Evaluation of Molecular Positive Margins Using Surrogate P53 and Retinoblastoma Protein Expression and Correlation with Surgical Outcomes in Oral Squamous Cell Carcinoma

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BACKGROUND
p53 mutation and Rb over-expression have been extensively studied in oral squamous cell carcinoma (OSCC) but their clinical relevance with respect to excision margins is still controversial. The purpose of the study was to determine the expression of molecular markers (p53 & Rb) for predicting early locoregional recurrence in oral cancer.

METHODS
Histopathological specimens of 93 patients of oral cavity squamous cell carcinoma were subjected to p53 mutation and Rb protein testing in tumour and at the closest negative margin on H &E using immunohistochemistry. The expression of p53 and Rb in tumour tissue and at excision margin was correlated with clinicopathologic parameters recurrence and survival over a 2 year follow up period.

RESULTS
p53 mutation expression in tumour tissue was associated with increased recurrence (22.5 % versus 11.3 % P = 0.13) and mortality (17.5 % versus 5.6 % P = 0.056). p53 expression at margins is also associated with higher recurrence and mortality. Rb overexpression in tumour tissue is not significantly associated with recurrence (15 % and 16.4 %). Rb overexpression at margins had higher recurrence (40 %; P = 0.627) and higher mortality (60 %) in comparison to Rb negative cases (16.4 % versus 6.8 % respectively).

CONCLUSIONS
Clinical and routine histopathological assessments of margins remain the standard method of prognosticating and planning adjuvant treatment. Determination of molecular positive margins using p53 & Rb in oral cancer may aid in identifying patients at high risk of development of recurrence despite negative pathological margins.

KEYWORDS
Rb, Margin, P53, Recurrence.

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Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck. It contributes to more than 90% of all malignant tumours of the oral cavity and is characterized by regional and distant metastasis. The key issue with surgical management of OSCC is predicting the risk of locoregional relapse which is reported to occur in up to 20% of cases. It is well known that positive or close margins are associated with an increased incidence of recurrence. However, recurrences do occur despite clear histopathological margins.

Demonstration of novel molecular markers may help in identifying patients who are prone to increased risk of relapse despite clear margins by routine H&E staining. This may aid in modifying therapy and adding appropriate adjuvant treatment on a personalized basis which may help to improve outcomes. A significant drawback is a considerable time required to perform such an assessment, which limits its intraoperative usefulness. To emphasize, the molecular assessment of surgical margins is currently investigational.

**Objectives**

The purpose of the study was to assess the use of molecular markers on negative margins post wide excision that may assist in identifying patients who are more likely to develop locoregional recurrence with treatment naïve oral squamous cell cancer.

The primary objective was to evaluate the locoregional recurrence in patients expressing p53, Rb in tumour tissue and at the excision margin. The secondary objective was to assess the survival among those expressing these markers in the tumour tissue and at the margins.

**METHODS**

All patients with operable treatment naïve oral squamous cell carcinomas presenting in head and neck unit at a tertiary cancer centre undergoing surgery and meeting the inclusion criteria were recruited over 1 year and followed prospectively for two years to detect recurrence as primary objective and survival as a secondary objective. This prospective observational study was conducted between April 2018 and April 2021. The study was started after obtaining clearance from the institutional ethical committee. A total of 100 cases were enrolled. 7 patients were lost to follow up. The data of the remaining 93 patients are presented here. The following parameters of patients were recorded: age, sex, history of tobacco chewing, smoking, alcoholism, tumour site, type of treatment and recurrence during follow up. Wide excision specimens were tested for expression of p53 and retinoblastoma proteins in tumours and at the closest margin tissue of oral squamous cell carcinoma using immunohistochemistry for patients who had negative margins on routine H & E staining. The patient was followed for 2 years for local recurrence / metastasis. The expression of p53 and Rb was correlated with local recurrence and survival.

**Inclusion Criteria**
1. Squamous cell cancer
2. Operable stage I to IVA
3. Treatment naïve
4. Margin negative on H & E
5. ECOG 1 - 2
6. Consenting to be part of the study and follow up

**Exclusion Criteria**
1. Non - squamous histology
2. Inoperable disease
3. Poor performance status
4. Positive margins on routine H & E

**Experimental Technique**

Paraffin-embedded tissues were sectioned, 4μm, using a microtome (Leica, Germany), and transferred to tissue bond-coated slides (Biocare, USA). After overnight incubation in a 60°C dry oven, paraffin-embedded sections were deparaffinised in xylene and rehydrated through graded ethanol series 100 %, 70 % and 50 %. Endogenous peroxidase activity was blocked with 3 % hydrogen peroxide in methanol for 30 min. Antigen retrieval was done by placing the slides in Tris - EDTA buffer (pH 9.0). These sections after cooling to room temperature were incubated with p53 and retinoblastoma primary antibody at room temperature for one hour, followed by treatment with polymer-based secondary antibody kit with dianinobenzidine (DAKO, Denmark). Positive reactions were visualized using dianinobenzidine, DAB (1:50). Sections were finally counterstained with 0.1 % Haematoxylin. The positive cells expressing the p53 and retinoblastoma were assessed for cytoplasmic as well as nuclear staining at lower and higher magnification (figure 1). Expression of p53 and retinoblastoma in negative histological margins was determined. Nuclear staining for p53 was both quantitatively and qualitatively evaluated. The intensity of staining was grouped into no staining (0), weak (1+), intermediate (2+), strong (3+) while the percentage of positively stained cells was calculated as a continuous variable. Intermediate to strong staining in more than 30 % cancer cells was taken as mutant type expression while weak to intermediate staining in less than 30 % tumour cells was interpreted wild type expression. No staining in cancer cells was taken as negative. The grade of any intensity and percentage staining for Rb was taken as positive.

**Follow Up and Data Collection**

Patients were followed for a period of 2 years for every 3 months and evaluated for recurrence or metastasis by examination which was confirmed by radiological or pathological investigations as per the merit of the case. Their clinical and pathological data were recorded in an MS excel sheet and updated periodically.

**Statistical Analysis**

The association between variables was assessed by chi-square test, with continuity correction for all 2 x 2 tables after pooling of data and by Fischer exact test for all 2 x 2 tables.
where P-value of chi-square test was not valid due to small counts despite pooling of data. In the presence of a small count in tables with more than two rows and / or columns, adjacent row / column data were pooled and the chi-square test was reapplied. Relative risk estimate was calculated in presence of various dichotomous variables. Comparison of quantitative data was done by using unpaired t-test if the data passed normality test or by Mann Whitney test if the data failed normality test. Statistical software SPSS 27.0.1.0 was used for statistical analysis.

RESULTS

Demographic and preoperative clinical factors like age, sex, smoking, alcohol, and tobacco habits were compared using standardized statistical tests and were found to be comparable in the present study (Table 1). An overall recurrence rate of 16.12 % (15 / 93) and a 2 - year mortality of 10.7 % (10 / 93) were observed. With respect to molecular margins, 43 % (40 patients) had p53 expression and 21.5 % (20 patients) had Rb protein overexpression, out of which 22.5 % (9 patients) were positive for p53 at margins and 50.0 % (10 patients) were positive for Rb at margins. 22.5 % (9) cases with p53 mutation developed recurrence while only 11.3 % (6) cases with wild type p53 had recurrence (Table 2). Recurrence was even more profound in those with mutant p53 at margins (33.33 %; 3 cases; P = 0.130). However, Rb overexpressing tumours had almost similar recurrence rates compared to normal Rb protein tumours, (20 %, 4 cases and 15.06 %, 11 cases) although those with Rb overexpression at margins had a higher chance of recurrence (30 %, 3 cases; P = 0.627). Similarly, mutant p53 expression tumours had higher mortality (17.5 %; 7 cases) than those with wild type p53 (5.6 %; 3 cases) (Table 3). Those with mutant p53 at margins had a further rise in mortality (44.44 %; 4; P = 0.007). Rb overexpression in tumours was also reported to have higher mortality (25 %; 5 cases) with margin positive cases showing the highest rate (60 %, 3 cases P = 0.035). However, Rb negative cases had significantly lower mortality rates (6.8 % 5 cases). Mean survival was significantly higher in those with wild type p53 expression (641.25 days in wild type, 498.63 days in mutant type; P = 0.035, hazard ratio = 0.029). Rb overexpression showed a similar but statistically insignificant decreased survival. Mean recurrence-free survival also showed decreasing trend with mutant p53 and Rb overexpression but was not statistically significant (figure 4). Recurrence was the major cause of mortality (7 cases), 1 patient succumbed to postoperative myocardial infarction while 2 cases experienced non-institutional natural death. 46.23 % of the patients received adjuvant radiotherapy or chemoradiotherapy, which was decided by routine pathological parameters. Our study found a higher p53 and Rb overexpression rate with increasing N stage but could not reach statistical significance. T stage and extranodal extension (ENE) were not different among different groups and were not found to be associated with either p53 mutant expression or Rb overexpression. The higher grade was found to have higher rates of p53 and Rb positivity but was not statistically significant. Lymphovascular emboli were associated with higher rates of mutant p53 expression and Rb overexpression. Perineural invasion was associated with higher rates of mutant p53 expression but not Rb overexpression. Other pathological factors like nuclear pleomorphism, mitotic rate, necrosis, tumour infiltrating lymphocytes, desmplasia, tumour budding and pattern of invasion were not found to be different between the different groups. 82.79 % of patients had an infiltrative pattern of invasion while pushing types were found in the rest (Table 4). Regarding the tumour site, there was no significant difference between the different groups. An overall recurrence rate of 16.16 % was observed, more in males (18.42 % in males and 5.8 % in females). Clear margins were found in 88.17 % of the patients while 11.82 % had close margins. Interestingly, all the recurrences were noted in those with clear margins, though it could not reach statistical significance. This reinforces the importance of the presence of premalignant genetic alterations in surrounding histological normal mucosa. Most of the recurrences were reported in gingivoibucal complex with no statistically significant difference compared to other sites. There was an increase in recurrence rates in higher T and N stages but could not reach statistical significance.

**Table 1. Summary of Clinical Factors and Molecular Markers**

| Factor                  | Wild Type | p53 Mutant Type | Rb Mutant Type | p53 Tumour & Rb Tumour | p53 & Rb margins | Total | P Value | Rb Tumour & Rb margins | Total | P Value |
|-------------------------|-----------|-----------------|----------------|------------------------|-----------------|-------|---------|------------------------|-------|---------|
| <60 Years               | 45        | 27              | 5              | 77                     | 0.072           | 61    | 9       | 7                      | 0.462 |         |
| >60 Years               | 8         | 4               | 4              | 16                     | 0.249           | 12    | 1       | 3                      |       |         |
| Males                   | 41        | 26              | 9              | 76                     | 0.249           | 61    | 9       | 6                      | 0.151 |         |
| Females                 | 12        | 5               | 0              | 17                     | 0.224           | 12    | 1       | 4                      |       |         |
| Non recurrent           | 47        | 25              | 6              | 78                     | 0.224           | 62    | 9       | 7                      | 0.070 |         |
| Recurrent               | 6         | 6               | 3              | 15                     | 0.224           | 11    | 1       | 3                      |       |         |
| No tobacco              | 1         | 0               | 0              | 0                      | 0.683           | 0     | 0       | 1                      | 0.015 |         |
| Tobacco chewer          | 52        | 31              | 9              | 92                     | 0.683           | 73    | 10      | 9                      |       |         |
| Non alcoholic           | 38        | 24              | 8              | 70                     | 0.760           | 56    | 8       | 6                      | 0.064 |         |
| Alcoholic               | 14        | 7               | 1              | 23                     | 0.224           | 17    | 2       | 4                      |       |         |
| No smoker               | 47        | 31              | 6              | 84                     | 0.029           | 67    | 10      | 7                      | 0.024 |         |
| Smoker                  | 5         | 0               | 3              | 0                      | 0.224           | 6     | 0       | 3                      |       |         |
| No mortality            | 50        | 28              | 5              | 83                     | 0.002           | 68    | 8       | 7                      | 0.052 |         |
| Mortality               | 3         | 3               | 4              | 10                     | 0.002           | 3     | 2       | 3                      |       |         |
| No ADJUVANT             | 29        | 17              | 4              | 50                     | 0.852           | 37    | 4       | 9                      | 0.064 |         |
| ADJUVANT                | 24        | 14              | 5              | 43                     | 0.224           | 36    | 6       | 1                      |       |         |
| Total                   | 53        | 31              | 9              | 93                     | 0.224           | 73    | 10      | 10                     |       |         |

**Table 2. p53 Mutant Type and Rb Overexpression in Tumours**

| p53 Mutant Type Expression in Tumour | Number of Patients | p53 Tumour & Rb Tumour | p53 & Rb margins | Total | Rb Protein Overexpression in Tumour | Number of Patients | Rb Tumour & Rb margins | Total |
|--------------------------------------|--------------------|------------------------|-----------------|-------|-------------------------------------|--------------------|------------------------|-------|
| Wild type p53                        | 53 (57 %)          | Negative               | 73 (70.5 %)     |       |                                     | 20                 | Positive               | 16 / 20 | 50 %     |
| Mutant p53                           | 40 (43 %)          | (22.5 %)               | 20              | 9 / 40| 16 (21.5 %)                         | (50 %)             |                        |       |           |
**Table 3. Association of p53 Mutant Type and Rb Overexpression with Recurrence and Mortality**

| Factor                          | Number of Cases (%) | Wild Type | P53 in Tumour only | P53 in Tumour & Margins | P Value | Rb Negative | Rb Positve | Rb Over Positive | Rb Positive Tumour & Margins | P Value |
|--------------------------------|---------------------|-----------|--------------------|-------------------------|---------|-------------|------------|------------------|-----------------------------|---------|
| N0                             | 58                  | 36        | 17                | 5                       | 0.189   | 45          | 7          | 6                | 0.579                       |         |
| N1                             | 19                  | 9         | 8                 | 2                       | 0.170   | 17          | 0          | 2                |                             |         |
| N2 - 3                         | 16                  | 8         | 6                 | 2                       | 0.114   | 18          | 4          | 3                | 0.548                       |         |
| ENE -                          | 25                  | 13        | 10                | 2                       | 0.449   | 40          | 4          | 5                | 0.348                       |         |
| ENE+                           | 10                  | 4         | 4                 | 2                       | 0.097   | 63          | 10         | 9                | 0.742                       |         |
| T0 - T1-T2                    | 49                  | 28        | 17                | 4                       | 0.254   | 30          | 2          | 2                | 0.803                       |         |
| T3-T4                          | 44                  | 25        | 14                | 5                       | 0.735   | 65          | 8          | 9                | 0.279                       |         |
| SCC                            | 82                  | 47        | 27                | 8                       | 0.029   | 55          | 5          | 5                | 0.277                       |         |
| VLS / SIN                     | 8                   | 4         | 3                 | 1                       | 0.064   | 55          | 6          | 7                | 0.669                       |         |
| NRL                            | 3                   | 2         | 1                 | 0                       | 0.574   | 30          | 3          | 3                | 0.755                       |         |
| Grade 1                        | 34                  | 25        | 8                 | 1                       | 0.563   | 59          | 5          | 7                | 0.211                       |         |
| Grade 2                        | 44                  | 24        | 15                | 2                       | 0.445   | 54          | 8          | 7                | 0.434                       |         |
| Clear margin                   | 82                  | 46        | 29                | 7                       | 0.494   | 8           | 1          | 1                | 0.699                       |         |
| Absent Rb                      | 65                  | 44        | 17                | 4                       | 0.410   | 39          | 4          | 4                | 0.482                       |         |
| Present Rb                     | 28                  | 9         | 14                | 5                       | 0.946   | 58          | 4          | 6                | 0.066                       |         |
| Absent PNI                     | 68                  | 42        | 23                | 3                       | 0.736   | 59          | 10         | 8                | 0.808                       |         |
| Present PNI                    | 25                  | 11        | 8                 | 6                       | 0.853   | 23          | 2          | 3                | 0.067                       |         |
| LowPle / nuclear grade         | 36                  | 24        | 10                | 2                       | 0.537   | 30          | 3          | 3                | 0.755                       |         |
| Intplo / nuclear grade         | 48                  | 28        | 17                | 4                       | 0.563   | 59          | 5          | 7                | 0.211                       |         |
| Highples / nuclear grade       | 9                   | 4         | 2                 | 3                       | 0.445   | 54          | 8          | 7                | 0.434                       |         |
| Absent, Few, Occ mitosis       | 71                  | 42        | 23                | 6                       | 0.494   | 8           | 1          | 1                | 0.699                       |         |
| Freq mitosis                   | 22                  | 11        | 8                 | 3                       | 0.410   | 39          | 4          | 4                | 0.482                       |         |
| No necrosis                    | 69                  | 46        | 21                | 6                       | 0.946   | 58          | 4          | 6                | 0.066                       |         |
| Nodular Tumour                 | 19                  | 10        | 5                 | 3                       | 0.736   | 59          | 10         | 8                | 0.808                       |         |
| Mod TIL                        | 38                  | 21        | 13                | 4                       | 0.853   | 23          | 2          | 3                | 0.067                       |         |
| Severe TIL                     | 45                  | 27        | 15                | 3                       | 0.574   | 30          | 3          | 3                | 0.755                       |         |
| NA + Desmoplasia               | 47                  | 26        | 19                | 2                       | 0.410   | 39          | 4          | 4                | 0.482                       |         |
| 2+3 Desmoplasia                | 46                  | 27        | 12                | 7                       | 0.946   | 58          | 4          | 6                | 0.066                       |         |
| No Budding                     | 88                  | 40        | 22                | 6                       | 0.736   | 59          | 10         | 8                | 0.808                       |         |
| Budding                        | 35                  | 16        | 9                 | 3                       | 0.853   | 23          | 2          | 3                | 0.067                       |         |
| Pre-infiltrative POI           | 29                  | 16        | 9                 | 3                       | 0.853   | 23          | 2          | 3                | 0.067                       |         |
| GB Suii                        | 19                  | 14        | 9                 | 2                       | 0.574   | 30          | 3          | 3                | 0.755                       |         |
| RMT                            | 21                  | 12        | 8                 | 1                       | 0.574   | 30          | 3          | 3                | 0.755                       |         |
| Tongue                         | 10                  | 15        | 6                 | 2                       | 0.574   | 30          | 3          | 3                | 0.755                       |         |
| LIP                            | 5                   | 6         | 5                 | 1                       | 0.574   | 30          | 3          | 3                | 0.755                       |         |
| Hard Palate                    | 1                   | 1         | 0                 | 0                       | 0.574   | 30          | 3          | 3                | 0.755                       |         |

**Table 4. Summary of Pathological Factors and Molecular Markers**

**DISCUSSION**

The surgical margins (SMs) or resection margins are boundaries of resection specimen excised by the surgeon to obtain local control and increase survival. The status of these resected SMs is an important and valuable tool to predict the treatment outcome. Apart from routine histopathology, the molecular assessment of resected margins referred to as molecular margins have recently gained value as tumours with histologically negative margins (HNMs) that are also known to demonstrate locoregional recurrence. Histological distance of > 5 mm from the invasive carcinoma to SMs is labelled as clear margin taking into account shrinkage at fixation and elasticity at resection. Various molecular markers have been employed to detect these fields of genetiically altered cells. These fields serve as fertile
grounds for the evolution of potentially malignant lesions as well as invasive cancer. Majority of these altered fields can be identified through immunohistochemistry (IHC) or genetic analysis. Determining the “molecular status” of the SMs is one of the newer diagnostic methods employed in OSCC and studies have shown that subjecting the SMs for molecular analysis helps to determine the adequacy of tumour tissue removal. Overexpression of tumour suppression genes (such as p53), oncogenes (such as epidermal growth factor receptor), and proto-oncogenes (like Her - 2) in margins reported to be cancer-free on routine histopathological examination explains the initiation of premalignant and malignant changes at these margins, which may further result in recurrence and second primary tumours. In a study by Von Houten et al. the absence of TP53 - mutated DNA in surgical margins was significantly associated with higher local and locoregional recurrence-free survival (P = 0.027 and P = 0.028, respectively). Moreover, about 20 % of the cases have p53 mutated DNA in the surgical margins related to the primary tumour and the presence of TP53 - mutated DNA in the surgical margins serve as an independent prognosticator for locoregional recurrence (relative risk = 7.1; P = 0.021). Brennan et al. identified 25 patients with primary squamous-cell carcinoma of the head and neck containing a p53 mutation with complete tumour resection based on a negative histopathological assessment. In 13 of these 25 patients, molecular analysis was positive for a p53 mutation in at least one tumour margin. In 5 of 13 patients with positive margins by this method (38 percent), the carcinoma had recurred locally, as compared with none of 12 patients with negative margins (P = 0.02). Huang X et al. found the probability of developing local recurrence was significantly higher for the group with p53 mutation-positive margins when compared with the group with clear margins (P = 0.048) and more strongly associated with p53 mutation-positive deep molecular margins than mutation-positive mucosal molecular margins or positivity at both sites (P = 0.009). In our study, several reports have suggested that Rb protein function was absent in malignant oral epithelium, whereas others have indicated raised Rb protein during oral cancer progression. The expression of Rb protein was found to increase from normal oral, precancerous lesions to cancer. Patients with combined habits of betel chewing, smoking, and alcohol consumption had a higher expression compared to those without habits. The management of tumours found to have a positive molecular margin is not clear. Radiation to the primary site did not prevent the development of local recurrence when the residual tumour harboured a p53 gene mutation. Surgical resection is impractical as the size of resection is large and ill-defined. Intense surveillance and individual case base modification in treatment protocol remain the best available option. The presence of genetic alterations in HNMs demands the refinement of the definition of tumour-free margins in OSCC and it is recommended to include molecular status along with histology. This would influence the therapeutic approach and predict local recurrence and survival rate.

**CONCLUSIONS**

Clinical and routine histopathological assessment of margins remains the standard method of prognosticating. Determination of molecular positive margins in oral cancer can aid in identifying patients at higher risk of development of recurrence. p53 and Rb expressions in histologically negative margins were associated with increased locoregional recurrence and mortality. Higher N stage, tumour grade but not T stage was associated with mutant type p53 expression and Rb protein overexpression in histologically negative margins. Further, large scale studies with longer follow up are needed to comment on the effect on overall and disease-free survival. Determination of molecular marker positivity can assist in determining the high risk of development of locoregional recurrences and mortality in selected cases.

**Limitations**

Even though many more molecular markers which have proven to be more accurate to predict the margin status have been implicated, our study only includes two markers. Presently the value of these markers in routine resection specimens of oral cancers is not well established yet. Nonetheless beholding the role of these genetic and molecular alterations to predict recurrence and survival, further studies, preferably well designed randomized trials are recommended that focus on validation and assessment of the clinical utility of these molecular markers.

Data sharing statement provided by the authors is available with the full text of this article at jemds.com.

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