, more specifically, has two main parts: core and cladding. The core is typically doped to obtain a refractive index slightly higher than the cladding's one, which guarantees total reflection by Snell law under certain conditions; however, there is always a small energy transmission from the core to the cladding. Talking in terms of electromagnetic fields, most of the signal is transmitted by core modes, although a tiny part of it is coupled into the cladding modes, also known as evanescent field.

2.2 Techniques for the development of sensors

Generally, biosensors are composed of three main components. These include a biological sensing element, physicochemical detector or transducer and a signal processing system [8]. Biological sensing elements are used to interact with the analyte of interest to generate a signal. Sensing elements normally include materials such as tissues, microorganisms, organelles, cell receptors, enzymes, antibodies, and nucleic acids. The signal generated through the interaction of the sensing element and the analyte of interest is then transformed to a measurable and quantifiable electrical signal via the transducer. The signal processing system thereforeamplifies the electrical signal and conveys it to a data processor that produces a measurable signal in the form of a digital display, print out or color change [9, 10].A successful biosensor is composed of two main components , mainly a biological receptor or sensor element and a transducer .

Biological receptor is also known as sensor or detector element, which serves as a biochemical receptor that specifically recognizes the target analyte. When the biological receptor interacts with the target analyte, it generates a signal in the form of light, heat,pH ,charge or mass change .This material should be highly specific , stable under storage condition and must be immobilized , the biological receptor should be capable of selectively detecting the target compound or analyte in the test sample . The biological receptor

determines the sensitivity of the entire device through the generation of the physiochemical signal that is monitored by the transducer.
Most orally administered drugs that are soluble in water and capable of penetrating biological membrane during the passage of gastrointestinal tract will eventually become bioavailable in the body , due to low solubility of drugs ,they cannot be administered intravenously and parenteral administration does not always increase bioavailability.Three factors govern the speed and degree of absorption of orally administered drugs: (i) dissolution rate, (ii) solubility and (iii) intestinal permeability, which are grouped according to the biopharmaceutical classification system (BCS, Biopharmaceutical Classification System) in the categories

2.3. The Methods of Biosensors Detecting Cell-Related Analytes

2.3.1Electric cell-substrate impedance sensing

Electric Cell-substrate Impedance Sensing (ECIS) is an in vitro impedance measuring system to quantify the behaviour of cells within adherent cell layers . A constant small alternating current is applied in cells grown in special culture chambers on top of opposing , circular gold electrodes . The insulating properties of the cell membrane create a resistance towards the electrical current flow resulting in an increased electrical potential between the electrodes

2.3.2 Surface plasmon resonance biosensor for real-time detection

The conventional methods in molecular biology to evaluate cell differentiation require cell lysis or fixation, which make investigation in live cells difficult. In addition, a certain amount of cells are needed in order to obtain adequate protein or messenger ribonucleic acid for various assays. To overcome this limitation, a unique SPR-based biosensing apparatus for real-time detection of cell differentiation in live cells according to the differences of optical properties of the cell surface caused by specific antigen-antibody binding.
The SPR system and the protocol reported in this study can rapidly and accurately define osteogenic maturation of MSCs in a live cell and label-free manner with no need of cell breakage. This SPR biosensor will facilitate future advances in a vast array of fields in biomedical research and medical diagnosis.

3. DETECTION OF ORAL CANCER BIOMARKERS

Salivary Biomarkers of Oral Cancer

Oral squamous cell carcinoma(OSCC) is the most common cancer in men and the third most common cancer in women.The early stages of this disease manifest as asymptomatic lesions that usually go unnoticed, resulting in a delayed diagnosis when the patient eventually presents with pain during later stages.Saliva which consists for more than 99% of water , contains a variety of electrolytes including potassium , magnesium , calcium , sodium , bicarbonate and phosphates , as well as biological elements , such as immunoglobulins , mucins , enzymes , proteins and nitrogenous products .

EXPERIMENTAL PROCEDURE

Study subject and source of sample

Serum Collection

Fasting Blood samples of patients were obtained from 8:00 AM to 11:00 AM , and centrifuged at 1000 x g for 15 minutes at RT and supernatant was collected into 1.5 ml sterile sample tubes . All seum samples were stored at -80^{o}C .

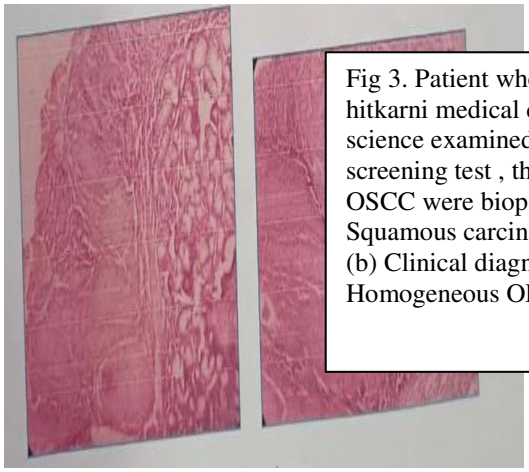
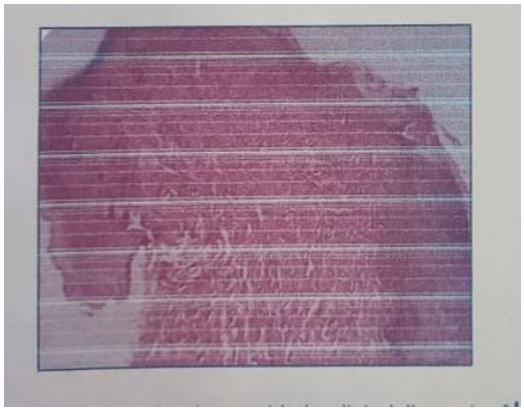


Fig 3. Patient who were presented to hitkarni medical college of dental science examined by oral mucosal screening test , the case of OPMD and OSCC were biopsy proven (a) Squamous carcinoma cell of patient. (b) Clinical diagnosis of Homogeneous OPMD

A.



B.

Saliva Collection

The subject are patients who presented to Hitkarni medical college of Dental science from 2022-2023 .The study included 90 subjects that were divided into three groups . Group A included 30 oral leukoplakia patients who were clinically and histopathologically diagnosed into different grades of oral epithelial dysplasia (OED).Group B included 30 patients of OSCC who were clinically and histopathologically diagnosed with varying grades i.e., well / moderately / poorly differentiated oral squamous cell carcinoma. Group C included healthy controls with age, sex match and periodontal status confirmed by Community periodontal index (CPI) matched to that of group A and B. All subject were examined by oral mucosal screening test . The cases of OPMD and OSCC were biopsy proven , and patients underwent routine check- ups according to standard protocols .

Subject were guided to refrain from either eating or drinking at least one hour before collection of salivary samples. Subjects were asked to rinse out their mouth with water at least 5 min prior to saliva collection. Samples were obtained by requesting subjects to swallow first, tilt their head forward, and expectorate all saliva into the sterile disposable tubes. The collected saliva is centrifuged at 3,500 rpm for 10 minutes to remove debris.

Table 1. Comparative data of 90 patients divided into three groups (i) Group I for controlled patients for comparative study (ii) Group II of 30 patients for leukoplakia patients (iii) Group III of another 30 patients for OSCC patients

DATA	Controls	OPMD	OSCC
Total no. of cases	30	30	30
Age	25-60 yrs	25-70 yrs	30-70 yrs
Males	15	25	
Females	15	5	10
Smokers	-	28	12
Tobacco chewer	-	5	18
Histological differentiation	-	-	-
Well Differentiated	-	-	13
Poorly Differentiated	-	-	7
Histological differentiation	-	4	2
Mild Dysplasia	-	10	-
Moderate Dysplasia	-	10	-
Severe Dysplasia	-	10	-

The samples/ test specimens were brought to the room temperature. Addition of 100 µl of test/specimen samples to each well of pre - TNF- α antibody coated 96 - well microtiter plate, which was s incubated for 60 minutes and rinsed . Followed by addition of 100 µl of detection antibody to each well of the microtiter plate and incubated for 60 minutes at 28°C followed by washing. Then adding of 100 µl of streptavidin - HRP to each well of the microtiter plate and incubate for 30 minutes at 28°C, followed by adding 100 µl of TMB substrate solution to each well of the microtiter plate and incubated for 15 minutes in dark at 28°C is done. To stop the reaction, 50 µl of stop solution (N/20 H_2SO_4) to each well was added. The resultant product is yellow. The changes in colour intensity and the absorbance at 450 nm and 600 nm was read using ELISA microplate reader (EMP emperor medical - M201 microplate reader).

Two -dimensional LC-MS for iTRAQ Analysis - The samples were loaded into a SCX-HPLC column (Luna® 10 m SCX 200 Å, LC Column 1.0 □ 400 mm, Phenomenex, Taipei, Taiwan) at a constant flow rate
of 2 □l/min in buffer A and separated with buffer B (0.2% FA, 60% ACN, and 0.2 M NH_4Cl). A linear
gradient of buffer B (5% for 2 min, 5–15% for 3 min, 15–35% for 13 min, 35–70% for 25 min, 70–99% for 28 min, and 99% for 32 min) was applied . The resulting peptide fractions were diluted in-line with 0.2% FA before trapping onto the column Zorbax 300SB-C_{18} (0.3 □ 5 mm, Agilent Technologies, Wilmington, DE). Each fraction was then loaded into a homemade column (HydroRP 2.5 □m, 75 □m □ 20 cm length) with a 15-□m tip using buffer C (0.1% FA) and

separated with buffer D (CAN containing 0.1% FA) at a flow rate of 0.3 □l/min. A linear gradient of buffer D (0–5% for 10 min, 5–28% for 60 min, 28–40% for 20 min, 40–60% for 8 min, 60–90% for 2 min, 90% for 6 min, 90–5% for 4 min, and 5% for 20 min) was applied.

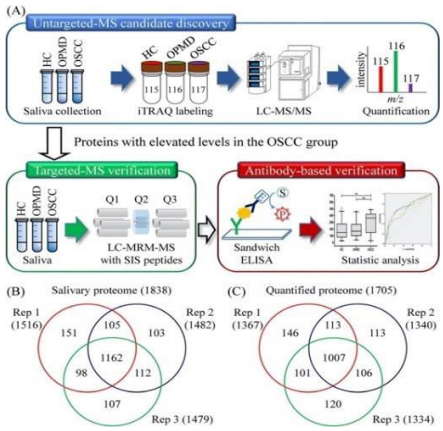


FIG. 1. Strategy for identification and verification of salivary biomarkers in OSCC. A, Untargeted-MS candidate discovery: Saliva samples from healthy controls (HC), individuals with oral po-tentially malignant disorders (OPMD), and OSCC patients (OSCC) were pooled. After tryptic digestion and iTRAQ reagent labeling, peptide mixture was analyzed by LC-MS/MS with three replicates. Tar-geted-MS verification: Proteins with ele-vated iTRAQ ratios between OSCC and noncancerous group were selected for verification using multiple reaction moni-toring (MRM)-MS assays with stable iso- tope-labeled standard (SIS) peptides in a small saliva cohort. Antibody-based veri-fication: Efficacy of biomarker candidates was then evaluated with sandwich ELISAs in an independent and larger cohort. Venn diagrams show overlaps between the proteins identified (B) and quantified (C) in each replicate (Rep) of the iTRAQ analy-sis. The total numbers of detected or quantified proteins are listed in brackets.

The levels of salivary and serum PrPc in OSCC, OPMD, and control groups

As shown in table 2, salivary and serum levels of PrPc in OSCC patients were higher than those in the OPMD and control groups (0.05) , but there were no significant differences between OPMD patients and normal controls (P>0.05) .

Observational studies reporting on the alteration of any cytokine salivary concentration in patients with OSCC were considered suitable for the present review. In particular, the inclusion criteria for study eligibility were: (1) the evaluation of the salivary concentration of cytokines in patients with a histological diagnosis of OSCC; (2) comparison of salivary cytokine concentrations of OSCC patients with a control group; (3) comparison of pre-operative with post-operative salivary cytokine concentrations of OSCC patients, or comparison of post-operative salivary cytokine concentrations measured at different times during the follow-up period; 4) statistical analysis for a comparison of the salivary levels of cytokines. The main findings extracted from each study were the comparisons between the salivary levels of cytokines in healthy and pathological conditions, including the *p*-value, when significant ($p \leq 0.05$).

Table 2. Salivary and serum PrPc levels in OSCC , OPMD and control groups.

Groups	Cases	SalivaryPrP^{C}	P value	Serum PrPc	P value
OSCC	30	1.920± 0.562	<0.0001[a]	1.521± 0.525	0.021[a]
OPMD	30	1.213±0.741	<0.651[b]	1.511[b]	0.625
Control	30	1.331± 0.680	<0.001[c]	1.245[c]	0.020

a Difference between OSCC and OPMD groups
b Difference between OPMD and control groups
c Difference between OSCC and control groups

Enzyme linked Immunosorbent Assay (ELISA)

The content of PrP^{c}in Saliva and serum samples was detected by ELISA . PrP^{c}WH2 antibody was added to the appropriate wells of ELISA plates ,incubated overnight at 4^{o}C and blocked with 10 % fetal bovine serum .After removal of the liquid from each well , 200µl aliquot of each saliva or serum sample was added into each well for 2 hours , then coated with biotin labelled secondary antibody . Next washed and added Streptavidin -HR into wells for 30 minutes at RT , then washed and added 100µl of TMB substrate solution to each well , after that the plates were kept at RT for 15 minutes .In the end , 150µl stop solution was added to each well to terminate the enzyme reaction , and absorbance was reas at 520-550 nm by using a spectrometer.

The studies analyzed demonstrate that numerous cytokines (e.g., IL-6, IL-8, TNF-α) are present in the saliva of OSCC patients at a significantly different concentration when compared to healthy persons. The epidemiology of oral squamous cell carcinoma outlines that the 5-year survival rate of patients diagnosed in stage I is 72–90%, in stage II is 39–85%, in stage III is 27–70%, and in stage IV is 12–50% [15,14]

Studies have outlined that IL-6, IL-8, IL-1b, and CCL2 are involved in the initial process of OSCC and that the present antibodies against IL-6 and p53 can be potential salivary biomarkers [16]. Although, there have been more than 100 salivary biomarkers identified, the need for a standardization method brings to light the different outcomes of research studies due to the lack of a collection and storage protocol [17]. Among proteinaceous salivary biomarkers, studies on the molecular role of cytokines in the tumor microenvironment (TME) revealed that they are involved in processes leading to the initiation, growth, invasion, and metastasis of cancer [18]

Discussion

Nanoparticles interact with host biology and immune function in complex ways that affect both the performance of nanoparticle-based pharmaceuticals and the diseased host [8-12]. Expectations raised by early preclinical successes, which have proven to be unreliable predictors of clinical performance for cancer nanomedicine, remain unmet when measured against impact on patient survival [7]. Disconnect between expectations founded on preclinical data and realized clinical performance highlights critical gaps in knowledge. With all the exquisite properties of plasmonic biosensors, the sensitivity has been further improved by introducing metamaterials. Metamaterial-based biosensors provide different geometric structures, each having its own sensing properties, which expands and

improves the use of conventional plasmonic biosensors were significantly correlated with the survival of OSCC patients, suggesting that these factors might serve as diagnostic biomarkers. IL-6 is implicated in various cancers in suppressing apoptosis and accelerating uncontrolled cell growth via activating growth factor and related signaling pathways [37]. Although the role of IL-6 in cancer development and progression is clear, studies on the serum levels of IL-6 in healthy controls and OSCC patients have shown contradictory results. The data showed no differences in the serum IL-6 levels in OSCC patients compared with those in healthy controls [38]. In contrast, two recent studies strongly suggested a positive correlation between IL-6 and cancer development by observing much higher levels of serum IL-6 in oral cancer patients than in healthy controls [29,39]. These contradictory results may be due to differences in the sensitivity of the assays used.

RESULT

All the included studies are observational (cross-sectional or longitudinal). Some of them, when evaluating the cytokine level, considered different OSCC histological grades and clinical stages. Based on the methodological design, studies were divided into three groups: Group I (n = 15)—cross-sectional studies comparing salivary cytokine levels of patients with OSCC to control subjects; Group II (n = 10)—cross-sectional studies comparing salivary cytokine levels of patients with OSCC to control subjects and evaluating their relationship to OSCC histological grading and/or clinical staging; Group III (n = 5)— longitudinal studies considering tumor excision as a treatment and comparing salivary cytokine levels before and after surgery.

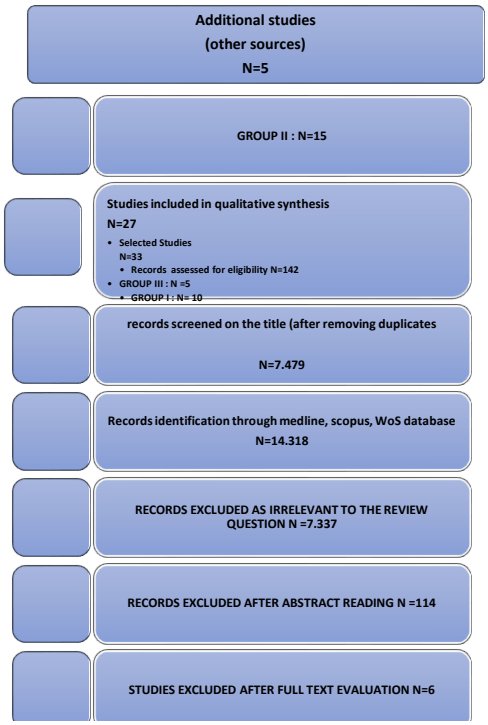


Figure 1. Flow diagram of the different phases of the systematic search and review. Phases are presented in accordance with the PRISMA statement. Group I and Group II articles include cross-sectional studies, while Group III includes longitudinal studies. Group I articles compare salivary cytokine levels of OSCC patients with control subjects; Group II articles include, together with the OSCC/control comparison, the comparison between salivary cytokine levels of different OSCC histological grades and clinical stages; Group III articles compare salivary cytokine levels before and after tumour excision treatment

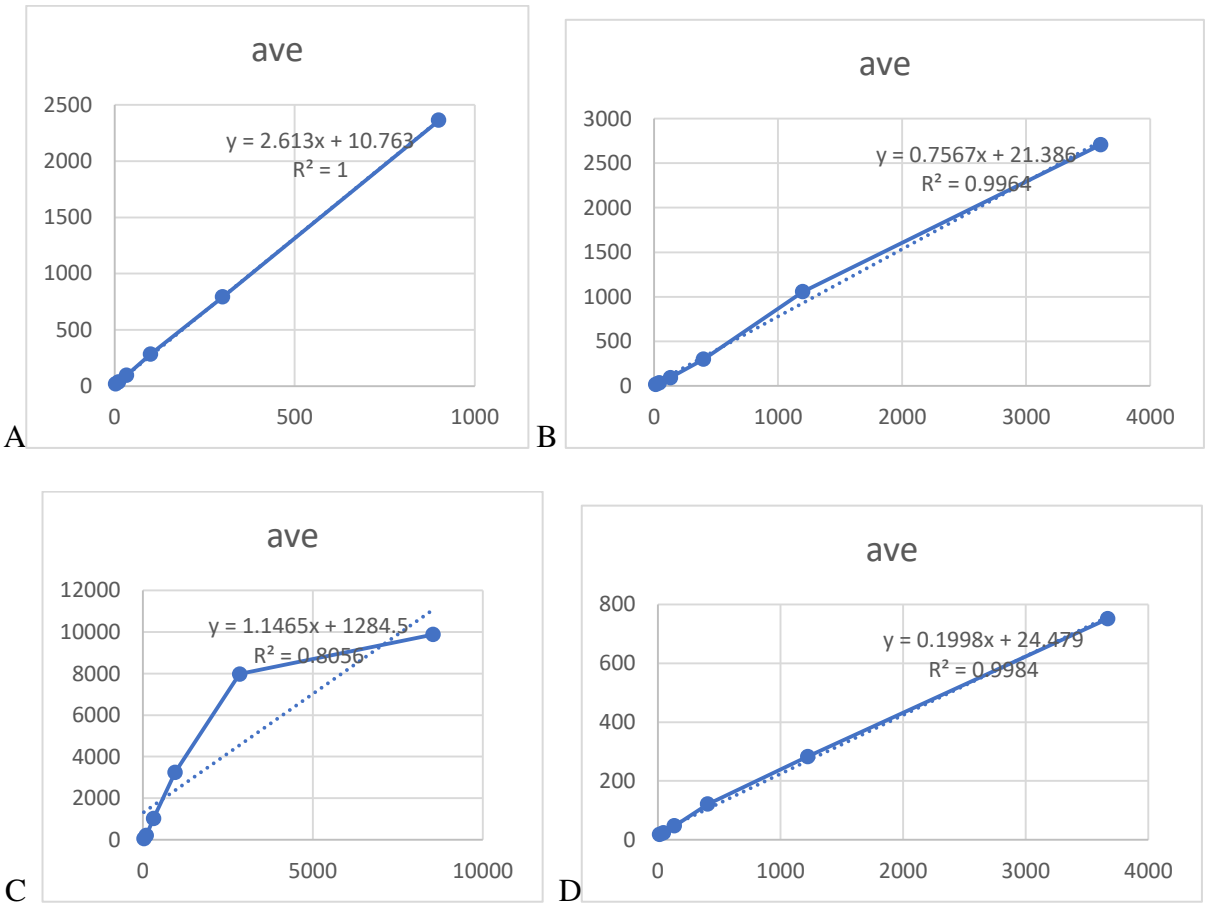
It is well accepted that inflammation and cell-mediated immunity are active players in the control of oral carcinogenesis progression. Cells' evasion from immune surveillance has been described as a primary step in oncogenesis. Pro-inflammatory cytokines (IL-6, IL-8, TNF-α, soluble interleukin-2 receptor (sIL-2R)) are key-molecules involved in the crosstalk between stromal and cancer cells, their expression being, to some extent, associated with tumor growth promotion or inhibition [19].

Table 3. ELISA for cytokine Analysis with reference to the graph

	Std	std	ave	conc	ave	
	2363	2361.5	2362.5	900	2362.25	
IL6	796.5	789	791.5	300	791.75	fig
	285.5	282	283.75	100	283.75	
	96	95	95.5	33.33	95.5	
	38	34	36	11.11	3.73	
	17	19	18	3.7	18	
	2777.5	2619	2698.25	3600	2698.25	
IL1B	1099	999.5	1049.25	1200	1045.25	fig
	289	293.5	291.25	400	291.25	
	84.5	83.5	84	133.33	84	
	27.5	23.5	25.5	44.44	25.5	
	8.5	10.5	9.5	14.81	9.5	
CCL2	9741.78	10019.24	3242.32	8540	9880.51	
	8585.79	7359.13	3244.03	2846.667	7972.46	fig
	3242.32	3244.03	3243.03	948.88	3243.175	
	1023.98	1019.94	948.88	316.296	1021.96	
	242.36	176.35	324.175	105.432	209.355	
	47.07	45.08	46.07	45.1	46.073	
	699	804	751.5	3670	751.5	
IL-8	268	298	283	1223.33	283	fig
	125	117	121	401.77	121	
	47	47	47	135.92	47	
	25	23	24	45.3	24	
	18	19	18.5	24	18.5	

According to the analysis of the 5-year survival rate, OSCC patients with lower serum levels of Il-6 had a better survival rate, whereas the serum levels of P. gingivalis or F. nucleatum IgG were not correlated with the survival rate. Interestingly, significant value in predicting the prognosis of OSCC patients was observed for the serum value of IL-6, which showed significantly different values in OSCC patients who survived for more than 5 years than in those who survived for less than 5 years (158.5 ± 75.5 pg/mL and 359.4 ± 323.11 pg/mL, respectively). Our study shows that high serum levels of the inflammatory cytokine IL-6 and P. gingivalis IgG are strongly correlated with OSCC. In addition, the serum IL-6 values were

significantly correlated with the survival of OSCC patients, suggesting that these factors might serve as diagnostic biomarkers



Conclusion

Early diagnosis of OSCC can save numerous lives , diminish burden of mobility arose from treatment of the disease at advance stage . However , the current approach to OSCC diagnosis, which includes visual examination of oral cavity succeeded by inspection with biopsy , is sometimes inefficient . OSCC comprises of the tumour epithelium and the surrounding connective tissue stroma which constitutes the tumour microenvironment (TME) within which varying populations of mesenchymal cells, extracellular matrix, and inflammatory cells are present .

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