Original Article

Immunoexpressionof p53, Bax and hTERT in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma – A Comparative Study

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ABSTRACT:
Objective: The development of oral cancer is a multistep process comprising genetic mutations and chromosomal abnormalities. There are several molecular markers concerned with carcinogenesis of oral squamous cell carcinoma. The aim of this article is to evaluate the immunoexpression pattern of p53, Bax andhTERT in oral epithelia dysplasia and oral squamous cell carcinoma. **Material and methods:**Tissue Samples of 210 formalin-fixed, paraffin-embedded biopsy specimens were retrieved. The samples were oral leukoplakia with dysplasia (n=90), oral squamous cell carcinoma (n=90) and normal oral epithelium (n=30). The quantitativeimmunohistochemistry analysis for p53, Bax and hTERT were performed for all selected samples. The percentage of positive cells, tissue and cellular localization were evaluated and analysed. **Results:** In oral epithelium,the expression of p53 (80%) and hTERT (82.3%)wasconfined to basal layer butBax (76.5%)expression was predominant in supra basal layers.In oral epithelial dysplasia, supra basal expression of p53 and hTERT were increased with histological grades but, it was decreased for Bax.p53 and Bax expressions were statistically significant among histological grades of dysplasia (p<0.001) whereas,hTERT expression was not significant (p-0.674). In oral squamous cell carcinoma, the expression of p53, Bax and hTERT were statistically significant between histological grades(p<0.05). Remarkably, nuclear expression of hTERT was completely shifted to nuclear and cytoplasm with grades of dysplasia and oral squamous cell carcinoma. The association of p53, Bax and hTERT expression was significant among oral epithelium, oral epithelial dysplasia and oral squamous cell carcinoma(p<0.05). **Conclusion:**Increased expression of mutant p53,hTERT along with decreasedBax expression were significantly associated with histological grades of oral epithelial dysplasia and oral squamous cell carcinoma. These expression pattern of markers suggest their role as surrogate markers of malignant transformation.
Key words: Tumor suppressor protein, Pro apotoic protein, Telomerase, Oral epithelial dysplasia, Oral squamous cell carcinoma.

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INTRODUCTION:
Oral canceris a progressive disease; fromnormal epithelium passes through stages of oral epithelial dysplasia and transforming into invasive carcinoma. Although, many types of carcinomas are presented in oral cavity, the most common form of oral cancer is squamous cell carcinoma.[1] The initial presence of precursor lesion subsequently developing into cancer is well recognized.[2] Oral leukoplakia is one of the best-known antecedent lesions with 16% to 62% of malignant transformation.[3-5] The development of oral cancer is a multistep process comprising genetic mutations and chromosomal abnormalities. Despite an accumulation of genetic alterations to potentiate this transition, the imbalance in

harmony between proto-oncogenes and tumor suppressor genes play a crucial role in triggering and promoting cancerous growth in oral squamous cell carcinoma.[6-8] p53regulates the cell cycle and it has been described as 'the guardian of the genome'.The tumor suppressor protein p53is known to be mutated in approximately 70% of adult solid tumors.[9-11] Two important events are regulated by p53 in connection with its function as tumor suppressor: induce growth arrest by holding the cell cycle (to repair the DNA) at $G_{1/}$ S regulation point and induces apoptosis if DNA damage evidences to be irreparable.[12,13] Although, most of the effects of p53 are recognized to its function as a transcription factor, the protein can also induce apoptosis independently through Baxproapoptotic protein which involves cytochrome c release and caspase activation.[14-16]

In addition to, another phenomenon, telomere, may be a potentially important target in progression of cancer.[17] It plays important role in maintaining genome stability by preventing the activation of DNA damage check points that induce p53 dependant cell cycle arrest and apoptosis.[18] Maintenance of telomeric length confers immortality of cells,which is a characterstic feature of malignant cells and is considered to be a crucial step in carcinogenesis. [19] The enzyme telomerase maintains telomeric repeats by elongating telomeric DNA through reverse transcription and its activity is determined by the expression levels of human telomerase reverse transcriptase (hTERT). The altered expression of hTERT may be an early event in oral carcinogenesis.[20]

Detection of molecular abnormalities before the consequences become clinically or histologically detectable, will significantly enhance the potential for early diagnosis. The finding of relation between p53,Bax and hTERT markers by immunohistochemistry may serve as a diagnostic tool for ealy diagnosis. So,the objective of the study was to analyse the pattern of immunoexpression of p53, Bax and hTERT in oral epithelial dysplasia and oral squamous cell carcinoma.

MATERIAL AND METHODS:

Two hundred and ten formalin-fixed, paraffin-embedded biopsy specimens were retrieved from the archives of Department of Oral Pathology, Vinayaka Mission's Sankarachariyar Dental College, Salem, which include 90 samples of oral leukoplakia with dysplasia, 90 samples of oral squamous cell carcinoma including 30 samples of normal oral epithelium. Hematoxylin and eosin staining was performed for the verification of histopathologic diagnosis and categorization of the degree of histopathologic differentiation. The samples were classified according to the criteria of World Health Organization [21,22]. Histologically, the oral epithelial dysplasia was graded as mild (n=30), moderate (n=30), and severe (n=30). The oral squamous cell carcinoma was classified as well-differentiated (n=30), moderately differentiated (n=30), and poorly differentiated (n=30). The study was approved by ethics committee of Vinayaka Mission's Sankarachariyar Dental College, Salem (VMSDC/IEC/Approval No.071).

Histopathology and Immunohistochemical staining

The streptavidin-biotin standard protocol was performed. Paraffin-embedded tissues were cut into 3 to 5 µm thick sections, placed over slides, deparaffinized in xylene and rehydrated with graded alcohol. Antigen retrieval was performed with target antigen retrival solution (EDTA with pH 7.4 -7.8 for p53, pH 6 for Bax and sodium citrate with pH 9 for hTERT) in a water bath, followed by incubation with 6% hydrogen peroxide to quench endogeneous peroxidase. The sections were then incubated in blocking solution (3% bovine serum albumin) for an hour at room temperature, followed by primary antibody incubation, previously diluted in blocking solution. Mouse monoclonal p53primary antibody (Clone BP -53-12, 1: 100 dilution; Path in Situ, US)and anti-hTERT (Novocatra, clone 44f 12, 1:75 dilution-leica microsystems, Berlin, Germany)antibodies were incubated for 30 minutes at room temperature, and Rabbit monoclonal Bax antibody (Clone E63, pre-dilution form- Bio SB, CA) were incubated for 3 hours. The antibodies were detected using diaminobenzidinechromogen and counterstained with Mayer's hematoxylin.

Evaluation of immunostaining

The following parameters were used to assess the expression of p53,Bax and hTERT:

1. Tissue localization of the stain: Layers of the section were assessed in basal and supra basal layers. It was considered as basal when confined to the basal layer; and suprabasal when both basal and suprabasal layers were positive [23].
2. Cellular localization of the stain: p53, Bax stained cells were defined as nuclear, cytoplasm respectively,whilehTERT expression was defined as being either nuclear or combined nuclear and cytoplasm.
3. Percentage of positive cells: The percentage of immunostained cells were calculated after counting 100 cells in 10 consecutive high-power fields using ocular grid. The percentage of positive cells was assigned according to the grading system given by KowichiNagawa et al [24].
   Negative : 0-5% of positive cells
   Weekly positive : 5% to 25% of positive cells
   Moderately positive : 25% to 50% of positive cells
   Strongly positive : >50% of positive cells

Statistical analysis

IBM SPSS statistics 21.0 Version was used for statistical analysis. The data were analysed using Chi-Square and Kruskal-Wallis tests. A p value < 0.05 was considered statistically significant.

RESULTS

Detection and Comparison of p53, Bax and hTERT in oral epithelium, oral epithelial dysplasia and oral squamous cell carcinoma

The result of immunoexpression of p53, Bax and hTERT in the oral epithelium, oral epithelial dysplasia and oral squamous cell carcinoma were summarized (Table 1,2,3).

The positive nuclear expression of p53 was confined to basal layer in oral epithelium (80%), while, in oral epithelial dysplasia supra-basal expression was increased as histological grade increases (mild: 21.8%, moderate: 40% and severe dysplasia: 68%). The expression between the histological grades of dysplasia was found statistical significant (p<0.001). Whereas, in oral squamous cell carcinoma, the expression of p53 was increased in both moderately and poorly differentiated (90%) than well differentiated tumors and also the histological grades of oral squamous cell carcinoma was found significant (p<0.001). The expression of p53 between oral epithelium, oral epithelial dysplasia and oral squamous cell carcinoma was statistically significant(p<0.001).

The cytoplasmic Bax expression was greater in supra basal layer (76.5%) of oral epithelium but it was reduced in mild dysplasia 34.7%,moderate dysplasia 34 % and severe epithelial dysplasia 23.5%. Likewise, the expression of Bax was gradually reduced with histological grades of oral epithelial dysplasia (p-0.009) and oral squamous cell carcinoma(p-0.002). There was a statistical significant expression between oral epithelium and oral epithelial dysplasia and oral squamous cell carcinoma(p<0.05).

In oral epithelium, hTERT expression was found throughout the epithelial layers (56.7%) but the expression was identified more in basallayer with predominant nuclear (76.5%) than nuclear and cytoplasam staining (23.5%). In oral epithelial dysplasia, supra basal expression was increases with grades of dysplasia.The nuclear expression were shiftedto combinednuclear and cytoplasmwith histological grade progresses (mild:73.3%, moderate: 76.7% and severe dysplasia:83.3%), but the expression of hTERT was not statistically significant (p-0.674) among histologic grades. Whereas, in oral squamous cell carcinoma, statistically significant expression were found between the grades (p<0.005) and also significant differences among oral epithelium, oral epithelial dysplasia and oral squamous cell carcinoma(p<0.005).

The expression ofp53, Bax and hTERT was found a positive association among oral epithelium, histological grades of oral epithelial dysplasia and oral squamous cell carcinoma(p<0.005).

Table 1: Expression of p53, Bax and hTERT in oral epithelium (n=30)

Percentage of positive cells	p53	Bax	hTERT	p-value
0-5%	25(83.3%)	13(43.3%)	13 (43.3%)	
5-25%	03(10.0%)	06(20.0%)	02(06.7%)	0.000
25-50%	02(06.7%)	04(13.4%)	13(43.3%)	
>50%	0	07(23.3%)	02(6.7%)	

Table 2: Expression of p53, Bax and hTERT in oral epithelial dysplasia

Oral epithelial dysplasia	Percentage of positive cells	p53	Bax	Htert	p-value
Mild epithelial dysplasia (n=30)	0-5%	07(23.3%)	04(13.3%)	08(26.7%)	0.063
	5-25%	09(30.0%)	06(20.0%)	02(06.7%)	
	25-50%	09(30.0%)	05(16.7%)	07(23.3%)	
	>50%	05(16.7%)	15(50.0%)	13(43.3%)	
Moderate epithelial dysplasia (n=30)	0-5%	12(40.0%)	12(40.0%)	07(23.3%)	0.000
	5-25%	4(13.3%)	01(03.3%)	02(06.7%)	
	25-50%	7(23.3%)	02(06.7%)	06(20.0%)	
	>50%	7(23.3%)	15(50.0%)	15(50.0%)	
Severe epithelial dysplasia (n=30)	0-5%	02(06.7%)	15(50.0%)	05(16.7%)	0.001
	5-25%	02(06.7%)	01(03.3%)	0	
	25-50%	05(16.7%)	06(20.0%)	10(33.3%)	
	>50%	21(70%)	08(26.7%)	15(50.0%)	

Table 3: Expression of p53, Bax and hTERT in oral squamous cell carcinoma

Oral squamous cell carcinoma	Percentage of positive cells	p53	Bax	hTERT	p-value
Well differentiated squamous cell carcinoma (n=30)	0-5%	18(60.0%)	08(26.7%)	02(6.7%)	
	5-25%	0	01(03.3%)	0	0.000
	25-50%	02(06.7%)	05(16.7%)	06(20.0%)	
	>50%	10(33.3%)	16(53.3%)	22(73.3%)	
Moderately differentiated squamous cell carcinoma (n=30)	0-5%	03(10.0%)	18(60.0%)	11(36.7%)	
	5-25%	08(26.7%)	05(06.7%)	0	0.115
	25-50%	15(50.0%)	01(13.3%)	06(20.0%)	
	>50%	04(13.3%)	06(20.0%)	13(43.3%)	
Poorly differentiated squamous cell carcinoma (n=30)	0-5%	03(10.0%)	22(73.3%)	16(53.3%)	
	5-25%	08(26.7%)	03(10.0%)	0	0.027
	25-50%	15(50.0%)	03(10.0%)	02(06.7%)	
	>50%	04(13.3%)	02(06.7%)	12(40.0%)	

DISCUSSION:

Oral epithelium constitutes the proliferative compartments of basal/ parabasal layers and differentiating compartments of superficial layers.[25] The p53 and hTERT are generally repressed in normal oral epithelial cell layers and up-regulated in immortal cells.[25,26] In case of positive p53 expressionin the normal oral epithelium, it is confined to the basal layer. This might have occurred due to the normal physiological response of cells to genotoxic stress (physical, chemical or microbiological). However, wild-type p53 never accumulate in supra-basal layer because of its short half-life and putative DNA got repaired prior to its replication. [23] But the presence of p53 in dysplastic cells is likely to reflect the presence of mutant protein and diminished turnover of cells will persist for a longer period of time. It also indicated, the presence of damaged DNA cells which have withdrawn from the cell cycle.[23,27]

The expression of Bax was greater in supra-basal layers in normal oral epithelium[28] but reduced supra-basal expression in dysplastic epithelial cells indicated that dysregulation of the apoptosis mechanism, preventing the death of genetically damaged cell.[28,29] WhereashTERT is up-regulated in immortal cells, and it is a primary determinant of telomerase activity in normal and cancerous cells. The expression of hTERT is considered to be the critical factor for the activity of dysplastic cells.[17,26]

Cellular localization of the stain was defined as being either nucleus or cytoplasm or both.[26] The p53 expression constrainted to the nucleus and theBax was expressed in the cytoplasm.[29,30] Whereas,hTERT expression was presented in nuclear or both nuclear and cytoplasm, which might help to recognize the cells on the path of malignant transformations.[26,30] The aim of the study was to analyze the pattern of imunoexpression of p53, Bax and hTERT in oral epithelial dysplasia and oral squamous cell carcinoma to assist as a surrogate marker.

In the present study, the percentages of p53, Bax and hTERT positive cells were 16.7%, 56.7%, and 56.7% respectively in oral epithelium. The result showed that normal oral epithelium expresses p53and hTERT limited to the basal layer, but Bax expression was greater in the supra-basal layer. In oral epithelium, cellular localization of hTERT was predominantly nuclear (76.5%) than combinednuclear and cytoplasm(23.5%) expression.

In our study, the expression p53 was greater in severe dysplasia than in moderate and mild epithelial dysplasia. These results were in consensus with the observations of Murti et al.,who indicated that p53 expression peaked close to the time of transition from the pre-cancer state to cancer.[31] The supra basal expression of p53 was increased in severe dysplsia than moderate and mild oral epithelial dysplasia. This is coincides with Cruz et al, he stated that supra basal immunoexpression pattern of p53 was associated with high grades of dysplasia and progress to oral squamous cell carcinoma, and can be considered for predictor marker for malignant transformation.[23]

The suprabasal expression of pro apoptotic Bax was significantly reduced in severe dysplasia than moderate and mild oral dysplasia. This fact can indicate a dysregulation of the apoptosis mechanisms when epithelial dyspastic grade increases. It also specified that preventing genetically damaged cells and consequently, increasing the malignant transformation risk.[29] Studies also suggested that reduced Bax expression is the critical step   in the development and progression of oral cancer.[29] The expression of hTERT was not found significant between the histological grades of epithelial dysplasia. But, the increased expression pattern revealed that this is to maintain chromosomal stability and to promote cell proliferation in oral epithelial dysplasia than in oral epithelium.

The immunoexpression of p53 and Bax and hTERT has been detected in a large percentage of oral squamous cell carcinoma, reflecting the altered status of p53 protein, dysregulation of apoptosis mechanism, telomere stabilization and cell proliferation. More than 70% of our oral squamous cell carcinoma samples were positive for p53,Baxand hTERT with the result described in the literature although, in this study, more cases of Bax and hTERT positive in well differentiated compared to poorly differentiated squamous cell carcinoma with an inverse pattern of expression for p53. The increased expression of p53 with higher histological grades of oral squamous cell was consistent with the results of previous studies which suggested that the mutation of p53 may be significant in the pathological differentiation of oral squamous cell carcinoma.[32] Whereas, reduced expression of Bax in less differentiated tumors coincide with study of Doi et al,whostated that decreased expression of Bax in the cancerous tissues may reduce apoptotic cell death as well as accelerate their growth.[33] These results of p53 and hTERT confirmed with observations of Abbas et al [27] and Jayanthi et al [17], who stated that increased expression of proteins from premalignant to malignant lesions of the oral cavity presumably reflects a series of genetic and cellular alterations.

The localization of hTERT expression in mild and moderate oral epithelial dysplasia was predominantly nuclear, whereas, severe oral epithelial dysplasia and oral squamous cell carcinoma had both nuclear and cytoplasm staining. The localization of hTERT expression in the cytoplasm which represents phosphorylated and in active form of the protein probably awainting nuclear translocation and it has been previously reported in carcinoma of breast, cervix and larynx.[34,35] Our results also concides with Chen et al., and showed a correlation between nuclear and cytoplasmichTERT expression in histological grades of oral squamous cell carcinoma.[36] Additionally, the number of hTERT positive cells in well differentiated had tendency to be higher than poorly differentiated squamous cell carcinoma. It indicated that other molecular mechanisms also might have involved in maintaining telomerse length in poorly differentiated squamous cell carcinoma.

On comparision of p53, Bax and hTERT expression pattern of were progressively increases from oral epithelium through oral epithelial dysplasia to oral squamous cell carcinoma.

Finally, the expression of mutant p53 was observed in oral epithelial dysplasia and oral squamous cell carcinoma, suggesting that p53 mutation has ability to suppress the function of oncogenes. Furthermore, mutant p53 may function as an oncogene to stimulate cell division and promote the growth of tumor cells.[32] The reduced expression of Bax in higher grades of dysplasia and oral squamous cell carcinomaindicates a dysregulation of the apoptotic mechanisms, preventing the death of genetically damaged cells and consequently, increasing the malignant transformation risk. Studies suggested that decrease in the expression of Bax is essential for the development and progression of malignant transformation.[37,38] The nuclear/cytoplasm hTERT expression was increased in dysplastic epithelium and oral squamous cell carcinoma when compared to oral epithelium, whereas nuclear hTERT was decreased in oral squamous cell carcinoma suggesting that hTERT expression can be a biomarker for this type of lesions which concides with Chen et al.[36,39]

The altered expression of p53, Bax and hTERT in oral epithlial dysplasia and oral squamous cell carcinoma reflecting the accumulation of mutated p53 protein, dysregulation of apoptosis, telomere stabilization which leads to cell proliferation and diminished apoptosis. Additionally, the increased supra-basal expression of p53, hTERT and reduced expression of Bax also indicated a malignant transformation in higher grades of dysplasia and oral squamous cell carcinoma. Based on this findings, the significant association of p53, Bax and hTERT was found among oral epithelial dysplasia and oral squamous cell carcinoma, and has the potential to be used as a surrogate markers of malignant transformation.

CONCLUSION:

In conclusion, this study showed a significantly altered expression of p53, Bax and hTERTin histological grades of oral epithelial dysplasia and oral squamous cell carcinoma.Dysregulation of cell cycle inhibitors and apoptotic proteins in oral carcinogenesis as evidenced by the expression of mutant p53 along with decreased Bax expression and increased expression of hTERT in oral epithelial dysplasia and oral squamous cell carcinoma. The significant expression of markers suggests their role as surrogate markers of malignant transformation.

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