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HSP90 EXPRESSION AND NECK METASTASIS IN TONGUE SCC

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ABSTRACT

In oral cavity, the tongue is the most common site prone to development of squamous cell carcinoma (SCC). Cervical lymph node metastasis is a sign of tumor with invasive and malignant nature; it is also important to specify the severity of tumor extension and its prognosis. Considering malignant transformation as a cellular stress, the expression of heat shock proteins (HSPs) may be affected in this process. Heat shock proteins (HSPs) have vital roles in normal cells in appropriate holding and folding of proteins. Moreover HSPs over express in a wide range of human cancers and play roles in proliferation, differentiation, angiogenesis, invasion, metastasis, apoptosis and helping the immune system. Targeting HSPs as a treatment for cancer is under clinical evaluation. One of the important proteins of this group is HSP90. The goal of present study is the comparison between HSP90 in two metastatic and non metastatic tongue squamous cell cancer groups in order to specify the relation of HSP90 expression with tongue SCC. 32 patients with tongue SCC that had undergone surgical treatment with lymphatic metastasis in half of them were selected. 2 sections were prepared from the paraffin blocks of patient samples. One of sections was colored with the commonly H&E staining and the other was used for immunohistochemical study (IHC) of HSP90 antigens. The intensity of HSP90 expression (the intensity of staining) was determined and its relation with lymphatic metastasis and parameters such as age and gender were analyzed; Data were analyzed using mannwhitney and correlation coefficient tests. There was no statistical correlation between HSP90 expression and metastasis in SCC. Also no statistical correlation between HSP90 expression with age and gender was observed. According to results obtained, HSP90 had no significant difference in two metastatic and nonmetastatic groups. Due to overlapped routes of metastasis signaling, the role of these proteins is sophisticated; so the relation between HSP90 expression and tongue SCC metastasis remains obscure. Paying attention to the contradictious results, repeating the tests, further studies with larger sample size and the use of more sophisticated methods seem necessary.

Keywords: Heat Shock Proteins, Molecular Chaprons, Secondary Proteins, Squamous Cell Carcinoma, Lymphatic Metastasis

INTRODUCTION

In oral cavity, the tongue is the most common site prone to development of squamous cell carcinoma (SCC) (1-2).

Cervical lymph node metastasis is a sign of tumor with invasive and malignant nature; it is also important to specify the severity of tumor extension and its prognosis (3).Considering malignant transformation as a cellular stress, the expression of heat shock proteins (HSPs) may be affected in this process. Heat shock proteins are a big family of proteins, which exist few in our normal cells and act as chaprone molecules helping in folding and translocation of proteins (4). They prevent misfolding of proteins and help in refolding of denatured ones (1). The members of this family are divided into the subclasses according to their molecular weight (5-6).

Among heat shock proteins, HSP90 family are presented in cancers at high levels, whereas in normal cells under oxidative stress (inflammation or infection) increased expression is induced (1). It has been shown that ectopic expression of HSP90 not only prevents apoptosis but also has the ability to enhance tumor
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genesis (7-10). On the other hand, these proteins can enter the bloodstream and stimulate the immune system, causing immune responses against cancer (1).
HSP90 is found in abundance in the cytosol and has two α and β isoforms which are almost in equal amounts in the cytosol (11).
Previous studies have shown that increased expression of HSP90 in breast cancer is associated with increased cell proliferation, poor differentiation, lymph node metastasis and poor response to therapy (1). Increased expression of HSP90 in human metastatic cancers of different types such as pancreatic and colon carcinoma have been reported (12-13).
HSP90 expression in endometrial carcinoma associated with a better prognosis than other HSPs have been reported (14). In ovarian carcinomas the increase of HSP90 mRNA was significantly higher in malignant tumor than benign and borderline tumors (15).
It has been shown that in cancerous cells the level of HSP90 of cell surface increases and is associated with metastatic activity (16). Although its mechanism is not clear yet, but it has been identified that HSP90 secretion is stimulated by environmental stress and growth factors (17,18). Extracellular HSP90 in cancer metastasis may act individually and unique or may overlap with operation of other intracellular chaperon involved (4). The first time it was shown that HSP90 has a role in cell motility and invasiveness of fibrosarcoma cells by means of a functional protein (7).
In 2003 Zuo and colleagues reported no statistically significant association between HSP90 expression and lymph node metastasis in gastric cancer (19).
In 2009 Giaginis and his colleagues reported there is no statistically significant association between HSP90 expression and lymph node metastasis in gastric cancer (20).
The best indicators of prognosis in patients with oral SCC are tumor size and metastasis (21-23). Metastasis is the result of a complex and highly organized processes, including changes in adhesion, cell Motility and capability of angiogenesis (7). During this process cancer cells migrate throughout the body (14). Most cancer related deaths are due to metastasis of the original tumor (7). In Carcinoma of the oral cavity the metastasis is not an early founding (8). However, due to delays in diagnosis, at diagnosis time 21% of patients have neckmetastasis (4).
How HSPs expression increases in cancersis still remains as a mystery (24). One hypothesis is that the tumor physiopathology microinviroment (reduced PH, Glucose and Oxygen) has a tendency to induce HSPs (25). But still it is not clear that this assumption is correct or not (26). Another hypothesis is that elevated levels of HSPs associated with malignancies may be related with genetic changes due to tumor progression (26).
The aim of this study was to evaluate HSP90 expression in metastatic and non-metastatic SCC in order to understand whether this molecular marker has a role in progression and metastasis of tongue SCC.

MATERIALS AND METHODS
Sampling
This cross sectional descriptive study was conducted on patients with tongue squamous cell carcinoma (SCC), half with and half without lymph node metastasis. This population does meet the criteria for inclusion and without exclusion criteria of the study.
At first those patients who had SCC and were cured at Imam Khomeini Hospital were identified and among them 30 samples including the inclusion and no exclusion criteria were selected;
Inclusion Criteria
-according to medical history, patient is healthy.
-Patient has undergone hemiglossectomy and radial neck dissection.
Exclusion Criteria
-tongue SCC and another tumor simultaneously.
-tongue SCC and a serious medical disease simultaneously.
-with the history of chemotherapy and radiotherapy.
-samples with inappropriate fixation, too broad areas of bleeding and necrosis.
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Demographic characteristics of samples such as age, sex, and metastatic lesions were achieved from patient documents. Paraffin blocks and grids of these patients were reassessed and those with appropriate fixation, with adequate tissue and no wide necrosis or hemorrhage were selected. Samples with lymph node metastasis were histologically proved and checked and were written in the medical records. 32 samples with inclusion criteria were selected and divided into two equal groups:

Group A: with cervical lymph node tumoral involvement.

Group B: with notumor in cervical lymph nodes.

So in each group 16 patients were studied from each Block, 2 sections with the thickness of 4μm were prepared. One of the sections was colored with the commonly H&E staining and was confirmed by the supervisor. The other section was used for immunohistochemical study (IHC).

Immunohistochemical Staining

The streptavidin-biotin complex technique was using in this study. First 4μm sections of paraffin blocks were prepared and placed on grids covered with poly-L. Lysine. Then the samples were deparaffinized in xylene and Rehydrated in different concentrations of alcohol (ethanol). Then in order to prevent Peroxidase activity, samples were placed in methanol containing 30% H2O2 for 15 minutes and then were washed with (PBS) Phosphate-buffered saline;

And then, according to the manufacturer’s instructions tissues were placed adjacent to a solution of 1/200 monoclonal antibody HSP90 (clone PB24; Novo-castra laboratories Ltd, Newcastle, uk)at the room temperature for 60 minutes. Tissue sections were then incubated for 10 min with PBS and after that the invasion system (secondary AB) were used and again 10 minutes washing with PBS was done. In order to cell staining, Sections were adjacent to 3.3 Diamino Benzidine hydrochloride (DAB).

After this step, samples were stained with Harrishematoxylinas counter stain, dehydrated and then cover slip was placed on the slide. The cytoplasm of stained tumor cells turned into brown. Endometrial tissue was the positive samples for HSP90. Staining without primary antibody was used as a negative control. Positive and negative controls were performed with each series.

The intensity of immunohistochemical staining was assessed independently by two blind pathologists without any knowledge about the clinical status and patients’ history. According to the methodology used in the Anastasi's study, the intensity of cytoplasmic HSP90 expression in samples was assessed (78). In comparison to the positive control, if the cytoplasmic staining intensity of tumor cells were similar to the control sample, it was given score 3 and has been identified as High Intensity. If cytoplasmic staining intensity was moderate, it was given Score 2 and has been identified as moderate intensity. If cytoplasmic staining intensity was low, it was given Score 1 and has been identified as low intensity. If the there was no staining, it was given score 0.

0-no staining
1- low staining (Low expression)
2- moderate staining (Moderate expression)
3- intense staining (High expression)

Image Analysis

Sample staining with HSP90Abin all specimens caused thecytoplasm of cancer cells turn into brown in (Figures 1 and 2). Only in 8th samplenuclear staining was observed in addition to cytoplasmic staining (Figures 3 and 4).

From the perspective of immunohistochemistry, samples were evaluated based on the quality or intensity of the color of cell staining. Also, muscles, muscular walls of vessels, vascular endothelial cells and minor salivary gland ducts were stained.
Statistical Analysis
In this study in order to assess the relationship between the intensity of HSP90 expression and metastasis, and patient gender and HSP90 expression mannwhitney test was used (P <0.05).
To investigate the correlation between the intensity of HSP90 expression and patient age, correlation coefficient test was used.

RESULTS AND DISCUSSION
Results
From a quality perspective, there was no unstained sample (score 0). Therefore, there are three score of low, moderate, and severe. HSP90 expression in metastatic group was weak in 6 cases (37.5%), moderate in 4 cases (25%) and severe in 6 cases (37.5%), and in non-metastatic group it was poor in 5 cases (31.3%), moderate in 6 cases (37.4%) and severe in 5 cases (31.3%) (Table 1).

Test between Parameters
Relationship between HSP90 expression and tongue SCC metastasis: In this study, in order to assess the relationship between the intensity of HSP90 expression and metastasis mannwhitney test was used. According to this test, there was no significant difference between the two metastatic and non-metastatic groups (p>0.999) (Table 2). Relationship between patient gender and HSP90 expression:
According to mannwhitney test, there was no statistically significant association between patient gender and HSP90 expression (P value> 0.05) (table 3).
Relationship between patient age and HSP90 expression:
No significant correlation between HSP90 expression and patient age was observed (P> 0.05) (table4).
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**Table 1: Staining intensity of samples (HSP90)**

<table>
<thead>
<tr>
<th>Patients in nonmetastatic group (%)</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients in metastatic group (%)</td>
<td>31.3</td>
<td>37.4</td>
<td>31.3</td>
</tr>
<tr>
<td></td>
<td>37.5</td>
<td>25</td>
<td>37.5</td>
</tr>
</tbody>
</table>

**Table 2: The intensity of HSP90 expression in two metastatic and nonmetastatic groups**

<table>
<thead>
<tr>
<th>Tumor condition</th>
<th>HSP expression intensity</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>With no metastasis</td>
<td>Score1</td>
<td>5</td>
<td>31.3%</td>
</tr>
<tr>
<td></td>
<td>Score2</td>
<td>6</td>
<td>37.5%</td>
</tr>
<tr>
<td></td>
<td>Score3</td>
<td>5</td>
<td>31.3%</td>
</tr>
<tr>
<td></td>
<td>Score4</td>
<td>6</td>
<td>37.5%</td>
</tr>
<tr>
<td>With metastasis</td>
<td>Score2</td>
<td>4</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>Score3</td>
<td>6</td>
<td>37.5%</td>
</tr>
</tbody>
</table>

**Table 3: Gender dispersion two metastatic and nonmetastatic groups**

<table>
<thead>
<tr>
<th>Gender</th>
<th>Tumor condition</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With no metastasis</td>
<td>9(56.3%)</td>
<td>7(43.8%)</td>
</tr>
<tr>
<td></td>
<td>With metastasis</td>
<td>4(25%)</td>
<td>12(75%)</td>
</tr>
</tbody>
</table>

**Table 4: Age dispersion two metastatic and nonmetastatic groups**

<table>
<thead>
<tr>
<th>Age</th>
<th>Tumor condition</th>
<th>Number</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Average</th>
<th>Std. deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With no metastasis</td>
<td>16</td>
<td>19</td>
<td>70</td>
<td>48.56</td>
<td>16.92</td>
</tr>
<tr>
<td></td>
<td>With metastasis</td>
<td>16</td>
<td>26</td>
<td>80</td>
<td>62.37</td>
<td>15.56</td>
</tr>
</tbody>
</table>

**Discussion**

Plasticity of tumor cells to cope with adverse circumstances is extraordinary that one of the ways it is typically included the increased resistance to apoptosis (4). Another cancer Hallmark that allows adaptation to environmental changes is heat shock protein upregulation in response to cellular stress. Special mechanism of HSPs in human cancer is still unknown. Expression of HSPs in response to cell proliferation and apoptosis are observed (27). A hypothesis has been proposed that these proteins act as molecular chaperons in reactive protein associated with stabilization of mRNA produced by several protooncogene (28). HSP90 on the surface of tumor cells are found to be antigenic indicators for T-cells and may be tumor antigens (27). Heat shock proteins (HSPs) are over expressed in a wide range of human cancers. Studies showed that these proteins are involved in proliferation, differentiation, invasion, metastasis, apoptosis of cancer cells and their detection by the immune system (26). Studies confirmed the role of HSPs in many aspects of tumor progression and response to treatment. Though there is not still sufficient information about the diagnostic value of HSPs, but it is known to be effective biomarkers in carcinogenesis, differentiation and invasion of tissues. Moreover, some researchers have known the HSPs and HSP antibodies in the sera of patients with cancer to be useful in diagnosis of some of cancers (26). Pathophysiological function of HSPs in tumorogenesis is associated with growth and differentiation (29, 30). It is clear that HSPs family member such as HSP90 are usually associated with cell cycle proteins such as P53 (31, 32). A hypothesis has been proposed that cancer cells are addicted to HSP90 and use this protein to facilitate many oncoproteins. There are also reports that HSP90 on the surface of cancer cells are associated with metastasis and motility (33). Because the presence of this molecular chaperons is the reason of telomere asstability, so it is important in transformation and is over expressed or activated in many cancers (34). HSP90 expression in cancerous tissue and the presence of HSP90 autoantibodies are associated with poor prognosis in breast cancer. In contrast, HSP90 expression is associated with good prognosis in...
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endometrial cancer also HSP90 expression in bladder cancer has not been associated with an increased risk of invasion and recurrence. HSP90 expression lacks prognostic value in oral and ovarian cancers (26). Based on different studies on the relationship between HSP90 expression and different tumor behavior, a variety of different results had been achieved. Although many studies have been conducted to clarify the association between HSPs and tumors in various organs, few studies were about oral squamous cell carcinoma (OSCC) (35).

As results are various and contradictory, the need to more study about HSP90 is obvious in order to answer to this question: does HSP90 really have any influence on cancer factors in prognosis especially oral cancer?

Therefore, in this study we assessed the expression of the above marker with one of the major factors, cervical metastasis, affecting the prognosis of tongue SCC.

Ito and colleagues (1998) did not observe HSP90 expression in normal oral epithelium, saying that the positive expression of HSP90 in tongue SCC may be the reason of substrates related to Tumor genesis or unusual stress associated with tumor formation (36). In the present study, the positive and mild cytoplasmic HSP90 was observed in the epithelium of tongue normal mucosa. Also in all samples of tongue SCC the positive expression of HSP90 was observed which was more severe in comparison to the normal epithelium.

In our study a very mild HSP90 expression was observed in the cytoplasm of normal epithelium. In tumor specimens HSP90 expression is mainly cytoplasmic and in only one case of metastatic and one case of nonmetastatic samples nuclear and cytoplasmic expression of HSP90 was observed. The explanation for the differences in the pattern of staining is not easy, and the limited number of samples in our study makes it difficult to judge on this issue (27). Despite the reports, Gandour states that as HSPs are natural components of cells, their presence in the tumor cells may be only a cell response to increased proliferation (27). So the selection of samples from different areas of the oral cavity in Gandour study or the small sample size of our study may partly explain the differences in the results of these two studies.

Giaginis and colleagues (2009) studied the HSP90 expression in gastric cancer. Its expression in tumor cells was mainly cytoplasmic and in some cases in membrane. The intensity of HSP90 expression had a significant association with tumor size and its histological type. But there was no statistically significant correlation with lymph node and distant metastasis (20). Zuo and colleagues (2003) showed that there is a statistically significant association between HSP90 expression and lymph node metastasis in gastric cancer (19, 20). In the field of cytoplasmic expression of HSP90, the result of this study is consistent with our but it was not the same as in association with lymph node metastasis.

Recent reports have shown that increased expression of HSP90 in malignant cells is associated with prognosis, metastasis and resistance to chemotherapy in malignancies such as breast, ovary, esophagus, endometrium, melanoma.

In our study, in order to gather samples we used some especial inclusion and exclusion criteria resulting restrictions on the number of samples. Among Exclusion criteria treatment such as radiotherapy and chemotherapy was mentioned. Thus, patients who had received treatment other than surgery were excluded from our study; therefore the lack of nuclear expression of HSP90 in this study compared with previous studies may be related to differences in the treatment technique applied. Evaluation of this protein expression in tongue SCC treated with radiotherapy and chemotherapy, as well as their association with different clinical and pathological criteria need further studies in the future.

Gulsum and colleagues investigated HSP90 expression in malignant ovarian tumors and evaluated their relationship with clinical pathological findings. They are present in both nuclear and cytoplasmic. Nuclear HSP90 expression was much less. They did not report a significant correlation with histopathologic findings (37).

No significant correlation between HSP90 expressions with metastatic ovarian cancer has been reported although there has been associated in more advanced stages (38).

The results of studies on HSP90 expression in ovarian cancer are consistent with the results obtained in oral cancer. Nuclear and cytoplasmic HSP90 expression in cancer cells suggests that these markers react
with different proteins in the nucleus and cytoplasm of epithelial cancer cells. Although in our study HSP90 expression was mainly as the form of obvious cytoplasmic granules which may be caused by mitochondrial localization of these proteins in tongue epithelial cancer cells. In contrast to the results of our study, Lee and colleagues (2008) compare HSP90 expression in normal oral epithelium and squamous cell carcinoma. In this study, 41 samples of OSCC and 10 normal epithelium samples were analyzed by immunohistochemistry and clinical-pathological profile analysis. Immunohistochemical results of this study showed that HSP90 expression was significantly higher in OSCC samples, but no significant association with gender, age, stage, T category was observed (39). The results of this study are inconsistent with ours. In Lee study, HSP90 expression in tumor islands was more severe than in normal epithelium. The membranous HSP90 expression in tumoral tissues but very weak intracellular HSP90 expression in normal epithelium was observed (39). Results from our study about the relation between HSP90 with age, gender and the intensity of expression in normal and tumoral tissues is consistent with Lee and Kaur study. However, in our study, membrane expression of these proteins was not observed in tumor cells, although patterns of expression in normal epithelium were similar to Lee study. This difference in the place of the above protein expression may be the result of sampling from different regions of oral cavity or laboratory protocols. It has been shown that HSP expression is various in different regions of the oral cavity. Unfortunately, with cells staining intensity and the number of stained cells in immunohistochemical studies there have been many limitations. Many factors have effect on the amount and the intensity of anti-genes expression which caused decrease in worldwide studies about them. It is known that depending on the type of cancer, the molecular profile, the type of tissue reactions of each HSP has an especial association with disease prognosis. The other reason of these differences may be due to variations in HSPizotypes (26). HSP studies on the cellular and molecular level in cancers are very limited and very little information about the effect of HSP on molecular events such as tumor growth, invasion and metastasis is in hand. Such studies are essential for targeting HSPs in cancer treatment (26). HSP90 antagonists act as pure factors in the treatment of cancer (40). HSP90 has been shown almost to be over expressed in all human tumors and has essential roles in controlling mitosis and inhibiting apoptosis. HSP90 dysfunction causes mitochondrial apoptosis and suppression of cell proliferation, suggesting potential treatment for cancer (40). On the other hand, further work on HSP90 in the last 20 years suggests that cancer may be an inducer for HSP90 inhibitors. Recently a study about HSP90 showed it as preoapoptosis protein inhibitor of cancer cells suggesting HSP90 inhibiting will kill cancer cells (33). Although the data from animal models and in ten years clinical trial has shown that such a simple strategy for targeting HSP90 as pharmacological therapy may not be an effective treatment (33). There are studies supporting an important role for HSP90 in the level tumoral cell surface, mitochondria, endoplasmic reticulum and nucleus, respectively. In addition, two cytosolic isoforms of HSP90 (HSP90α, HSP90 β) may have different roles on cancer cells and their metastasis. Within 5 to 10 years later the discovery of the importance of isoforms and the cellular location of inhibitors will develop the therapeutic effect of HSP90 inhibiting (33).

Conclusion and Recommendations
The amount of HSP90 expression represents no significant correlation with cervical lymph node metastasis in tongue SCC. Due to the impact of various factors on the different stages of metastasis and the overlapped routes of metastasis signaling, contradiction of conclusions from different studies, the existence of different types of HSP90 azyotypes and various HSPs functions, the biological significance of HSP90 in metastatic tongue SCC remains obscure and future studies are needed. To clarify and better understand the issue, repeating the tests, further studies with larger sample size, the use of more sophisticated methods and simultaneous studies of other factors influencing metastasis seem necessary.

REFERENCES
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