RESEARCH COMMUNICATION

p53 Expression as a Marker of Microinvasion in Oral Squamous Cell Carcinoma

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Abstract

Introduction: Oral squamous cell carcinoma (OSCC) has high local recurrence, partly caused by the lack of clear margin identification on surgical removal of cancerous tissues. Direct visualization by immunostaining and fluorescent *in situ* hybridization (FISH) in tissue sections gives more definite information about genetic damage at margins with appropriately selected biomarkers. Aims: To determine the usefulness of immunohistochemical techniques and FISH of the tumour suppressor TP 53 gene to identify microinvasion in marginal tissue sections and to relate the possible correlation between protein expression and genetic aberrations in OSCC cases in Malaysia. Methods: Immunohistochemistry and FISH of TP 53 genes were applied on 26 OSCC formalin fixed paraffin embed (FFEP) blocks selected from two oral cancer referral centers in Malaysia. Results: For p53 protein immunohistochemistry, 96% of the 26 OSCC studied showed positive immunostaining at the excision margins. In FISH assay, 48.9±9.7% of the cancerous cells were monoploid for p53 probe signals, 41.0±9.5 % were diploid, and 10.2±7.8 % were polyploid. A correlation between p53 immunostaining and TP53 gene aberrations was noted ($p<0.05$). Conclusions: Immunohistochemical analysis of p53 protein expression and FISH of TP53 gene could be applied as screening tool for microinvasion of OSCC.

Keywords: Oral SCC - tumor margins - TP 53 - FISH - immunohistochemistry - Malaysia

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Introduction

p53 is a product of TP53 gene, has been called "the guardian of the genome" has a number of roles, in particular conferring DNA stability by halting the G1/S regulation point in cell cycle if DNA damage is present and activating DNA repair proteins. TP53 gene can also activate apoptosis if the DNA is beyond repair. A strong correlation has been shown between TP53 gene overexpresssion with neoplastic development and progression (Keswani et al., 2006; Reshmi and Gollin, 2005). In addition, high frequency of mutation of the TP53 gene has been shown in some human solid tumours example like ovarian carcinoma (Nezhat et al., 2008), colon carcinoma (Mollevi et al., 2007) and bladder carcinoma (Malats et al., 2005).

In view of TP53 gene has been shown to control the cell cycle and maintenance of genomic stability (Vogelstein et al., 2000) to prevent accumulation of genetic damage (Lu-Hesselmann et al., 2004). Its gene mutations have been found in early cancerous development in most of head and neck squamous cell carcinoma (HNSCC). Moreover, its overexpression due to mutation is detectable in tumour distant, mucosal biopsies that appear histological normal (Stoehr et al., 2002). If this correlation existed, it would strengthen the diagnosis usefulness of p53 immunohistochemical staining in tumour biopsies. Furthermore, the incidence of overexpression of wild-type and functionally active TP53 gene might be indicated by those cases not showing aneuploidy. Therefore, immunohistochemistry for p53 overexpression was combined with FISH for TP53 gene to examine the existence of genetically altered cells in excised marginal tissue specimens for microinvasive detection of OSCC (Braakhuis et al., 2010; van der Toorn et al., 2001). In the present study, apoptosis-related oncoprotein-like TP53 gene expression status was investigated by immunohistochemistry and FISH techniques. In addition, TP53 gene overexpression and chromosomal instability detection was also evaluated in Malaysian OSCC cases.

Materials and Methods

For the study, 10% neutral buffered formalin-fixed, paraffin-embedded tissues of 26 oral SCC cases were obtained retrospectively from the archives of two Oral Cancer Referral Centers in Kuala Lumpur, the Institute for Medical Research and the Department of Oral Pathology, Oral Medicine and Periodontology, Faculty of Dentistry, University of Malaya. These cases were obtained from 3

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Malays, 5 Indians and 18 Chinese with an overall mean age of 54.6 years (range 40-85 years). Fifteen (57%) cases were excised from site of tongue, five (19%) of the maxilla, three (12%) of the buccal mucosa, a case (4%) each in of the buccal sulcus, alveolus and unspecified site. All these cases represented primary tumours in the study. Sections from a tissue block containing OSCC with overexpression of TP53 gene were used as positive control. The stained Hematoxylin and Eosin (H&E) sections were reviewed by an Oral Pathologist, and then representative specimens from resection marginal sections were selected for further analysis. The tissue specimens and five normal buccal mucosa specimens that previously fixed in 10% neutral buffered formalin were selected and processed with histological embedding techniques. For all cases, 4 μm thick paraffin sections were cut by microtome and stained for H&E staining for detailed histopathological investigation. p53 labelled streptavidin biotin (LSAB) using DO-7 antibody that recognizes epitopes expressed by the wild and mutant TP53 genes was used for immunohistochemistry (Hawes et al., 2009). FISH assay was followed modified Hopman's (1991) protocol using specific probe of LSI p53 (17p13.1).

Immunohistochemistry

Labelled streptavidin biotin technique (LSAB) (Hawes et al., 2009) was applied with 4 μm thick sections prepared on silanized slides, and air dried overnight. Sections were deparaffinized using xylene and rehydrated with graded alcohols. Microwave pretreatment for 20 mins by commercially available DAKO target retrieval solution (pH 6.1), for enhancing the immunolabeling and reducing non-specific background staining for antibodies. Endogenous peroxidases were quenched with 3% hydrogen peroxide in phosphate buffered saline (pH 7.4) for 5 minutes. Immunohistochemistry was performed following the manufacturer's instructions (LSAB technique, Dako, USA). Slides were then incubated with the primary antibody for 30 minutes at room temperature, Mouse anti-human p53 protein (DO-7, Dako, USA) was diluted 1:50 for this step. The biotinylated secondary antibody (rabbit anti-mouse) was applied to the sections for 15 minutes at room temperature. The streptavidin-horseradish peroxidase complex was then applied at room temperature for 30 min. Chromogen Diaminobenzidine (DAB) was applied to observe the peroxidase activity after the antibody-antigen reaction. Sections were then counterstained with 10% Harris Haematoxylin, dehydrated, cleared and mounted. Negative controls were included from sections of p53 overexpression which processed by excluding primary antibody in each staining series.

Analysis of staining intensity (Piffkò et al., 1998)

Only nuclear staining intensity of tumour cells were observed, this scoring was assessed by Siar CH and Khor GH after inter-examiner calibration and variability were carried out and approximately graded following the modified criteria of four point scale: 0 (negative), tumour showing no immunoreactivity; 1+ (weak), faint and focal nuclear staining of less than 50% of tumour cells, or any cells proportion shows pale nuclear staining or not easily seen the staining colour; 2+ (moderate), focal moderate nuclear staining of more than 50% staining of tumour cells; 3+ and 4+ (strong), dark nuclear staining that is easily seen and involves more than 50% of the tumour cells.

Fluorescent In Situ Hybridization (FISH)

A LSI p53 directly labeled probe (17p13.1; Vysis, IL, USA) (Hopman et al., 1991) and other hybridization reagents were purchased from Vysis. Color hybridization steps were performed using a supplement probe kit (Vysis, IL, USA) by following the manufacturer's protocols.

Analysis of chromosome copy number

The pathologist compared the hybridized test slides to a corresponding H&E stained section when selecting areas for chromosome copy number analysis. The hybridized signals appeared as small nucleus spots since a chromosome region containing only a small region of the interphase nucleus. In the defined histological area, 200 nuclei were counted and scored under microscopic magnification of 1000X. Then the hybridized signals in each nucleus were counted for the chromosome copy number. TP53 gene aberration was defined as cell number showing three or more signals in each nucleus.

Image analysis

The fluorescent signals were evaluated in at least 200 nuclei per slide using a Nikon fluorescent microscope equipped with single band filters for 4,6-diamidino-2-phenylindole (DAPI) and Spectrum Orange to discriminate the colour signals of orange signals for TP53 gene during scoring. Hybridization Images were acquired by using a photometrics cooled Charge-Coupled Device (CCD) camera and processed by Image-Pro Express version 4.01.

FISH evaluation

An evaluation criterion of 200 nuclei per slide was conducted following the description by Soder (1995). Normal controls yielding disomic hybridization signals were obtained from mucosal layer of specimen for non tumour patients. For the tumour specimens, a significant divergence of disomic hybridization signals in the normal control was observed in this study. A significant deviated cells percentage calculated based on the respective number of FISH signals which was more than the mean+2SD (standard deviation) of the normal controls.

For TP53 gene aberration, the resulting cutoff value for monosomy was set at 48% (30.2±2x8.9). Therefore, when the cell numbers with single signal in nucleus was counted above 48%, it was considered as p53 monosomy. Otherwise, p53 polysomy was deliberated when nucleus presented 3 or 4 signals that above value of 5 (3.2±2x0.8). Tumour polyploidy was concluded if more than three signals per cell were observed.

Statistical analysis

Statistical analysis was conducted using the descriptive statistic, the Pearson Chi-square test in Statistical Package of Social Sciences (SPSS) software program, student

version 17.0. The significance level for this study was set at p value less than 0.05 in relation to the p53 protein expression and TP53 gene aberrations.

Results

p53 expression (Figure 1)

Twenty-five (96%) of the 26 OSCC studied showed a clearly positive reaction to p53 at the marginal excision tumour site. Nuclear p53 staining either occurred in all epithelial cells throughout the tumour or as positive stained cells scattered among negative stained cells. Ten (38%) cases of OSCC cases showed strong p53 staining, 9 (35%) weak staining, and 6 (23%) cases demonstrated moderate staining, whereas only 1 (4%) case was non-reactive for p53 staining.

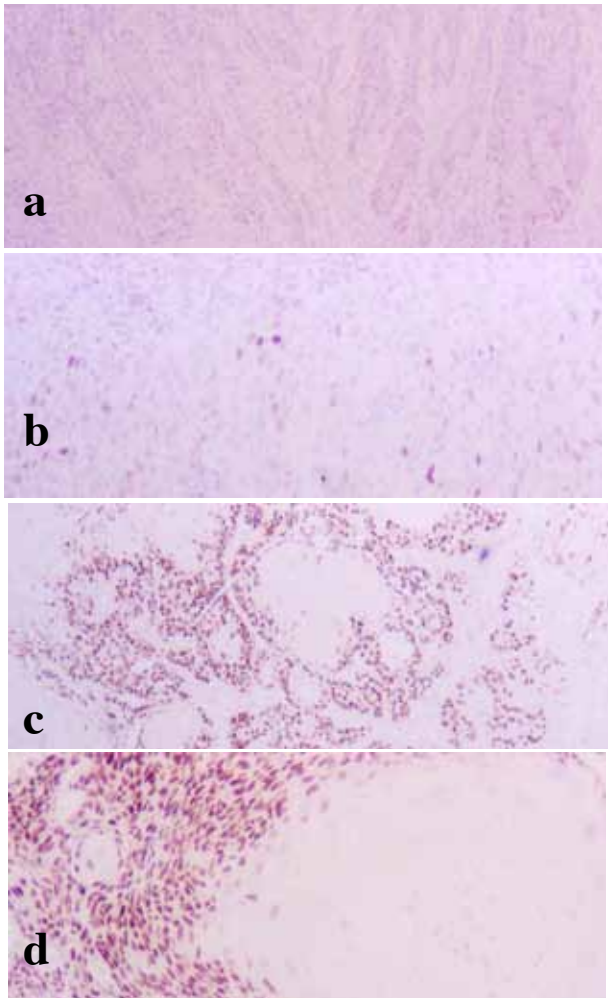

Figure 1. IP p53 Staining. a) Negative (x 40); b) Weak (x100); c) Moderately nuclei staining at the infiltrating margins and periphery of invading epithelial nests (x 40); Strong p53 nuclei staining in tumor cells. Keratinized cells are not stained (x 200)

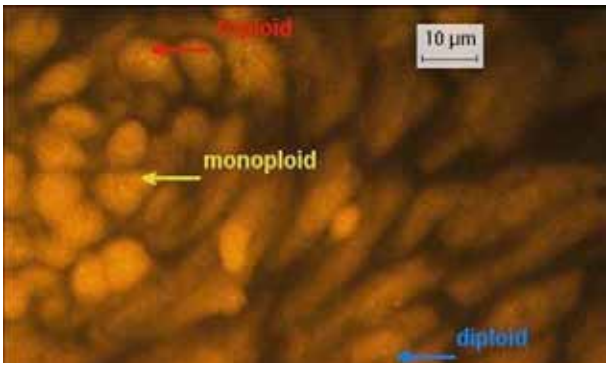

Figure 2. Various Signal Probes of p53. Monoploid, diploid and triploid orange signals were observed in the tumor cells. (Original magnification x 400)

Table 1. Cross-tabulation for p53 Immunoreactivity and p53 Aberrations of Oral SCCs

p53 Immunoreactivity	p53 Aneusomy		
	Monoploid	Diploid	Tripoid
Negative	0 (0)	1(100)	0 (0)
Weak	1 (11)	3 (33)	5 (56)
Moderate	1 (17)	0 (0)	5 (83)
Strong	0 (0)	5 (50)	5 (50)

TP53 gene aberrations (Figure 2)

In the 5 normal control cases, majority of the cells (mean value: 66.2 ± 8.6%) had two hybridized signals in single nuclei that shown diploid status, otherwise cells (mean value: 3.2 ± 0.8%) with more than two signals were rare. The remaining cells in the control samples (mean value: 30.2 ± 8.9%) demonstrated single hybridized signals (monoploid). In the test samples, most of the cancerous cells were monoploid (mean value: 48.9±9.7%) for p53 probe signals. Whereas only 41.0±9.5 % were diploid, and 10.2±7.8 % were polyploid TP53 gene.

On a per case basis, this study showed that TP53 gene aberrations was observed in 17/26 (65%) cases of OSCC where 2 (8%) cases demonstrated monoploid TP53 gene and 15 (57%) cases were polyploid. The remaining 9 (35%) cases were diploid status.

Correlative analyses p53 expression vs. TP53 gene aberration (Table 1):

The correlative analysis between p53 immunoreactivity and TP53 gene aneusomy in all 26 cases of OSCC studied is shown in Table 1. Polyploid TP53 gene was the dominant TP53 gene aberrations found in all strong staining intensities for p53 overexpression. Chi-square statistic provides the association between 2 independent variables is suitable to investigate the small sample size. Statistically significant correlation was detected between p53 immunoreactivity and TP53 gene aberrations in the cases of OSCC examined ($p<0.05$).

Discussion

A number of studies have applied immunohistochemistry to directly assess the TP 53 gene in OSCC, evidenced with various positive staining results ranging from 54 to 80.9% of the tumours examined (Ögmundsdóttir et al., 2002; van der Toorn et al., 2001). In the study, p53 protein expression was observed in 96% of OSCC cases, higher that other those reported in the literatures. One possible reason may be due to our criteria of case selection where the resection margins of tumour tissue blocks were only selected in the study.

In our study, 10 cases of OSCC showed p53 overexpression, recognized as strong nuclear staining in majority of cancerous cells. In all of our other OSCC

cases, variable levels of expression were detected. Occasionally only a few cells were found positive for p53 immunostaining. Similar findings have also been reported in previous studies (van der Toorn et al., 2001; van Oijen and Slootweg, 2000). These contrasting phenotypes may indicate differences in biological activity. This observation is due to the transcribed protein is more stable than the wild type of p53, which tend to pool in the nucleus, and therefore is not difficult to detect with p53 immunohistochemistry.

In HNSCC, the existence of abnormal TP53 gene expression is correlated with increased risk of local recurrence, resistance to radiotherapy; and developing a second primary tumour (Thomas et al., 2005). The similar observation also found in invasive breast cancers, more than 50% cases have been shown to contain gene mutations which lack of functional TP53 gene is corrected with aggressive biological behavior and poor clinical outcome (Olivier, 2006). These studies have clarified the molecular basis for loss function of wild-type and mutant TP53 gene overexpression in malignant cells. Half of the tumours analyzed showed changes of both TP53 gene alleles, either by gene deletion and point mutation (Curtin et al., 2004; Olivier et al., 2010). A point mutation stabilizes the p53 protein and together with the normal gene loss may lead to mutant protein accumulation within a cell. This accumulation alters the normal function of TP53 gene which negatively regulates cell growth and transforms TP53 gene into a dominantly acting oncogene. The most common type of TP53 gene mutation is missense mutation, where change in one or more bases of a codon results the shift of affected codon into specifying a different amino acid. This only affect the areas of p53 protein outside the tetramerisation domain in the C-terminus, most of the TP53 gene are still able to form tetramers (Nylander et al., 2000). However, not all mutation processes cause loss of normal gene functions. Therefore the role of TP53 gene is still uncertain in the specific mutations of neoplasia biology.

Some authors have reported TP53 gene overexpression in mucosa adjacent to OSCC tumour tissues (Liu et al., 2003; Ögmundsdóttir et al., 2002; van Oijen and Slootweg, 2000), consistent with findings in our study. There are several views forwarded to explain this phenomenon of TP53 gene alterations may: i) represent an early event in oral carcinogenesis (Cruz et al., 2002); ii) serve as a possible basis for cancerization (Liu, et al., 2003; Ögmundsdóttir, et al., 2002); iii) simply indicate a normal TP53 gene working system that activated in genetically-stressed cells. These stressed cells may represent those either restored, directed towards apoptosis, or to malignant transformation (Louis, 2006).

p53 overexpression can be easily monitored by immunohistochemistry, reliably indicating functional inactivation, either due to mutations in the DNA-binding domain (Pavletich et al., 1993; Walker et al., 1999) or binding by cellular or viral oncoproteins (Roth et al., 1998). Hence p53 overexpression may be linked with enhanced genomic instability and cancerous progressions (Reid et al., 2001). In the present study, a well-characterized antibody clone DO-7 for p53 was applied onto the samples. (Clone DO-7 recognizes an epitope located between amino acids 19 and 26 of wild type and mutant human p53 proteins).

The disadvantages of immunohistochemistry are due to the possibility barrier of antigenic alterations additional to the processes of tissue fixation and embedding. This barrier can be overcome by FISH analysis. Furthermore, FISH assay can provides a quantitative assessment of TP53 gene status in the cancerous cells. In view of the inherent limitations of immunocytochemistry, FISH assay was carried out as a complementary tool in our study.

In the study, we used DNA probes specific for TP53 gene to both 5 cases of normal control, and 26 archival tissue blocks of OSCC. The results demonstrated that the FISH technique allows the evaluation and detection of gene aberrations in interphase cells in each cases of OSCC. We found 15 (57%) cases of OSCC demonstrated TP53 gene polyploidy. The finding was significantly different from those of normal mucosa, which had two signals in nucleus. Similar results were reported in bladder cancer (Brandau and Böhle, 2000), gastrointestinal cancer (Takahashi et al., 2000; Khayat et al., 2009), and HNSCC (Patel et al., 2001).

FISH was performed on the normal buccal mucosa to make sure the hybridization technique was achieved and also to obtain baseline values of the hybridization signals distribution within the nuclei. We noticed that in FISH assay of TP53 gene for normal control, most of the cells (mean: 66.2±8.6%; range: 49-83%) had two hybridization signals that indicative of TP53 gene dipoidy, followed by cells (mean: 30.2±8.9%; range: 12-48%) with single hybridized signal (TP53 gene monoploidy) and the least common were cells (mean: 3.2±0.8%; range: 2-5%) with more 3 or more signals (TP53 gene polyploidy). The findings in normal sample cells tend to yield disomic signals and the cutoff value is 61%. These results showed that the tissue materials generated from formalin-fixed, paraffin-embedded are suited for FISH analysis.

A high frequency of chromosomal abnormalities has been previously observed in HNSCC by a variety of techniques, such as comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) assays (Nylander et al., 2000; Worsham et al., 2003). Moreover, all these techniques demonstrated similar results in an extensive genomic imbalance, which was also found in our study.

Genomic instability is involved in the multistep process of tumourigenesis in most cancers (Hanahan and Weinberg, 2000; Jefford and Irminger-Finger, 2006; Shin et al., 2001), that occurs at two levels: the nucleotide level and the chromosome level (Hoeijmakers, 2001). Genomic instability may also lead to chromosome non-disjunction and the generation of cells with zero, one, two, and three or more chromosome copies. Hence, the presence of cells exhibiting three or more chromosome copies (chromosome polysomy) might be considered as a quantitative marker for accumulated genomic instability in tumours. Gains or losses of whole or significant portion of chromosome in cancerous cell are observed in most cancer cases (Bharadwaj and Yu, 2004; Rajagopalan and Lengauer, 2004). This is a main driving force to determine the in several human cancers (Bellacosa, 2003; Duesberga

et al., 2005). In the study, a significant population of aneuploid cells was detected in most of the cancerous cells, and this was frequently represented by gain in chromosome rather than loss. There were 15 (57%) cases of TP53 gene polypoidy and only 2 (8%) of TP53 gene monosomy detected in this study, and this implies that chromosome gains was more frequently encountered than chromosome loss in the OSCCs studied here. In hence, p53 aneusomy in the excised margin of tumours can be detected by FISH.

Genetically aberrant cells as marked by either p53 overexpression and TP53 gene aberrations, was identified in the resection margins of 17 of 26 OSCCs in our study. In most of these resection margins, genetically aberrant cells were detected by immunohistochemistry and FISH techniques, which provide strong evidence of presence of premalignant or malignant cells beyond the morphological tumour invasive margin (Xu et al., 2002; Lear-Kaul et al., 2003; Blancato et al., 2004).

Aneuploidy is proportional to the degree of instability in cells. It influences the alteration of normal cellular phenotypes that become destablise leading to karyotypic and phenotypic heterogeneity of cancer cells (Duesberga et al., 2005). The possibility of TP53 gene alterations causing increases in the levels of genomic instability was studied by determining the relationship between p53 expression statuses in OSCCs in this study. We found that TP53 gene aneusomy showed a positive correlation with the rates of genomic instability. P support the points that TP53 gene plays a key in regulating genomic stability (Grady and Carethers, 2008; Hahn and Weinberg, 2002). In fact, the correlation between TP53 gene abnormality and genomic instability has been previously reported in malignancies involving the thyroid follicular cell (Kondo et al., 2006), renal cell (Jones et al., 2005), breast (Børresen Dale, 2003; Gisselsson et al., 2002), and head and neck (Gisselsson et al., 2002).

Although dysregulation levels of p53 proteins were usually associated with increased genomic instability in the cases studied, but in few cases of OSCC, there were no apparent increases in p53 immunoreactivity. The possible explanation is that mutant TP53 occurred in a gene site did not cause p53 protein accumulation. Otherwise, genomic instability during tumourigenesis may also happen in p53-independent pathways, which also influence cell cycle regulatory pathways (Charames and Bapat, 2003; Shin et al., 2001). Similarly, interruption controls of cell cycle regulatory which also induces genomic instability in the cells (Emdad et al., 2005; Kanu et al., 2009).

In the study, there were 2 cases of OSCC characterized by p53 immunoreactivity but showed no evidence of TP53 gene aberrations. This means that functional wild-type of TP53 gene or other biological functions such as methylation may be involved but not leading to genomic instability and aneuploidy. An alternative explanation would be that wild-type TP53 gene overexpression is responsible for maintaining genomic stability in these 2 cases. In our study, 15 cases of OSCC with aberration of TP53 gene, also simultaneously showed p53 immunoreactivity. This suggests that overexpression of p53 proteins are caused by the increase in TP53 gene copies of these cases. The correlation was statistically significantly detected between p53 immunoreactivity and TP53 gene aberrations in the cases of OSCC examined ($p<0.05$).

Our study has shown that: (a) Immunohistochemical analysis of p53 proteins and FISH of TP53 gene may be used as a routine screening for microinvasion of OSCC in individuals at high risk through the detection of TP53 gene aberrations. (b) Early TP53 gene aberrations can be detected by FISH technique at excised marginal section of OSCC cases. (c) OSCC progression involves aneuploidy/chromosomal instability which involved the loss of TP53 gene. (d) There is a correlation between p53 protein expression and aberrations of the TP 53 gene OSCC cases in Malaysia.

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