ORIGINAL ARTICLE

Heat shock protein (HSP70) as a marker of epithelial dysplasia in oral dysplastic lesions: A clinicopathological study

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ABSTRACT

Objective: In the present study, expression of heat shock protein (HSP70) was evaluated and compared in oral dysplastic lesions, in particular leukoplakia (study group) and in normal mucosal tissues (control group). Additionally, correlation of HSP70 expression with clinical disease status was investigated. **Subjects and Methods:** A total of 60 fresh tissue specimens were obtained from the oral cavity, consisting of 30 dysplastic cases and 30 normal mucosal tissues. The presence of epithelial dysplasia and its histologic grading was evaluated. Immunohistochemistry was carried out with the monoclonal HSP70 antibodies and expression of cytoplasmic HSP70 within the epithelium was compared between dysplastic and normal mucosal samples using Student's *t*-test. **Results:** Expression of HSP70 was detected in 93% of the oral dysplastic tissues and 20% of the normal mucosal tissues. Statistical significant difference in the HSP70 expression was seen between oral dysplastic tissues and normal oral mucosal tissues ($P < 0.000$). The interexaminer reliability was 93.3%. Statistical significant difference was seen in the HSP70 expression between controls and different grades of dysplasia (mild, moderate and severe). There was no relationship of HSP70 expression with clinical parameters like age, sex, site of the lesion, history of adverse habits and duration of adverse habits. **Conclusion:** In the present study, HSP70 activity was significantly higher in oral dysplastic (leukoplakia) group than in the control group. Further, as the grade of dysplasia increased, the staining intensity and/or distribution increased, indicating that enhanced HSP70 expression occurs during oral carcinogenesis. Hence, it is concluded that increased HSP70 immunoexpression could be an objective marker for the presence of epithelial dysplasia.

Key words: Epithelial dysplasia, heat shock protein, leukoplakia, markers of dysplasia

INTRODUCTION

Many oral cancers are preceded by precancerous lesions which have varied presentations in the oral cavity. In a World Health Organization Workshop held in 2005, these oral lesions with predisposition to malignant transformation were renamed as 'potentially malignant disorders' and this term includes both precancerous lesions as well as precancerous conditions.[1] These potentially malignant disorders are characterized histologically by various degree of epithelial dysplasia. The term 'epithelial dysplasia' is assigned to histopathological changes associated with an increased risk of malignant development. Oral epithelial dysplasia is not associated with any specific clinical appearance. However, leukoplakia is the most common lesion classically associated with dysplastic changes. It should be recognized that some of the dysplastic lesions may remain clinically unchanged or may even show complete regression. Furthermore, carcinomatous transformation may also take place in non-dysplastic leukoplakia.[2] Hence, clinical and histological features alone cannot accurately predict whether such a lesion would remain

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stable, regress or progress to malignancy. Such behavior of premalignant disorders necessitates the identification of molecular markers, which can predict the disease progression. Previously, proteomic technologies have been used to analyze the malignant transformation from precancerous oral leukoplakia (OL) to oral cancer and approximately 85 different proteins have been identified with altered expression levels in oral squamous cell carcinoma (OSCC) in the transformation, of which 52 were upregulated and 32 downregulated.[3] HSP70 is one among such protein which is upregulated in the premalignant lesions and OSCC. Alteration of HSP expression in precancerous lesions has been reported in endometrium and uterine cervix.[4] Also, alterations of HSP expression have been reported in oral lichen planus and OL, with the highest intensity in severe dysplastic changes.[5-8] Previously, very few studies have been done to detect expression of HSP70 in oral dysplastic lesions and correlate it with the clinical parameters. Accordingly, the objectives of the present study are to determine the expression of cytoplasmic HSP70 in potentially malignant lesions, in particular, OL with diverse degrees of epithelial dysplasia (mild, moderate and severe) and in normal oral mucosal tissue. Additionally, correlation of HSP70 with clinical disease status is investigated to evaluate the clinical significance and validity of HSP70 as a useful biomarker.

SUBJECTS AND METHODS

Clinicopathologic characteristics of patients

The study was carried out on 30 patients with OL, diagnosed according to the clinical and pathologic criteria (study group); and on 30 normal samples, which were obtained from patients undergoing third molar extraction (control group). A written informed consent was obtained from all the patients.

Oral dysplastic group consisted of four females and 26 males. Control group also consisted of four females and 26 males. In the study group, the age of patients ranged from 36 to 78 years with mean of 52 years. In the control group, the age ranged from 35 to 52 years with the mean of 45 years. The surgically resected tissue specimens were fixed in 10% buffered formalin, dehydrated and embedded in paraffin blocks. Serial sections, 4 μm thick, were mounted on 3-amino-propyl-tri-ethoxy-saline-coated slides. The paraffin blocks were sectioned, deparaffinized and processed for immunohistochemical analysis and then counterstained with hematoxylin and eosin. The presence of epithelial dysplasia and its histologic grading was evaluated according to the World Health Organization classification: Mild, moderate and severe squamous epithelial dysplasia. The lesions were independently scored by two pathologists.

Immunohistochemicalanalysis of HSP70

The protocol for the sections embedded in paraffin was as follows:

Firstly, the sections were mounted on poly-L-lysine slides. Before incubation of the antibodies, the slides were immersed in sodium citrate 0.1 M and preheated in a 750 W microwave oven for 7 min to expose antigens. The primary antibody was mouse monoclonal antibody to HSP70 (AM2890409, Biogenex Life Sciences Limited CA, USA) diluted 1:100 in phosphate buffered saline. The secondary antibody was a biotinylated antimouse immunoglobulin G (Ig G; used in a 1:400 dilution in phosphate buffered saline). Further treatment with peroxidase coupled avidin allowed immunostaining with diaminobenzidine. As a positive control, a section of breast carcinoma was used (following BioGenex recommendations).

The stratified squamous epithelium was divided into three layers namely basal, suprabasal and superficial.

Each layer was evaluated for:

- The intensity of overall staining
  - 0 = negative staining
  - 1 = light staining
  - 2 = moderate staining
  - 3 = intense staining.
- The proportion of stained cells
  - 0 = no stained cells
  - 1 = less than 25% stained cells
  - 2 = 25–50% stained cells
  - 3 = more than 50% stained cells.

An immunostaining intensity distribution (IID) index was calculated for each specimen for the cytoplasmic expression of HSP70 as follows:
IID index = Overall staining intensity for each layer × proportion of stained cells in that layer.

The resulting scores for the three layers (basal, suprabasal and superficial) were then added to provide an IID index for each specimen. All specimens were examined manually independently by two experienced oral pathologists in a blinded fashion. Five fields in a representative section from each specimen were counted and the numbers were averaged. The observers were blinded for the grades of dysplasia.

Statistical analysis

HSP70 levels were compared between oral dysplastic lesions and normal oral mucosa using Student's *t*-test. Relationship between the level of HSP70 and clinicopathologic factors such as age, sex, adverse habits, duration of habit and site of the lesion and histopathologic grades of dysplasia were analyzed. Student's *t*-test and analysis of variance (ANOVA) test were applied to validate the significance of the difference between groups. Pair-wise comparison among the grades of dysplasia and HSP70 levels was done by Tukey's post-hoc procedure.

Interexaminer variability was calculated using rank correlation coefficient.

RESULTS

Cytoplasmic HSP70 expression was seen in 93% (28/30) of oral dysplastic lesions and in 20% (6/30) of normal oral mucosa. The mean IID index score for oral dysplastic lesion was 12.7 (SD = 8.91) and for normal oral mucosa was 1.977 (SD=4.52). IID index score differed significantly between oral dysplastic lesions and normal oral mucosa (t = 5.879, P = 0.0000). Also, significant differences in mean HSP70 expression were seen in different layers of epithelium between oral dysplastic lesions and controls [Table 1]. Interexaminer reliability using rank coefficient was 99.33%. Significant difference was seen in the HSP70 expression between controls and mild, moderate and severe dysplasia with P value of 0.0443, 0.0066 and 0.0134, respectively. Relationship of HSP70 expression and various clinical parameters are summarized in Table 2.

In normal epithelium the staining was present only in the basal cells [Figure 1a]. Among 30 cases of dysplasia in the present study; 12 cases had mild dysplasia, 16 had moderate and two had severe dysplasia. Two cases of mild dysplasia were negative for HSP70. In few mild dysplastic cases, although the staining was seen in the entire epithelium, the staining intensity was low [Figure 1b]. In few other cases of mild dysplasia, the staining was limited to the basal cells. In moderately dysplastic epithelium, the staining was seen in basal and suprabasal layer with no staining in superficial layer [Figure 2a]. In severe dysplastic cases, the staining intensity and distribution was more when compared to the mild and moderate cases and involved entire thickness of the epithelium [Figure 2b].

Table 1:IID index score in different layers of epithelium

Layer of epithelium	Mean IID index score		*P* value*
	Cases	Controls	
Basal layer	4.300	0.831	0.000*
Suprabasal layer	4.767	0.824	0.000*
Superficial layer	3.633	0.322	0.000*
Specimen (total)	12.700	1.977	0.000*

*Student's *t*-test. IID index score was computed by multiplying the score of staining intensity by the score of proportion of stained cells. IID=Immunostaining intensity distribution

DISCUSSION

HSP70 belong to stress inducible group and is perhaps the most well-studied group of cytoprotective HSPs.[9] Members of the HSP70 family are believed to act as molecular chaperones accompanying newly synthesized proteins through various intracellular compartments, facilitating passage of proteins across various intracellular barriers, maintaining proper folding of newly synthesized proteins, refolding of misfolded and aggregated proteins and aiding in the elimination of incorrectly synthesized or assembled proteins.[10] Protein misfolding is a major cause of a number of human diseases. By maintaining cellular proteins in a folding-competent state, HSP70 in coordination with other chaperones play an important role in cellular viability.[11] HSP70 is constitutively and gradually expressed in a broad range of normal tissues and neoplasms and their expression has been assessed as markers for oral epithelial dysplasia. It is known to play a specific role in the pathogenesis of oral cancer. Experimental evidence suggests that HSPs may promote tumorigenesis by suppressing apoptosis.[12] Also, it appears to be associated with mutations of the p53 gene and interact with Bcl2, lending support to the proliferation effect.[13]

The present study was aimed at detection of HSP70 expression in normal oral mucosal tissue and oral dysplastic lesions, in particular leukoplakia, since it is the most common oral lesion classically associated with dysplastic changes. We also investigated the relationship of HSP 70 expression with clinical parameters like age, sex, history of adverse habits, site of the lesion and in patients with oral dysplastic lesions.

Table 2:Relationship of IID index score with various clinical parameters in oral dysplastic group

Parameter	Subgroups	Number	Mean IID index score	*P* value
Sex	Males	26	12.4615	0.7158*
	Females	4	14.2500	
Age (years)	<50	12	11.4615	0.4158*
	>50	18	15.542	
Habit	Smoking	8	11.83	0.5997†
	Smokeless tobacco	10	11.88	
	Both smoking and chewing tobacco	12	15.50	
Duration of habit (years)	1-10	9	9.33	0.1504†
	11-20	7	12.41	
	>20	14	18.00	
Site of lesion	Palate	5	11.50	0.4785†
	Buccal mucosa	15	15.00	
	Retrocomissure	5	12.73	

*Student's *t*-test, †analysis of variance. IID=Immunostaining intensity distribution

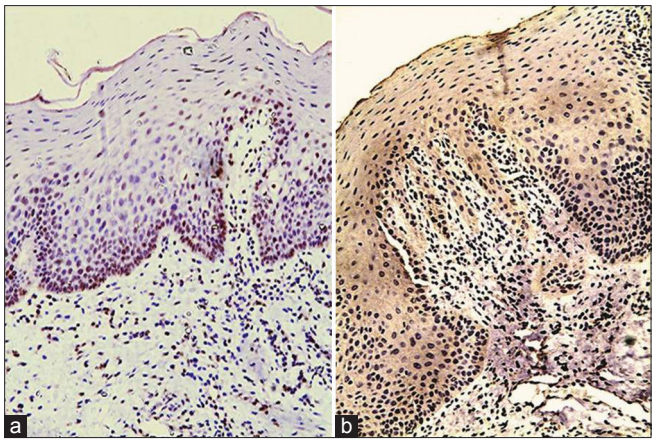

Figure 1: (a) Normal epithelium showing heat shock protein (HSP70) immunoexpression in the cytoplasm of basal cells of epithelium (IHC stain, ×100). (b) Mild dysplastic epithelium showing weak immunoexpression of HSP 70 in the entire width of epithelium mainly in the cytoplasm of the cells (IHC stain, x200)

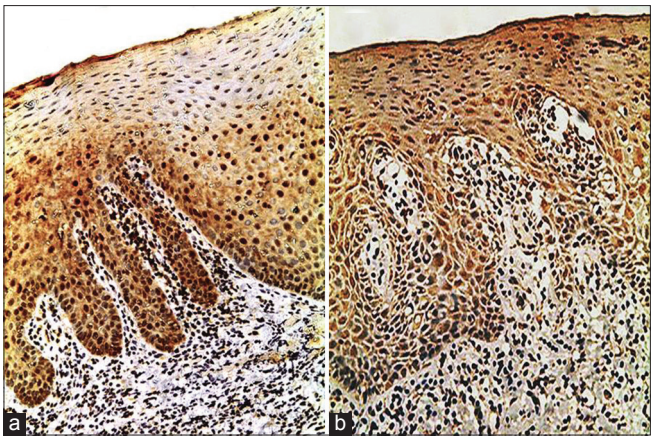

Figure 2: (a) Moderate dysplastic epithelium showing strong immunoexpression of HSP 70 in the cytoplasm of basal and suprabasal cells of the epithelium (IHC stain, x100). (b) Severe dysplastic epithelium showing strong immunoexpression of HSP 70 in the cytoplasm of basal, suprabasal and superficial cells the of epithelium (IHC stain, x200)

Previously only two studies have tried to find the correlation between HSP70 expression and clinical pathological features in oral dysplastic lesions.[5,7]

In the present study, cytoplasmic HSP70 expression was significantly overexpressed in oral dysplastic lesions (mean IID index score = 12.7) when compared to normal oral mucosal tissue (mean IID index score = 1.977). Overexpression of HSP70 may reflect a state of biological stress experienced by premalignant or dysplastic cells. Sugerman *et al.,* (1995);[8] Ito *et al*., (1998);[14] Seoane *et al*., (2004);[15] Seoane *et al*., (2006)[16] and Markopoulos *et al*.,(2009)[17] also have reported increased HSP70 expression in oral dysplastic lesion when compared to normal epithelial mucosa in their studies. Among similar studies conducted in Indian population, Kaur *et al.,* in 1996[18] and in 1998[5,7] showed increased HSP70 expression in oral dysplastic lesions when compared to normal mucosa.

The immunoreactivity of HSP70 may reflect a state of biologic stress or may be associated with a state of increased cellular activity. This may well justify the weak cytoplasmic staining for HSP70 in the epithelium of normal oral mucosa in the present study (mean IID index score for normal mucosal tissues = 1.977). This finding is consistent with results of Kaur *et al*., who found only five patients positive for HSP70 out of 96 normal oral mucosal controls[7] and Seoane *et al*., who found weak cytoplasmic expression of HSP70 protein in normal oral epithelium in their study.[16]

Among 30 cases of dysplasia in the present study, 12 cases had mild dysplasia, 16 had moderate and two had severe dysplasia. In few mild dysplastic cases, although the staining was seen in the entire epithelium, the staining intensity was low. In few other cases of mild dysplasia, the staining was limited to the basal cells. In moderately dysplastic epithelium, staining was seen in basal and suprabasal layer with unstained superficial layer. In severe dysplastic cases, the staining intensity and distribution was more compared to the mild and moderate dysplastic cases and involved entire thickness of the epithelium. This indicated that as the grade of dysplasia increased the intensity and/or distribution of the staining increased; suggesting a positive association between HSP70 expression and severity of dysplastic lesions. However, there was no statistical significant difference in HSP70 expression in mild, moderate and severe dysplasia subgroups which could be attributed to smaller sample size and unequal distribution of different grades of dysplasia.

As seen in Table 2, there was no significant relationship between HSP70 expression and clinical parameters like age, sex, site of the lesion, history of adverse habits and duration of the adverse habits. Kaur *et al.,* reported no correlation between HSP70 expression and age, sex and site of the lesion.[5,7] However, Kaur *et al*., in 1998 reported significant association between HSP70 overexpression and habit of consumption of tobacco and/or betel with areca nut and found a positive trend in the incidence of HSP70 overexpressing premalignant cases across the groups: Nonconsumers, betel and arecanut consumers and consumers of tobacco as well as betel and areca nut.[7] In the present study, all the patients in case group were either tobacco chewers or smokers. Previously, none of the studies have tried to correlate the HSP70 expression with duration of adverse habits. A positive association in the HSP70 expression and duration of the habit was seen in the present study; although, this finding was not statistically significant ($P = 0.1504$).

CONCLUSION

In the present study, cytoplasmic HSP70 activity was significantly higher in oral dysplastic (leukoplakia) group than in the control group. Further, as the grade of dysplasia increased, the staining intensity and/or distribution increased;

indicating that enhanced HSP70 expression occurs during oral carcinogenesis. This supports the critical role of HSP70 in the development of human oral cancer. Hence, it is concluded that increased HSP70 immunoexpression could be an objective marker for the presence of epithelial dysplasia. Further studies with larger sample size and with equal distribution of different grades of dysplasia are required to substantiate these results.

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