

Expression of heat shock proteins 27, 60 and 70 in oral carcinogenesis: An immunohistochemical study

Oral karsinogenezde ısı şok proteinleri 27, 60 ve 70 ekspresyonu:
İmmünohistokimyasal çalışma

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OBJECTIVES

Heat shock proteins (HSPs) are a class of functionally related proteins, the expression of which is increased when cells are exposed to elevated temperatures or other stresses, including infection, irradiation, heavy metals, ethanol, and oxidants. The aim of this study was to investigate the significance of HSP60, HSP70 and HSP27 as prognostic factors in different stages of oral carcinogenesis by immunohistochemical analysis.

METHODS

Twenty specimens of oral squamous cell carcinoma (OSCC) of the oral cavity were immunostained for HSPs to expose differences in stainability among normal epithelium (n=20) and leukoplakia (n=20) as dysplasia.

RESULTS

Immunohistochemistry demonstrated that OSCC cells were positive for all of HSP60, HSP70 and HSP27. Leukoplakia cases were positive for HSP70 and HSP27, but stained with variability for HSP60. Normal epithelium expressed HSP60 and to a lesser extent HSP70, while HSP27 were hardly ever expressed. The HSP70 and HSP27 stainings in OSCC were significantly higher than in normal epithelium, and demonstrated almost the same staining character as with leukoplakia.

CONCLUSION

Our results demonstrated that HSP immunochemistry revealed changes in especially HSP70 and HSP27 expression during tumorigenesis of squamous epithelium of the oral cavity.

Key words: Carcinogenesis; dysplasia; heat shock protein; oral cancer; squamous cell carcinoma.

AMAÇ

Isı şok proteinleri (HSPs), hücrelerin enfeksiyon, radyasyon, ağır metaller, etanol ve oksidan gibi streslere veya yükselmiş ısıya maruz kaldıklarında üretimleri artan bir grup proteindir. Bu çalışmanın amacı, immünohistokimyasal olarak HSP60, HSP70 ve HSP27 ekspresyonunun bir prognostik faktör olarak oral karsinogenezisin farklı aşamalarındaki önemini incelemektir.

GEREÇ VE YÖNTEM

Yirmi adet oral skuamöz hücreli karsinom (OSHK), 20 adet sağlıklı epitel ve 20 adet displazik lezyon olarak lökoplaki olguları arasında immünohistokimyasal olarak HSP reaksiyonlarının farklarına bakıldı.

BULGULAR

OSHK'da tümör hücreleri HSP60, HSP70 ve HSP27'nin hepsinde immünohistokimyasal olarak pozitif boyanma gösterdi. Lökoplaki olguları HSP70 ve HSP27 ile pozitif boyanma gösterirken, HSP60 ile değişik derecelerde boyandı. Sağlıklı epitel HSP60'ı eksprese ederken, HSP70 ile daha az oranda reaksiyon verdi ve HSP27'yi ise hemen hemen hiç eksprese etmedi. OSHK, HSP70 ve HSP27 ile sağlıklı epitelde anlamlı ölçüde daha fazla boyanma gösterirken, lökoplaki olguları ile benzer boyanma karakteri gösterdi.

SONUÇ

HSPs ile immünohistokimyasal olarak yaptığımız çalışma, oral kavitenin skuamöz epitelinin tümörögenesi sırasında özellikle HSP70 ve HSP27 ekspresyon değişikliklerinin olduğunu ortaya çıkarmıştır.

Anahtar sözcükler: Karsinogenez; displazi; ısı şok proteinleri; oral kanser; skuamöz hücreli karsinom.

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MATERIALS AND METHODS

Sample Selection

A total of 60 cases, diagnosed between November 2003 - November 2008, were collected from the tissue block archive of Department of Tumor Pathology, Institute of Oncology, Istanbul University. The study was performed in 20 cases of oral squamous cell carcinoma (OSCC) at the T1N0M0 stage (8 women, 12 men, and mean age of 53.7), leukoplakia with moderate dysplasia (12 women, 8 men, and mean age of 48.6) and 20 cases of normal oral mucosa as the control group (10 women, 10 men, and mean age 36.5).

Immunohistochemistry

For immunohistochemistry, the paraffin blocks were cut serially into approximately 5 µm thick sections on charged slides. Firstly, the sections were penetrated and dried overnight in an autoclave (56 °C). They were deparaffinised with xylene for 30 min, and washed with 99% alcohol for 15 min, then 96% alcohol and distilled water. Ultravision Large Volume Detection System Anti-Polyvalent HRP Kit (Rabbit-mouse, Lab Vision Corporation, Fremont, CA, USA) was used in this study. For antigen retrieval, the sections were microwaved four times for 5 min in citrate buffer (Ph 6.0), cooled to room temperature and then washed in phosphate buffered saline (PBS) for 5 min. Endogenous peroxidase activity was blocked by incubating the sections with 3% H₂O₂ and washed distilled water and waited in PBS for 5 min. To prevent non-specific reactions, sections were incubated with block solution. Heat shock protein 27 (HSP27) antibody ready to use (Lab Vision Corporation Neomarkers, Fremont, CA, USA), heat shock protein 60 (HSP60) antibody ready to use (Lab Vision Corporation Neomarkers, Fremont, CA, USA), heat shock protein (HSP70) antibody ready to use (Lab Vision Corporation Neomarkers, Fremont, CA, USA) were used as primary antibodies. Slides were incubated 60 min with HSP27, HSP60, and HSP70. The secondary antibody was reacted for 25 min., followed by streptavidin peroxidase reagent for 25 min. AEC (Lab Vision Corporation, Fremont, CA, USA) chromogen was used to visualize the reaction. Finally, the sections were counter-

Squamous epithelium is the primary surface structure of the skin, lips, and mucous membranes of the oral cavity and 86% to 95% of head and neck malignancies originates from the surface epithelium.^[1] Squamous cell carcinoma is an invasive neoplasm arising from surface epithelium with varying degrees of squamous differentiation and occurring predominantly in smoking and alcohol consumption.

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies worldwide. The individual steps in the etiopathogenesis of OSCC are not clearly identified. Carcinogenesis is a complex process which is controlled by different kinds of genes and mechanisms. Many of the oncogenes including Ras, myc, c-erbB1; antiapoptotic proteins such as Bcl-x and Bcl-2; and several tumor suppressor genes have been recognized in pathogenesis of oral carcinomas. Also, precancerous lesions such as leukoplakias and erythroplakias play a role in oral carcinogenesis.^[2,3]

Heat shock proteins (HSPs) form the most ancient defence system in all living organisms. They are a class of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures or other stresses, including infection, irradiation, heavy metals, ethanol and oxidants. These proteins can be divided into different families according to their molecular weight such as HSP27 (27KDa), HSP70 (70KDa), HSP60 (60 KDa), and HSP90 (90KDa).^[4-7]

However HSPs are beneficial to the normal cells. Cancer cells can also use HSPs in response to stress, leading to increased expression.^[8] HSPs are detected in neoplasms arising from many tissues and organs such as prostate,^[9] adrenal gland,^[7] bladder^[10] and oral carcinomas.^[5,11,12] Also recent studies have revealed that HSPs are expressed in cardiovascular diseases.^[13]

HSPs are considered useful as diagnostic or prognostic predictive factors in a variety of tumors;^[14] therefore it aimed to investigate the presence of HSPs in different stages of oral carcinogenesis in this study.

stained with Mayer's haematoxylin, and evaluated by a light microscope.

Evaluation Methods

Immunoreactivity of HSP27, 60, and 70 were detected in the cytoplasm of oral epithelial cells. The immunostained slides were evaluated on a subjective estimate 4 point scale, negative or faint stain (-), focal or diffuse weak stain (+), focal strong stain (++), diffuse strong stain (+++), by two independent oral pathologists. The HSP27, 60, and 70 levels in the slides with a score of (-) was taken basal level of expression whereas a score of (+) or higher were taken to represent overexpression of these antibodies.

Statistical analysis was performed using the Graph Pad Instant Version 2.02. To test the significance of the differences in HSP27, 60, and 70 expressions between OSCC, leukoplakia and normal oral mucosa, chi-square test was used and p value of < 0.05 was considered to indicate statistical significance.

RESULTS

The expressions of HSP27, 60 and 70 were observed in different stages of oral carcinogenesis; normal, premalignant and malignant oral tissues. Among 20 normal mucosa; 3 cases (15%) showed elevated staining of HSP27, 17 cases (85%) showed no HSP27 staining, whereas 19 cases of OSCC (95%) and 15 cases of dysplastic lesions (75%) showed high staining of HSP27 and no HSP27 staining was observed in only one case of OSCC (5%) and 5 cases of dysplastic lesions (25%).

HSP27 staining in OSCC and dysplastic lesions (Fig. 1a and 1b) was significantly higher than normal epithelium ($p < 0.001$, $p = 0.001$, respectively).

Nine cases of oral mucosa (45%), 19 cases of OSCC (95%) and 20 cases of leukoplakia (100%) showed HSP70 overexpression. No HSP70 staining was observed in 11 cases of normal mucosa (55%) and just one case of OSCC (5%) and none of dysplastic lesions. HSP70 was expressed more strongly in dysplastic lesions and OSCCs (Fig. 1c and 1d) than in normal mucosa ($p < 0.001$, $p = 0.002$, respectively).

Twenty of the OSCC cases, 17 cases (85%) showed overexpression of HSP60, and 3 cases (15%) showed no HSP60 staining (Fig. 1e). In dysplastic lesions, only 2 cases (10%) showed elevated HSP60 expression and 18 cases (80%) showed no HSP60 staining (Fig. 1f), whereas 19 cases (95%) of normal mucosa showed HSP60 overexpression and no HSP60 staining was observed in only one case of normal mucosa (5%). Expressions of HSP60 in normal mucosa and OSCC were significantly higher than dysplastic lesions ($p < 0.001$).

DISCUSSION

HSP27 is a member of the small HSP family and acts as a protein whose main function is to prevent the aggregation of nascent and stress-accumulated proteins. In addition to its chaperoning functions, HSP27 plays important roles in cytoskeleton dynamics, cell differentiation and embryogenesis.^[15] HSP27 expression is associated with increased tumorigenicity, growth rate or invasiveness of some carcinomas. Although there are many studies about HSP27, the function of HSP27 in head and neck squamous cancer has not been fully understood. In the current study, expression of HSP27 was higher in leukoplakia and OSCC than in normal mucosa. No difference in HSP27 expression was apparent between OSCC and dysplastic lesions. Zhu et al.^[16] observed HSP27 overexpression in a head and neck squamous cell carcinoma from a metastatic lymph node, but it was rarely expressed in the primary cancer cells from the same patient. It was shown that reduced HSP27 expression in primary tongue squamous cell carcinoma was associated with poor differentiation; furthermore the higher expression of HSP27 was correlated with better overall survival.^[12] However it was observed that a high level of HSP27 expression was associated with tongue squamous cell cancer invasion and metastasis.^[17] Lo Muzio et al.^[18] investigated HSP27 expression in OSCC and they found similar results with our study and they suggested that HSP27 reduced expression is an early marker of poor prognosis in OSCC.

HSP60 accumulates specifically in mitochondria where its major functions are protein chaperoning and folding. Also HSP60 is known to act as

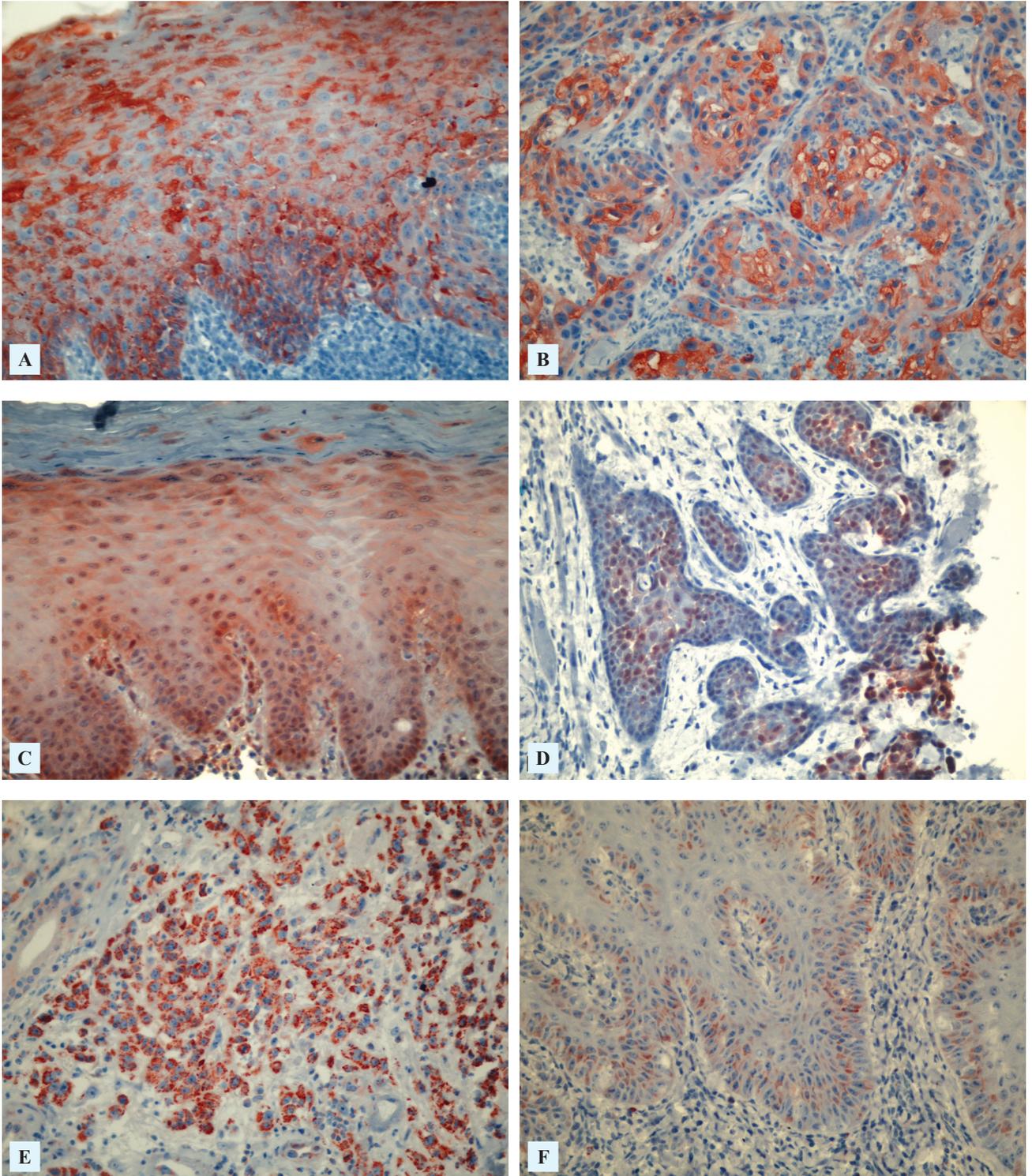


Fig. 1. Immunohistochemical staining for HSP27, HSP70, and HSP60. The strong cytoplasmic staining of HSP27 was demonstrated full thickness of epithelium of leukoplakia (A) and the tumor island of OSCC (B). The strong cytoplasmic staining was found in leukoplakia (C) and the moderate cytoplasmic staining was demonstrated in OSCC (D). The strong cytoplasmic staining of HSP60 was found in OSCC (E) and the moderate cytoplasmic staining of HSP60 was observed in the basal epithelial cells of leukoplakia (F), (x400).

a positive modulator of apoptosis by accelerating the maturation and activation of caspase-3.^[4,19] In the present study, normal mucosa and OSCC expressed HSP 60, while dysplastic lesions did not. In contrast to our result, Fan et al.^[20] that observed the expression of HSP60 was higher in leukoplakia and OSCC than in normal epithelium. They found mild significant data about HSP60 expression associated with histopathological characteristics and clinical features.

HSP70 regulates a wide range of protein-associated activities and elevated levels of HSP70 protect cells from apoptotic death.^[4,19] In OSCC, immunostaining intensity for HSP70 is suggested to be related to the degree of tumor cell differentiation.^[21] In the present study, expression of HSP70 was higher in leukoplakia and OSCC than in normal epithelium. Kaur et al.^[22] showed that HSP70 overexpression in premalignant oral lesions are correlated with a high risk of transition to malignancy and patients with OSCCs show poor prognosis. Elevated levels of HSP70 have also been shown in oral verrucous carcinoma, oral verrucous hyperplasia,^[23] oral lichen planus^[24] and ameloblastomas.^[25]

Oncologic investigations have observed that HSPs might be crucial for carcinogenesis^[26] and, also expression of the differential HSPs have been shown in oral tumorogenesis.^[16] HSPs may be involved in cell proliferation by interaction with the proteins required for the proliferation process.^[27] Our research showed that HSPs were found in normal, dysplastic and neoplastic oral tissues, suggesting that HSPs play a certain role in their cellular functions.

In general, our results agree with certain results of the literature. On the other hand, a correlation between HSP immunostaining and survival period or prognostic factors such as lymph node metastasis, clinical stage has not been shown clearly. Further investigations with the use of other methods could help to determine the real levels of these mediators in these lesions.

REFERENCES

1. Werning JW. Oral cancer: diagnosis, management, and

rehabilitation. 1st ed. New York: Thieme Medical Publishers; 2007.

2. Silverman S Jr, Sugarman PB. Oral premalignancies and squamous cell carcinoma. *Clin Dermatol* 2000;18(5):563-8.
3. Llewellyn CD, Johnson NW, Warnakulasuriya KA. Risk factors for squamous cell carcinoma of the oral cavity in young people--a comprehensive literature review. *Oral Oncol* 2001;37(5):401-18.
4. Sreedhar AS, Csermely P. Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy: a comprehensive review. *Pharmacol Ther* 2004;101(3):227-57.
5. Ciocca DR, Oesterreich S, Chamness GC, McGuire WL, Fuqua SA. Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. *J Natl Cancer Inst* 1993;85(19):1558-70.
6. 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(1):18-22.
7. Pignatelli D, Ferreira J, Soares P, Costa MJ, Magalhães MC. Immunohistochemical study of heat shock proteins 27, 60 and 70 in the normal human adrenal and in adrenal tumors with suppressed ACTH production. *Microsc Res Tech* 2003;61(3):315-23.
8. Gibbons NB, Watson RW, Coffey RN, Brady HP, Fitzpatrick JM. Heat-shock proteins inhibit induction of prostate cancer cell apoptosis. *Prostate* 2000;45(1):58-65.
9. Glaessgen A, Jonmarker S, Lindberg A, Nilsson B, Lewensohn R, Ekman P, et al. Heat shock proteins 27, 60 and 70 as prognostic markers of prostate cancer. *AP-MIS* 2008;116(10):888-95.
10. Leuret T, Watson RW, Molinié V, O'Neill A, Gabriel C, Fitzpatrick JM, et al. Heat shock proteins HSP27, HSP60, HSP70, and HSP90: expression in bladder carcinoma. *Cancer* 2003;98(5):970-7.
11. Markopoulos AK, Deligianni E, Antoniadis DZ. Heat shock protein 70 membrane expression in oral cancer: a possible new target in antineoplastic therapy? *Chemotherapy* 2009;55(4):211-4.
12. Wang A, Liu X, Sheng S, Ye H, Peng T, Shi F, et al. Dysregulation of heat shock protein 27 expression in oral tongue squamous cell carcinoma. *BMC Cancer* 2009;9:167.
13. Grundtman C, Kreutmayer SB, Almanzar G, Wick MC, Wick G. Heat shock protein 60 and immune inflammatory responses in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2011;31(5):960-8.
14. Ciocca DR, Calderwood SK. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment

- implications. *Cell Stress Chaperones* 2005;10(2):86-103.
15. Parcellier A, Gurbuxani S, Schmitt E, Solary E, Garrido C. Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. *Biochem Biophys Res Commun* 2003;304(3):505-12.
 16. Zhu Z, Xu X, Yu Y, Graham M, Prince ME, Carey TE, et al. Silencing heat shock protein 27 decreases metastatic behavior of human head and neck squamous cell cancer cells in vitro. *Mol Pharm* 2010;7(4):1283-90.
 17. Ye H, Wang A, Lee BS, Yu T, Sheng S, Peng T, et al. Proteomic based identification of manganese superoxide dismutase 2 (SOD2) as a metastasis marker for oral squamous cell carcinoma. *Cancer Genomics Proteomics* 2008;5(2):85-94.
 18. Lo Muzio L, Campisi G, Farina A, Rubini C, Ferrari F, Falaschini S, et al. Prognostic value of HSP27 in head and neck squamous cell carcinoma: a retrospective analysis of 57 tumours. *Anticancer Res* 2006;26(2B):1343-9.
 19. Bukau B, Horwich AL. The Hsp70 and Hsp60 chaperone machines. *Cell* 1998;92(3):351-66.
 20. Fan GK, Chen J, Ping F, Geng Y. Immunohistochemical analysis of P57(kip2), p53 and hsp60 expressions in premalignant and malignant oral tissues. *Oral Oncol* 2006;42(2):147-53.
 21. Sugerman PB, Savage NW, Xu LJ, Walsh LJ, Seymour GJ. Et al. Heat shock protein expression in oral epithelial dysplasia and squamous cell carcinoma. *Eur J Cancer B Oral Oncol* 1995;31B(1):63-7.
 22. 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(6):496-501.
 23. Lin HP, Wang YP, Chiang CP. Expression of p53, MDM2, p21, heat shock protein 70, and HPV 16/18 E6 proteins in oral verrucous carcinoma and oral verrucous hyperplasia. *Head Neck* 2011;33(3):334-40.
 24. Seoane J, Ramírez JR, Romero MA, Varela-Centelles P, Garcia-Pola MJ. Expression of heat shock protein (HSP70) in oral lichen planus and non-dysplastic oral leucoplakia. *Clin Otolaryngol Allied Sci* 2004;29(2):191-6.
 25. Kumamoto H, Suzuki T, Ooya K. Immunohistochemical analysis of inducible nitric oxide synthase (iNOS) and heat shock proteins (HSPs) in ameloblastomas. *J Oral Pathol Med* 2002;31(10):605-11.
 26. Fuller KJ, Issels RD, Slosman DO, Guillet JG, Soussi T, Polla BS. Cancer and the heat shock response. *Eur J Cancer* 1994;30A(12):1884-91.
 27. Pechan PM. Heat shock proteins and cell proliferation. *FEBS Lett* 1991;280(1):1-4.