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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ABSTRACT

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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BACKGROUND

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.^{1,2} 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 diaminobenzidine (DAKO, Denmark). Positive reactions were visualized using diaminobenzidine, DAB (1:50). Sections were finally counter-stained 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 expressing 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, desmoplasia, 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 (Table4). 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 gingivobuccal 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.

Factor	p53				P-Value	RB			
	Wild Type	Mutant Type in Tumour	Mutant Type in Tumour & Margins	Total		Rb Negative	Rb + Tumour	Rb + Tumour & Margins	P-Value
<60 Years	45	27	5	77	0.072	61	9	7	0.462
>60 Years	8	4	4	16		12	1	3	
Males	41	26	9	76	0.249	61	9	6	0.151
Females	12	5	0	17		12	1	4	
Non recurrent	47	25	6	78	0.224	62	9	7	0.870
Recurrent	6	6	3	15		11	1	3	
Non tobacco	1	0	0	01	0.683	0	0	1	0.015
Tobacco chewer	52	31	9	92		73	10	9	
Non alcoholic	38	24	8	70	0.760	56	8	6	0.064
Alcoholic	14	7	1	23		17	2	4	
Non smoker	47	31	6	84	0.029	67	10	7	0.024
Smoker	5	0	3	09		6	0	3	
No Mortality	50	28	5	83	0.002	68	8	7	0.052
Mortality	3	3	4	10		5	2	3	
No ADJUVANT	29	17	4	50	0.852	37	4	9	0.064
ADJUVANT	24	14	5	43		36	6	1	
Total	53	31	9	93		73	10	10	
Table 1. Summary of Clinical Factors and Molecular Markers									

p53 Mutant Type Expression in Tumour			Rb Protein Overexpression in Tumour		
	Number of Patients	Mutant p53 Expression on Margins		Number of Patients	Rb Protein Overexpression in Margins
Wild type p53	53(57 %)		Negative	73(78.5 %)	
Mutant p53	40 (43 %)	9 / 40 (22.5 %)	Positive	20 (21.5 %)	10 / 20 (50 %)
Total	93		Total	93	

Table 2. p53 Mutant Type and Rb Overexpression in Tumours

Mutant p53 Expression and Recurrence						Relative Risk of Margin Positivity	Rb Protein Overexpression and Recurrence					
	p53		Total	P Value	Mutant Type p53 at Margins	(P-Value)	Rb		Total	P Value	Rb Over expression at Margins	Relative Risk of Margin Positivity (P-Value)
	Wild Type	Mutant Type					Negative	Positive				
No recurrence	47	31	78	0.130	6	2.3 (0.224)	62	16	78	0.627	7	2.3 (0.870)
Recurrence	6	9	15		3		11	4	15		3	
	(11.3 %)	(22.5 %)	(16.12 %)		(33.33 %)		(15.06 %)	(20 %)	(16.12 %)		(30 %)	
Total	53	40	93		9		73	20	93		10	
	Mutant p53 expression and mortality						Rb protein overexpression and mortality					
Alive	50	33	83	0.007	5	6.2 (0.002)	68	15	83	0.035	4	3.5 (0.052)
Deaths	3	7	10				5	5	10		6	
	(5.6 %)	(17.5 %)	(10.75 %)		4 (44.44 %)		(6.8 %)	(25 %)	(10.75 %)		(60 %)	
Total	53	40	93		9		73	20	93		10	
Table 3. Association of p53 Mutant Type and Rb Overexpression with Recurrence and Mortality												

Factor	Number of Cases (%)	P53			P Value	RB			P Value
		Wild Type	Mutant Type in Tumour only	Mutant Type in Tumour & Margins		Rb Negative	Rb Positive Tumour	Rb Positive Tumour & Margins	
N0	58	36	17	5	0.189	45	7	6	0.579
N1	19	9	8	2		17	0	2	
N2 - 3	16	8	6	2		11	3	2	
ENE -	25	13	10	2	0.114	18	4	3	0.548
ENE+	10	4	4	2		7	2	1	
T0 - T1-T2	49	28	17	4	0.449	40	4	5	0.340
T3-T4	44	25	14	5		33	6	5	
SCC	82	47	27	8	0.973	63	10	9	0.742
VL / SIN	8	4	3	1		7	0	1	
NRL	3	2	1	0		3	0	0	
Grade 1	34	25	8	1	0.254	30	2	2	0.803
Grade 2	44	24	15	2		36	4	4	
Grade 3	15	4	8	0		7	4	4	
Clear margin	82	46	29	7	0.735	65	8	9	0.279
Close margin	11	7	2	2		8	2	1	
Absent LVI	65	44	17	4	0.029	55	5	5	0.277
Present LVI	28	9	14	5		18	5	5	
Absent PNI	68	42	23	3	0.064	55	6	7	0.669
Present PNI	25	11	8	6		18	4	3	
LowPleo / nuclear grade	36	24	10	2	0.574	30	3	3	0.755
Intpleo / nuclear grade	48	25	19	6		39	6	3	
Highpleo / nuclear grade	9	4	2	3		4	1	4	
Absent, Few, Occ mitosis	71	42	23	6	0.563	59	5	7	0.211
Freq mitosis	22	11	8	3		14	5	3	
No necrosis	69	42	21	6	0.445	54	8	7	0.434
Necrosis	24	11	10	3		19	2	3	
Mild TIL	10	5	3	2	0.949	8	1	1	0.699
Mod TIL	38	21	13	4		28	4	6	
Severe TIL	45	27	15	3		37	5	3	
NA, 1+ Desmoplasia	47	26	19	2	0.410	39	4	4	0.482
2+3+ Desmoplasia	46	27	12	7		34	6	6	
No Budding	68	40	22	6	0.946	58	4	6	0.066
Budding	25	13	9	3		15	6	4	
Infiltrative POI	77	44	25	8	0.736	59	10	8	0.808
Pushing POI	16	9	6	1		14	0	2	
Buccal Mucosa	28	16	8	4	0.853	23	2	3	0.067
GB Sulci	19	8	9	2		14	2	3	
RMT	21	12	8	1		16	5	0	
Tongue	23	15	6	2		19	1	3	
LIP	1	1	0	0		0	0	1	
Hard Palate	1	1	0	0		1	0	0	

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.³ 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.³ Various molecular markers have been employed to detect these fields of genetically altered cells.^{1,4,5} These fields serve as fertile

grounds for the evolution of potentially malignant lesions as well as invasive cancer.^{6,7} Majority of these altered fields can be identified through immunohistochemistry (IHC) or genetic analysis.⁷ 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.^{8,9,10,11} 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).¹² 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; $P = 0.021$). J.A. 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.¹³ 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 ($P = 0.009$).¹⁴ 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.^{15,16,17} The expression of Rb protein was found to increase from normal oral, precancerous lesions to cancers. A consistently high expression of Rb protein was seen in oral cancers, with an increase in well-differentiated and moderately differentiated tumours. Patients with combined habits of betel chewing, smoking, and alcohol consumption had a higher expression compared to those without habits.^{18,19} 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.²⁰ 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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