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Research Article

Evaluation of mRNA Expression of p16 Gene in Blood Samples of Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma Patients-A Reverse Transcriptase-Polymerase Chain Reaction Study

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Abstract

Aim: The aim of the study was to determine the expression level of p16 gene mRNA in blood samples of normal healthy, OSMF and OSCC patients by the Quantitative Real Time RT-PCR method.

Materials and Methods: 90 human participants of both genders were selected from the Outpatient Department of Oral Pathology and Microbiology. The participants were divided into three groups: Group I - Normal: Not having tobacco and areca nut habit and any Oral potentially malignant disorder or Cancerous changes in oral mucosa (n = 30), Group II - Patients with Oral Submucous Fibrosis (OSMF) (n = 30) and Group III - Patients with Oral Squamous Cell Carcinoma (OSCC) (n = 30). 1ml of peripheral blood samples were collected from each subject. Total RNA extracted and subjected to Real time RT-PCR for quantification.

Results: Mean age of Group I was 34 years , Group II was 34.6 years and in Group III was 47.6 years. In both the groups, there was male predominance being 80% of males & 20% of females in group II and 63.3% of males and 36.7% of females in group III (Graph 2). In 30 cases of OSCC, lesions were located predominantly on buccal mucosa 12 (40%), followed by alveolobuccal complex 8 (26.7%), tongue 7 (23.3%), Palate 2 (6.7%) and lip 1 (3.3%) (Graph 3). A -2.86 fold downregulation of p16 m RNA expression level was found in OSMF samples as compared to Normal samples, a -7.72 fold down-regulation of p16 m-RNA expression was found in OSCC samples as compared to Normal samples and -2.69 fold down-regulation of p16 mRNA expression was found in OSCC group, as compared to OSMF group.

Conclusion: There was downregulation of p16 mRNA expression in OSCC cases in comparison to both Normal and OSMF cases. In OSCC group, p16 mRNA expression was progressively down regulated in Well Differentiated Squamous Cell Carcinoma (WDSCC) followed by Moderately Differentiated Squamous Cell Carcinoma (MDSCC) and in Poorly Differentiated Squamous Cell Carcinoma (PDSCC) cases.

Keywords: *p16 mRNA Expression; Quantitative Real Time RT-PCR; Oral Cancer; Oral Squamous Cell Carcinoma; Oral Submucous Fibrosis*

Abbreviations

OSCC: Oral Squamous Cell Carcinoma; OSMF: Oral Submucous Fibrosis; WDSCC: Well Differentiated Squamous Cell Carcinoma; MDSCC: Moderately Differentiated Squamous Cell Carcinoma; PDSCC: Poorly Differentiated Squamous Cell Carcinoma; IIO: Interincisal Opening

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide and the third most common form of cancer in the developing countries. Squamous cell carcinoma occurs due to multiple genetic changes leading to formation of either abnormal proteins or altered amount of normal proteins [1].

Clinical OSCC is often preceded by stepwise transition from potentially malignant states like leukoplakia and Oral Submucous Fibrosis (OSMF) to the metastatic tumour phenotype. Numerous alterations accumulate to potentiate this transition to malignancy [2].

Oral submucous fibrosis is a potentially malignant and crippling condition of the oral mucosa. The etiopathogenesis of OSMF believed to be multi-factorial; includes areca nut chewing, ingestion of chillies, genetic and immunologic processes, nutritional deficiencies and other factors [3]. In patients with OSMF, the oral epithelium becomes atrophic and thereby becomes more vulnerable to carcinogens [4].

Oral potentially malignant lesions have shown a rate of progression to cancer upto 17% within a mean period of 7 years after diagnosis. Amongst the premalignant conditions, Oral Submucous Fibrosis (OSMF) has a high rate of morbidity rate due to its progressive inability to open the mouth, resulting in nutritional deficiencies along with a significant mortality rate due to its transformation into OSCC at a rate of 3% - 19% (7.6%) over a period of 17 years [5].

Given this aggressive nature of the potentially malignant lesions, identification of a suitable biomarker is imperative for timely diagnosis, prognosis and treatment [6]. Mutations in tumour suppressor genes namely p53, pRb, p16 and pro-apoptotic genes namely bcl2, bax have been stated as contributing factors to the development and transformation of precancer to cancer. It has been found that inactivation of p16 occurs early in the development of OSCC [7].

The p16 gene (located on chromosome 9p21) plays a key role in cell cycle regulation and codes for a protein which binds and inhibits cyclin-dependent kinases (CdK-4/6) and phosphorylates serine and threonine residues of retinoblastoma (RB) protein [8]. The inactivation of p16 allows the cell to enter in the S phase after a pause at the G1 checkpoint [9]. Down-regulation of p16 has been described in oral cancer and its inactivation is believed to be a precursor event in oral carcinogenesis. Additionally, the absence of p16 is an early and often critical event in tumor progression, if deletion occurs in at least one copy there are quite high chances of premalignant lesions giving selective advantage to neoplastic cells [10].

Purpose of the Study

The purpose of this study was to evaluate and compare the mRNA expression of p16 gene in OSMF and OSCC cases within the population using blood as an important surrogate material by utilizing Quantitative Real Time RT- PCR and to emphasize the potential role of p16 gene in pathogenesis of OSMF and its biological and prognostic significance in OSCC.

Materials and Methods

The present study was carried out at Government Dental College and Hospital, Nagpur in collaboration with Molecular Research Laboratory. 90 human participants of both genders were selected from the Outpatient Department of Oral Pathology and Microbiology. An

informed consent was obtained from all the cases included in the present study after explaining them about the study pattern. The study was approved by Institutional Ethics Committee. (Ethics number : GDCHN/SS/Ethics 2017/5866/2017)

The blood samples from the included individuals were divided into three groups; group I, normal (NOM); group II, OSMF; and group III, OSCC; depending on the clinical state of the corresponding individual. The blood samples were included in the NOM group, if the individual had no history or current findings of any potentially malignant or malignant lesion or any systemic disease, had no medication in the last one week, and had no habit of consuming tobacco or related products. Blood samples included in OSMF group were obtained from the patients who were clinically diagnosed and histopathologically confirmed of suffering from OSMF, reported a history of consumption of areca nut, tobacco, or other related products, had no history of any other malignant lesion or systemic condition, and had not received any treatment for their current condition. Lastly, blood samples included in OSCC group were obtained from the patients who were confirmed with OSCC histopathologically, had no other systemic condition, and had not received any treatment for their current condition. In all the three groups, individuals with a history of chronic alcoholism or those diagnosed with HIV infection, other systemic conditions, or cancers of any other regions of the body were excluded from the study. Thus, 30 samples each of NOM, OSMF, and OSCC were obtained to study the differential expression level of p16 mRNA through RT-PCR.

Histopathologic examination

Group III (OSCC) samples were subjected to routine formalin-fixed hematoxylin and eosin (H and E) stained histopathological examination as well for diagnostic confirmation and grading in cases of OSCC (Fig.1)(Fig.2)(Fig.3).

RT-PCR

Blood samples stabilized in DNA/RNA Shield™ were taken from storage temperature and incubated with proteinase K mix for 30 minutes following which isopropanol in equal volume was added and vortexed. mRNA extraction was done directly from whole blood using the Zymoresearch Quick-RNA Whole Blood extraction kit (USA) and evaluated for purity and concentration in DeNovix DS-11 Spectrophotometer. The mRNA was then reverse transcribed into complementary DNA using the 2X TOPreal™ One-step RT qPCR Reaction Mix (with low ROX and includes SYBR® Green).

p16 and HPRT1 (Housekeeping gene, Reference Gene) were selected from the literature-based values of self-complementary and Tm, and the primer sequences were as follows:

p16:

Forward Primer 5’ AGCCTTCGGCTGACTGGCTGG 3’

Reverse Primer 5’ CTGCCCAT CATCATGACCTGGA 3’

Product size: 139 bp PCR product

HPRT1: Forward Primer 5’ CCTGGCGT CGTGATTAGTG 3’

Reverse Primer 5’ TCAGTCCTGTCCATAATTAGTCC 3’

Product size: 125 bp PCR product.

The primers were diluted as per company’s instruction and stored at -20ºC in a deep freezer.

Semi-quantitative PCR

Following were the semi-quantitative PCR conditions: 50°C for 30 min, hold; reverse transcription: 95°C for 10 min, hold; initial denaturation: 95°C for 5 seconds; denaturation -40 cycles, and annealing and elongation at 60°C for 30 sec, 40 cycles in Bio-Rad, CFX 96 Real-Time PCR detection system (USA) to obtain the CT value (Figure 4).

The final reverse transcribed DNA formed was confirmed by agarose gel electrophoresis using proxiO 100bp DNA ladder. (Fig.5) (Fig 6.).

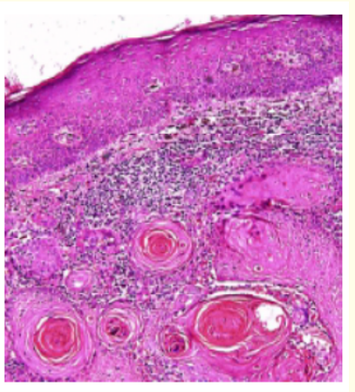


Figure 1: *Histopathological picture of WDSCC .*

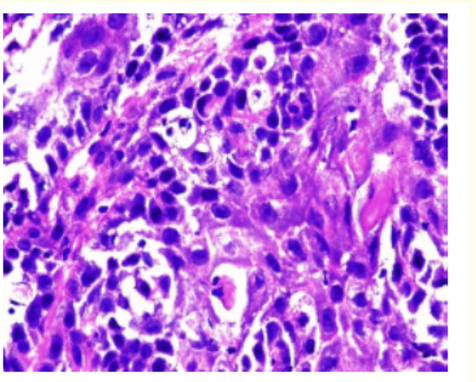


Figure 2: *Histopathological picture of MDSCC*

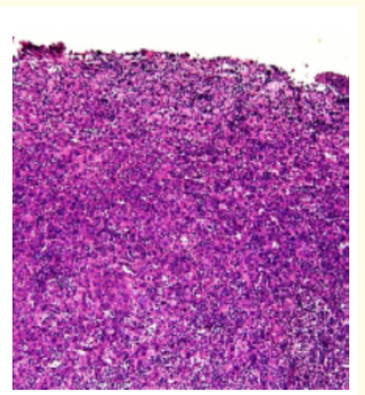


Figure 3: *Histopathological picture of PDSCC*

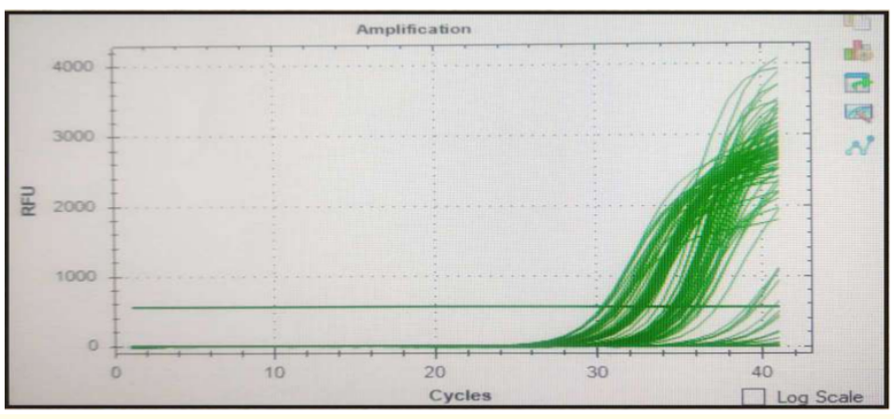


Figure 4: *RT- PCR results (PCR Amp/cycle graph for SYBR)*

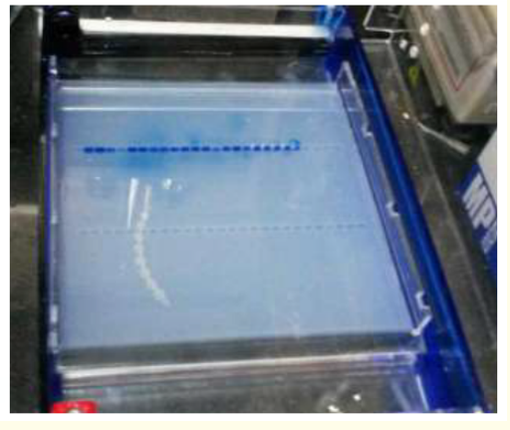


Figure 5: *Agarose gel , wells filled with ProxiO 100bp DNA ladder (DNA Marker)*

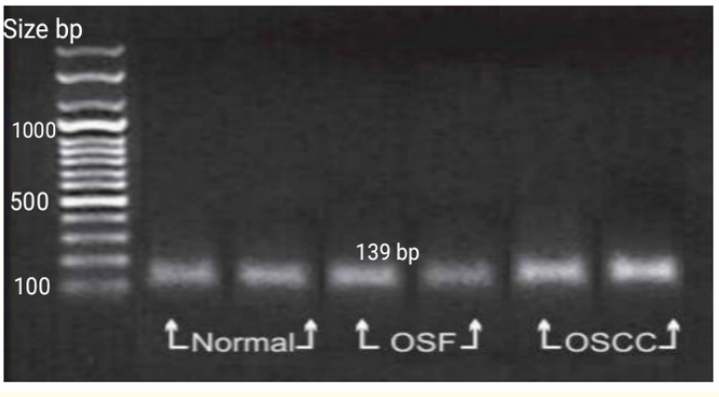


Figure 6: *Gel electrophoresis image*

Statistical analysis

The statistical analysis was performed using $2^{-\Delta\Delta Ct}$ (Livak) method, relative quantification (RQ) = $2^{-\Delta\Delta Ct}$, to get the relative expression of target gene in the target group in comparison with the control group in terms of fold change. Log_{10} of RQ was calculated to know the upregulation or downregulation of the target gene:

- Data was subjected to statistical analysis using Statistical package for social sciences (SPSS v 23.0).
- Inter group comparison (2 groups) was done using Mann Whitney U test.
- Inter group comparison (> 2 groups) was done using Kruskal Wallis ANOVA followed by pair wise comparison using Mann Whitney U test.
- Intra group comparison was done using Friedman's test followed by pair wise comparison using Wilcoxon Signed rank test.
- For all the statistical tests, $p < 0.05$ was considered to be statistically significant

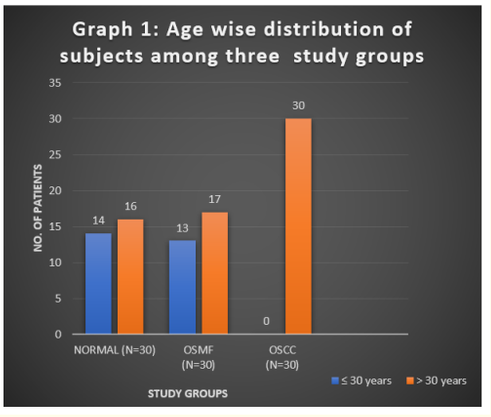
Results

Demographic data

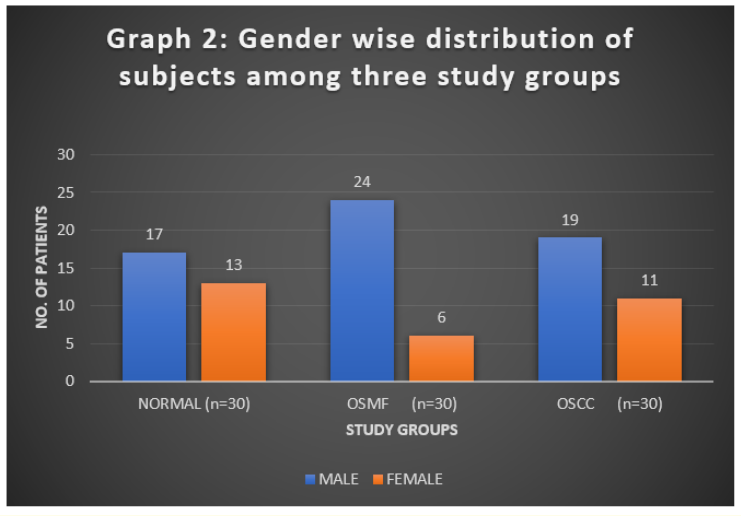
The mean age of Normal (Group I) was 34 years, mean age of OSMF (group II) was 34.6 years and in OSCC (group III) was 47.6 years respectively. There was a male predominance observed in the study comprising of 24 males (80%) and 6 females (20%) in group II and 19 males (63.3%) and 11 females (36.7%) in group III (Table 1). Of all the individuals under 30 years of age 14 (46.7%) were normal, 13 (43.3%) were diagnosed with OSMF, and 0 (0%) with OSCC; while, of the individuals above 30 years of age, 30 (100%) were diagnosed with OSCC, 17 (56.7%) with OSMF, and 16 (53.3%) were healthy normal individuals. Thus, OSMF and OSCC were found more in individuals above the age of 30 years (Graph 1 and 2). In 30 cases of OSCC, lesions were located predominantly on buccal mucosa 12 (40%), followed by alveolobuccal complex 8 (26.7%), tongue 7 (23.3%), Palate 2 (6.7%) and lip 1 (3.3%) (Graph 3 and table 2).

Gender	Group wise number (%)		
	Group I Normal (n = 30)	Group II OSMF (n = 30)	Group III OSCC (n = 30)
Male	17 (56.7%)	24 (80%)	19 (63.3%)
Female	13 (43.3%)	6 (20%)	11 (36.7%)

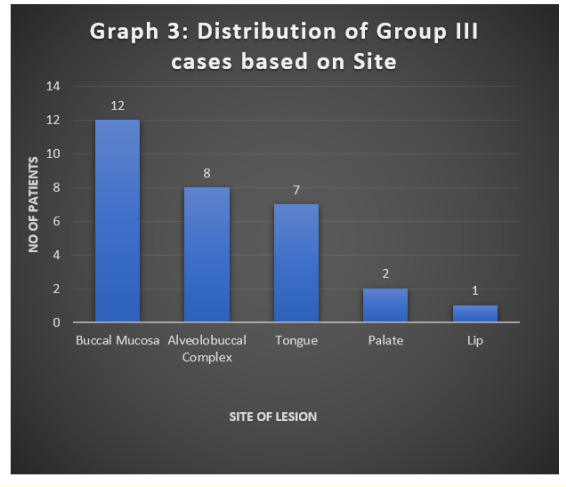
Table 1: *Distribution of study population according to gender in three study groups.*



Graph 1: *Graphical visualization of study population according to age in different study groups.*



Graph 2: *Graphical visualization of study population according to gender in different study.*



Graph 3: *Graphical visualization of distribution of group III study population based on site of lesion.*

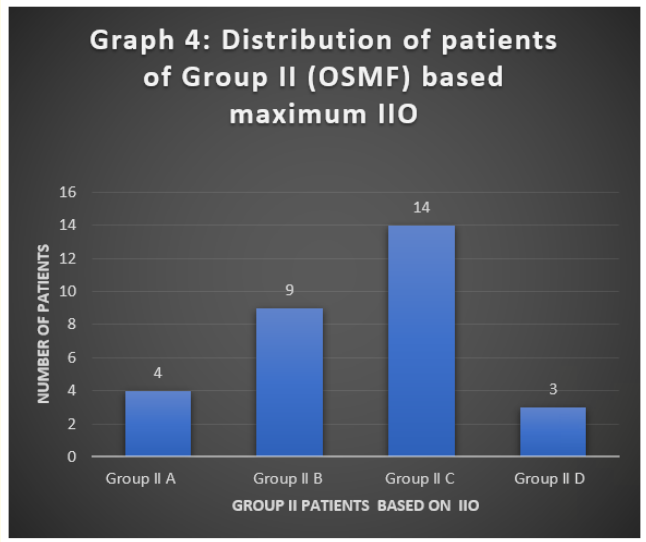
Site	Number of subjects	Percentage
Alveolobuccal complex	8	26.7%
Buccal mucosa	12	40%
Tongue	7	23.3%
Palate	2	6.7%
Lip	1	3.3%
Total	30	100%

Table 2: *Distribution of group III subjects based on site.*

The OSMF Patients (Group II) were sub-grouped based in maximum interincisal opening (IIO). Out of these 30 cases of OSMF, maximum number of cases 14 (46.7%) belonged to Group II C (IIO: 25 mm - 15 mm), followed by 9 (30%) cases of Group II B (IIO: 26 mm - 35 mm), 4 (13.3%) cases of Group II A (IIO > 35 mm) and lastly 3 cases (10%) of Group II D (IIO < 15 mm) (Table 3 and graph 4).

Maximum Inter-Incisal opening (mm)	No of Patients
Group II A (IIO > 35 mm)	4 (13.3%)
Group II B (26 mm - 35 mm)	9 (30%)
Group II C (25 mm - 15 mm)	14 (46.7%)
Group II D (IIO < 15 mm)	3 (10%)

Table 3*: Distribution of patients of group II (OSMF) based on maximum inter-incisal opening (IIO).*

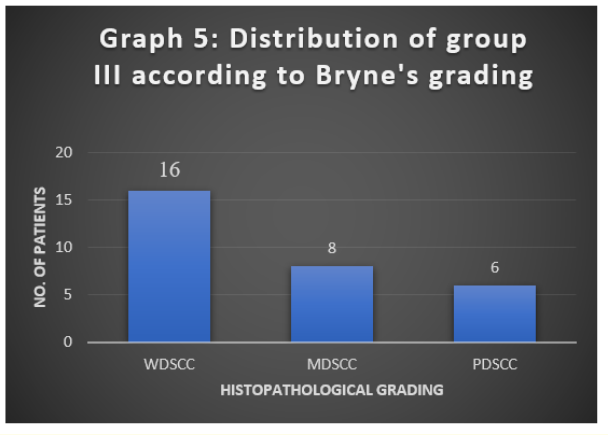


Graph 4: *Graphical visualization of distribution of group II study population based on maximum inter incisal mouth opening (IIO).*

Similarly, OSCC cases were graded as WDSCC (n=16) (Fig.1), MDSCC (n=8) (Fig.2) and PDSCC (n=6) (Fig.3) based on Bryne's grading system (Table 4) (Graph 5).

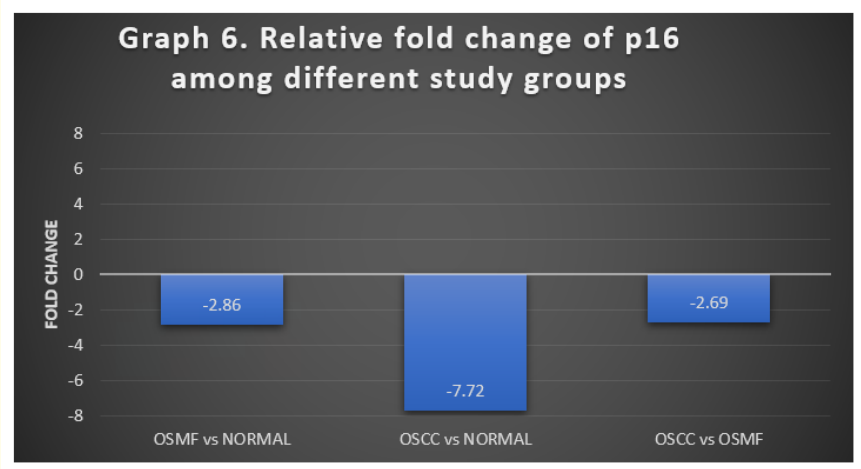
	Bryne's grading (n = 30)			
	WDSCC	MDSCC	PDSCC	Total
Number	16	8	6	30

Table 4: *Distribution of group III study population according to Bryne's grading.*



Graph 5: *Graphical visualization of distribution of group III study population based on Bryne's grading.*

The main observation was that p16 mRNA expression was downregulated from NORMAL to OSMF to OSCC (Graph 6). The relative quantification value (RQ) for the comparison of OSMF vs. Normal was obtained as 0.34 and fold change was -2.86, indicating downregulation of p16 in OSMF as compared to Normal. Thus, the change in the expression level of p16 between groups was clinically relevant. Statistically, the difference of mean dCt values between OSMF and Normal group was highly significant. (p-value < 0.001).

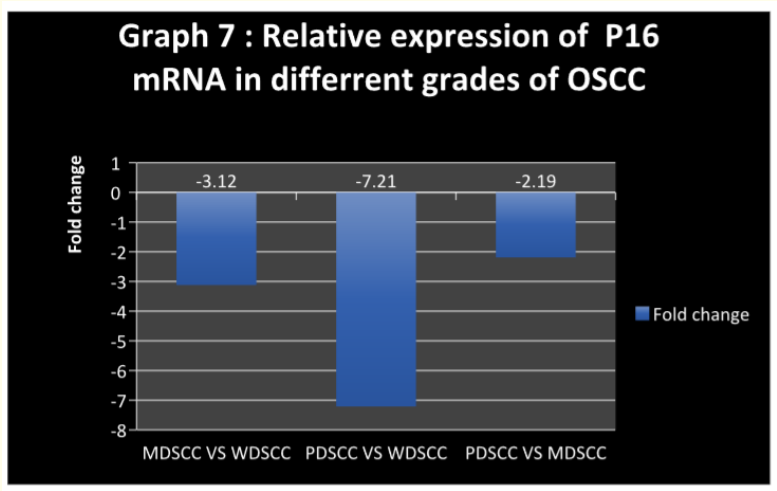


Graph 6: *Graphical visualization of relative fold change of p16 gene in different study groups.*

The RQ value for the comparison of OSCC vs. Normal was 0.129 and fold change was -7.72, implying significant downregulation of p16 in OSCC group, as compared to Normal group. These findings were clinically and statistically highly significant.

The RQ value for the comparison of OSCC vs. OSMF was 0.37 and corresponding fold change was -2.69, implying significant downregulation of p16 in OSCC group, as compared to OSMF group. The change was clinically relevant and statistically highly significant (p-value of < 0.001).

Among (Group II) OSMF patients, there was a progressive downregulation of p16 mRNA expression with decrease in interincisal opening which was also statistically significant and in OSCC group, p16 m-RNA expression was progressively downregulated in WDSCC followed by MDSCC and highest rate of downregulation was seen in PDSCC cases and all these findings were also statistically significant (Graph 7).



Graph 7: *Relative expression of P16 mRNA in differrent grades of OSCC.*

Discussion

The incidence of OSCC has increased due to increased risk factors such as tobacco and alcohol. OSCC usually arises from cells undergoing genetic and epigenetic alterations particularly in oncogenes or tumour suppressor genes. OSCC occurs as a consequence of multiple molecular events that develop from the combined effects of an individual's genetic predisposition and exposure to environmental carcinogens such as, tobacco, alcohol, chemical carcinogens, ultraviolet or ionizing radiation and microorganisms [11].

The risk of malignant transformation of patients with OSMF increasing day by day and hence it is very important to monitor these patients to identify early transformation into OSCC. If identified at initial stages, the incidence of death rates due to OSCC can be reduced considerably [12].

The results of p16INK4a expression in OSCC showed variable results between different research studies, showing both subexpression as well as overexpression. The decrease of p16INK4a expression and its epigenetic silencing is associated with oral cancer and precancer and its inactivation is believed to be an early and gradual event as the tumor stage and the grade of dysplasia advances in premalignant lesions, which is occasionally associated with certain clinical and pathological parameters or the expression of other cell cycle regulatory proteins [13].

Blood is one of the most easily available clinical samples and tumor DNA is known to be present in blood stream as cell free DNA circulating in the plasma or serum of cancer patients. Genetic and epigenetic alterations including point mutations, microsatellite instabilities (MI), losses of heterozygosity (LOH), and DNA hypermethylations have been reported in circulating DNA. Thus, blood may serve as an important surrogate material for cell free DNA-based molecular analysis in cancer and Oral Potentially Malignant Disorders (OPMD) patients [19].

Hence this study was conducted to measure the relative m RNA expression level of p16 gene as the target gene while keeping HPRT1 gene as the reference gene by RT-PCR method using Bio-Rad CFX 96, Fast Real Time PCR, in fresh blood samples of Normal, OSMF and OSCC patients with 30 samples in each group.

The mean age of patients with OSMF was 34.6 ± 9.29 (Range = 18 - 53 yrs), and was more common in 4^{th} decade of life, showing much higher prevalence in young patients. This was similar to findings of J G Ray., *et al*. [14] who also found similar occurrence. For OSCC, the mean age was 47.6 ± 10.94 (Range = 31 - 68 yrs), most common being in 5^{th} decade of life. This finding was in accordance with Shenoi R., *et al*. [15] who reported the mean age of OSCC to be 49.73 years (Graph 1).

Out of randomly selected 30 subjects each in OSMF and OSCC group, a male predominance was observed. In OSMF group 24 were males (80%) and 6 (20%) were females (Graph 2) while in OSCC group, 19 were males (63.3%) and only 11 were females (36.7%). Our findings go in hand with the studies of J G ray., *et al*. [14] and Warnakulsuriya S., *et al* [16]. Usually, a male predilection is observed in the literature when it comes to most OPMD conditions and OSCCs. This can be attributed to the risk habits which are being practiced more commonly among males, relative to the female community. This may also account for multiple simultaneous habits such as smoking and alcohol, which have a synergistic effect on carcinogenesis [17].

Majority of OSCC occurred in the Buccal mucosa (40%) followed by alveolobuccal complex (26.7%) and tongue (23.3%). Very few cases were found in palate (6.7%) and lip (3.3%). Our observation was in accordance with Tandon A., *et al* [18]. This finding of increased occurrence of OSCC can be attributed to the tobacco and quid keeping habit in the buccal vestibule found commonly in India (Graph 3).

Comparative evaluation of p16 expression level

Normal and OSMF

In the present study, a -2.86-fold downregulation of p16 m RNA expression level was found in OSMF samples as compared to Normal samples. Our study was in accordance with the study done by Bhatia V., *et al.* [19] where they found significant downregulation of p16 mRNA expression in OSMF group (~ 3 - 4 folds).

These epigenetic alterations i.e. hypermethylation-induced transcriptional silencing of gene caused by tobacco, arecanut may be the reason for the above-mentioned downregulation of p16 gene in Group II patients.

Normal and OSCC

In the present study a -7.72-fold down-regulation of p16 m-RNA expression was found in OSCC samples as compared to Normal samples. Our study was in accordance with the study conducted by Bhatia V., *et al*. [19] where expression of p16 gene was assessed by Methylation-specific PCR (MSP) and gene expression by real-time PCR (qPCR) in 76 OSCC patients and they found significant down-regulation of p16 mRNA expression in both tissue samples (~ 7-8 folds) and blood samples (~ 4-5 folds). Our present study also goes in hand with the study conducted by Allameh A., *et al*. [20], where expression of p16 gene was determined in 67 OSCC patients using Methylation-specific PCR (MSP) and real-time PCR (qPCR). Their results also showed downregulation of p16 gene expression in OSCC and found that there was a significant association between p16 expression and pathological grade and stage of the tumors.

The reason for this downregulation of p16 that we obtained in Group III (OSCC) can be justified on the basis of the study done by Kim HS., *et al*. [21], where they reported that methylation of the CpG islands of tumor suppressor genes leading to their transcriptional inactivation to be a highly consistent feature of tumorigenesis. Studies of primary tumors and OSCC cell lines that they did indicate that methylation may constitute an alternative mechanism in silencing of the $p16^{INK4a}$ gene.

OSCC and OSMF

A -2.69-fold down-regulation of p16 mRNA expression was found in OSCC group, as compared to OSMF group. Sharada., *et al*. [22] through a study concluded that p16INK4a expression demonstrated increased expression in normal group while there was reduction in expression as the disease progressed from Oral epithelial Dysplasia to OSCC and the expression pattern in OSMF was intermediate in between normal and mild dysplasia.

Relative quantification of p16 based on Histopathological grading in OSCC group revealed a -3.12-fold down-regulation of p16 m RNA expression was found in MDSCC group as compared to WDSCC group. Our study goes in hand with the study done by Allameh A., *et al*. [20], where they found significant downregulation of p16 mRNA expression between MDSCC/PDSCC groups when compared to WDSCC group. A -7.21 down-regulation of p16 m RNA expression was found in PDSCC as compared to WDSCC group while a -2.19-fold down-regulation of p16 m RNA expression was found in PDSCC group as compared to MDSCC group. These above-mentioned findings of our study also can be justified quoting the study Perez- Sayans., *et al*. [13], they observed a gradual reduction in p16INK4a expression as tumor stages advanced.

Conclusion

Tumors release tumor DNA into the peripheral blood circulation. This finding can be utilized for detection of genetic alterations and methylation abnormalities in the blood sample of patients and can have a profound impact on noninvasive diagnosis of cancers among high-risk populations.

The results of p16 expression vary greatly between the different studies that have been reported in the literature. The actual applicability of the determination of p16INK4a is therefore a bit controversial. Some studies reveal that p16 can be a clear potential marker in recognizing dysplasia in squamous mucosa of the head and neck. However, other authors state that p16 is not actually a correct marker for mucosa dysplasia, molecular staging of surgical margins, or malignant transformation. On the basis of our findings, it appears that tumor progression is partially attributed to downregulation of the p16. Thus, the expression of p16 is reduced as the tumor grade advances and this was statistically significant also. So, more studies should be done targeting the upregulation of p16 in cancer patients which could be considered as a therapeutic option.

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