RESEARCH ARTICLE

p53 Expression Helps Identify High Risk Oral Tongue Pre-malignant Lesions and Correlates with Patterns of Invasive Tumour Front and Tumour Depth in Oral Tongue Squamous Cell Carcinoma Cases

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Abstract

Oral tongue squamous cell carcinoma (OTSCC) is the most common oral cancer subtype with a maximum propensity for regional spread. Our objective was to study if p53 expression might have any correlation with aggressive patterns of invasion within oral tongue cancers as well as with the histologically identified degree of oral tongue dysplasia. p53 immunoexpression was studied using immunohistochemistry in early staged OTSCCs (n=155), oral tongue dysplasias, (n=29) and oral tongue normal specimens (n=10) and evaluated for correlations with histological and clinicopathological parameters. Our study (n=194) showed a pattern of p53 expression increasing with different grades of tongue dysplasia to different grades of invasive OTSCC (p=0.000). Among the OTSCC tumours, positive p53 expression was seen in 43.2% (67/155) and a higher p53 labelling index was significantly associated with increased Bryne's grade of the tumour invasive front (p=0.039) and increased tumour depth (p=0.018). Among the OTSCC patients with tobacco habits, (n=91), a higher p53 labelling index was significantly associated with increased risk of local recurrence (p=0.025) and with lymphovascular space involvement (p=0.014). Evaluation of p53 through varying degrees of dysplasia to oral tongue cancer indicates that p53 expression is linked to aggressive features of oral tongue cancers and tongue precancers entailing a closer monitoring in positive cases. Among the OTSCCs, p53 expression is associated with tumour aggressiveness correlating with increased grading of invasive tumour front and tumour depth.

Keywords: Oral tongue carcinoma - oral tongue dysplasia - p53 expression - increased tumour depth - local recurrence

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Introduction

Oral Tongue squamous cell carcinoma (OTSCC) is one of the most common sub-site of oral cancers in the world. The trends in epidemiology of oral cancer in Asia in the past decade (2000-2012) shows oral tongue as the most frequently affected site (Krishna et al., 2013). Earlier studies report a higher incidence of OTSCC in India compared to other countries (Moore et al., 2000; Elango et al., 2006; Krishnamurthy et al., 2013; Mishra et al., 2014).

The incidence of OTSCC in the population based cancer registry (PBCR) of Chennai has been showing an increasing trend. The age adjusted incidence rate (AAR) for carcinoma tongue has increased from 3.6 to 5.7 per 100,000 persons over the above 25 years. OTSCCs has been traditionally believed to be associated with a poor prognosis and this may be linked with its maximum propensity of regional spread compared to other oral sub-sites (Yeole et al., 2011). Our earlier studies reported an increase in tongue cancers among the non-tobacco

users (Krishnamurthy et al., 2013). A HPV independent mechanism of p16 over-expression conferring a poorer prognosis in OTSCCs was reported by us (Ramshankar et al., 2014).

Studies have shown that prognosis of patients with oral cancer can be improved if detected and treated early with a five year survival as high as 80% (Mehrotra R et al., 2006).

Studies on early staged OTSCCs show that about 20-40% present with occult metastasis and therefore clinical stage is insufficient to depict good prognosis (Santii et al., 2007). It is therefore important to have parameters that can identify patients at higher risk and adverse outcome during presentation. The well-established concept of oral potentially malignant lesions (OPML) shows a step wise transition towards oral squamous cell carcinoma (OSCC) (Tsantoulis et al., 2007). OPML have the transformation rate ranging from 0.6%-18% (Gupta et al., 1980). The transformation rate is dependent on the severity of the dysplasia, which needs to be carefully assessed to

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determine the risk of malignancy. Some studies have shown that presence of Oral Epithelial Dysplasia (OED) in OPML as the most reliable factors predicting malignant transformation (Abbey et al., 1995). But there are other studies contradicting the presence of epithelial dysplasia in the biopsy not being a significant factor for malignant development (Holmstrup et al., 2007). Histopathological assessment many times is subjected to inter-observer variation. Therefore there is a necessity for tumour markers that can help identify lesions that needs to be addressed immediately and require closer monitoring.

p53 gene behaves as a multifunctional transcription factor and is involved in cell cycle, programmed cell death, senescence, differentiation, development, transcription, DNA replication, DNA repair, and maintenance of genomic stability. (Ogden et al., 1992, Hainaut, 2000; Maddocks et al., 2011) p53 is a biomarker studied extensively in oral cancers as well as in other cancers like breast, lung, colon (Nigro et al., 1989). In fact, p53 is the most common mutated gene in OSCC and mutations have been found in >50% of oral tumours. (Maddocks et al., 2011).

In the current study (n=194), we evaluated p53 immunoeexpression in normal tongue tissue, tongue dysplasias and early staged oral tongue cancers to evaluate its role in predicting the higher risk of aggressive behaviour in early staged oral tongue cancers and tongue pre-cancers. We have further correlated the expression with the pattern of invasion grading and histological features of exclusive subsite Oral mobile tongue presenting with cancer and precancer.

Materials and Methods

Patient materials

The current study was done on an exclusive cohort of early staged tongue cancers (clinical stages, T1 and T2 and N0) (n=155) presenting to Head and Neck Oncology clinic between the years 1995 and 2007, oral tongue dysplasias (n=29) and oral tongue normals (n=10). The study was approved by the University Research Council of Tamil Nadu Dr. MGR Medical University and conducted as per the Institutional ethical guidelines with informed consent, maintaining the patient confidentiality. The FFPE blocks from oral tongue dysplasia samples and tongue normals were obtained from patients presenting to the Dental outpatient clinic of Sree Balaji Dental College and Hospital and Saveetha Dental College and hospital with an informed consent and ethical committee clearance of respective Institutional IRB (SBDCECM 106/14/130), (IHEC/SDMDS/20MP2) respectively.

Variables recorded and evaluated for the study included age, sex, site, size of the tumour, pattern of the lesion, depth of invasion, clinical stage, histological grade (both Broders grading and Bryne's grading) and tobacco habits.

Patient treatment methods

Comprehensive history and physical examination of the oral cavity for the precancers and additionally upper aerodigestive tract with neck imaging was done

using ultrasound for the OTSCC patients. The patients had undergone standard treatment consisting of either wide excision glossectomy or brachytherapy, with or without selective neck dissection (Levels I to IV). Patients unwilling/unfit for surgery were treated using External Beam Radiotherapy as per the decisions of multidisciplinary tumour board of the Institution. Pattern of failure and good outcome was recorded for each patient. Death due to the disease was included in the definition of the overall survival (OS), while time to disease recurrence in months was used to calculate disease free survival (DFS).

Histopathological analysis

All the patients had undergone a routine evaluation which included a biopsy for histological confirmation of cancer and grades of dysplasia seen in tongue leukoplakia lesions. All the samples used in the study were histologically confirmed for their tissue status before inclusion in the study. Grading of H and E staining of OTSCC tumours was done as per the invasive tumour front according to criteria of degree of keratinisation, nuclear Pleomorphism, pattern of invasion and Lymphoplasmocytic infiltrate giving the scores from 1-4 as Bryne's grade as described previously (Bryne et al., 1992). When true invasive tumour front (ITF) was absent in the section, the deepest portion of the tumour was graded. The depth of invasion, measured from the highest basement membrane to the deepest portion of the tumor was obtained as described before (Tan et al., 2012) directly in micrometers using ProgRes CapturePro 2.8.8 software (JENOPTIK optical systems) at 4x objective magnification.

p53 Immunohistochemistry

The IHC detection of p53 expression was performed on five-micron sections of FFPE tissues. The sections were de-paraffinised in xylene and rehydrated in absolute ethanol. Antigen retrieval was done with 0.05M Tris Buffer (pH – 9) in pressure cooker for 20 minutes. Endogenous peroxidase activity was blocked by incubation in 0.03% hydrogen peroxide in distilled water for 10 minutes and then washed with phosphate buffered saline (PBS). Sections were pre-incubated with power block (BioGenex) for 10 minutes and then incubated with BioGenex ready-to-use mouse monoclonal antibody against p53 (1801) at room temperature for 90 minutes. p53 expression was observed using the SuperSensitive™ Polymer-HRP IHC Detection System (BioGenex Laboratories, San Ramon, CA). Sections were counterstained with hematoxylin, dehydrated, and mounted in DPX. Positive controls included sections of oral buccal mucosa squamous cell carcinoma previously known to show over-expression of p53. Primary antibody was replaced with 2% BSA in negative control. Immunostaining of the sections was reviewed along with corresponding Haematoxylin and Eosin stained sections.

P53 scoring

The p53 positivity was seen as nuclear brown stain with varying intensity. The p53 IHC stained slides were

graded into Grade 0-4. The grading was done by counting total of 1000 cells from 5 different representative sites in the mid tumor using eye piece grid. All the counting was done at the objective magnification of 40x. Grade 0 = none of the malignant epithelial cells showing nuclear positivity, Grade I=1-10% of the cells showing positive nuclear stain, Grade II=11-25% of the cells with positive nuclear stain, Grade III= 26-50% nuclear positivity, and grade IV= >50% of the cells showing nuclear positivity. p53 expression in >10 % of tumour cells was considered as positive as shown previously (Gorgoulis et al., 1995). p53 labeling index was calculated as ratio of number of p53 positive tumour cells for every 1000 tumour cells counted.

Statistical analysis

Statistical analysis of the data was done using SPSS version 16. Distribution of categorical variables was assessed by Pearson chi squared test. P value of less than 0.05 was considered statistically significant.

Results

All the tongue normal tissues were found to be negative for p53 immunopositivity, showing no detectable p53 staining. Grades of p53 expression was very significantly different ($p=0.000$) with proportion of lower percentage positivity of p53 expression observed in hyperplasia, and increasing percentage positivity for p53 expression with increasing grades of tongue dysplasia. Over-expression of p53 in >50% of tumour cells being found in OTSCC only. (Figure 1)

p53 expression vs. differentiation grades in Oral Tongue dysplasia, OTSCC

Positive immunopositivity for p53 was found in 43.2% of OTSCC (67/155) and 37.9% (11/29) Oral tongue pre-malignant lesions (Table 1). Figure 2 shows total p53 percentage positivity in different grades of oral tongue dysplasias and OTSCC. Higher percentage positivity of p53 immunopositivity was observed with increasing grades of dysplasia and degree of differentiation, as a progressive trend through the different histological grades of OTSCC with a high statistical significance ($p=0.000$). Among the patients presenting with severe dysplasia, 2/5 (40%) had p53 expression in >10% of tumour cells with one patient expressing nuclear p53 in up to 50% of

Figure 1. Relationship of p53 Expression in OTSCC and Tongue Dysplasia

Figure 2. Expression of p53 in Different Grades of Tongue Dysplasia and OTSCC

Figure 3. (A) Photomicrograph of Hyperplastic Epithelium Showing Grade 1, Intense p53 Nuclear Positivity at The Basal Cells. (IHC, 10x) (B) Photomicrograph of Intense p53 Nuclear Positivity (grade II) at The Basal and Parabasal Cells in Mild Epithelial Dysplasia (IHC, 10x) (C) Photomicrograph of Intense p53 Nuclear Positivity (grade II) at The Basal and Suprabasal cells in Mild Epithelial Dysplasia (IHC, 20x) (D) Photomicrograph of Intense p53 Nuclear Positivity ((grade III) in all the Layers of The Epithelium in Severe Epithelial Dysplasia (IHC, 10x)

Figure 4 : Expression of p53 and Staining in Tongue Dysplasias

Table 1. Clinicopathological Features Classified with P53 Immunoexpression

Clinical Parameters	p53 Immuno-expression in OTSCC (n=155)		p53 Immuno-expression in Oral Dysplasia (n=29)	
	p53 Negative (n=88; 56.8%)	p53 Positive (n=67; 43.2%)	p53 Negative (n=18; 62.1%)	p53 Positive (n=11; 37.9%)
AGE				
20-40 years	13(59.1)	9 (40.9)	7(77.8)	2(22.2)
41-60 years	50(58.1)	36(41.9)	9(60)	6(40)
61-80 years	25(53.3)	22(46.8)	2(40)	3(60)
GENDER				
MALE	66(61.7)	41(38.8)	16(69.6)	7(30.4)
FEMALE	22(45.8)	26(54.2)	2(33.3)	4(66.7)
STAGE				
STAGE 1	35 (59.3)	24(40.7)		
STAGE 2	53(55.5)	43(44.8)		
GRADE				
Well diff	73(58.4)	52(41.6)		
Mod Diff	12(46.2)	14(53.8)		
Poorly Diff	3(75)	1 (25)		
Hyperplasia			6 (75)	2(25)
Mild dys			2(50)	2(50)
Mod dys			7 (58.3)	5(41.6)
Severe Dys			3(60)	2(40)
BRYNES GRADE (p=0.037)				
Grade 1	6(100)	0		
Grade 2	33(63.5)	19(36.5)		
Grade 3	37(54.4)	31(45.6)		
Grade 4	12(41.4)	17(58.6)		
TOBACCO HABITS				
Chewer				
Smoker	23(52.3)	21(47.7)	6(75)	2(25)
Chewer+Smoker	14(46.7)	16(53.3)	6(66.7)	3(33.3)
Non User	12(66.7)	6(33.3)	5(83.3)	1(16.7)
39(61.9)	24(38.1)	1(16.7)	5(83.3)	
TREATMENT				
Iridium	62(58.5)	44(41.5)		
EBRT	21(48.8)	22(51.2)		
Surgery	5(83.3)	1(16.7)		
OUTCOME				
Alive/no disease	52(58.4)	37(41.6)		
Alive with Disease	3(75)	1(25)		
Dead	33(53.2)	29(46.8)		

Table 2. p53 Grading and Intensity of Expression in OTSCC

P53 Grading	Low intensity of Staining (n=45)	High intensity of staining (n=37)
1-10% (n=16)	15 (93.8%)	1 (6.2%)
11-25% (n=4)	3 (75%)	1 (25%)
25-50% (n=11)	8 (72.7%)	3 (27.3%)
> 50% (n=51)	19 (37.3%)	32 (62.7%)

*P=0.000

the cells.

Pattern of p53 expression in Oral Tongue Dysplasia

Figure 3 (a-d) show the pattern and grade of p53 staining in oral tongue dysplasia. Positive p53 immunoexpression was observed in 11/29 (37.9%) oral tongue dysplasias. Interestingly, p53 expression in up to 25% of cells was observed in 2/8 (25%) hyperplasias, 1/4(25%) mild dysplasias, 2/12 (16%) moderate

Figure 6. p53 Expression Association with Tumour depth

dysplasias and 1/5 (20%) severe tongue dysplasias. Figure 4 shows the pattern grade of p53 expression in oral tongue dysplasia. As the percentage positivity of p53 increased upto 50% of dysplastic epithelium, the pattern

Table 3. p53 Labelling Index in Tobacco Users

Recurrence pattern	P53 LI LOW	P53 LI HIGH	OR (95%CI)
Local Recurrence	6 (30)	14 (70)	2.8 (0.908-8.629) * (p=0.025)
Nodal Recurrence	6 (66.6)	3(33.3)	0.6 (0.132-2.70)
Locoregional Recurrence	14 (77.7)	4 (22.2)	0.3 (0.09-1.20)
LVI Present	11 (36.6)	19 (63.3)	3.062 (1.235-7.592) (p=0.014)

*Figures within brackets indicate percentages

Figure 5. (A) Photomicrograph of Well Differentiated Squamous Cell Carcinoma Showing Negative Staining for p53 (IHC, 10x) (B) Photomicrograph of Well Differentiated Squamous Cell Carcinoma Showing Less than 10% Cells Positive for p53 (IHC, 20x) (C) Photomicrograph of Well Differentiated Squamous Cell Carcinoma Showing Less than 25% Cells Positive for p53 (IHC, 10x) (D) Photomicrograph of Moderately Differentiated Squamous Cell Carcinoma Showing more than 50% Cells Positive for p53 (IHC, 10x)

Figure 7. (A) The Depth of Invasion was Measured in Micrometres. The Shallow Depth of Invasion and Less Than 10% Cells Showing p53 Nuclear Positivity. (IHC, 10X) (B) The Maximum Depth which Could be Measured at 4x Objective Magnification. The Total Depth of Measurement was 5003 Micrometres and p53 Nuclear Positivity was Seen in More than 50% of the Cells (grade IV). (IHC, 4x)

of expression was observed above parabasal layers up to suprabasal layers (p=0.000).

p53 correlates to increased invasion and tumour depth in OTSCC

Figure 5 (a-d) shows photomicrograph of p53 expression grades in various OTSCC tumour samples. Over-expression of p53 was found in 67/155 (43.2%) of OTSCC tumours. Of these, p53 over-expression in >50% of tumour cells was observed in 51/67 (76.1%) OTSCCs. Table 1 shows association of p53 status with the clinico-

pathological profile of patients with OTSCC and tongue dysplasia. In OTSCC, over-expression of p53 had a statistically significant positive correlation with a higher invasive front scoring by Bryne's grade (p=0.037). Table 2 shows the p53 expression in different grades of OTSCC vs. the intensity of staining. Higher the percentage of tumour cells expressing p53, higher was the intensity of staining which was evident in OTSCC (p=0.000). We found a statistically significant positive correlation between p53 over-expression and increasing tumour depth (p=0.018) (Figure 6). Tumours with p53 over-expression in >50% had increased tumour depth, which is a parameter known to depict prognosis in tongue cancers (Figure 7a,b). There was no association between p53 expression and other clinico-pathological parameters like age, gender, clinical stage, tobacco habits, treatment and outcome. Also, there was no correlation between presence of HPV DNA, p16 expression and p53 expression (Data not shown).

p53 vs tobacco use in patients with Oral tongue Dysplasia

Among the tobacco users with tongue dysplasia, 11/23 (47.8%) were negative for p53 expression indicating that, with cessation of tobacco habits, the risk of developing oral cancer in these patients can probably be decreased. Among the patients with lesions expressing p53, 3/23 (13%) had p53 over-expression in up to 25% of the cells, indicating a higher risk of malignancy with p53 over-expression as well as tobacco addiction. One patient presenting with severe dysplasia, with tobacco habits had p53 expression in up to 50% of tumour cells, indicating a probably higher risk of progression. These results suggest that patients with p53 over-expression in >10% of cells within dysplastic lesions with or without habits may have to be followed closely for early detection of transformation.

Role of Age and Tobacco usage in patients with OTSCC

Median age of the patients in the current series was 55 years. Among the tobacco users, (n=91) we found that increased age (> 55 years) was associated with increased p53 labeling index significantly (p=0.000). However this was not observed among the non-users. This association was significant for both tongue dysplasia and OTSCC. Interestingly, increase in patients with no tobacco related habits was seen in patients with OTSCC compared to patients presenting with lack of tobacco habits and with tongue dysplasia [64/155 (41.3%) vs 6/29 (20.6%); (p=0.039)], suggestive of the trend that OTSCC is increasing among the non tobacco users.

p53 expression among tobacco users indicates local recurrence and increased lymphovascular space

Among the tobacco users presenting with tongue cancer (n=91), higher p53 labelling index was significantly associated with increased local recurrence (OR=2.8; p=0.025) and lymphovascular space involvement (OR=3.062; p=0.014), suggestive of increased aggression of the tumour in the presence of tobacco habits (Table 3)

Discussion

Expression and mutation of p53 is the most common genetic event studied in oral cancer. It is well known that wild type p53 has a short half-life and cannot be detected immunohistochemically in contrast to the mutant forms that are more stable and have an extended half-life that can be detected using immunohistochemical methods (Ogden et al., 1992).

All the normal oral tongue tissues expressed no detectable p53 protein similar to the findings obtained before (Rich et al., 1999; Ogden et al., 1992). Earlier studies suggest that p53 positivity in dysplastic oral lesions ranges from 17% (Warnakulasuriya KA and Johnson, 1992) to 89% (Lippman et al., 1995). The current study showed positive p53 protein immunorexpression in (11/29) of tongue dysplasias, as suggested in previous studies. We hypothesize that expression of p53 in oral tongue dysplasia may help to identify patients who may have a higher risk of malignant transformation. We found these lesions with p53 expression in up to 25% of cells had suprabasal layer positivity. Previous reports suggest that suprabasal positive expression of p53 is an early event and can be an indication of a developing carcinoma (Kerpdon et al., 1997; Cruz et al., 2002).

Studies by Cruz et al (2002) with a follow up of dysplastic lesions for a period of 16 years show that p53 expression pattern could show higher specificity than histopathological assessment of dysplasia (96 % vs. 54 %) and higher positive predictive value (86 % vs. 44 %) for correct prediction of the malignant transformation of the lesions suggesting that clear expression of p53 above the basal cell layer could be an early event in oral carcinogenesis and an indicator of a developing carcinoma, even preceding morphological tissue alterations (Nylander et al., 2000). Conversely, studies also show that patients with negative p53 expression may not have the risk of oral tongue cancer provided they quit the tobacco habit, the known risk promoting factor as confirmed previously (Rich et al., 1999). Another study in which patients with precancers were followed up to cancer over 4-25 years, showed up to 30% positive for p53 (Cruz et al., 1998, Murthy et al., 1998).

The current study suggests that it may be important to consider p53 expression for risk assessment, in addition to the histopathological severity of oral tongue dysplasia. We had 20.6% tongue dysplasias with p53 over-expression in up to 25% of the cells and one of these was histologically hyperplasia. These lesions therefore would require a closer monitoring as a high risk for malignant transformation.

Expression pattern of p53 had significant differences comparing tongue tumours and tongue dysplasia. Our observation that p53 expression increases with severity

of dysplasia has been reported in earlier studies as well. (Girod et al., 1998; Iwasa et al., 2001; Kovesi et al., 2003; 2012). None of the tongue dysplasia showed p53 nuclear positive expression in >50% of cells unlike tumours where we had p53 overexpression identified in 76.1% of the positive tumours. Our study has shown overall positive p53 expression in 43.2% of oral tongue cancers. The results are similar to studies reported by Danieli et al., 2005 where they showed a 44% p53 positive expression in OTSCC. This is similar to the earlier studies from Indian population, 46%, 45%, 36% respectively (Yan et al., 1996; Saranath et al., 1999; Pillay et al., 2003).

Current study shows a positive correlation between higher p53 labelling index and higher Bryne's grade, and increased depth of tumour indicating the aggressiveness of the tumour. Tobacco users in current study with high p53 labelling index in the tongue tumours were significantly associated with local recurrence and lymphovascular space invasion. Earlier studies have shown that p53 overexpression screened by the same antibody used in the current study to evaluate positive tumour margins could be used to predict local recurrence. This study additionally showed that a small cluster of p53-positive carcinoma cells could be observed that was already present in the routine H&E staining but was missed by routine histology (Houten et al., 2004).

Tobacco users with higher p53 labelling index in their respective tumours are therefore suggested to have an increased risk of local recurrence. Tobacco users >55years of age had an increased p53 expression, which could be due to the genotoxic stress accumulating during the course of years of habits and this phenomenon was not seen among the non-tobacco users. This was significant for both patients with dysplasia as well as OTSCC. This implies that with prolonged use of tobacco in these patients over the period of years could have increased the genotoxic stress that is depicted by the p53 expression, as shown previously (Humanyun et al., 2011). Conversely, in our series of cases, only 20.6% of patients with tongue dysplasias were non tobacco users compared to 41.3% of non-tobacco users among patients with OTSCC. We have shown previously that OTSCC is increasingly evident in non-tobacco users as a changing trend and presence of p53 overexpression is important factor to be considered for closer monitoring in both tongue dysplasias and OTSCCs.

Use of biomarkers to increase the sensitivity of histology in predicting cancer development will help in identifying high risk patients, including those with lower risk histology. With these quantified cancer risk assessments factors, investigators and clinicians can offer the most appropriate, tailored cancer prevention strategies to each individual. Our study suggests that p53 immunorexpression may be a potential marker for tongue precancer lesions that may have a higher risk of progression and these patients need be monitored more closely. Among the OTSCC, p53 expression is associated with tumour aggressiveness as depicted by the invasive tumour front and tumour depth. Among tobacco users, p53 overexpression is correlated to increased risk of local recurrence and aggression. The results of our study will aid in better understanding the potential role of p53 in the

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