Detection and comparison of microsatellite marker D9S1747 with clinical stages and grades of oral squamous cell carcinoma

Deepa Babji1, Ramakant Nayak1, Kishore Bhat2, Vijayalakshmi Kotrashetti1, Harsha Babaji3, Manohar S Kugaji2

1Department of Oral Pathology and Microbiology, Maratha Mandal’s NG Halgekar Institute of Dental Sciences and Research Centre, Belgaum, Karnataka, India
2Department of Microbiology, Maratha Mandal’s NG Halgekar Institute of Dental Sciences, Belgaum, Karnataka, India
3Department of Maxillofacial Surgery, College of Dental Sciences, Davangere, Karnataka, India

Abstract

Background: One of the main characteristics of oral squamous cell carcinoma (OSCC) is genetic alteration in specific target regions. 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. To redefine more clearly the role of D9S1747 (p16 microsatellite marker) and its expression in OSCC, the study was designed with the aim to check the detection of D9S1747 in OSCC and to compare the same with histopathological grades and tumor node metastasis staging.

Materials and Methods: Forty cases of paraffin-embedded tissue section which was histologically confirmed as OSCC and 10 cases of normal tissues were retrieved from the archives. DNA was extracted from the tissue sections and subjected for polymerase chain reaction to detect p16 microsatellite marker D9S1747. Data were analyzed using Chi-square test and Fisher’s exact test.

Results: Twenty-seven cases (67.5%) showed p16 microsatellite marker positivity for OSCC. It was observed that 44.4%, 51.9% and 3.7% p16 microsatellite markers were positive in Stage 1, Stage 2 and Stage 4 OSCC cases, respectively. p16 microsatellite marker positivity was found in 77.8%, 22.2% and 0% for well-differentiated, moderately differentiated and poorly differentiated OSCC cases, respectively.

Conclusion: The observations of the present study revealed D9S1747 marker as an early event in OSCC, and this can be used as a prognostic marker.

Keywords: D9S1747, normal mucosa, oral squamous cell carcinoma, p16 microsatellite marker, polymerase chain reaction
INTRODUCTION

Cancer is a genetic disease which results due to a variety of genetic alteration which induces normal cells to transform into malignancy. Similar genetic alterations are found to occur in oral squamous cell carcinoma (OSCC). Among such genetic alterations, gene deletion, gene amplification or rearrangements, leading to either mutation or overexpression of oncogenes, activation of protooncogenes along with mitotic recombination of nondisjunction events which might cause loss of tumor suppressor genes (TSGs) appears to be main events.[1–3]

The key event in tumorigenesis of OSCC is inactivation of TSG. The loss of heterozygosity (LOH) and homozygous deletion at polymorphic loci are the types of allelic losses which are the chief events played in TSGs. Most TSGs are linked to cyclins, cyclin-dependent kinase (CDKs) and CDK inhibitors, and the retinoblastoma-susceptibility gene (RB1) which takes part in control of cell cycle progression from G1 to S-phase. p16 gene, inhibitor of CDK4a, or CDK inhibitor 2A is most commonly involved in carcinogenesis as p53. p16 is considered as the first TSG to be inactivated in OSCC, and it is an early event that occurs during initial stages of OSCC.[4–6] More than 80% of head and neck tumors show early inactivation of p16, whereas deregulation of p53 and cyclin D1 occurs later in sequence of molecular progression to frank carcinoma. In head and neck cancers, p16 protein is often inactivated by any one or a combination of homozygous deletions, promoter methylations or point mutations.[7]

In recent years, microsatellite biomarkers have been observed as new diagnostic and therapeutic targets for screening genetic alterations in OSCC tissues.[8] Microsatellite biomarkers are repeat DNA sequences which are of two types: LOH and microsatellite instability (MSI). Microsatellite markers on different chromosomes (2q, 3p, 4q, 5q, 7q, 8p, 10q, 11q, 13q, 18q, 20q, 9p) were studied at 9p21–22, the locus for p16. p16 protein is often inactivated by any one or a combination of homozygous deletions, promoter methylations or point mutations.[9]

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MATERIALS AND METHODS

After obtaining the Institutional Review Board and Ethical Committee approval, a retrospective study was conducted. Forty diagnosed cases of OSCC who underwent tumor resection along with radical neck dissection were retrieved from the department archives, and 10 cases of normal tissues were also retrieved from the department archives who had no history of tobacco in any form. 4-µm thick section was obtained from paraffin-embedded tissue sample and stained with hematoxylin and eosin to reevaluate the cases. Demographic data and other related clinical history and habit history were obtained from the archives. Clinical staging was determined for OSCC cases as per the American Joint Committee for Cancer (8th edition) TNM staging.[11] Histopathologically, the cases were graded as well-differentiated squamous cell carcinoma (WDSCC), moderately differentiated squamous cell carcinoma (MDSCC) and poorly differentiated squamous cell carcinoma (PDSCC) according to the WHO (Broder’s criteria).[12]

DNA extraction

5-µm thick paraffin-embedded sections were collected for DNA extraction for both OSCC and normal cases. The sections were deparaaffinized using xylene and alcohol. DNA extraction was done using modified proteinase-k method. For 10 mg/ml proteinase k, lysis buffer I containing 1M Tris and 0.5M EDTA and lysis buffer II containing 50 mM Tris-HCl, 2.5 mM MgCl₂, 0.45% tween 20 and 0.45% Nondient P-40 were added. Subsequently, tubes were kept at 60°C for 2 h and then in the boiling water bath for 10 min to deactivate the enzyme. The centrifugations of tubes were done to obtain supernatant containing DNA. Later, the DNA was collected in fresh tube and stored at −20°C until polymerase chain reaction (PCR) was performed.[13]

Polymerase chain reaction

For PCR analysis, p16 microsatellite marker D9S1747 with the sequence of forward 5’-ATTCAACG AGTGG GATGAAG-3’ and reverse 5’-TCCAGGTTGCTGCAAATGCC-3’ was selected. The PCR product size expected was 130–150 bp. Ampli Taq red master mix containing 2 mM MgCl₂, 0.2 mM of dNTPs and 1 U/µl of Taq DNA polymerase was prepared as reaction mixture. Primer concentration of 0.4 μM and 3 µl of DNA template was added to the mixture. To carry out amplification, a Veriti thermal cycler (Applied Biosystems, CA,
USA) was used. After this, thermal cycling conditions were used for running the PCR which was 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 54°C for 1 min and 72°C for 1 min. Finally, 2% agarose gel was prepared and the 15 µl samples were loaded on to the gel which was further subjected to electrophoresis at 80 V for 1 h. The gel was subsequently stained with 0.5 µg/ml ethidium bromide and observed under ultraviolet gel documentation system (Major Science, Saratoga, CA, USA).[14]

The molecular weight of the band was confirmed by comparing the location with standard molecular weight marker ladder (100–1500 bp).[12]

Statistical analysis
Cross-tabulations were done to show association of p16 microsatellite marker D9S1747 between OSCC cases along with clinical staging and histopathological grading. The comparisons were done using Chi-square test. Fisher’s exact test was applied for small expected values, \( P < 0.05 \) was considered statistically significant.

RESULTS
Clinical and histopathological evaluation
Among the 40 cases of OSCC, 67.5% were males and 32.5% were females. The age of the patients ranged from 16 to 75 years, with a mean age range of 49.92 years. Buccal mucosa was most common site for OSCC (47.5%) followed by tongue (32.5%). Majority were in Stage II. On the basis of histologic grading, 70% cases were of WDSCC, 20% cases were MDSCC and 10% cases were PDSCC.

p16 microsatellite marker D9S1747 was positive for 27 (67.5%) cases of OSCC and 13 (32.5%) cases were negative for OSCC. In normal cases, only 2 (20%) cases showed positivity for p16 microsatellite marker D9S1747 and 8 (80%) cases were negative [Table 1]. Part of these data has been published in 2018.

When TNM classification for staging was analyzed, higher positivity was observed in Stage II - 14 (51.9%), whereas in Stage I, it was 12 (44.4%) and in Stage IV it was 1 (3.7%). None of the samples were Stage III patients [Table 2].

On histopathological grade showed 21 (77.8%) cases showed positivity for p16 in WDSCC followed by 22.2% cases for MDSCC where as PDSCC did not show any positivity [Table 3].

DISCUSSION
Microsatellites are repetitive DNA in which DNA motifs (1–6 base pairs) are repeated 5–100 times. They have a higher mutation rate than other areas of DNA leading to high genetic diversity. MSI and LOH both are considered being separate pathogenic mechanisms in the development of carcinoma. Failure to correct inaccurate repetition producing insertion or deletion of repeated DNA microsatellite sequences results in MSI. Loss of the entire gene and the surrounding chromosomal region results in LOH. In cancer, it indicates the absence of a functional TSG in the lost region.[15] MSI and LOH most frequently altered on chromosomal arm 9p21 ± 22 which includes the putative TSG and cell cycle regulatory gene, p16INK4a. Moreover, these genes have been reported as an early event in oral cancers.[16] MSI plays a secondary role as compared to LOH in oral cancers.[16,17]

The aim of this study was to detect one of the p16 microsatellite markers D9S1747 on locus 9p21 in various stages and grades of OSCC and compare to normal oral mucosa. Several studies have been conducted on OSCC for changes in p16 using microsatellite markers on different chromosomes, methylation and immunohistochemistry. It was observed that these markers that have provided

**Table 1: p16 microsatellite positivity in oral squamous cell carcinoma and normal group**

| PCR_positivity_T | Tumor | Normal | Total | Significance |
|------------------|-------|--------|-------|--------------|
| Negative         | 13    | 8      | 21    | Fisher’s exact test \( P=0.011 \), significant |
| Positive         | 27    | 2      | 29    |              |
| Total            | 40    | 10     | 50    |              |

**Table 2: p16 microsatellite positivity according to stages of oral squamous cell carcinoma**

| TNM   | PCR_positivity_T | Total | Significance |
|-------|------------------|-------|--------------|
| Stage 1 | 1 (7.7%) | 12 (44.4%) | 13 (32.5%) | Pearson |
| Stage 2 | 10 (76.9%) | 14 (51.9%) | 24 (60.0%) | Chi-Square, \( P=0.04 \), significant |
| Stage 3 | 2 (15.4%) | 1 (3.7%) | 3 (7.5%) |             |
| Total   | 13 (100.0%) | 27 (100.0%) | 40 (100.0%) |              |

**Table 3: p16 microsatellite positivity in histopathological grades of oral squamous cell carcinoma**

| HP_grade | PCR_positivity_T | Total | Significance |
|----------|------------------|-------|--------------|
| WDSCC    | 7 | 21 | 28 | Pearson |
| MDSCC    | 2 | 6 | 8 | Chi-Square, Value \( 9.231, P=0.009 \), significant |
| PDSCC    | 4 | 0 | 4 |              |
| Total    | 13 | 27 | 40 |              |


evidence up to 87% of oral cancers show inactivation of p16 and in that homozygous deletion is the most common mechanism of inactivation.\textsuperscript{[8,9,17]}

In the present study, we observed high p16 microsatellite marker D9S1747 positivity in OSCC than normal cases [Figure 1]. LOH in 9p region has been reported in several human cancers.\textsuperscript{[18,19]} In head and neck cancer, LOH was studied in different chromosomal loci by el-Naggar et al., and they showed high incidence of LOH in 9p, 8p, 3p, 9q and 11q loci.\textsuperscript{[20]} We found 27 (67.5%) of OSCC cases positive for p16 microsatellite marker. Our results were corroborative to the previous studies which showed highest frequency of homozygosity on 9p chromosome (72%) in carcinoma of head and neck.\textsuperscript{[20]}

This indicates that homozygous deletion is the frequent mechanism of inactivation in the development of OSCC. Similar observations were made by el-Naggar et al. in their analysis on young adults; OSCC patients’ highest frequency of alteration of microsatellite marker found was D9S168 on locus 9p23-22. However, in one of the cases, it was found that MSI with the marker similar to our D9S1747 was present in tumor but not in matched normal tissue.\textsuperscript{[21]} Wang et al. conducted molecular analysis of TP53, D9S1747, D9S162 and RPS6 in OSCC.\textsuperscript{[22]} The LOH and MSI frequency was 59% on 9p21. Tokugawa et al. used six microsatellite markers at chromosome 9p13–22 on esophageal squamous cell carcinoma, dysplasia and normal cases.\textsuperscript{[18]} Microsatellite marker D9S1747 LOH was commonly detected near p16 in superficial as well as deeper parts. In the present study, 32.5% of the OSCC samples were negative for p16 microsatellite marker D9S1747. The possible reasons could be genetic alterations on chromosome band p21–22 in head and neck cancer not restricted to p16 and the microsatellite marker we used (D9S1747) did not include the mutation of one within p16.\textsuperscript{[18]} Our results showed 80% of cases negative for p16 microsatellite marker in histologically normal squamous epithelium and 20% of the histologically normal tissue showed p16 microsatellite marker positivity. This finding is similar to the study done by Lee et al. and they related it to LOH for 13q loci.\textsuperscript{[23]} The possible reasons could be that there is increased cell turnover rate and p16 plays a role in cell senescence in normal oral mucosa also.

In our study, we correlated p16 microsatellite marker with stages and histological grades of OSCC. The correlation of p16 microsatellite marker D9S1747 with TNM staging in the present study, we found 44.4%, 51.9% and 3.7% positivity in Stage 1, Stage 2 and Stage 4 OSCC cases, respectively. Sargolzaei et al. found expression of p16 higher in Stage I compared to Stage II–IV.\textsuperscript{[24]} Our study showed higher positivity in Stage 2 OSCC cases. Hiroshi et al. found LOH at chromosome 9p and suggested that LOH at chromosome 9p could be detected in early stage and the frequencies of LOH tend to be higher in later clinical stages. Partridge et al. analyzed allelic imbalance and clinicopathological features on the key chromosomal loci such as 3p24–26, 3p13 and 9p21. A fractional allelic score was calculated for all the TNM stages. They found the hazard ratios for survival analysis showed poor prognosis with hazard ratios of 3.93, 2.60 and 2.48 for 3p24–26, 3p13 and 9p21, respectively. Also found that allelic imbalance was a better prognostic marker than TNM staging. Allelic imbalance at each of these regions has shown 25 times increase in cases in morbidity rate relative to patient showed LOH at these loci.\textsuperscript{[24]} With detecting more of critical aberrations associated with tumorigenesis, it is possible to develop a molecular staging system, which will further help in identifying patients and their prognosis.

In the present study, 77.8% and 22.2% WDSCC and MDSCC cases were positive for p16 microsatellite marker. Wang et al. found that the LOH and MI frequency at 9p21 was significantly associated with WHO grading (P < 0.01) and lymph node metastasis.\textsuperscript{[25]} Sargolzaei et al. found that expression of p16 was 33.3%, 31.6% and 33.3% in WDSCC, MDSCC and PDSCC which were consistent in all grades when compared to our findings.\textsuperscript{[6]} In contrast to this study, Mahale and Saranath found 83% positivity in PDSCC as compared to 46% of MDSCC to WDSCC.\textsuperscript{[16]} Tsai et al. found an elevation in histological grades showing 54% in WDSCC, 56.5% in MDSCC and 58% in PDSCC.\textsuperscript{[26]} To our knowledge, as far as grade and stage are concerned, only few studies have been conducted to understand the correlation between the alterations and clinicopathologic characteristics of OSCC and have shown different results.\textsuperscript{[6,22,23]} The difference in the results could be attributed to the diversity.
in the distribution of different grades and stages in all the studies. In our study, WDSCC showed higher positivity p16 microsatellite marker this suggests that there is an early change seen in the development of OSCC and also it may be involved in tumorigenesis.

CONCLUSION

Genetic alterations of the p16 gene lead to its inactivation, resulting in deregulation of cell proliferation and tumorigenesis. Our results indicate that the detection of p16 microsatellite marker D9S1747 is the most common genetic change delineated in OSCC and its detection decreases as the grade increases suggesting that it is an early event in OSCC. Studies of different chromosomal regions or genome-wide analysis are necessary to define the unique genetic abnormalities. In the present study, the p16 microsatellite positivity was higher in Stage II specimen. The constraint of our study is correlation with the survival of the patient postsurgically was not conducted. Additional studies are required to understand the critical alterations at molecular level which are involved in carcinogenesis. This will possibly help us to develop a molecular staging system for patients which will further help us to identify patients with unfavorable genetic and clinical profile so that aggressive treatment can be provided for such patients who are identified with genetic abnormalities.

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Conflicts of interest
There are no conflicts of interest.

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