Original Article

Potential of phosphatase and tensin gene polymorphisms as salivary biomarkers in oral squamous cell carcinoma – A cross-sectional study

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Abstract

Background: *PTEN* (phosphatase and tensin homologue) is a tumour suppressor gene which is well known for its negative regulation on phosphatidylinositol 3-kinase (*PI3K*) pathway, thereby controlling the cellular growth and proliferation in the process of carcinogenesis. In the present study, the frequency of the genotypes of the *PTEN* gene, (*rs2943773*, *rs1234224*, *rs9651495*, *rs3827678*, and *rs11202600*) has been observed in individuals with oral squamous cell carcinoma (OSCC) using real-time polymerase chain reaction (RTPCR).

Methodology: Saliva samples were collected from healthy individuals and individuals with OSCC. DNA extraction was done, followed by PCR using fluidigmn technique to observe the frequency of the genotypes.

Results: Variation was observed in the distribution of frequencies of the alleles (*rs9651495* and *rs1234224*) of the *PTEN* gene between the healthy individual and those with occurrence of OSCC. The other genotypes did not show any statistically significant difference in the distribution between the study group and the control group, nor any association was observed with OSCC.

Conclusion: The variation in the frequency of *rs9651495* and *rs1234224* of the *PTEN* gene suggests an association between the "*PTEN*" gene and "OSCC" and hence its use in the panel of diagnostic markers. This also opens the field for future research in the therapeutic applications of the *PTEN* gene.

Keywords: Oral squamous cell carcinoma, polymorphism, *PTEN* gene

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INTRODUCTION

Oral neoplasms make up the majority of the subgroup of head and neck cancers, which is the sixth most common type of malignancy worldwide. The most frequent type of oral neoplasm is oral squamous cell carcinoma (OSCC), accounting for almost 90%.[1,2] The combined effects of environmental and genetic factors are responsible for the occurrence of OSCC. The environmental factors such as tobacco, alcohol, viral infections, fungal infections, and chronic inflammation are well documented and lead to changes in the functioning of oncogenes and tumour suppressor genes.[3,4] The most common genetic changes are somatic mutations, single nucleotide polymorphism, and gene deletions.[5]

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PTEN (phosphatase and tensin homologue), the second most common tumour suppressor gene, is essential in the mechanism of the cell cycle pathway. It controls the signalling pathway of *AKT*, which promotes cell survival by suppressing the apoptosis of the cells. One of *PTEN*'s primary roles is to obstruct the *AKT* signalling pathway, which further reduces cell viability and encourages apoptosis. Similar to p53, *PTEN* also acts as a protector against the damaged cells, which eliminates these cells from the process of cell division.[6] Alterations in the *PTEN* gene, like downregulation, polymorphisms, gene silencing, and promoter methylation, have been observed often in many neoplasms such as that of breast, cervix, endometrium, colon, and glioblastoma.[7-9] Thus, methylation of the *PTEN* gene may be included as a molecular biomarker for detection of cancer. Apart from its role in maintaining the rhythm of the cells in the cell cycle, *PTEN* is also implicated in the aging process and plays a crucial role in how cells respond to certain hormones, including insulin and other growth factors. Somatic *PTEN* polymorphisms have been found in numerous sporadic malignancies, including lung, prostate, and breast cancers.[10-12]

The *PTEN* gene's genetic bit in head and neck cancers has been observed, and its association with oral cancer has been a subject of interest in the era of precision medicine. Earlier studies have been done to detect the gene alterations in tissues, cell lines, and serum. With the availability of the newer technologies, it has been possible to use saliva as a collection tool. It also has an added advantage of being a non-invasive method. With this background, this study was done with a purpose to ascertain the genotype frequency of the *PTEN* gene in OSCC and to look for a relationship between the polymorphisms in the *PTEN* gene and OSCC staging in saliva.

METHODOLOGY

The study observed the single-nucleotide polymorphism in specified *rs* numbers of the *PTEN* gene in the saliva of histopathologically diagnosed cases of OSCC. Prior to the start of the study, institutional ethical committee approval was secured. Two groups of participants were formed. The people having OSCC were included in the study (cases) group, and the control (healthy) group included healthy individuals with no apparent oral lesion and no habit.

The demographic data of all the participants were noted in a pre-designed proforma. Detailed case history included presence of tobacco chewing, smoking, or both and duration and the presence of any other associated habits like alcohol use. The saliva samples were collected from 150 healthy individuals and 150 histopathologically confirmed OSCC cases under aseptic conditions using a standard protocol. After obtaining the informed consent from the individuals participating in the study, 3 ml of drooling saliva was collected in a disposable plastic saliva collector. DNA extraction from the saliva sample was done using a Qiagen Kit. The variants were observed by real-time polymerase chain reaction (RTPCR) using the specific primers. The primers were designed and validated by Applied Biosystem. After DNA extraction, quantification was done using the Nanodrop technology and stored at -20 ºC. This was followed by RTPCR using the Fluidigm Microfluidic technique. SNP Genotyping Analysis software v. 3.1.1 and Fluidigm Data Collection software were used for the analysis of the data obtained.

Statistical analysis

Using the Chi-square test, the Hardy–Weinberg equilibrium (HWE) was determined. In the control group, every genotype complied with the Hardy–Weinberg equilibrium. For statistical analysis, IBM SPSS (Statistical Package for the Social Science) Version 21 was employed. To compare the continuous variable between the cases and controls, an independent *t*-test was used. The odd's ratio was calculated for both the genotypes to assess the association between the alleles and oral cancer. Only statistically significant results ($P < 0.05$) were considered for all statistical tests, which were conducted using a 95% confidence interval.

RESULTS

The demographic details were recorded, which included age, gender, and habitual risk, which are associated with OSCC. Out of 300 samples, 59 did not pass the quality criteria on DNA quantification and therefore could not be used for further analyses. Finally, there were 91 cases and 142 controls. In the study group, there were 67 male participants and 24 female participants, whereas the control group had 80 male participants and 62 female participants. The research group's average participant age was 50.86 years, while the control group's average age was 44.61 years. The most common site of OSCC in this study was buccal mucosa, followed by tongue. Based on TNM staging, the participants were categorised in four stages. There were 31 cases in stage 1, 23 cases in stage 2, 29 participants in stage 3, and 8 participants in stage 4.

Observation of the frequencies of the genotypes

The frequency of the wild-type genotype (*T: T* of *rs2943773_TG*; *G: G* of *rs11202600_GC*; *G: G* of *rs9651495_GA*; *C: C* of *rs3827678_CT* and *T: T* of *rs1234224_TC*) was found

in the control group to be considerably greater than the other variant of genotype [Table 1].

In the study group, the frequency of wild-type homozygous genotype was higher in *rs2943773(T: T)*, *rs11202600 (G: G)*, and *rs3827678(C: C)* compared to the heterozygous variant. With respect to *rs9651495(G: G)* and *rs1234224 (T: T)*, the frequency of the homozygous genotype was less compared to the heterozygous genotype. This infers polymorphisms with these genotypes in cases of OSCC [Table 2].

The variation in the frequency of *rs9651495* genotype of the *PTEN* gene was significant between OSCC and control groups. The homozygous genotype *G: G* of *rs9651495* showed statistical significant results between the groups with a *P* value of 0.00. There was a statistically significant difference in the frequency of the heterozygous genotype *A: G* between the study group and the control group ($P = 0.00$). With respect to homozygous variant *A: A*, an increased frequency in the study group was observed than the control group with a statistical significant difference ($P = 0.00$). The homozygous variant *A: A* and the heterozygous *A: G*, genotype of *rs9651495*, showed an increased association of risk of OSCC with odd's ratios of 5.8310 and 1.8810, respectively, the *P* value being < 0.05. The frequency of the homozygous genotype *G: G* of *rs9651495* was found to be decreased in OSCC [Table 3, Figure 1].

Table 1: Frequency of the genotypes in the control group

rs number	Genotype	Frequency	Percent (%)	*P*
rs2943773_TG	T:T	132	93.0	0.00*
	G:T	10	7.0	
rs11202600_GC	G:G	134	94.4	0.00*
	C:G	8	5.6	
rs9651495_GA	G:G	79	55.6	0.00*
	A:G	48	33.8	
	A:A	15	10.6	
rs3827678_CT	C:C	132	93.0	0.00*
	C:T	10	7.0	
rs1234224_TC	T:T	71	50.0	0.00*
	C:C	37	26.1	
	C:T	34	23.9	

rs-Reference SNP cluster ID. *$P<0.05$ is considered statistically significant

Table 2: Frequency of the genotypes in the cases of OSCC. rs-Reference SNP cluster ID

rs number	Genotype	Frequency	Percent	*P*
rs2943773_TG	T:T	85	93.4	0.00*
	G:T	6	6.6	
rs11202600_GC	G:G	86	94.5	0.00*
	C:G	5	5.5	
rs9651495_GA	G:G	28	30.7	0.3
	A:G	32	35.1	
	A:A	31	34.2	
rs3827678_CT	C:C	86	94.5	0.00*
	C:T	5	5.5	
rs1234224_TC	T:T	33	36.3	0.00*
	C:C	6	6.5	
	C:T	52	57.2	

*$P<0.05$ is considered statistically significant

The homozygous genotypes *T: T* and *C: C* of *rs1234224* showed a statistically significant difference between the study group and the control group with a *P* value of 0.00. With a statistically significant difference of 0.00, the heterozygous variant *C:T* was more common in the study group than in the control group.

The frequency of homozygous genotype *T: T* of *rs1234224* was found to be decreased in OSCC cases. The frequency of *C: T* genotype was increased in OSCC cases [Table 4, Figure 2]. It also showed an increased association with risk of OSCC, with an odd's ratio of 3.2906 (the *P* value is < 0.05). There was no difference observed in the frequencies of the other genotypes between the control group and the study group.

In addition, the prevalence of the genotypes was also observed in the stages of OSCC. The statistical analysis between the subgroups showed that *PTEN rs9651495 A: A* showed an increase prevalence in all the stages – I–IV (OR = 5.8310, CI = 2.7482 to 12.3717). Similarly, *PTEN rs1234224 C: T* showed a higher prevalence in all the stages of OSCC cases, stage I–IV (OR = 3.2906, CI = 1.8098 to 5.9827).

DISCUSSION

OSCC possesses a multi-factorial aetiology, and OSCC remains an enigma for health care professionals though there are diagnostic and therapeutic advances over many years. The 5-year survival rate is around 45% only.[13,14]

Table 3: Comparison of rs9651495_GA between study and control groups

			rs9651495_GA						Total
			G:G	*P*	A:G	*P*	A:A	*P*	
Group	Study	*n*	28	0.000*	32	0.000*	31	0.000*	91
		%	26.2%		40.0%		67.4%		39.1%
	Control	*n*	79		48		15		142
		%	73.8%		60.0%		32.6%		60.9%
Total		*n*	107		80		46		233
		%	100.0%		100.0%		100.0%		100.0%

*$P<0.05$ is considered statistically significant, *n* is the frequency of the genotype

Table 4: Comparison of rs1234224_TC between study and control groups

			rs1234224_TC						Total
			T:T	*P*	C:C	*P*	C:T	*P*	
Group	Study	*n*	33	0.000	6	0.000	52	0.000	91
		%	31.7%		14.0%		60.5%		39.1%
	Control	*n*	71		37		34		142
		%	68.3%		86.0%		39.5%		60.9%
Total		*n*	104		43		86		233
		%	100.0%		100.0%		100.0%		100.0%

*$P<0.05$ is considered statistically significant, *n* is the frequency of the genotype

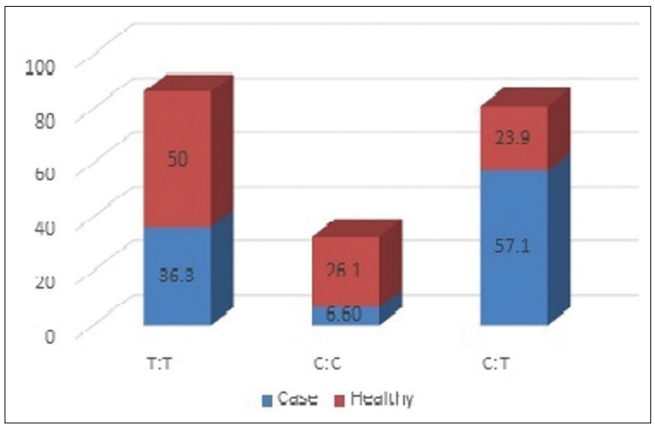

Figure 1: Comparison of rs9651495 between study (Case) and control(Healthy) groups

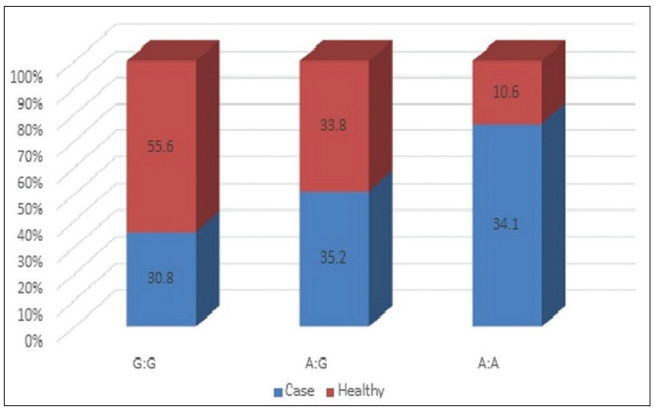

Figure 2: Comparison of rs1234224 between study(Case) and control(Healthy) groups

In this study, an association was observed with the polymorphism of the variants of the '*PTEN* gene' and 'OSCC'. Previous studies have established the association of alcohol consumption, tobacco smoking, or chewing and betel nut usage as important precipitating factors contributing to the increased number of cases of OSCC.[15-17] These factors were prominently seen among men, which could explain the gender bias of OSCC in male patients, compared with that seen in female patients. The average age of the participants with OSCC was 50.86 ± 10.25. The majority of studies have also shown that people with OSCC often age between 50 and 60 years old.[18] In the present study, buccal mucosa was the most often affected area by OSCC due to erroneous habits of areca nut and tobacco chewing. Whilst in America and European countries, the lateral side of the tongue is considered the most common site of involvement for OSCC.[19,20]

The study group exhibited a statistically significant difference in the frequency of the *G: G* genotype of *rs9651495* when compared to the control group, the study group showing reduced frequency. The odd's ratio also showed a correlation between the frequency of *rs9651495* and OSCC, and it was observed that the homozygous variant *A: A* and the heterozygous *A: G*, genotype of *rs9651495*, showed an association with increased risk of OSCC with an odd's ratio of 5.8310 and 1.8810, respectively, the *P* value being < 0.05.

This change in frequency of the alleles could be due to the polymorphisms occurring in OSSC individuals. A similar study done by Liu M *et al.* using serum to observe the correlation between *PTEN* gene polymorphism and OSCC suggested that the *C/C* genotype frequency of *rs9651495* was much higher in OSCC subjects (50.15%) than in healthy subjects (23.71%) ($P < 0.05$). The frequency of *T: T* genotype of *rs9651495* was lower (31.33%) in OSCC patients than in healthy individuals.(57.19%).[8] They found that compared to patients with other genotypes, those with the *C/C* genotype had considerably decreased *PTEN* protein expression. A relationship between OSCC and *PTEN* gene polymorphism was observed. The higher frequency of the *C/C* genotype corresponds to the lower level of *PTEN* protein expression, which induces OSCC.[8] The other genotypes *rs2943773*, *rs11202600*, and *rs3827678* did not show any association with polymorphism of the *PTEN* gene and OSCC.

The frequency of *C: T* genotype of *rs1234224* was increased in OSCC cases and showed an increased association with risk of OSCC, with an odd's ratio of 3.2906 (the *P* value is <0.05). On the contrary, a study done in Chinese population by Xu-Dong Yang in 2015 observed that the heterozygous *C: T* genotype (OR = 0.89, 95% CI = (0.55–1.42), *P* = 0.83) and *T: T* genotype (OR = 1.01, 95% CI = (0.58–1.74), *P* = 0.74) of *rs1234224* was not associated with significant increased risk with OSCC compared to the homozygous *C: C* genotype. In

comparison to the *C* allele, the *T* allele did not show significant increased risk (OR = 0.99, 95% CI = 0.72–1.58, *P* = 0.69). This difference could be due to the polymorphisms which differ in populations of different origin and also can be attributed to the epigenetic factors.[21]

Liu M *et al*. found a significant association between OSCC and the *PTEN* gene's *rs9651495* locus polymorphism, highlighting the importance of the *PTEN* gene in OSCC.

Thus, polymorphism of *rs9651495* may be used as one of the diagnostic markers in the early diagnosis of OSCC.[8] Immunohistochemical analysis done by Squarize *et al*.[22] concluded that the *PTEN* expression was associated with the advancement of OSCC and decreased in tumours that progressed more quickly, suggesting that it could be used as a prognostic indicator. Analogous immunohistochemical investigations have additionally demonstrated statistically significant variations in *PTEN* protein expression between OSCC tissues and normal tissues (*P* = 0.0104), indicating a potential decrease in *PTEN* protein expression in OSCC.[8,11] A positive association has also been observed between the polymorphism of *rs3830675* of *PTEN* gene and OSCC.[23,24] Additionally, recent research has indicated that the variant of *PTEN* gene *IVS4* (*rs3830675*) is linked to higher possibility of cancer (OR = 1.45) and a few gastrointestinal neoplasm subgroups (OR = 1.67).[23,24] More than 1000 coding-region single-nucleotide polymorphisms have been identified in the genes of the *PTEN*/*AKT*/*mTOR* pathway. Some of these are *rs2295080* in the promoter area of the *mTOR* gene, *rs2494750* and *rs2494752* in the 5'UTR region of the *AKT1* gene, *rs2536* in the 3'UTR of *mTOR7*, and *rs701848* in the 3'UTR region according to Sun *et al*.[25]

A number of studies in different populations of the world have shown variable results concerning the mutations in the *PTEN* gene. According to Poetsch *et al*., the *PTEN* gene in the German population under study has a 13% mutation frequency. However, a small number of investigations have not revealed *PTEN* gene alterations in the Israeli population. This suggests that these polymorphisms need to be studied in different populations to eliminate the bias of ethnic and epigenetic changes.[26,27]

Saliva was used in this study to extract DNA as saliva collection is a non-invasive procedure and does not require any skill to master. To the best of our knowledge, saliva has never been used in any previous investigation to observe polymorphisms. The majority of the studies have used serum for DNA extraction, and many studies have been done on cell lines *in vitro* and by using immunohistochemical analysis. Saliva, being a mirror of the body, can be used and compared with the results obtained from the serum.

In addition, an association was observed of the polymorphisms of the above studied SNPs with the clinical pathological characteristics based on the TNM staging of OSCC. Prevalence of the genotypes was also observed among the four stages of OSCC. The statistical analysis between the subgroups showed higher prevalence of *PTEN rs9651495 A: A* than the wild type *G: G* (OR = 5.8310, CI = 2.7482 to 12.3717). Similarly, *PTEN rs1234224 C: T* showed an increased prevalence in the four stages of OSCC cases, stages 0–IV (OR = 3.2906, CI = 1.8098 to 5.9827). Thus, we found an association of the polymorphism of *PTEN* gene with the stages of OSCC, further emphasising its role as a prognostic marker.

The spotting of markers both prognostic and predictive markers is essential from the clinician point of view because OSCC is a multi-factorial disease with varied clinical characteristics. An elaborative knowledge of the molecular drivers of OSCC in the context of precision medicine could improve the therapeutic results through the customised selection of targeted therapies. Based on the above discussion, it may be said that the progression of cancer not only is due to the single genetic inheritance of a variant of protein but also is a plethora of multiple factors, which includes the interaction of multiple genes involved in various metabolic pathways, epigenetic events, and environmental factors. Thus, it is the need of the hour to identify the specific changes in these genes which are associated with OSCC.

There were some limitations of the study as well. The study does not include all the genotypes of the *PTEN* gene and does not observe its genetic expression due to lack of funding.

CONCLUSION

To conclude, polymorphism in *PTEN* gene has a definite role in the process of carcinogenesis and hence can be used in the early diagnosis and prognosis of OSCC. It can be added in the panel of biomarkers in diagnosis of OSCC. Moreover, *PTEN* has also been used for therapeutic purposes in a few cancers to improve the prognosis. By using a combination of molecular markers, healthcare professionals can thereby gain a comprehensive understanding of the underlying biological and clinical factors driving the process of OSCC and select the most effective personalised treatment options for each patient. Thus, this study opens the avenue for further research on *PTEN* restoration and implementation in the treatment of OSCC.

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Conflicts of interest
There are no conflicts of interest.

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