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SALIVA BASED ORAL CANCER SCREENING SYSTEM USING GFP EXPRESSING YEAST CELLS

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Abstract

Purpose: Oral cancer poses a great threat to an individual's life, which can be mitigated effectively if detection and confirmation occur earlier during the disease. Transforming growth factor-β (TGFβ) plays an integral role as a regulator in governing many biological processes. TGFβ level rises in saliva of oral cancer patients. Thus, it could be used as a diagnostic biomarker. This study aimed to develop a reporter system in yeast cells to detect TGFβ as a tool for the non-invasive screening of oral cancer. **Methodology:** Yeast cells were transformed with transforming growth factor beta receptor II (TGFβRII), and collagen type II alpha chain I (col2a1) (intronic region) containing vector and confirmed through restriction digestion. The recombinant proteins were expressed, optimized and established through gel electrophoresis analysis. Furthermore, recombinant yeast cells were mixed with saliva of oral cancer patients and assessed through enzyme-linked immunosorbent assay (ELISA) and fluorescence microscopy through green fluorescence protein. **Results:** The significant difference in expression (P value < 0.05) in the expression of TGFβ was found between transformed and non-transformed yeast cells. The presence of TGFβ was confirmed through ELISA in saliva samples of oral cancer patients, while solid-phase sandwich ELISA was performed to verify TGFβ presence in saliva-treated yeast samples. The highest fluorescence was observed in the yeast samples with expression vectors for both Col2a1 and TGFβRII. **Conclusion:** In the present study, a yeast based green fluorescent protein reporter system was constructed for molecular reporting of early oral cancer through saliva. TGFβRII protein expression in *S. cerevisiae* was optimized and detected through ELISA and fluorescent microscopy. This co-transformed system can be used as non-invasive diagnosis of oral cancer.

Keywords: Collagen type II alpha 1 chain, Enzyme-linked immunosorbent assay, Gel electrophoresis, Intronic Region, Transformation, Tumor growth factor beta, Tumor growth factor receptor-II

INTRODUCTION

Oral cancer is one of the leading cancers common among developed and underdeveloped countries equally. Transforming growth factor expression (TGFβ) is found in all the human body's cells, which play aplay a vital role in maintaining homeostasis and many developmental processes at the cellular level [1,2]. These growth factors play an important part in regulating the number of biological processes during embryonic growth and development [3]. A review of the literature and the experimentations performed by many experts suggested that the receptors involved in TGFβ signaling are interlinked such as the kinase activity of TGFβRI and TGFβRII associated with each other.

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However, experimentations through in vitro and in vivo analysis proposed that TGFβ signaling pathway is related to the enhancement or initiation of cancer [4].

The *Col2a1* gene, which is specifically marked to have 54 exons and approximately 31.5 kb of genomic DNA found at 12q13.11, ~ 47 Mb from p-telomere directly linked to the encoding of a pro-alpha-1 (II) series of type II collagen, which is collagen primarily found in cartilage and vitreous indulge in the eye. Therefore, it has been reported that defects in the *Col2a1* gene could manifest as the correlating etiology of multiple disorders, namely type II collagen disorders [5-7]. The transcriptional reporter system Col2a1 is designed for checking up the expression that is a quantitative indicator of the activity of the associated gene which can be measured by luciferase assay and green fluorescent proteins [8].

Yeast *S. Cerevisiae* is proven to be highly efficient in responding, to complex signalling pathways. Yeast transformation with different genes for the study and analysis of signaling pathways has been manipulated to understand many diseases, such as cancer and metabolic syndromes [9]. This study involved co- transform of TGFβRII and Col2a1 in a single yeast cell for the detection of non-invasive oral cancer and single gene has been transformed in yeast successfully [10].

The detection of oral cancer was performed by analyzing the detectable tumour DNA in biological fluids such as blood, serum, and saliva in head and neck cancer patients. However, saliva sample analysis for detecting the biomarker in oral cancer was a non-invasive and less painful method [11].

In our research, we have used saliva samples of oral cancer patients because of their non-aggravated nature. Human saliva has proved to be a more beneficial sample due to ease of availability, and it was an accessible mode to conduct a study in large population groups. [12].

This project constructed a yeast-based expression system for early detection and rapid diagnosis of oral cancer patients through saliva.

MATERIALS AND METHODS

Study Design and Sample Collection:

We have conducted a retro-prospective nature cohort study. Our experimental study mainly had two core steps. The first step was to co-transform TGFβRII and GFP genes containing vectors in saccharomyces cerevisiae and analyze expression kinetics of TGFβR protein in a yeast cell. Further, optimize reporter system assay for detection of TGFβ cytokine. The second was mainly data collection step in which we collected data from 60 patients with the provisional diagnosis of oral cancer from various tertiary and palliative care hospitals in Lahore, Pakistan. According to the statistical configuration of our study, out of the 60 patients that participated with the provisional diagnosis of oral cancer, 20 were categorized as normal, and 40 were diagnosed with oral cancer. The project was approved by the ethical committee of the Institute of molecular biology, and biotechnology (IMBB/UOL/20/137), and research experiments have been conducted according to international and institutional guidelines.

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The collected samples from both cohort have gone through under the vigorous evaluation of 6 parameters that mainly constituent solid phase sandwich ELISA. Medically certified pathologist has given the review report to aid the diagnosis of differentiating cancer containing section from healthy section of the specimen on slide. Patient that was diagnosed had the clinical findings of oral cancer that has invaded many anatomical regions of head and neck. We used a very distinctive approach for our inclusion criteria. Only participants who freely consented to participate in our study and who gave us formal authorization for it have been included in our study.Patients who are at least 30 years old, have never had cancer before, and have never received either therapeutic or palliative care for the disease were included.

Moreover, only patients with no prior history of diabetes or cardiac-related issues or have had any medications for such conditions. All participants who did not meet our inclusion criteria were excluded from this study.

Strains, Vectors and Media

Two vectors with eukaryotic promoter system were purchased from ADD GENE. One vector had a DNA sequence of TGFβRII gene (Figure S1) and the other contained green fluorescence gene with upstream region of Col2a1 enhancer (Figure S2).

E. coli DH5 alpha cells were used to propagate plasmid vectors. *E. coli* DH5 alpha cells were in stock at Center for Research in Molecular Medicine, Institute of Molecular Biology and Biotechnology (CRiMM/IMBB), The University of Lahore, Pakistan. For eukaryotic expression, yeast strain *S. Cerevisiae* was used. For growth of *E. coli* DH5 alpha bacterial strain LB agar has been used while *S. Cerevisiae* was maintained in YPD medium. The cells with vector were maintained on agar plate supplemented with ampicillin, following the standard methods [13]. Plasmids of TGFβRII and Col2a1were isolated and the results were confirmed using 1% agarose gel electrophoresis.

Transformation of TGFβRII and Col2a1 Containing Vectors

Yeast cells were grown in the lab for transformation. The LiAc/SS Conveyor DNA/PEG transformation technique described by Gietz and Schiestl, 2007 was utilized for the *S. Cerevisiae* competent cells transformation with little modification [14]. The growth of the transformed colonies with TGFβRII plasmid was marked by growing on ampicillin supplemented medium. TGFβRII containing yeast was transformed with Col2a1 plasmid. The selection on ampicillin supplemented medium separated the transformed colonies.

Confirmation of Transformation

For the confirmation of transformation of yeast, the single enzyme digestion was performed. The isolated plasmid was incubated with 1µl restriction enzyme *BamH*I at 37°C for 1 hour. The digested DNA was visualized on gel electrophoresis under UV light. Colony PCR was performed to confirm transformation of TGFβRII and Col2a1 plasmid. Polymerase chain reaction (PCR) was made in 20µl final volume, using 1X PCR buffer (5µl), $MgCl_2$ (3µl), dNTPs (0.5µl) of each primer, Taq polymerase (0.5µl) and 10µl of supernatant of boiled transformed colony. PCR thermocycler was set for following

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scheme designed; initial DNA denaturation at 95^{o}C for 5 minutes and 30 cycles of DNA denaturation for 1 minute at 94^{o} C annealing temperature of 62^{o}C for *Col2a1* and 60^{o}C for "TGFβRII specific primers for 40 second and extension of 1 minute at 72^{o} C for DNA elongation. At last, 5 minutes at 72^{0} C was given to complete amplification of each PCR reaction. The results of each PCR products were visualized by 1% gel electrophoresis under UV light.

Gene Expression Analysis of TGFβRII and GFP

To check the expression of TGFβRII protein, 12.5% SDS-PAGE was run with a pre-stained protein ladder (LC5925, Invitrogen). Transformation-confirmed yeast culture samples were taken at intervals of 2 hours, processed and run at 12.5% SDS-PAGE.

Serum and Saliva Samples Collection

Blood samples from oral cancer patients and controls were centrifuged at 1500 rpm for 15 min at room temperature and the serum was collected and frozen at -80°C until analysis, moreover 5ml of whole saliva was also collected from oral cancer patients and controls.

Evaluation of Cytokines Level

Saliva and serum samples were collected from patients and healthy individuals to evaluate 6 parameters through sandwich ELISA (enzyme linked immunosorbent assay). These parameters were TGFβ, vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF-α), P53, interleukin-6 (IL-6) and interleukin-1 (IL-1). Moreover, TGFβRII activity also checked through ELISA by exploiting antigen-antibody interaction of the receptor protein TGFβRII from yeast lysate with TGFβRII antibody. ELISA protocol as given by Wajid et. al., 2016 was followed as under [15].

Antibodies for these were obtained from Santa Cruz Biotechnology USA., as anti-P53 (catalog no. sc-393031), anti-TNFα (catalog no. sc-515765), anti-VEGF (catalog no. sc-57496), anti- TGFβ (catalog no. 65378), anti-IL-6 (catalog no. ab9324), anti-IL-1 (catalog no. ab2105). Coating buffer (100µl) was used for the dilution of the solution, and the cultured plate was washed with TBS (tris buffered saline) solution several times and was incubated for 24 hours at 4°C along with the blocking solution. Wells have then rewashed with Tris-buffered saline (TBS) and incubated overnight with Horseradish Peroxidase (HRP) conjugated secondary antibody (Santa Cruz Biotechnology, USA). The plate was washed several times for the removal of a secondary antibody. The chromogenic solution 3,3',5,5'- Tetramethylbenzidine (TMB) (Invitrogen Inc., USA) was processed to detect a secondary antibody. To stop the chromogenic reaction sulphatic acid was processed. Absorbance was taken at 450nm along with 650nm as reference value.

Flouresence Microscopy

Fluorescence microscopy was performed as previously described [16,17]. Harvested cells are rinsed in distilled water and rearranged; Cell suspension was observed using a BIOREVO BZ-9000 fluorescence microscope with a 40X objective lens (Keynes, Osaka, Japan). Green fluorescence images were obtained with a 470/40 band-pass filter for

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excitation and a 535/50 band-pass filter for emission. Scale bar: 20µm. The exposure time was 1/15s. The collected cells were normal yeast cell.

Statistical Analysis

The comprehensive results obtained from our experimental cohort study of normal and cancer individual was statistically analyzed. Descriptive statistical analysis and t-test analysis of six parameters of TGFβ, VEGF, TNF-α, P53, IL-6 and IL-1 were performed to access the normality distribution and reliability of the data. All the values were expressed as mean ±SEM. The experiment was carried in triplicates wherever required.

RESULTS

Transformation of TGFβRII and *Col2a1*

The presence of plasmid cells was confirmed by screening the pCMV5B-TGFβ plasmid on gel, which later signifies the presence of large bands under gel electrophoresis. Later, the colonies containing TGFβRII and Col2a1 were screened on media by growing colonies coated with ampicillin since plasmid had ampicillin resistance gene. The later band was bit heavier compared to pCMV5B- TGFβ plasmid band.

Confirmation of Transformation

The isolated plasmids were subjected to enzyme digestion to confirm the presence of both pCMV5B- TGFβ plasmid and pTGF1-Col2a1plasmid. The common enzyme *BamH*1 was selected for the digestion, the plasmids incubated with enzyme for 3 hours, the single cut by this enzyme on both the plasmids has shown two bands on agarose gel shown in figure 1.

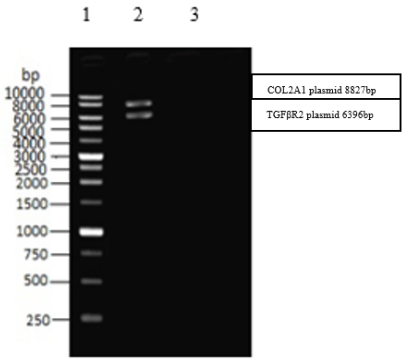

Figure 1: Lane 1: Restriction digestion product of the two plasmids inserted isolated from transformed yeast cell. Lane 2: The size of the upper first band is at 8827bp and the second band is at 6396bp position in lane gene ladder. Lane 3: Negative control

Further confirmation of co-transformation was carried out by performing end-point PCR. TGFβRII receptor gene was amplified the band of 1800bp was presented on gel electrophoresis shown in figure 2a. Similarly, transformed yeast cells were run for PCR

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to confirm the *Col2a1* enhancer intronic region. The band of 407bp was presented on gel electrophoresis shown in figure 2b.

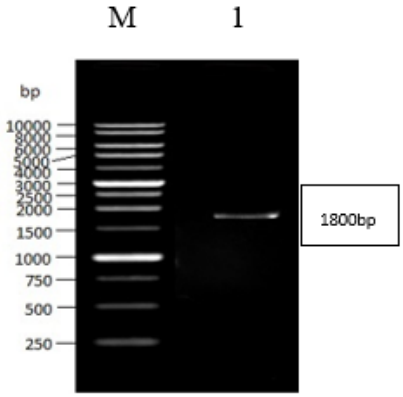

Figure 2 (a): The band size is 1800bp in lane 1 whereas the gene ladder was run in lane M. The insert size has confirmed the presence of *TGFβRII* transformation in yeast cells.

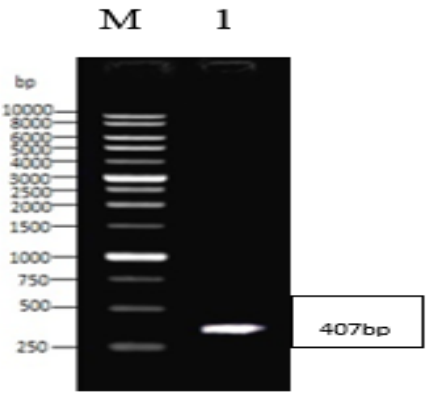

Figure 2 (b): The band size is 407bp in lane M whereas the gene ladder was run in lane 1. The insert size has confirmed the presence of Col2a1 transformation in yeast cells.

The above Enzyme digestion and PCR results confirmed the co-transformation of TGFβRII gene and Col2a1 gene.

Gene Expression Analysis of TGFβRII and GFP

Proteins were extracted following an increase in incubation time (lane 1 to lane 10). There was rise in recombinant protein production at incubation time 120hr shown in figure 3.

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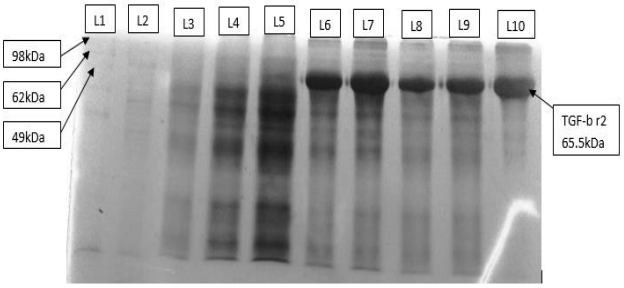

Figure 3: 12.5%SDS PAGE of TGFβRII protein. Lane 1: pre-stained protein ladder, Lane 2: un-transformed yeast cells (15µL), Lane 3: TGFβRII in co-transformed yeast cells at 24hr, Lane 4: TGFβRII in co-transformed yeast cells at 48hr (15µL), Lane 5: TGFβRII in co-transformed yeast cells at 72hr, Lane 6: TGFβRII in co-transformed yeast cells at 96hr (15µL), Lane 7: TGFβRII in co-transformed yeast cells at 120 hr. Lane 8: TGFβRII in co-transformed yeast cells at 120 hr (15µL). Lane 9: TGFβRII in co-transformed yeast cells at 120 hr. Lane 10: TGFβRII in co-transformed yeast cells at 120 hr.

Fluorescence Microscopy

TGFβ will be used as substrate to detect activation of Col2GF reporter activity in transformed yeast cells through fluorescence microscopy after treatment with oral cancer patient's saliva and results have been obtained with the optimized incubation of 96 hours shown in figure 4.

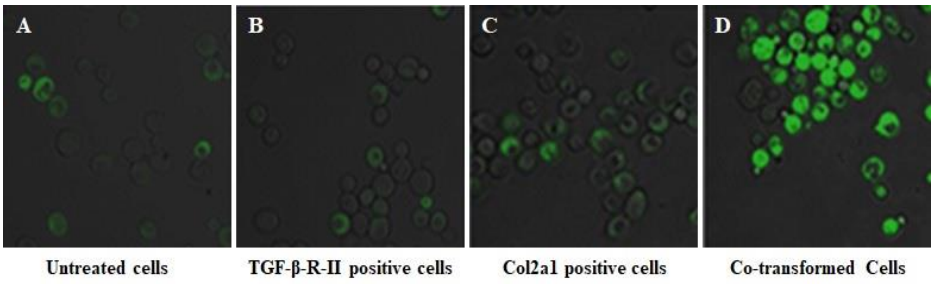

Figure 4: Col2GF expression plasmids were observed in oral cancer patients' saliva. (a) Untreated expression of yeast cells without subjected to patients' saliva. (b) Reaction of patient's saliva with TGFβ-receptor II plasmid. (c) Reaction of patient's saliva with Col2a1 plasmid. (d) Highest expression of fluorescence seen in oral cancer patients' saliva treated with co-transformed plasmids. The images were acquired with a fluorescence microscope equipped with a 40× objective lens. Scale bar: 20 µm. The exposure time was 1/15s.

Statistical Analysis

The expression of cytokines (TGFβ, VEGF, TNF-α, P53, IL-6, and IL-1) in both saliva and serum was higher in patients than in controls ($p<0.005$). Diseased samples were 40, and normal were 20 in size. The P value of yeast cells with co-transformation was significant

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(p<0.05), but yeast cells cloned with TGFβRII and Col2a1 plasmid individually were non-significant (p=0.216). Samples were measured on an Elisa plate reader set at 450nm.

Level of Growth Factor Cytokines in Saliva of the Oral Cancer Patients

Sandwich ELISA was used to quantify growth factors (TGFβ, VEGF, P53, TNF-α IL-6, IL-1) differently in both normal and cancerous patients' saliva. p53 levels were significantly lower in the saliva of oral cancer patients (0.074 ± 0.0039) compared to normal patients' saliva (0.16 ± 0.0072). However, the levels of TGFβ (1.6 ± 0.030), were significantly elevated in oral cancer patients as compared to normal patients (0.17 ± 0.011), the level of VEGF (1.32 ± 0.0835) was significantly elevated in oral cancer patients as compared to normal patients (0.130 ± 0.00601), and level of TNF-α was also significantly elevated in oral cancer patients (1.6 ± 0.043) as compared to normal patients, (0.27 ± 0.020). The level of IL-1was higher in the saliva of oral cancer patients (1.8 ± 0.037) as compared to the saliva of normal patients (0.29 ± 0.016) similarly, IL-6 was also higher in the saliva of oral cancer patients (1.5 ± 0.060) as compared to normal patients' saliva (0.14 ±0.010). However, the difference among them was statically significant shown in figure 5.

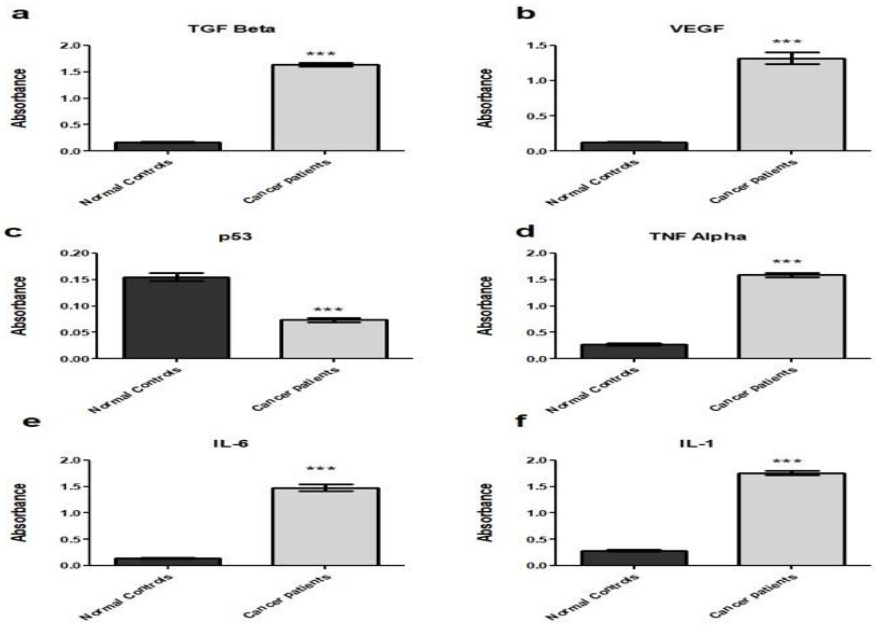

Figure 5: The Bar Graph Indicates Saliva Cancer Markers Expression in Normal and Oral Cancer Patients. (a) Shows enhanced expression of TGFβ in saliva. (b) Shows increased expression of VEGF in saliva. (c) Shows decreased level of P53 in saliva. (d) Shows upregulation of saliva in TNF-α. (e) Shows enhanced expression of IL-6 in saliva. (f) Shows upregulation of IL-1 in saliva. A bar graph is drawn to display the frequency distribution graphically. The height of the bar shows the relative distribution of each category of cancer markers. T-test (Nonparametric) was applied and values were expressed as ±SEM. (p<0.05 consider as significant shows the significant difference between normal and cancer patients. Sample size is 60 (20 Normal and 40 Cancer patients) is used.

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TGFβRII ACTIVITY

TGFβ-receptor II receptor activity checked through enzyme linked immunosorbent assay (ELISA) by exploiting antigen-antibody interaction of the receptor protein TGFβRII from yeast lysate with TGFβ-receptor II antibody. We used transformed and un-transformed groups of yeast cells treated with saliva of oral cancer patients shown in figure 6.

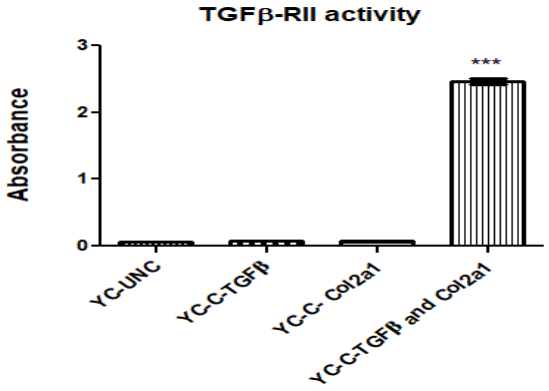

Figure 6: The bar graph indicates TGFβ Lysate expressions according to plasmids after reaction with saliva of oral cancer patients. A bar graph is drawn to display the distribution graphically. The height of the bar shows relative distribution of each category of TGFβ yeast lysate expression. The last bar shows that the Yeast cells with co-transformation 2 plasmids are higher in number as compare to un-cloned Yeast cells, cloned with TGFβ plasmid and Yeast cells cloned with Col2a1 plasmid individually. One way ANOVA with Bonferroni's posttest was applied and values were expressed as mean ±SEM. p<0.05 consider as significant.

Level of Growth Factor Cytokines in Serum of the Oral Cancer Patients

Sandwich ELISA was used to evaluate the growth factors (TGFβ, VEGF, P53, TNF-α, IL-6, IL-1) in serum of both normal and cancerous patients differently. The level of p53 was significantly lower in the serum of oral cancer patients (0.051 ± 0.0028) compared to the serum of normal patients (0.089 ± 0.0020). On the other hand, the levels of TGFβ in oral cancer patients (1.8 ± 0.053), were significantly elevated as compared to the serum of normal patients (0.13 ± 0.0098) and the level of VEGF (2.33 ± 0.0401) was significantly increased in serum oral cancer patients as compared to the normal patients (0.0793 ± 0.00345). The level of TNF-α in serum of oral cancer patients was also significantly increased (2.4 ± 0.061) as compared to serum in normal patients (0.071 ± 0.0043). Similarly, the level of IL-1 was higher in the serum of oral cancer patients (2.5 ± 0.054) as compared to the serum of normal patients (0.073 ± 0.0038). The level of IL-6 was also upregulated in the serum of oral cancer patients (2.4 ± 0.056) as compared to normal patients' serum (0.099 ±0.0051). However, the difference among them was statically significant shown in figure 7.

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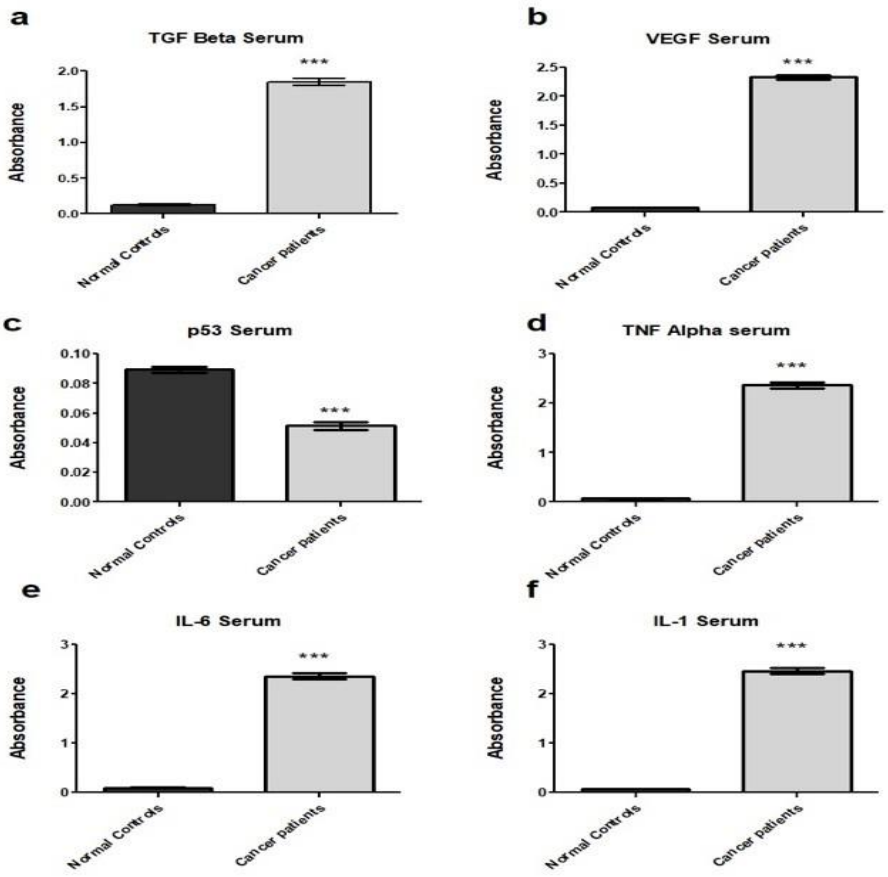

Figure 7: The bar graph indicates serum cancer markers expression in normal and oral cancer patients. (a) Shows increased expression of TGFβ in serum. (b) Shows enhanced expression of VEGF in serum. (c) Shows decreased level of P53 in serum. (d) Shows enhanced expression of serum in TNF-α. (e) Shows up regulation of IL-6 in serum. (f) Shows increased expression of IL-1 in serum. A bar graph is drawn to display the distribution graphically. The height of the bar shows the relative distribution of each category of cancer markers. T-test was applied and values were expressed as mean ±SEM. (p<0.05 consider as significant shows the significant difference between normal and cancer patients. Sample size is 60 (20 Normal and 40 Cancer patients) is used.

DISCUSSION

Oral cancer is the 6th most frequent cancer with higher mortality rate. Oral squamous cell carcinoma accounts for > 90% of all oral cancers. The use of saliva for early cancer detection is encouraging due to noninvasive and easy sampling and collection techniques. More than 100 salivary biomarkers included cytokines (IL6, IL-1, TNF alpha and P53) have already been identified [1].

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In this study, we constructed the yeast transformed with two circular plasmids; pGF1-4eCol2a1 (intronic region) and pCMV5B-TGFβ receptor II (TGFβ receptor II gene), the later protein TGFβ receptor II induced the expression of former one Col2a1 [2].

It has been reported that TGFβ has excessive affinity for binding with kind II receptor, a transmembrane protein with a cytoplasmic serine/threonine kinase domain [3, 18-19]. It had been seen that type II receptor required association of TGFβ binding protein and activity of kinase and type I receptor inhibit the early gene response [20]. Although the TGFβ signalling pathway is efficient when bound with TGFβRI and TGFβRII receptors for its activation the TGFβRII constructed, plasmid is sufficient for mediating BUB1 (budding uninhibited by benzimidazoles 1) phosphorylation of the SMAD2-TGFβR structural fragmentation, thus regulating the TGFβ signature event [21].

In this study we discussed 6 parameters for oral cancer detection in serum and saliva samples. These parameters are TGFβ, VEGF, TNF alpha, P53, IL-6 and IL-1.

In oral cell carcinoma it is believed that, at first changes occur in oral epithelium that leads to oral cells dysplasia and then cancer of cells. Several factors may involve in this process. Transforming growth factor-beta (TGFβ) is an important cytokine that induces epithelial mesenchymal transition (EMT). TGFβ belongs to a superfamily that consists of more than thirty proteins including activins, growth factors and bone morphogenetic proteins. TGFβ is a diversified cytokine that plays an important role in proliferation, differentiation, and migration of cancer cells [24]. Our study revealed that the TGFβ level in oral cancer patients is greater than the normal patients and it's also predicted by the study of Xin-Pang et al., 2018 that TGFβ plays the role of tumor suppressor in early stages of carcinogenesis and it has contradictory effects on cancer.

In two-hybrid system of yeast, TGFβRII (Transforming Growth Factor Receptor Type II) is a decoy as, the effect of TGFβ is mediated by a heteromeric complex of type I and type II threonine kinase receptors. TGFβRI can't bind its ligand without TGFβRII due to the downstream substrate while TGFβRII binds with TGFβ and phosphorylated constitutively [23]. Yeast has signal transduction mechanism just like a signaling pathway mediated by human TGFβ that's why 25-amino acid insertion sequence of TGFβRII is obtained in cloned subtype relative to the receptor sequence of type-II receptor [24].

Under culture conditions of physiological signalling of TGFβ superfamily can drive the expression of Col2a1. It increases TGFβ2 mRNA expression, TGFβ bioactivity and protein secretion. Gene expression of Col2a1 was induced upon knockdown of TGFβ2 isoform under enhanced bioactivity of TGFβ [2].

Collagen type II transcription is implicated via TGFβ in chondrocytes. TGFβ ligand bind with TGFβRII (TGFβ type II receptor) and then Transcriptional activity is regulated by the recruitment of ALK5 (type I receptor activin-like kinase 5) and phosphorylation of intracellular effector molecules SMAD-2 and -3 [25].

TGFβ is used as a substrate to detect the activation of Col2GF reporter activity in yeast cells transformed by fluorescence microscopy. Various dilutions (24hrs, 48hrs, 96hrs and 120hrs) of TGFβ substrates have been used against the transformed yeast culture to

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optimize the fluorescence signal. TGFβ expression in saliva increases in the early stages of cancer so we measured the presence of TGFβ cytokine in the saliva of cancer patients and allow it to react with recombinant yeast cells. A GFP signal indicates the presence of TGFβ. Saliva from healthy individuals is used as a negative control, while TGFβ substrate is used as a positive control.

The presented results derived from designing a model of a reporter system in yeast cells for the detection of TGFβ that can be used as a tool for quantification and spatial assessment of oral cancer by saliva sample, which would be an innovative, non-invasive approach for the early detection of oral cancer.

CONCLUSIONS

In the present study, a yeast hybrid system was constructed containing both TGFβRII and Col2a1 plasmid with *TGFβ* and *Col2a1* genes, both of them had the efficiency of optimized protein expression successfully integrated in eukaryotic expression system *S. cerevisiae* through which recombinant protein has been obtained inhibited GFP signal. This transformant system is more beneficial and has a positive response of early disease detection in oral cancer patients because currently there is no other biological system.

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Conflict of Interest: Sidra Hasnain, Sana Khurshid and Sana Javaid Awan declare that they have no conflict of interest.

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