Expression of Heat Shock Protein 70 in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma: An Immunohistochemical Study

Abstract

Introduction: Heat shock proteins are biomarkers regulating the degree of differentiation and aggressiveness in oral carcinogenesis. The study was carried out to evaluate the expression of HSP 70 in histological grades of epithelial dysplasia and oral squamous cell carcinoma using immunohistochemistry. Methodology: A sample of 40, which included 10 (Normal), 15 (epithelial dysplasia) and 15 (oral squamous cell carcinoma) were selected. The biopsy samples were stained using H and E staining and HSP 70 IHC. Results: There was a statistical significant difference in expression of HSP 70 between normal oral mucosal tissue, leukoplakia and OSCC. Discussion and Conclusion: This study concludes the critical role of HSP70 as an oncogene in the development of oral cancer. HSP70 inhibition is, therefore, a promising therapeutic approach for cancer treatment.

Keywords: Heat shock proteins 70, immunohistochemistry, oral cancer, tumor node and metastasis staging

Introduction

Histopathological grading of leukoplakia and squamous cell carcinoma alone cannot predict the progression of the lesion, therefore, in the recent years, a number of molecular-based studies have been undertaken to predict the outcome of the lesion.[2]

Previously, proteomic technologies have been used to analyze the transformation from precancerous lesion to cancerous lesion. Approximately 85 different proteins have been identified with altered expression levels in oral squamous cell carcinoma (OSCC) in the metastatic transformation, of which 52 were upregulated and 32 downregulated. Heat shock proteins 70 (HSP70) is one among such protein which is upregulated in different histological grades of premalignant lesions and OSCC.[3,4]

HSP70 is a highly conserved group of protective cellular proteins whose synthesis is increased in response to a variety of environmental or pathophysiological stresses. These proteins play an important role in the maintenance of cellular homeostasis, both under normal conditions and during stress.[5] Therefore, in the absence of HSP70, apoptosis of the cell will take place. HSP70 is overexpressed in a wide range of human cancers and are implicated in tumor cell proliferation, differentiation, invasion, and metastasis. Therefore, they are useful biomarkers for carcinogenesis in tissues and signal the degree of differentiation and the aggressiveness of cancers.[5,6] HSP70 is one among such protein which is upregulated in the premalignant lesions and OSCC with the highest intensity in severe dysplastic changes.

Till now, only few studies have been done to detect the expression and its correlation of HSP70 in leukoplakia and OSCC with the clinical and histopathological parameters.[7]

Therefore, the aim of the study is to evaluate the expression of cytoplasmic HSP70 as a prognostic biomarker in three histological variants of leukoplakia (mild, moderate and severe) and squamous cell carcinoma (well differentiated [WSCC], moderately differentiated [MSCC], poorly differentiated [PSCC]) and to correlate between the two variants using immunohistochemistry (IHC), in adjunct with the demographic, tumor node and metastasis (TNM) staging, and histopathological grading.

How to cite this article: Priyanka KP, Majumdar S, Kotina S, Uppala D, Balla H. Expression of heat shock protein 70 in oral epithelial dysplasia and oral squamous cell carcinoma: An immunohistochemical study. Contemp Clin Dent 2019;10:185-90.
**Aim**
The aim of this study is to evaluate the expression of HSP 70 in normal, leukoplakia, and OSCC.

**Objectives of the study**
1. To evaluate the expression of HSP70 in various histological grades of leukoplakia and OSCC using IHC
2. To compare and correlate TNM staging, histological grading, and expression of HSP70 regarding prognosis of leukoplakia and OSCC patients.

**Methodology**
This study was carried out on 15 patients with leukoplakia and 15 patients with OSCC diagnosed according to the clinical and pathologic criteria (study group); and on 10 normal samples, which were obtained from patients undergoing third molar extraction, periodontal surgeries (control group). All these patients were obtained from the Outpatient Department of Gitam Dental College and Hospital and peripheral cancer hospitals located in and around the Visakhapatnam. Written consent was obtained from all the patients after explanation.

A detailed case history, including medical, clinical, and habit was taken followed by thorough clinical examination was done before the commencement of the study. Informed consent was obtained from all individuals before the study. Incisional biopsy was performed in clinically diagnosed cases after proper medical and hematological investigations. Routine hematoxylin and eosin staining were done to confirm the clinical diagnosis of three groups, followed by the histological differentiation in epithelial dysplasia (ED) and OSCC. Tissues from the normal controls were collected from the buccal mucosa/gingiva obtained during the extraction of the lower third molars. Formalin fixation and tissue processing were performed. Later, slides obtained from paraffin-embedded blocks of three groups were subjected to IHC using antibody against HSP70. The present study was conducted in accordance with Ethical Committee guidelines and clearance.

**Reagents**
The primary antibody was mouse monoclonal antibody to HSP70 (AM2890409, Biogenex Life Sciences Limited CA, USA) (diluted in phosphate buffered saline (PBS), pH 7.6, containing 1% bovine serum albumin and 0.09% sodium azide)

The secondary antibody was a biotinylated anti-mouse immunoglobulin G (Ig G; used in a 1:400 dilution in PBS).

The mounted sections on poly L lysine slides were immersed in sodium citrate 0.1 M and preheated in a 750 W microwave oven for 7 min to expose antigens. All the reagents stored in refrigerator at 2–8°C were brought to room temperature before immunostaining. At no time, the tissue sections were allowed to dry during the staining procedure. It is Followed by treating with power block for 10 min to prevent nonspecific staining and then blotted. The slides were covered completely with primary antibody, mouse monoclonal antibody to HSP70 (AM2890409, Biogenex Life Sciences Limited CA, USA) for 1 h and then washed with wash buffer. Slides were then incubated with secondary antibody, i.e., biotinylated antimouse (Ig G; used in a 1:400 dilution in PBS) for 30 min and then washed with wash buffer for 3 times. Slides were incubated with 3-amino-9-ethyl-carbazole chromogen substrate for 10 min to develop immunostaining and then washed under running tap water. Counterstaining was done with Harris hematoxylin for 1 dip. The slides were dehydrated in alcohol followed by xylene for 5 min. Then, the slides were mounted using dibutyl phthalate in xylene.

The presence of dark brown color immunostained cells was considered as positive immunoreactivity [Figures 1 and 2]. Cells with cytoplasmic staining were taken into consideration.

- The labeled cells index was calculated as the percentage of labeled cells out of the total number of tumor cells counted

\[
L.I = \frac{\text{Number of positive cells}}{\text{Total number of cells counted}} \times 100
\]

IDI was determined by the multiplication of labeling index (L. I) and staining intensity (S. I)

![Clinical, histopathological, and immunohistochemistry images of leukoplakia. SCC: Squamous cell carcinoma, HSP 70: Heat shock protein 70](image)
IID = L. I × S. I

- Grading of L. I was done in the following manner:
  - 1 = 0%–25% of positive cells
  - 2 = 25%–50% of positive cells
  - 3 = 50%–75% of positive cells
  - 4 = 75%–100% of positive cells.

### Results and Discussion

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 = 0.5). This finding is consistent with results of Kaur et al.,[8] who found only five patients positive for HSP70 out of 96 normal oral mucosal controls, and Seoane et al., who found weak cytoplasmic expression of HSP70 protein in normal oral epithelium in their study.

Among 15 cases of leukoplakia in the present study, 4 (26%) cases had mild dysplasia, 5 (34%) had moderate, and 6 (40%) had severe dysplasia. In cases of mild dysplasia, the staining was limited to the basal cells (1.25%) In moderate dysplasia, staining was seen in basal and suprabasal layer with unstained superficial layer (1.6%, 2.8%, and 0.2%). In severe dysplastic cases, the S I and distribution was more compared to the mild and moderate dysplastic cases and involved entire thickness of the epithelium (2.33%, 3%, 3.67%) [Graph 1]. This indicated that as the grade of dysplasia (mild, moderate, and severe dysplasia) increased the intensity and/or distribution of the staining increased; suggesting a positive association between HSP70 expression and severity of dysplastic lesions. However, those were not statistically significant ($P = 0.87$) [Table 1 and Graph 2]. This could be attributed to smaller sample size and unequal distribution of different grades of dysplasia.

Among 15 cases of squamous cell carcinomas in the present study, 7 (46.7%) cases had WSCC, 5 (33.3%) cases had MSCC, and 3 (20.0%) cases had PSCC. There is a significant difference in the expression of HSP70 in different histological grades of OSCC [Table 2 and Graph 3]. These results are in consistent with the studies conducted by the Lee et al.[9] and Kaur (2010)[10] which showed a significant difference in the expression of HSP 70 in that PSCC showed increased expression of HSP70 when compared to the well and moderately differentiated SCC and in contrast with the studies conducted by the Ito et al. (1998),[11] Deyhimi and Azmoudeh.[12]

Among similar studies conducted in Indian population, Kaur et al. in 1998[13] and in 2000[10] showed increased HSP70 expression in oral premalignant and malignant tissues when compared to normal mucosa.

The ANOVA test is used for the comparison of HSP70 expression between the three study groups. Mean IID scores of normal and leukoplakia are 0.5% and 4% showed the significant difference with the $P < 0.05$ [Table 3 and Graph 4]. Mean IID scores of normal and OSCC are 0.5% and 9.73% showed highly significant difference with the $P < 0.01$ [Table 4 and Graph 5]. Mean IID scores of leukoplakia and OSCC are 3.56%, 4.23% showed the significant difference with the $P < 0.01$ [Table 5 and Graph 6] finally all the three groups showed a significant difference with the $P < 0.001$ [Table 6 and Graph 7], suggesting that there is a significant difference in the expression of HSP70 in controls, leukoplakia, and OSCC.

In this study, cytoplasmic staining in all the cases of positivity was observed, which was similar to other
Overexpression of HSP70 may reflect a state of biological stress experienced by premalignant and malignant cells.

In the present study, there is a significant increased HSP70 expression from control to leukoplakia and OSCC cases indicating that enhanced HSP70 expression occurs during oral carcinogenesis. According to Tang et al.,[14] the physiopathological features of the tumor microenvironment (low glucose, pH, and oxygen) tend toward HSP induction. This was confirmed by a recent study which indicates that when cells are transferred from tissue culture to growth as xenografts in vivo, HSP expression declines markedly.[15] According to Ciocca and Calderwood,[16] increased HSP70 expression is correlated

Table 1: Comparison of HSP70 expression in three histological grades of leukoplakia

| Histological grading | IID   | SD   | P   | Inference |
|----------------------|-------|------|-----|-----------|
| Normal               | 0.5   | 0.53 | <0.05 | NS        |
| Leukoplakia          | 4     | 3.56 |      | Inference (S) |

SD: Standard deviation; NS: Not significant

Table 2: Comparison of HSP70 expression in squamous cell carcinoma groups with histological grading

| Histological grading | Percentage | IID   | SD   | P   | Inference |
|----------------------|------------|-------|------|-----|-----------|
| WSCC                 | 30.14      | 6.29  | 2.36 | <0.01 | HS        |
| MSCC                 | 39.72      | 11.6  | 2.88 |      |           |
| P.SCC                | 30.14      | 14.67 | 2.31 |      |           |

SD: Standard deviation; WSCC: Well-differentiated squamous cell carcinomas; MSCC: Moderately differentiated squamous cell carcinomas; P.SCC: Poorly differentiated squamous cell carcinomas

Table 3: Comparisons of HSP70 expression in controls, leukoplakia

| IID    | Mean | SD   | P   | Inference |
|--------|------|------|-----|-----------|
| Normal | 0.5  | 0.53 | <0.05 | NS        |
| Leukoplakia | 4  | 3.56 |      | Inference (S) |

SD: Standard deviation

Table 4: Comparison of HSP70 expression in controls and oral squamous cell carcinoma groups

| IID    | Mean | SD   | P   | Inference |
|--------|------|------|-----|-----------|
| Normal | 0.5  | 0.53 | <0.01 |           |
| SCC    | 9.73 | 4.23 |      | Inference (HS) |

SD: Standard deviation; SCC: Squamous cell carcinomas; HS: Highly significant

Table 5: Comparison of HSP70 expression in leukoplakia and oral squamous cell carcinoma groups

| IID    | Mean | SD   | P   | Inference |
|--------|------|------|-----|-----------|
| Leukoplakia | 4  | 3.56 | <0.05 | S: Significant |
| SCC    | 9.73 | 4.23 |      |           |

SCC: Squamous cell carcinomas; SD: Standard deviation; S: Significant

Table 6: Comparisons of HSP70 expression in controls, leukoplakia, and squamous cell carcinoma groups with histological grading

| IID    | Mean | SD   | P   | Inference |
|--------|------|------|-----|-----------|
| Normal | 0.50 | 0.53 | <0.01 |           |
| Leukoplakia | 4.00| 3.56 |      | Inference (HS) |
| SCC    | 9.73 | 4.23 |      |           |

SCC: Squamous cell carcinomas; SD: Standard deviation; HS: Highly significant

studies conducted by Deyhimi and Azmoudeh,[12] Patil et al.[17]
cell carcinoma of the bladder. This is consistent with the HSP70 associations with poor differentiation, lymph node metastasis, increased cell proliferation, block of apoptosis, and higher clinical stage, which are markers of poor clinical outcome. There also have been a number of studies suggesting an anti-apoptotic role of HSP70. HSP70 may affect apoptosis through its interaction with co-chaperone Bag-1, which is known to interact with antiapoptotic protein bcl-2. Elevated HSP70 protects cells from cytotoxicity induced by apoptosis-inducing agents; tumor necrosis factor, monocytes, radiation and chemotherapeutic agents. HSP70 binds directly to apoptotic protease activating factor-1, and prevents the formation of functional apoptosome.\[17,18\]

All these studies support the critical role of HSP70 as an oncogene in the development of human oral cancer and also increased HSP70 expression is critical for tumor growth and might be important as a prognostic factor for patients with oral cancer. HSP70 inhibition is, therefore, a promising therapeutic approach for cancer treatment.

**Conclusion**

HSP70 belong to stress-inducible group. Members of the HSP70 family are believed to act as maintaining proper folding of newly synthesized proteins, refolding of misfolded and aggregated proteins, and aiding in the elimination of incorrectly synthesized or assembled proteins. 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 homeostasis. 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 ED. 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 acting on the caspases-dependent pathway at several steps both upstream and downstream of caspase activation and on the caspases independent pathway. Overproduction of HSP70 leads to increased resistance against apoptosis-inducing agents. In addition, it appears to be associated with mutations of the p53 gene and interact with Bcl2, lending support to the proliferation effect.

HSP70 expression was significantly higher in oral dysplastic (leukoplakia) and OSCC groups than in the control group. There is a significant difference in the expression of HSP70 in different histological grades (well, moderate, and poor) of OSCC. Significant increased expression of HSP70 was observed from control to leukoplakia and OSCC cases. This supports the critical role of HSP70 in the development of human oral cancer and also increased HSP70 expression is critical for tumor growth and might be important potential biomarker for evaluating the role in treatment and prognosis.
So to conclude, the present study showed positive results with small sample size and limited period, further elaborated studies with larger cross section of population, posttreatment follow-up, and survival period should be included to reach conclusive decision on HSP70 as a potential biomarker for evaluating the role in treatment and prognosis.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References
1. Nehalben DP. Oral potentially malignant disorders of oral cavity – An update. J Res Adv Dent 2014;3:189-95.
2. van der Waal I. Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. Oral Oncol 2009;45:317-23.
3. Kumar V, Abbas AK, Fausto N, Mitchell R. Neoplasia. Robbins Basic Pathology. 9th ed., Ch. 5. Philadelphia, PA: Saunders, Elsevier; 2015. p. 161-214.
4. Wang Z, Feng X, Liu X, Jiang L, Ji N, et al. Involvement of potential pathways in malignant transformation from oral leukoplakia to oral squamous cell carcinoma revealed by proteomic analysis. BMC Genomics 2009;10:383.
5. Thubashini M, Malathi N, Kannan L. Expression of heat shock protein70 in oral submucous fibrosis and oral squamous cell carcinoma: An immunohistochemical study. Indian J Dent Res 2011;22:256-9.
6. Whitley D, Goldberg SP, Jordan WD. Heat shock proteins: A review of the molecular chaperones. J Vasc Surg 1999;29:748-51.
7. Patil P, Nandimath K, Prabhu S, Naikmasur VG. Heat shock protein (HSP70) as a marker of epithelial dysplasia in oral dysplastic lesions: A clinicopathological study. J Oral Maxillofac Pathol 2015;19:53-7.
8. Kaur J, Srivastava A, Ralhan R. Expression of 70-kDa heat shock protein in oral lesions: Marker of biological stress or pathogenicity. Oral Oncol 1998;34:496-501.
9. Lee SS, Tsai CH, Ho YC, Chang YC. The upregulation of heat shock protein 70 expression in areca quid chewing-associated oral squamous cell carcinomas. Oral Oncol 2008;44:884-90.
10. Kaur J, Kaur J, Ralhan R. Induction of apoptosis by abrogation of HSP70 expression in human oral cancer cells. Int J Cancer 2000;85:1-5.
11. Ito T, Kawabe R, Kurasono Y, Hara M, Kitamura H, Fujita K, et al. Expression of heat shock proteins in squamous cell carcinoma of the tongue: An immunohistochemical study. J Oral Pathol Med 1998;27:18-22.
12. Deyhimi P, Azmoudeh F. HSP27 and HSP70 expression in squamous cell carcinoma: An immunohistochemical study. Dent Res J (Isfahan) 2012;9:162-6.
13. Kaur J, Das SN, Srivastava A, Ralhan R. Cell surface expression of 70 kDa heat shock protein in human oral dysplasia and squamous cell carcinoma: Correlation with clinicopathological features. Oral Oncol 1998;34:93-8.
14. Tang D, Khaleque MA, Jones EL, Theriault JR, Li C, Wong WH, et al. Expression of heat shock proteins and heat shock protein messenger ribonucleic acid in human prostate carcinoma in vitro and in tumors in vivo. Cell Stress Chaperones 2005;10:46-58.
15. Kawanishi K, Shiozaki H, Doki Y, Sakita I, Inoue M, Yano M, et al. Prognostic significance of heat shock proteins 27 and 70 in patients with squamous cell carcinoma of the esophagus. Cancer 1999;85:1649-57.
16. Ciocca DR, Calderwood SK. Heat shock proteins in cancer: Diagnostic, prognostic, predictive, and treatment implications. Cell Stress Chaperones 2005;10:86-103.
17. Pockley AG. Heat shock proteins as regulators of the immune response. Lancet 2003;362:469-76.
18. Udono H, Srivastava PK. Heat shock protein 70-associated peptides elicit specific cancer immunity. J Exp Med 1993;178:1391-6.