Original Article

Differential Expression of Heat Shock Protein 27 in Oral Epithelial Dysplasias and Squamous Cell Carcinoma

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the most devastating neoplasm with dramatic increase in morbidity and mortality. The detection and prognostic evaluation of precancerous lesions could aid in early control of cancer. Heat shock protein (HSP) 27 has found to be a biomarker and therapeutic target in different types of cancer. **Aim:** This study aims to investigate the role of HSP 27 as prognostic molecular indicator of malignant transformation in oral epithelial dysplasias. **Materials and Methods:** Thirty samples of epithelial dysplasia (10 mild dysplasia, 10 moderate dysplasia, and 10 severe dysplasia/carcinoma *in situ* cases), 10 samples each of well-differentiated OSCC and normal oral mucosa were routinely processed, formalin-fixed, paraffin-embedded, and analyzed for HSP27 expression by immunohistochemistry. Statistical analysis was done by one way-ANOVA and Mann–Whitney test to assess the differences between two individual groups. **Results:** Normal mucosa showed intense, but nonuniform, expression of HSP27. An initial decline was noted in dysplasias. A significant correlation of HSP27 expression was observed with the severity of dysplasia and well-differentiated OSCC ($P < 0.05$). **Conclusion:** Low HSP 27 expression can be considered as early molecular indicator of initial dysplastic change in normal mucosa. An overexpression of HSP 27 in clinically and histologically confirmed dysplasia could indicate likely transformation to well-differentiated OSCC and could be of prognostic value. However, further studies with a larger sample size are required to confirm the role of HSP 27 as predictive indicator.

Keywords: Heat shock protein, heat shock protein 27, oral epithelial dysplasias and oral squamous cell carcinoma

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer in the world with a 5-year survival rate of <50%.[1] India has now become hub for oral cancer patients with over 80,000 new cases are diagnosed annually and 14 deaths occur per hour.[2,3] Tissue immunological, biochemical diagnostic, and prognostic markers pave the way for early detection. Heat shock proteins (HSPs) are one the proteins which play a role in carcinogenesis thereby can act as target molecules in cancer therapy.[4] Most of the studies with HSPs have been on the prognosis of OSCC but only few on its expression in different gradations oral epithelial dysplasias.[5] The present study was aimed at investigating the role of HSP 27 as a prognostic molecular indicator of malignant transformation in oral epithelial dysplasias. The objective was to compare the expression of HSP 27, both quantitatively and qualitatively, in normal mucosa (control), different gradations of dysplasia and well-differentiated OSCC. We hypothesize an increase in the expression of HSP 27 from normal mucosa to OSCC.

Materials and Methods

This was a retrospective case–control study. Formalin-fixed, paraffin-embedded blocks of the selected cases were retrieved from the archives of the Department of Oral Pathology,

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Drs. Sudha and Nageswara Rao Siddhartha Institute of Dental Sciences, Gannavaram. The study sample size was 40, which included: Ten cases each of mild, moderate and severe epithelial dysplasia/carcinoma *in situ* and well-differentiated OSCC. The previously established diagnoses of the selected cases were confirmed according to the WHO criteria (2005) for oral epithelial dysplasias and Broder's classification for OSCC.[6] The control group consisted of ten tissue samples of normal mucosa obtained from archives, patients undergoing prophylactically impacted 3rd molar tooth removal and healthy volunteers after obtaining a written consent. Individuals with clinical signs of inflammation or history with smoking habits and wound healing were excluded. The study was approved by Institutional Ethics Committee and ethical standards were in accordance to Declaration of Helsinki.

Immunohistochemistry

4 μ thick sections were de-paraffinized in xylene, rehydrated in graded alcohol and rinsed with distilled water. Antigen retrieval was done using trisodium citrate buffer in Antigen Retrieval System (Biogenex®) with 2 cycles; First cycle was at 95°C for 10 min and the second cycle 98°C for 5 min. Then phosphate-buffered saline (PBS) was used as a rinse. Sections were immersed in a peroxidase-blocking solution for 10 min. Anti-HSP27 antibody was added and incubated for 30 min at room temperature. The negative control was obtained by omitting the primary antibody from the assay. Slides were then incubated with secondary antibody-polyHRP. Visualization of the immunoreaction was completed using 3, 3-diaminobenzidine for 10 min as the peroxidase chromogenic substrate. The slides were then counterstained with hematoxylin for 30s, cleared in xylene and mounted with DPX mountant. Between each step, several rinses with PBS were carried out.[7]

Brown color staining was considered a positive staining. Blinding was followed and HSP27 expression was measured by quantifying the number of immune-stained cells. Five different fields were selected and hundred cells in each field (totally 500 cells) were evaluated. Qualitatively measurement was done from the intensity of the staining of epithelial layers. Image capturing was done using Jenoptik ProgRes® C14*plus* Image Capture software, Germany. Scoring was given according to staining intensity: Zero-No staining, 1-Very low staining, 2-Low staining, 3-Intermediate staining, and 4-Highly intense staining.[8] Breast carcinoma was used as a positive control. Statistical analysis was done by One-way ANOVA and Mann–Whitney test. Statistical significance was at $P < 0.05$. Statistical software Sigma XL® version 6.23 (Sigma XL, Toronto, Canada) was used for statistical analysis.

Results and Observations

HSP 27 stains epithelial cells, lymphocytes, vascular endothelial lining, glandular epithelium, and skeletal muscle. In our study, HSP staining of epithelium only was taken into consideration. The mean values and standard deviation of the percentage of cells positive for HSP 27 of different groups are shown in Table 1. The mean value was the least for mild epithelial dysplasia (64.38) and maximum for OSCC (98.98).

One way ANOVA test was performed to compare the mean values of the percentage of cells positive for HSP 27 in the five groups. These findings showed high statistically significant difference between the various groups with a P value of 0.000285×10^{-15} [Table 1]. Pair-wise comparison by Mann–Whitney test was performed to know if there were any statistically significant differences between two individual groups, i.e., between normal and mild dysplasia, between moderate and severe dysplasias, etc. Among these various groups, statistically significant differences ($P < 0.05$) were observed between all groups except between normal and severe epithelial dysplasia [Table 1].

Qualitative analysis was done by observing HSP 27 staining intensity. Staining varied among different layers of the epithelium. Of all the different groups, mild epithelial dysplasia had the least amount staining intensity in all the individual layers, in contrast to OSCC, which had the maximum. The statistically significant differences in the lower, middle, and upper layers are given in Table 2.

When all the layers were considered as a whole, and the scoring for staining intensity for HSP 27 was done, it was not surprising to find that mild epithelial dysplasia had the least amount staining intensity (mean value 1.2) and OSCC had the maximum (mean value 3.1). There was statistically significant difference ($P < 0.05$) in the staining of all the layers for HSP 27 between various groups, except for the following: normal versus severe epithelial dysplasia and mild epithelial dysplasia versus moderate epithelial dysplasia [Table 2].

Moreover, the mean value of normal mucosa was higher than that for mild epithelial dysplasia, which was highly significant statistically. The mean values of staining intensity for HSP 27 gradually increased from mild to moderate to severe epithelial dysplasia, peaking maximum at OSCC, which was more than that for normal mucosa.

Discussion

OSCC constitutes one of the most common malignant neoplasms with an evidence of increase in incidence and mortality. Since malignancy can endanger one's life, its early detection can save the life of an individual. The development of cancer is sometimes preceded by the presence of potentially malignant disorders. Histologically, these potentially malignant disorders are characterized by the presence of epithelial dysplasia which may vary from mild to severe. The degree of dysplasia also plays a key role in malignant transformation. The rates of malignant transformation for mild, moderate, and severe dysplasias are 3%, 4%, and 43%, respectively.[9,10]

In epithelial dysplasia, initially atypical cells appear in the basal layers and gradually dysplastic features involve the entire epithelium. Early detection of these cellular changes by

Table 1: Comparison of mean values of the percentage of cells positive for heat shock protein 27 of five groups by One-way ANOVA and Mann-Whitney test

One way ANOVA test				
Sources of variation	**Degrees of freedom**	**Sum of squares**	**Mean sum of squares**	***F***
Between groups	4	7442.1	1860.5	66.197
Within groups	45	1264.8	28.106	
Total	49	8706.9		
Mann-Whitney test				
	Normal versus mild	**Normal versus moderate**	**Normal versus severe**	**Normal versus OSCC**
Median	96.30-65.70	96.300-86.20	96.30-93.40	96.30-99
Mann-Whitney	55.00	145.00	130.50	66.00
P (two-sided)	0.0002*	0.0028*	0.0581	0.0034*
Mann-Whitney test				
	Mild versus moderate	**Mild versus severe**	**Mild versus OSCC**	
Median	65.70-86.20	65.70-93.40	65.70-99	
Mann-Whitney	55.00	55.00	55.00	
P (two-sided)	0.0002*	0.0002*	0.0002*	
Mann-Whitney test				
	Moderate versus severe	**Moderate versus OSCC**	**Severe versus OSCC**	
Median	86.200-93.40	86.200-99	93.400-99	
Mann-Whitney	69.50	55.00	55.50	
P (two-sided)	0.0080	0.0002*	0.0002*	

*$P<0.05$. OSCC: Oral squamous cell carcinoma

molecular markers not only enables a better understanding of the degree of malignant change of altered cells but also may help in the prevention of transformation. HSP27 is one of such markers which may have the potential to be used for detection as well as a therapeutic target.

HSPs are a group of proteins that are expressed in all organisms to ensure correct folding of polypeptide chain into functional protein. When cells are subjected to various stresses, these protein levels are elevated and they, in turn, assist in the repair or degradation of denatured proteins.[11]

HSP27 plays a role in cell growth/differentiation, cell migration, and cell adhesion. Under stressful conditions, it prevents cell death by inhibiting apoptosis. HSP 27 has a dual role in cancer:

1. It promotes the development of cancer by suppressing various anti-cancer mechanisms such as apoptosis and senescence, as well as by facilitating the expression of metastatic genes; and
2. It facilitates tumor rejection by immune system.

Recently "*addiction to chaperones*" hypothesis has been suggested to explain increased levels of HSP in carcinogenesis. The ability of HSPs to hold large amounts of altered proteins, including protein kinase and transcription factors, may significantly affect vital biologic processes. Cancer cells co-opt this capacitance machinery to protect an array of mutated and overexpressed oncoproteins from misfolding and therefore from proteasomal degradation. Hence, in contrast to their beneficial role in degenerative and inflammatory diseases, they protect cancer cells against apoptotic or other types of death triggered by the immune system. They also provide cancer cells with the ability to counteract host anticancer response which leads to aggressive cell growth, metastasis, dissemination, and poor prognosis.[12-17]

HSP 27 role was also implicated in periapical lesions, aphthous ulcers, lichen planus, ameloblastomas, and salivary gland tumors.[18-22] Although many studies have been done on HSP 27 expression in epithelial dysplasia and OSCC, very few studies have actually graded oral epithelial dysplasias and observed its expression. In our study, HSP 27 expression was observed in all normal oral mucosa specimens, similar to the previous studies [Figure 1]. This supported the hypothesis that HSP27 is involved in the normal maintenance of tissue, i.e., in cell differentiation and cell cycle regulation. However, staining intensity in normal mucosa varied among different layers. The middle layer of the epithelium showed more intense staining compared to the lower and upper layers of the epithelium. The difference in staining in different layers can be explained by the role of HSP27 as predifferentiation marker. As the differentiation increases from basal layer toward upper layer, the staining intensity increases. In the most superficial layers, the presence of keratin may result in poor absorption of HSP27.

Most studies have shown no or low staining in the basal layer of normal mucosa.[5,23,24] However, in our study, we have

Table 2: Mean value and standard deviation staining intensity of heat shock protein 27 in each layer and their comparison

Summary information	Normal	Mild	Moderate	Severe	OSCC
Staining intensity for HSP 27 in the lower layer of different groups					
Count	10	10	10	10	10
Mean	2.600	1	1.600	2.200	3.100
SD	1.0749677	1.155	0.843274	1.0327956	0.737865
Pairwise probabilities					
Normal		0.0007*	0.0274*	0.3667	0.2604
Mild			0.1782	0.0089*	0.0000*
Moderate				0.1782	0.0013*
Severe					0.0461*
Staining intensity for HSP 27 in the middle layer of different groups					
Count	10	10	10	10	10
Mean	2.800	1.900	2.400	2.900	3.100
SD	0.788811	1.197	0.699206	0.737865	0.737865
Pairwise probabilities					
Normal		0.0226*	0.2997	0.7943	0.4354
Mild			0.1963	0.0119*	0.0029*
Moderate				0.1963	0.0729
Severe					0.6024
Staining intensity for HSP 27 in the upper layer of different groups					
Count	10	10	10	10	10
Mean	1.600	0.700000	0.900000	1.700	3.100
SD	0.699206	1.059349905	0.994429	1.252	0.737865
Pairwise probabilities					
Normal		0.0439*	0.1138	0.8189	0.0012*
Mild			0.6472	0.0259*	0.0000*
Moderate				0.0719	0.0000*
Severe					0.0023*
Staining intensity for HSP 27 of all layers together of different groups					
Count	30	30	30	30	30
Mean	2.333	1.200	1.633	2.267	3.100
SD	0.994236	1.215	1.0333518	1.112	0.71196
Pairwise probabilities					
Normal		0.0000*	0.0092*	0.8019	0.0044*
Mild			0.1045	0.0001*	0.0000*
Moderate				0.0182*	0.0000*
Severe					0.0020*

*P<0.05. SD: Standard deviation, OSCC: Oral squamous cell carcinoma, HSP 27: Heat shock protein 27

observed this only in two cases. The normal cases where the basal layer showing intense staining were essentially from the buccal mucosa. What could possibly explain this difference in our study? Do different areas of oral mucosa show differential expression of HSPs?

Most of the earlier studies did not mention the location. In a few studies, the normal mucosal specimens were restricted to either the tongue and/or the gingival mucosa which are keratinized mucosae unlike buccal mucosa which is nonkeratinized.[23-26] We do not know the exact reason for the intense expression of HSP27 in the basal layers of buccal mucosa, but since HSP27 is a known marker for keratinocyte differentiation it is possible that its expression is related to the lack of normal keratinization of the buccal mucosa.

In a study attempting to localize HSP27 in adult rat gingiva, it was found that HSP27 was localized in all the layers. Immunoreactivity in the basal layer was confined to oral gingival and sulcular epithelium, but no immunoreactivity was found in the horny layer.[27] As we know, sulcular epithelium is nonkeratinized. Further studies are necessary to know whether there is differential expression of HSP27 in different regions of oral cavity and whether this is related to keratinization.

Thirty cases of oral epithelial dysplasia (10 each of mild, moderate, and severe epithelial dysplasias) were examined in the present study. Increased expression of HSP27 was observed with increasing gradations of dysplasia [Figure 2]. The percentage of positive cells for HSP27 showed statistically significant increase with increasing gradations of dysplasia [Table 1].

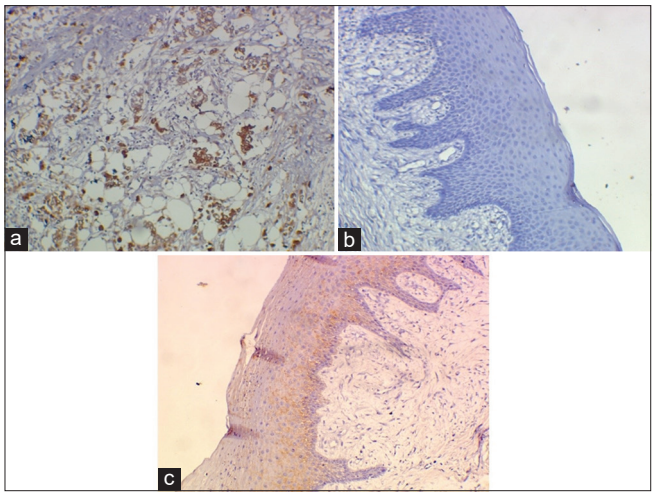

Figure 1: Photomicrograph showing immunohistochemistry analysis of heat shock protein 27 expression (a) breast carcinoma, positive control (obj. ×20) (b) normal mucosa, negative control (obj. ×20) (c) normal mucosa with moderate staining in lower and middle layers but low staining in the upper (obj. ×20)

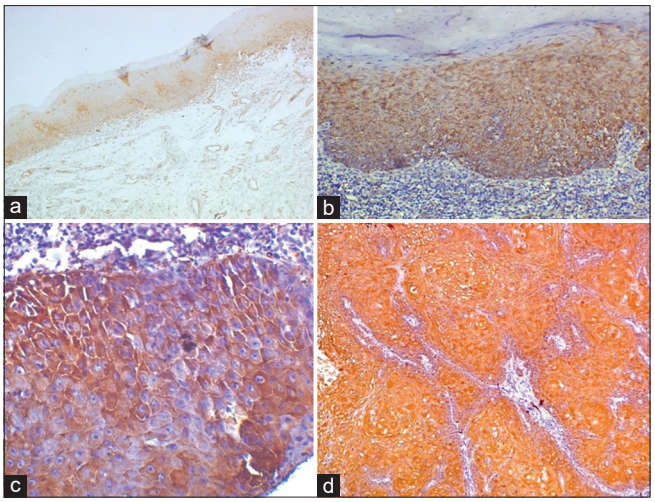

Figure 2: Photomicrograph showing heat shock protein 27 expression (a) mild dysplasia with low staining (lower, upper layers) moderate staining (middle layer obj. ×10) (b) moderate dysplasia with moderate staining (lower, middle layer obj. ×20) (c) severe dysplasia with intense staining (all layers obj. ×40) (d) well-differentiated oral squamous cell carcinoma with intense staining (obj. ×20)

Although earlier studies have observed the expression of HSP27 in oral epithelial dysplasia, to our knowledge, only three studies have investigated HSP27 expression in different grades of oral epithelial dysplasias.[28-30] They did not find any statistically significant differences between different gradations of dysplasia. Although the number of cases of epithelial dysplasia ($n = 30$) studied by Epivatianos *et al*. was similar to our study, they did not have equal number of samples (mild = 8, moderate = 15, and severe = 7).[28] In the study of Leonardi *et al*., not only the sample size ($n = 17$) of premalignant lesions was lesser than our study (5 mild, 7 mild-to-moderate, 2 moderate, 1 moderate-to-severe, 1 severe, and 1 simple keratosis), the categorization was also different.[29]

The HSP27 expression was less in the lower and most superficial layers of the epithelium when compared to the middle layer of the epithelium. There was no staining in 16% of cases in basal cell layer and 33.3% of cases in most superficial layers. The low levels of HSP27 in the superficial layers would suggest loss of differentiation and more susceptibility to mutagens. The overall HSP27 staining intensity correlated with increasing degree of dysplasia.

In dysplasias, HSP27 expression was low in mild dysplasia compared to normal mucosa [Table 1]. This finding was similar to the studies by Leonardi *et al.* and Seoane *et al*.[29,30] In our study, the decline was more marked in mild dysplasias. Initial down regulation of HSP27 compared to normal mucosa may occur due to the action of mutagens resulting in the impairment of the protective mechanism of HSP27. However, as dysplastic features progress, the tumor cells start utilizing the chaperone activity of HSP27 for their survival leading to carcinogenesis.

In well-differentiated OSCC, the entire epithelium showed a positive staining for HSP27, though there was variation in the intensity of staining. Intense staining of HSP27 was seen in 30% of cases, moderate staining in 50% of the cases, and low staining in 20% cases [Figure 1]. Similar observations (both low and high expressions in well-differentiated OSCC) were witnessed in the studies by Deyhimi and Azmoudeh and Suzuki *et al*.[8,31]

Compared to the oral epithelial dysplasia, the staining intensity and the mean number of positive immunostained cells were more in well-differentiated OSCC. These observations were similar to the studies by Ito *et al*. and Wang *et al*.[23,24] In our study, there was statistically significant difference ($P < 0.05$) between different gradations of dysplasias and well-differentiated OSCC [Tables 1 and 2] in contrast to the studies by Tekkesin *et al*. and Epivatianos *et al*.[28,32] The low levels in dysplasias appear to be an early event in the process of carcinogenesis and elevated levels of HSP27 levels in well-differentiated OSCC may be due to the addiction of tumor cells to HSP27 thereby preventing apoptosis.

HSP27 expression between normal mucosa and well-differentiated OSCC showed statistically significant differences in our study [Table 1, $P = 0.0034$]. In well-differentiated OSCC, HSP27 prevents the function of cytochrome C and procaspase and prevent mitochondrial apoptosis. Increased HSP27 expression also enhances tumor cell survival by increased production of free radicals and therefore increases cell resistance to oxidative damage. Figure 3 succinctly depicts schematically our hypothesis on the varying levels of HSP 27 and their prognostic significance in dysplastic change and malignant transformation.

Conclusion

Low HSP27 expression can be considered as an early event in the transformation from normal mucosa to oral epithelial

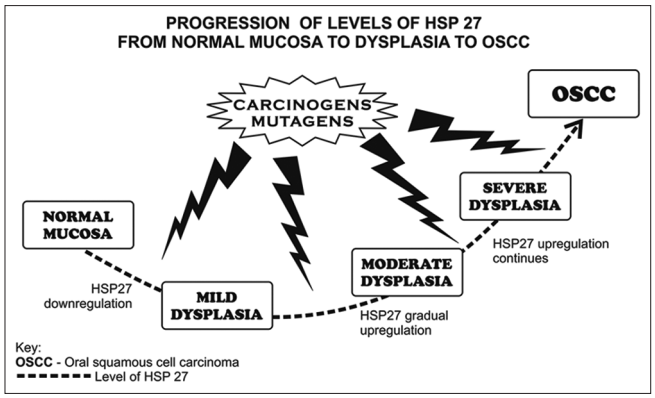

Figure 3: Hypothesis explaining the role of varying levels of heat shock protein 27 in the progression of epithelial dysplasia to oral squamous cell carcinoma. In normal mucosa, the cells utilize heat shock protein 27 for cell growth and cytoprotection. In dysplasias, the initial down regulation of heat shock proteins could be due to the action of carcinogens on the epithelium. This decline of these proteins impairs their protective mechanism thereby leading to cellular alterations. This decline was also noted in the study by Leonardi *et al*. As the dysplasia progresses toward oral squamous cell carcinoma, to maintain protein homeostasis, the tumor cells get addicted to heat shock proteins and utilize heat shock proteins to protect the mutant proteins against degradation and promote their growth by suppressing various anticancer mechanisms

dysplasia. An overexpression of HSP 27 in clinically and histologically confirmed dysplasia could indicate likely transformation to well-differentiated OSCC and could be of prognostic value. However, as our sample size was small, further studies with larger sample size are needed to confirm the role of HSP 27 as a prognostic molecular indicator of malignant change in oral epithelial dysplasias.

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Conflicts of interest

There are no conflicts of interest.

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