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ORIGINAL ARTICLE

Evaluation of Yes-associated protein 1 (YAP-1) expression in Squamous cell carcinoma of tongue

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ABSTRACT
Cell proliferation is one of the most important processes in the organ development, a new considered pathway in this process known as Hippo Pathway. This pathway consists of several components including a protein called Yes - associated Protein 1 (YAP-1) that increased in certain cancers and associated with cancer cell invasion and metastasis abilities. Although tumor-suppressor role in some papers has been expressed. Few studies about influence of this factor on Squamous cell carcinoma is done with variable results. The aim of the present study is to investigate the incidence of YAP-1 in squamous cell carcinoma of the tongue and its correlation with some clinico-pathologic parameters. In this cross-sectional study, 87 paraffin blocks of the primary tongue squamous cell carcinoma were retrieved from archives during 2006-2015. The expression of immunohistochemical marker and its relationship with clinico-pathologic factors were examined by semi-quantitative method. After recording, the data analyzed by statistical tests. Nuclear and cytoplasmic expression of YAP1 in tumoral tissue was increased significantly than in adjacent normal tissues, (P = 0.02 and P <0.001). Cytoplasmic YAP expression had a significant relationship with lymph node metastasis (P = 0.03) and sex (P = 0.04). No significant correlation was seen between clinico-pathologic factors and nuclear expression. The present study suggested that YAP-1 could pretend as either anti-apoptotic or tumor suppressor, based on the type of tissues and the presence or absence of specific binding tissue proteins. The intracellular localization of the marker may dictates it's function.
***Keywords**: Hippo Pathway - Squamous cell carcinoma - YAP1 (Yes associated Protein-1)*

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INTRODUCTION

Squamous cell carcinoma (SCC) is the sixth most common malignant cancer in the world. It encompassing more than 90% of all oral malignancies. As the name suggests, caused by malignant transformation in squamous cells of oral mucosa [1]. This cancer is more common in male than female and also occurs at older ages. Oral squamous cell carcinoma affect more frequently tongue, the critical anatomical site, in the oral cavity [2].

Despite improvement in diagnostic and therapeutic methods, this tumor is still characterized by a high rate of mortality. Deregulation of cell cycle, apoptosis and cell-cell/cell-matrix adhesions are considered pathways mainly influencing this multistage event [3]. Researches over the recent decade have been performed in order to investigate the biological diagnostic and prognostic parameters related to this event. The biological and immunological methods and effective factors on tumor growth have contributed to better understand the mechanisms involved in the oral carcinogenetic process. Among these factors could be noted Yes-associated protein 1 (YAP-1) which presents in Hippo pathway. The Hippo pathway

recognized for the first time in flies [3]. This pathway was unchanged based on rules of evolution and it's possible function is tissue homeostasis by regulation of cellular proliferation and apoptosis. The pathway consists of kinase cascade that phosphorylates the final effector known as YAP-1(4). Studies on YAP-1 suggesting an important role for the marker in various human disease such as cancers [4]. Dephosphorylation of the marker leads to entrance of YAP/TAZ to the nucleus and then start a transcriptional cascade promotes cellular proliferation and migration and suppresses anoikis [5]. While, phosphorylated YAP bind to the 14-3-3 proteins that results in cytoplasmic location of YAP and it's separation from nuclear transcription factors that leads to prevent from entering cells to cell cycle. Nowadays YAP-1 was considered as a true oncogene in solid tumors but limited information available about it's expression and precise molecular mechanism in carcinogenesis [4]. YAP is a 65 kDa protein (sometimes termed YAP65 or YAP-1) that classified to two main isoforms; YAP-1 and YAP-2 [4,6].
It's gene located on 11q12 chromosomes that often amplified in cancers and is a common site for loss of heterozygosity (LOH) in breast cancer. In mice, YAP synergically works with myc oncogene for increasing tumor growth. But contrary to the overexpression of this marker in many tumors, it's loss of function has only been seen in breast cancer and LOH of chromosome related with poorer survival rate in patients with breast cancer and suggested it's tumor suppressor role in cancer [7]. Also, some studies reported that YAP reacts with p53 binding protein-2 and is an important regulator of apoptotic activity of p53. So due to critical role of Hippo pathway in growth process and it's opposite function that dictated by final effector ie. YAP, as an oncogene or apoptosis stimulator, we decided to evaluate YAP-1 expression in squamous cell carcinoma of tongue, which is common site for oral cancer.

METHODS AND MATERIALS

In our descriptive cross-sectional study, 96 proven samples of squamous cell carcinoma of tongue were retrieved from archives of cancer institute of Emam-khomeini hospital of Tehran university of medical sciences, although included and excluded criteria such as no secondary tumor or recurrence, no treatment history like radiotherapy and chemotherapy and complete excision with radical neck dissection were considered. Clinical information such as age, sex and lymph node metastasis were collected and noted in a chart. Then Paraffin blocks that had adequate tumoral tissue were cut to 4µm section and stained by Hematoxylin and Eosin staining. The histopathologic grade of each slide was defined by two pathologist based on WHO classification. In the next step, blocks were cut again for IHC staining. For IHC staining, YAP-1(Rabbit polyclonal antibody, santa cruz, USA) were used. Immunohistochemistry was performed, according to the manufacturer's instructions antibodies with Envision method on paraffin blocks. In microscopic evaluation, the slides were examined at x400 magnification and YAP-1 immunoexpression in tumor cells was assessed semi quantitatively in terms of percentage and intensity and final immunoreactivity score.
The percentage of immunostaining was categorized into 4 groups based on positive stained cells, regardless of intensity:
Negative : 0 , 1 : <10% of cells , 2 : 10-≤25% of cells , 3 : >25-50% of cells
Moreover, the staining intensity of the samples were categorized according to the cytoplasmic and nucleus staining of cells separately into:
0= negative, 1=weak, 2=intermediate, 3=strong
Finally to determine the expression level of the YAP-1 marker (both nuclear and cytoplasmic), total Score was calculated by crossing the score for percentage to the score for intensity for each case. The samples were further classified into 4 groups:
0= negative expression , 1-3=low expression , 4-6=intermediate expression , 7-12=high expression
For positive control, colon adenocarcinoma was used. For negative control, the monoclonal primary antibody was substituted for PBS(4,8,9,10). After recording, the data analyzed by different statistical tests.

RESULTS

Finally, because of technical and laboratory difficulties 9 out of 96 samples were excluded from this study. So, 87 SCC blocks compared with normal epithelium of adjacent mucosa which lacking dysplastic changes. YAP-1 expression in cytoplasm and nucleus of all samples observed. Out of 87 cases, nuclear expression in 39 samples(44.8%), 35 samples(40.2%), 10 samples(11.5%) and 3 samples(3.4%) was negative, low, intermediate and high, respectively. Nuclear expression of YAP-1 in tumoral tissue was significantly higher than adjacent normal tissue(p=0.02)(Figure-1). Out of 87 cases, cytoplasmic expression in 11 samples (12.6%), 62 samples(71.3%), 14 samples(16.1%) was low, intermediate and high, respectively. Mann-Whitney analysis revealed that cytoplasmic expression of the marker in tumoral tissue is also

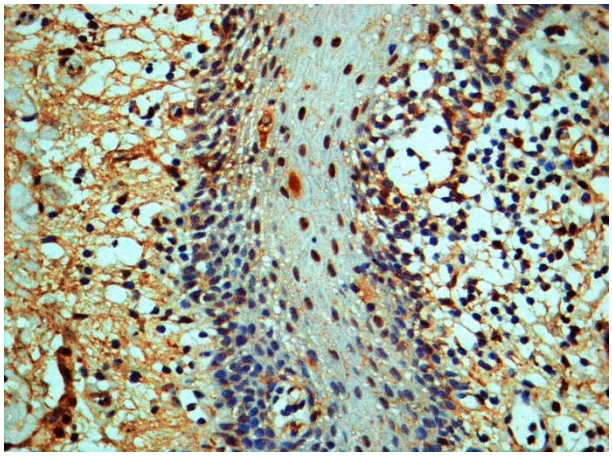
significantly higher than adjacent normal tissue (p<0.001)(Figure-2). Analysis show significant relationship between sex and cytoplasmic expression of the marker in tumoral tissue by the Mann-Whitney test(p=0.04).

The Chi-square exact test and Fisher's analyzes showed significant relationship between cytoplasmic expression of the marker and lymph node metastasis status(p=0.034). Clinical and histopathologic data and analytical results were shown in Table-1. It is noteworthy that expression of the marker in fibroblasts and endothelial cells was also observed in connective tissue adjacent to the tumor.

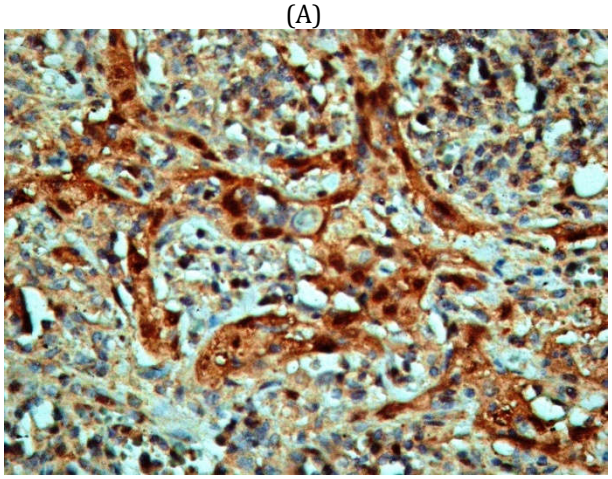
Table-1: Nuclear and cytoplasmic expression of YAP according to clinico-pathological factors

	NO=87	**Cytoplasmic expression**	**Nuclear expression**
Age			
≤50	30(%)	0.73[a]	0.36[a]
>50	57(%)		
Sex			
male	58(%)	**0.04*[b]**	0.5[b]
female	29(%)		
Histopathologic grade			
Garde I	55(%)	0.4[a]	0.6[a]
Grade II	25(%)		
Grade III	7(%)		
Metastatic lymph node			
positive	45(%)	**0.03*[c]**	0.14[c]
negative	42(%)		

a)Spearman correlation; b)Mann-whitney test; c)Fisher's exact test; *)P-Value<0.05

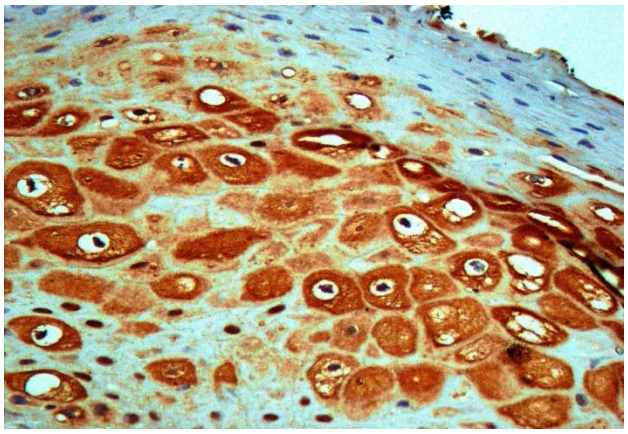


(A)

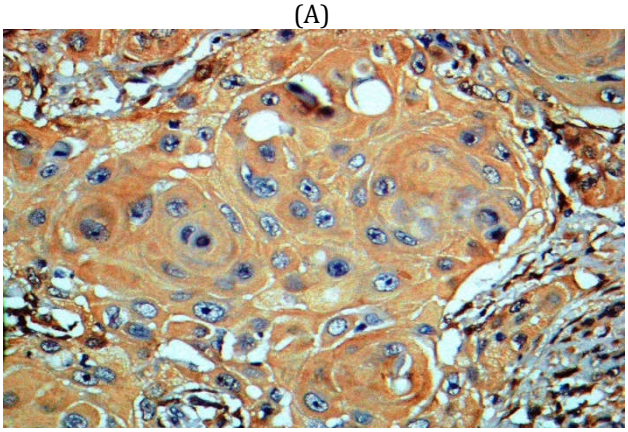


(B)

Figure-1: A) Nuclear expression of the YAP marker in adjacent normal tissue, B) Nuclear expression of the YAP marker in tumor.



(A)



(B)

Figure-2: A) Cytoplamic expression of the YAP marker in adjacent normal tissue, B) Cytoplasmic ()A) expression of the YAP marker in tumor.

DISCUSSION

Previous studies using cell carcinogenesis model, identified several genes that are guided different paths involved in cancer. Among these genes, YAP-1 gene is the one that it's expression increases in cancers, so evaluation of this marker can be determined new therapeutic strategies in the field of cancer [11]. As mentioned before, study on the Drosophila identified a Hippo signaling pathway that regulates both processes of proliferation and apoptosis. This way has been linked Merlin and Expanded cytoskeletal proteins to causing activation of kinase complexes Warts/Mats and Hippo/Sav. The ultimate goal of this pathway is transcriptional co-activator known as YAP/Yorkie (in drosophila) [7,12]. As described in detail before in our study, evaluation of the samples was defined that YAP-1 had a significant increase in the incidence in tumoral tissue compared to normal tissue and this increase mainly presented in the cytoplasm of the cells and simultaneously in a small number of nucleus. In general, a significant increase in the incidence of either nuclear or cytoplasmic YAP marker in malignant cells in the SCC was consistent with most previous studies on tumor specimens of tissues, including lung tissue, liver, breast, and etc [11,13,14,15,16]. But most of this increase occurred mainly in the nucleus compared to cytoplasm and researchers explained that the presence of this protein in nucleus represents it's proliferative role in the cancers. In fact, the potential impairment in protein kinases of Hippo signaling pathway caused dephosphorylation and accumulation of YAP in the nucleus and subsequently enhanced the transcription of target genes such as Cyclin D1. In fact YAP is an important intermediary in the transfer of G0 to S cell cycle [7,13,17]. Results of a research on neural tissue were presented that high expression of YAP may be involved in neoplastic transformation of the tissue(14). The other research revealed moderate to severe nuclear expression of YAP in hepatocellular carcinoma and its high expression was correlated with it's proliferative role, although significant relationships between marker expression and age, sex and TNM were not detected. At the same time in this article, overexpression of YAP was accompanied by overexpression of jag-1 (ligand for Notch signaling pathway) that results in activation of Notch signaling pathway and then high proliferation rate [18]. One study [19] was described that the nuclear and cytosolic localization of Yap linked to invasive lobular breast cancer which shows lack of E-cadherin. This study revealed Yap is not just transferred to the nucleus in this tumor and its overall level in this situation

increases. Also, mentioned that the YAP expression may be adjusted through YAP degradation. Probably membrane expression of E-cadherin eliminates YAP and vice versa loss of E-cadherin inhibits the YAP phosphorylation and subsequently inhibits ubiquitin-dependent dissolution of Yap and ultimately increases it's level. Interestingly, overexpression of YAP could change the E-cadherin expression in breast cancer cells so, results in loss of epithelial features, expression of mesenchymal markers, invasive and anchorage-independent growth characteristic. Accordingly, irrespective of upstream signaling cascade, nuclear YAP may have a central role in the regulation of invasion in breast cancer [19]. However, these results were in line with our findings about cytoplasmic expression of the marker and other study on adenocarcinoma and squamous cell carcinoma of the lung that it's findings were indicated significant increase in cytoplasmic YAP expression along with low histopathological grade of tumor and decreased pTNM. Also, the correlation between cytoplasmic overexpression of the marker and lymph node metastasis was negative but not significant. Moreover in this study high nuclear expression was associated with cyclin A and MAPK expressions and also EGFR gene amplification. It should be noted that the increased incidence of cytoplasmic YAP was associated with histologic dedifferentiation and lymph node metastasis in squamous cell carcinoma of lung [16]. However, some researchers have suggested that cytoplasmic expression of YAP specifically associated with neoplastic phenotype and this expression rarely seen without nuclear expression. It seems malignant cells due to genomic amplification produce higher level of YAP and by overcome the normal physiological regulatory systems leads to abnormal cytoplasmic accumulation of YAP protein [16]. In another study in 2013, evaluation of YAP expression in OSCC was shown increased level of YAP expression in tumoral tissue especially in higher grade than normal tissue. This higher expression was seen mainly in the cytoplasm and in small percentage of nucleus. This research suggested that the high expression of YAP in malignant tissue is probably due to activation of this pathway and accordingly more YAP translocation to cytoplasm [4]. We found meaningful increase in the incidence of cytoplasmic YAP marker in malignant cells in the SCC that was significantly associated with lymph node metastases. Our results suggest that in addition to the proliferative role, cytoplasmic expression of the marker is involved in the migration of tumor cells. This hypothesis is also was confirmed in another study [20] with a new role for cytoplasmic expression of this marker in epithelial cells. This study stated that YAP bonds to the AMOTL-1, angiomotin like 1, (which is involved in angiogenesis and cell migration) in the cytoplasm and prevent it's dissolution by E3 ubiquitin ligase Nedd 4.2 that is a member of the Nedd 4 (neural precursor cell expressed developmentally down-regulated protein 4) [20,21]. Our results in this study were shown absence or weak nuclear and/or cytoplasmic expression in normal tissue and also most expression were in basal and prickle layer of squamous epithelium. These findings are in accord with studies that suggest that the YAP is involved in physiological tissue homeostasis and tissue repair [16,18]. According to the findings of a one study on the expression of the marker in both nucleus and cytoplasm of normal tissue and nuclear expression specially in basal and prickle layer should be explained that basal expression of the marker is basically consistent with the role of YAP in conjunction with the homeostasis regulation and proliferation [4]. The results of one study [9] shown that cytoplasmic expression of YAP in well differentiation SCCs have increased than poorly differentiated ones like our findings in this article. The inverse relationship between YAP and histopathological grade of tumor was confirmed also in some other cancer studies including breast cancer research but this relationship didn't exist in liver cancer [9]. In addition, the relationship between the expression of YAP and age, sex, site and stage of tumor was not observed [9]. As well as to determine the effect of YAP on factors involve in epithelial-mesenchymal transition(EMT) and ultimately it's effect on lymph node metastasis, YAP was knocked down and little change was seen in the expression of EMT proteins such as E-cadherin and vimentin [9]. In the aforesaid study, the cytoplasm expression of the marker significantly associated with lymph node metastasis which is consistent with our results. Finally, consistent with our observations, precise analysis of the mentioned study [9] revealed that the structural component of stroma such as fibroblasts and endothelial cells adjacent to tumoral tissue were shown increase in YAP expression than structural component of stroma in benign oral tissue. In summary, we can say that YAP-1 is an important marker in cell growth and apoptosis and acts as an oncogene and/or as a tumor suppressor. The Hippo/YAP pathway cooperating with a lot of other signaling pathways for amplify cell growth or apoptosis based on different types of tissue, presence or absence of specific binding partners in tissues. Moreover sub-cellular localization of YAP may dictate it's function. In further studies to determine the prognostic value of YAP in clinical experiences and assessment of nuclear and cytoplasmic expression of YAP, it is important that each type of tumor to be specified separately. In addition, further studies are necessary to assessment of molecular partners that interact with YAP before treatment of lesions. Since YAP-1 has a dual function as both amplify and inhibit cell growth in different tissue, the treatment methods used to focus on YAP, probably successful in some tumors.

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