ORIGINAL RESEARCH

Expression of Microsatellite Marker D9S1747 in Potentially Premalignant Oral Epithelial Lesions and Oral Squamous Cell Carcinoma using qRT-PCR: A Comparative Study.

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Abstract

Aim: This is a cross sectional comparative study, aimed to quantify the expression of Microsatellite Marker D9S1747 in Potentially premalignant oral epithelial lesions, well differentiated oral squamous cell carcinoma, Normal mucosa and to compare their expressions.

Material and Methods: Genomic DNA was extracted, quantified and the expression of the Microsatellite Marker D9S1747 was done in 12 cases of Potentially premalignant oral epithelial lesions, 12 cases of well differentiated Oral squamous cell carcinoma and 12 cases of Normal mucosa by Quantitative Real Time Polymerase chain reaction (RT-qPCR).

Results: It was observed that there was an over expression of microsatellite marker D9S1747 in both potentially premalignant oral epithelial lesions and well differentiated oral squamous cell carcinoma with good mean cycle threshold (CT) value of 30.23±1.732 and 29.68±0.895 respectively. When comparing the microsatellite marker D9S1747 expression in three groups, potentially premalignant oral epithelial lesions (PPOELs), well differentiated Oral squamous cell carcinoma (WDOSCC) showed higher expression than normal mucosa and in between two groups PPOELs showed higher expression than WDOSCC and the difference is statistically significant with p value less than 0.025.

Conclusion: Our findings suggest that there is over expression of Microsatellite Marker D9S1747 in Potentially premalignant oral epithelial lesions, and in well differentiated Oral squamous cell carcinoma, compared to normal mucosa and highly expressed in PPOELs compared to WDOSCC. Over expression of microsatellite marker D9S1747 in PPOELs and WDOSCC may suggest the early event of tumorigenesis. Hence it can be used as a valuable marker for early diagnosis, prognostic marker and in the identification of therapeutic targets.

Keywords: Microsatellite marker, oral squamous cell carcinoma; Potentially premalignant oral epithelial lesions, D9S1747, Polymerase chain reaction

Introduction

Oral cancer is a multifactorial disease with a variety of genetic alterations which induces normal cells to transform into malignancy.[1] Despite, advances in recent years in detection, prevention and treatment of oral squamous cell carcinoma (OSCC) overall survival rate is still modest.[2]

A new term potentially premalignant oral epithelial lesions (PPOELs) is used to define both histologic and clinical lesions that have malignant potential.[3] Early identification and management of PPOELs is essential for secondary prevention of OSCC, thereby reducing morbidity and mortality.[4]

The key event in tumorigenesis of OSCC is inactivation of Tumor Suppressor Gene (TSG) and p16 is found to be the first TSG inactivated in OSCC.[5] D9S1747 is a p16 microsatellite marker and study of their expression helps to detect early changes in OSCC.[6] Hence, this study has been undertaken to determine the level of Microsatellite Marker D9S1747 in the subjects with PPOELs, OSCC and in healthy controls to correlate with the occurrence of OSCC.

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How to cite the article: Suganya G, Sahana N S, Renuga S, Akalya P, TahasildarJ, Venugopal S S. Expression of Microsatellite Marker D9S1747 in Potentially Premalignant Oral Epithelial Lesions and Oral Squamous Cell Carcinoma using qRT-PCR: A Comparative Study. Oral MaxillofacPathol J2024; 15(2);151-155.

Source of Support: Nil

Conflict of Interest: None

This study aims at evaluating the potential role of Microsatellite marker D9S1747 in PPOELs, WDOSCC and Normal mucosa and comparing the same with each other

thereby providing novel information about diagnostic as well as treatment modalities. It would help in specific targeted therapy and serve as novel target for chemotherapeutic intervention or other molecular targeted therapy to prevent or limit the extensive surgical intervention and prognosis. Hence, Microsatellite marker D9S1747 expression may prove useful in early diagnosis of potentially premalignant oral epithelial lesions and therapeutic targets for screening genetic alterations in OSCC.

Materials and Methods:

The study was conducted at the Department of Oral Pathology and Microbiology, Government Dental College and Research Institute, Bangalore, India.

The study protocol was approved by the Institutional Ethical Committee of Government Dental College and Research Institute, Bangalore, India. A total of 36 cases were selected from the archives of the Department of Oral Pathology, GDCRI Bangalore. Cases were divided into three groups, Group I: 12 histopathologically diagnosed cases of Potentially premalignant oral epithelial lesions, Group II: 12 histopathologically diagnosed cases of Well differentiated Oral squamous cell carcinoma. Group III: 12 histopathologically diagnosed normal oral mucosa.

Method: 10µm sections were cut from each formalin-fixed paraffin-embedded tissue block using a microtome and subsequently placed in 1.5 ml Eppendorf tubes. The sections were then deparaffinized by keeping the tissue section in the microwave oven at 65°C for 15 mins. Later 1ml of xylene was added to the tissue sections in the Eppendorf tubes to dissolve the paraffin, centrifuged at 14000 rpm for 3 mins after which the supernatant was carefully pipetted out and the process was repeated thrice. Residual xylene was removed and tissue rehydration was done by adding 1ml of absolute ethanol centrifuged at 14000 rpm for 3 mins, later the supernatant was removed by pipetting and this process was also repeated thrice. The pellets were vacuum dried completely. The tissue pellets then were re-suspended by adding 180 microlitre of lysis solution (AL) (DS0015) and incubated at room temperature for 30 minutes.

DNA Extraction: DNA was extracted from deparaffinized tissue pellets by adding 20 micro litre of proteinase K solution (20mg/ml) and mixed by vortexing and incubated at 56°C overnight. After overnight incubation, it was centrifuged at the speed of 20000x g at 15000 rpm for 5-10 minutes and the supernatant was transferred into a new 2.0ml capped microcentrifuge tube. 20 micro litre of RNase A solution (DS0003) was added and mixed by pulse vortexing thoroughly for 15 secs and incubated for 2 mins at room temperature. 200 microlitre of lysis solution (C1) (DS0010) was added mixed by vortexing thoroughly for 15 seconds incubated at 70°C for 10 minutes. Later 200 microlitre of absolute ethanol was added to lysate and mixed thoroughly by vortexing for 15 seconds. The lysate obtained was transferred to the HiElute Miniprep spin column and incubated at room temperature for 5 minutes. Later the lysate was centrifuged at 6500xg at 10000 rpm for 1 minute. After this, centrifugation flow was reloaded through liquid on to the column again and centrifuged at 10000 rpm for 1 minute.

TABLE 1:

Tests of Normality							
Groups		Kolmogorov-Smirnova			Shapiro-Wilk		
		Statistic	Df	p value	Statistic	df	p value
WDOSCC	CT VALUES	.224	12	.097	.905	12	.182
	Δct	.153	12	.200*	.940	12	.493
	ΔΔct	.153	12	.200*	.940	12	.493
	FOLD INCREASE	.183	12	.200*	.911	12	.217
PPOELs	CT VALUES	.174	12	.200*	.938	12	.471
	Δct	.191	12	.200*	.929	12	.368
	ΔΔct	.191	12	.200*	.929	12	.368
	FOLD INCREASE	.233	12	.072	.846	12	.330
NORMAL ORAL MUCOSA	CT VALUES	.285	12	.008	.845	12	.320
	Δct	.202	12	.188	.965	12	.851

TABLE 2: Comparison of the Parameters among the Groups Using ANOVA

	Groups	N	Minimum	Maximum	Mean	S.D	p value
CT	WDOSCC	12	28.496	31.721	29.683	0.895	0.001*
	PPOELS	12	26.933	33.148	30.238	1.732	
	NORMAL ORAL MUCOSA	12	31.554	33.016	32.200	0.422	
ΔCT	WDOSCC	12	-7.085	-5.285	-6.2311	0.6126	0.013*
	PPOELs	12	-6.198	-4.364	-5.3483	0.5556	
	NORMAL ORAL MUCOSA	12	-7.981	-4.001	-6.2653	1.1289	
ΔΔct	WDOSCC	12	-.819	.980	0.0342	0.6126	0.001*
	PPOELs	12	.067	1.902	0.9170	0.5556	
	NORMAL ORAL MUCOSA	12	0.000	0.000	0.0000	0.0000	
SCC	WDOSCC	12	.567	1.973	1.1124	0.4751	0.001*
	PPOELs	12	1.048	3.736	2.0289	0.8571	
	NORMAL ORAL MUCOSA	12	1.000	1.000	1.0000	0.0000	

*significant

The flow through was discarded and paced into the same column. Wash solution is prepared and 500 microlitre of wash solution was added to the column and centrifuged at 10000rpm for 1minute. The flow through liquid was discarded and the column was placed in same collection tube. Later another 500 microlitre of diluted wash solution was added to the column and centrifuged at 20000xg (15000rpm) for 3 minutes. The collection tube containing flow through liquid was discarded and the column was placed in a new 2.0ml uncapped collection tube. DNA Elution was done by adding 50 – 100 microlitre of the elution buffer directly onto the column and incubated for 5 minutes at room temperature and centrifuged at 6500x g (10000rpm) for 1minute.

DNA Amplification: The specific primer sequences of both Forward (F) and Reverse (R) primer were commercially obtained. Total of 10µl reaction volume per well was added in the 96 well-plate. 10µl reaction volume constitutes 1µl primer, 1µl extracted DNA, 5µl of master mix (SYBR Green), 3µl of nuclease free water. 96 well-plate with samples was sealed and loaded in an applied Biosystem Step One Plus TM Real-Time PCR system Thermal Cycling Block (S/N: 2720010242). Initial denaturation at 98°C for 5minutes, then 35 cycles of final denaturation at 94°C for 30 seconds, annealing at 58°C, extension at 72°C for 45 seconds and final extension at 72°C for 5mins followed by cooling for 5 mins was set to run and final amplification graph of microsatellite marker D9S1747 was collected from PCR machine.

Statistical analysis: Parametric data of CT value of Microsatellite Marker D9S1747 in PPOELs, WDOSCC and normal mucosa were expressed as mean and standard deviation (M [SD]). And for comparison of Microsatellite Marker D9S1747 expression between PPOELs, WDOSCC and normal mucosa ANOVA test was applied to compare the CT scores among the groups with post-hoc Bonferroni for inter-group comparison. Statistical significance level was defined at P =0.05.

TABLE 3: Inter-Group Comparison of the Groups using Post-Hoc Bonferroni

		Mean Difference	p value	95% Confidence Interval	
				Lower Bound	Upper Bound
CT VALUES	W D O S C C Vs OPMD	-.554916	.739	-1.7406	.63078
	W D O S C C Vs Healthy	-2.51689	.001*	-3.7025	-1.3312
	OPMD Vs Healthy	-1.96198	.001*	-3.1476	-.77629
ΔCT	W D O S C C Vs OPMD	-.882792	.034*	-1.7147	-.05085
	W D O S C C Vs Healthy	.034189	1.000	-.79775	.86613
	OPMD Vs Healthy	.916981	.027*	.08504	1.74892
ΔΔCT	W D O S C C Vs OPMD	-.882792	.001*	-1.3744	-.39114
	W D O S C C Vs Healthy	.034189	1.000	-.45747	.52584
	OPMD Vs Healthy	.916981	.001*	.42533	1.4086
FOLD INCREASE	W D O S C C Vs OPMD	-.916528	.001*	-1.4991	-.33392
	W D O S C C Vs Healthy	.112358	1.000	-.47025	.69496
	OPMD Vs Healthy	1.02888	.001*	.44628	1.6114

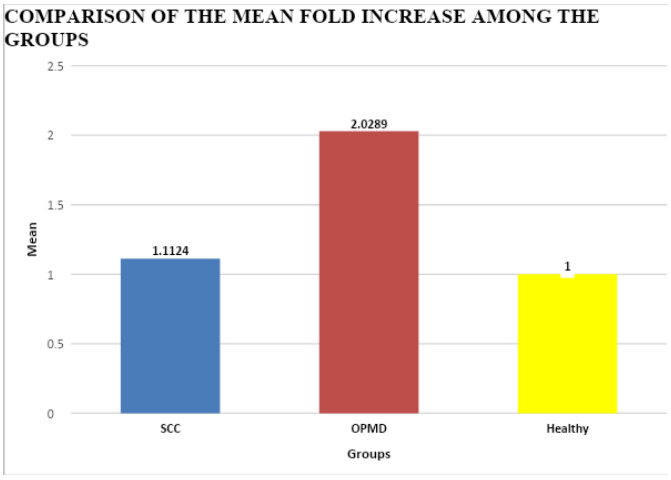
*significant

Results

To characterize the expression of Microsatellite Marker D9S1747 in Potentially premalignant oral epithelial lesions, Well differentiated oral squamous cell carcinoma and normal mucosa, we used qRT- PCR on genomic DNA isolated from 12 cases of Potentially premalignant oral epithelial lesions,12 cases of well differentiated oral squamous cell carcinoma and 12 subjects of normal mucosa. Both PPOELs and WDOSCC expressed microsatellite marker D9S1747 with good mean cycle threshold (CT) value of 30.23±1.732 and 29.68±0.895 respectively. On comparison with the three groups, the expression Microsatellite marker D9S1747 expressed more in PPOELs and WDOSCC with good CT value than in Normal mucosa (Graph 1) and within the two groups PPOELs showed higher expression compared with OSCC and the difference is statistically significant with the P value 0.025 [Table 1, Table 2 and Table 3]. Data was subjected to Normalcy test (Shapiro Wilk test). Data showed normal distribution. Hence parametric tests (ANOVA with Post-hoc Bonferroni) was applied.

The Shapiro–Wilk test is more appropriate method for small sample sizes (<50 samples) although it can also be handling on larger sample size while Kolmogorov–Smirnov test is used for n ≥50. For both of the above tests, null hypothesis states that data are taken from normal distributed population. If the p

GRAPH 1:



value of the Shapiro-Wilk Test is greater than 0.05, the data is normal. If it is below 0.05, the data significantly deviate from a normal distribution. SPSS (Statistical Package For Social Sciences) version 21. (IBM SPASS statistics [IBM corporation: NY, USA]) was used to perform the statistical analysis. Data was entered in the excel spread sheet. Descriptive statistics of the explanatory and outcome variables were calculated by mean, standard deviation for quantitative variables. Inferential statistics like ANOVA test (based on data distribution) was applied to compare the mean CT, ΔCT, ΔΔCT and Fold increase among the groups with post-hoc Bonferroni for inter group comparison. The level of significance is set at 5%

Discussion

Oral cancer is a multifactorial disease with a variety of genetic alterations such as gene deletion, gene rearrangement leading to mutation or over expression of oncogenes, activation of protooncogenes which causes loss or inactivation of tumor suppressor genes. Microsatellites are an extension of DNA in which 1–6 base pairs are tandemly repeated 5–100 times. They have higher mutation rates than any other areas of DNA. Microsatellite alterations are of two types- LOH (loss of heterozygosity) and MSI (microsatellite instability). Loss of entire gene and surrounding chromosomal region results in LOH which is a common genetic abnormality in early head and neck tumorigenesis. LOH can inactivate tumor suppressor genes and lead to uncontrolled cell growth.[7,8] Microsatellite markers were studied on different chromosomes (2q,3p,4q,9p) in OSCC cases. Allelic imbalance in tumor suppressor genes is the key event in OSCC which is associated with loss of heterozygosity mostly on chromosome 9p21 locus which includes p16 marker. p16 (D9S1747) is a microsatellite marker which detects early changes in OSCC.[9]

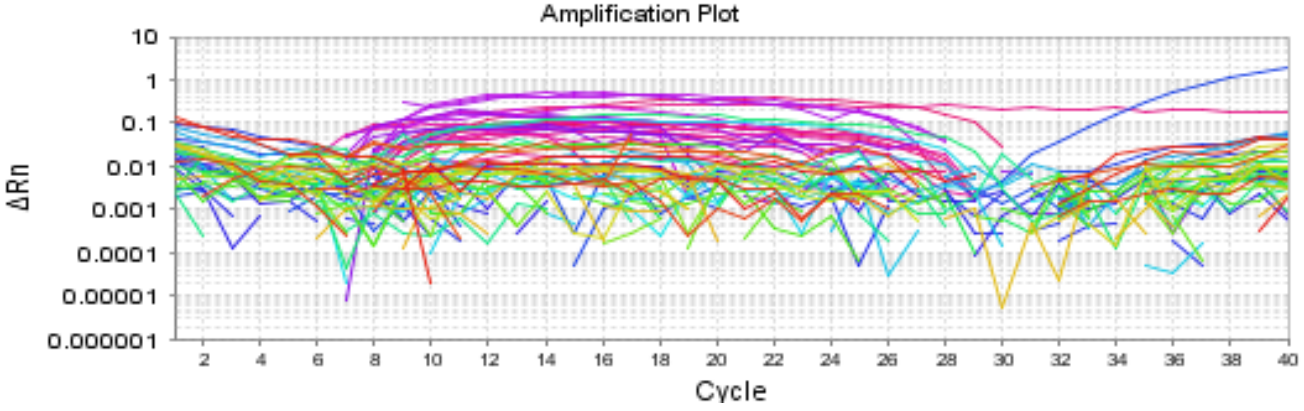
The aim of this study was to detect one of the microsatellite marker D9S1747 in PPOELs and WDOSCC and to compare with normal oral mucosa. Several studies have been conducted in different grades of OSCC for detection of microsatellite marker D9S1947 and concluded that D9S1747 expression is more in early grades of OSCC and homozygous deletion is the most common mechanism of inactivation. To best of our knowledge, this was the first study have been conducted on PPOELs and OSCC to understand the correlations between the alterations.

A study by Babji et al investigated different grades of oral squamous cell carcinoma for detection and comparison of microsatellite marker D9S1747 using polymerase chain reaction reported that 77.8% positivity in well differentiated, 22.2% positivity in moderately differentiated and 0% in poorly differentiated OSCC cases and concluded that detection of D9S1747 decreases as the grade increases suggesting that genetic alterations in D9S1747 has an role in tumorigenesis and occur as an early event in OSCC.[1] El-Naggar et al conducted a study in young adults OSCC patients compared with normal tissue and found that highest microsatellite alteration found on D9S168 on focus 9p23-22. But, in one of the cases, it was found that MSI with the marker similar to our D9S1747 was present in OSCC but not in matched normal tissue.[9] Wang et al conducted a molecular analysis TP53, D9S1747, D9S162 and RPS6 in oral squamous cell carcinoma. LOH and MI frequency at chromosomes 17p13 and 9P21 were 56% (35/63) and 59% (40/68) respectively and concluded that the prognosis was poor in the LOH and MI positive group of chromosomes 17p13 and 9p21.[10] Sargolzaei et al. found expression of p16 higher in Stage I OSCC compared to Stage II–IV OSCC and concluded that loss of p16INK4a expression occurred in initial stages of oral squamous cell carcinoma.[11]

Through our study we are reporting that microsatellite marker D9S1747 expressed more in PPOELs and WDOSCC with mean fold increase of 2.20 and 1.11 comparative to normal oral mucosa. (Graph 2). Our study showed that expressed more inPPOELs compared to WDOSCC suggesting that it is an early change in OSCC and plays an role in tumorigenesis. Hence, microsatellite biomarker D9S1747 will be useful in early diagnosis of potentially premalignant oral epithelial lesions and therapeutic targets for screening genetic alterations in OSCC.

Conclusion

The quantification of Microsatellite Marker D9S1747 gene expression using the qRT-PCR showed that there exists a



Graph 2: Amplification graph for Microsatellite marker D9S1747 expression

significant expression of D9S1747 gene in both potentially premalignant oral epithelial lesions and well differentiated OSCC. Comparatively Microsatellite Marker D9S1747 gene expression is expressed higher in PPOELs than well differentiated OSCC suggesting that early event in OSCC. The quantification and elaborate molecular analysis of gene expression which involved in tumorigenesis by inactivating tumor suppressor gene. D9S1747-first TSG to be inactivated in OSCC can be utilized for early diagnosis, prognostic or therapeutic predilection.

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