RESEARCH COMMUNICATION

HER2/neu Expression in Head and Neck Squamous Cell Carcinoma Patients is not Significantly Elevated

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Abstract

Background: HER2/neu, a member of EGFR family, is over expressed in some tumors .The purpose of this study was to determine the salivary level and tissue expression of HER2/neu in patients with head and neck squamous cell carcinoma (HNSCC) and any correlation with clinicopathologic parameters. Methods: An enzyme-linked immunosorbent assay (ELISA) was used to evaluate the salivary level and immunohistochemistry (IHC) to assess tissue expression of HER2/neu in 28 patients with HNSCC and 25 healthy controls. Results: The salivary levels of HER2/neu in HNSCC patients was not significantly higher than in the healthy controls (p>0.005). There was no apparent correlation in salivary HER2/neu level with clinicopathological features such as age, sex, grade, tumor size and nodal status. All HNSCC specimens were positive (membranous or/and cytoplasmic) for HER2/neu, except one sample. Only one HNSCC specimen was stained in cytoplasm purely. All control specimens were membranous and cytoplasmic positive for HER2/neu. There was a significant difference between cytoplasmic staining in case and control groups (p-value<0.05). Conclusion: In our cases, no overexpression of HER2/neu was observed. Thus, our findings suggested that the use of Her-2 as a salivary marker of HNSCC cannot be recommended.

Keywords: HER2/neu - salivary level - tissue expression - head and neck squamous cell carcinoma

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Introduction

Squamous cell carcinoma is the most common head and neck cancer (Patel and Shah, 2005). SCC has a focal invasion and its behavior depends on the region that it originates. Each anatomic area has its own growth patterns and prognosis (Anthony et al., 2008).

Head and neck squamous cell carcinoma (HNSCC) has been had a challenging treatment for a long time because of the high rates of recurrence and its advanced disease at the time of diagnosis. Molecular identification of tissue biomarkers in diagnostic biopsy specimens may not only identify patients at risk for developing of HNSCC but it may also select patients that benefit from more aggressive treatment modalities (Thomas et al., 2005).

The HER2/neu (ErbB) protein or epidermal growth factor receptor (EGFR) is a family of four structurally related receptor tyrosine kinases (NDBI official site). The c-erbB-2 proto-oncogene (HER/NEU/neu) encodes a 185 transmembrane protein product of tyrosine kinase family, with an extensive homology to the epidermal growth factor receptor, which has been mapped to the 21 region of chromosome 17 (Albuquerque et al., 2003) and can be activated by hetero oligomerization with the other members of the ErbB family (Silvaa et al., 2004).

Activation of EGFR family (HER2/neu) by a variety of ligands is necessary for normal growth and differentiation (Seifi et al., 2009). Increased levels of receptor ligands, co expression of EGFR mutants, and cross-talk with HER2 or other receptors are mechanisms that can enhance EGFR signaling output and potentially alter the response to EGFR inhibitors (Arteaga, 2002). The dysregulation of these receptors is linked to multiple features of malignant tumors, including a loss of cell cycle control, resistance to apoptotic stimuli, invasiveness, chemo-resistance, and the induction of angiogenesis (Salem et al., 2006; Woodburn JR, 1999).

The use of targeted agents against molecular markers belonging to the epidermal growth factor receptor (EGFR) family has recently become integrated into the treatment protocols of many malignancies, such as breast cancer (Ciardiello, 2001; Crombet et al., 2004; Soulieres et al., 2004; Cavalot et al., 2007; Radpour et al., 2009). Despite recent improvements in the diagnosis and treatment of cancer, there are still many difficulties in evaluating the prognosis of the head and neck carcinomas. Thus, an

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attempt to find more significant information to predict the biological behavior of this neoplasm and to look at the possible relationship between the tumoral progression and the products of genes which regulate cell proliferation and differentiation, such as the proto-oncogenes, anti oncogenes and apoptosis-regulating genes, has been reported recently.

However, there is a doubt over the prognostic significance of oncogene EGFR in these tumors, and thus its utility as a target of new therapy is still unclear (Xia et al., 1999; Shiga et al., 2000; Werkmeister et al., 2000; Camp et al., 2005).

Thus, the aim of this study was primarily to examine the expression of Her2/neu in human normal oral epithelium and HNSCC cases to validate the controversial results of various studies and to determine whether or not Her2/neu could be considered as a useful marker for head and neck cancer. Although we have investigated salivary levels of Her2/neu in healthy and HNSCC patients and also compared them with tissue expression of protein.

Materials and Methods

In this study, 28 cases of HNSCC (22 males and 6 females, 57.96±12.39 years of age) registered in Khalili and Chamran Hospital affiliated to Shiraz University of Medical Sciences and 25 healthy persons consist of 16 males and 9 females with 56.85± 13.41 years of age, were evaluated. Case and control groups were matched by the age and sex. Patients and controls who showed signs of significant morbidity, active medical problems, and any systemic or inflammatory disease were excluded from the study. Salivary samples were taken, before any surgical procedures or chemotherapy protocols. All subjects were informed about the research and agreed to participate on the study by signing the free and informed consent form. Patients with a histopathological diagnosis of HNSCC with enough tissue sample size in which their H & E stained slides were evaluated and 25 normal oral epithelial tissue , were enrolled in the research. Clinical data, such as age, gender, location of the tumor, TNM, and tobacco habits were obtained from medical records.

Saliva Collection and Analysis

Saliva Collection, prior to the collection of unstimulated whole saliva, subjects were asked to refrain from eating, drinking, and smoking or oral hygiene procedures for 30 minutes. The lip area was cleaned and each subject rinsed its mouth once with plain water. Typically, patients donated 5-10 ml of saliva. Samples were then centrifuged at 2,600 ×g for 15 minutes at 4ºc. The supernatant then stored at -80ºc until use(Bernardes et al., 2010). Salivary protein levels were measured by sandwich ELISA, in accordance with the procedures recommended by the manufacturer (BMS 207: Bender Med System GmbH, Germany).

Immunohistochemistry (IHC) Method, sections with 4 μ thick, were mounted on positive charged microscope slides. After dewaxing in xylene, sections were dehydrated in ethanol and rinsed in distilled water. Antigen retrieval was performed by DAKO Target Retreival solution (DAKO, Carpinteria, CA). The endogenous peroxidase was quenched by 3% H_2O_2. The peroxidase-labeled polymer conjugated to goat antimouse HER2 method was used to detect antigen-antibody reaction (DAKO EnVision_ System; DAKO Corporation, Carpinteria, CA).

Antibodies were made at the room temperature for 1 hour at the HER2 (ErbB 2 antibody ab2428, DAKO Corporation, Denmark) 1:200 dilution. Sections were then visualized with 3,3_diaminobenzidine as a chromogen for 5 minutes and counterstained with Harris's hematoxylin. Slides were washed in tap water, dehydrated, and mounted with glass coverslips. Positive controls were the sections of breast cancer tissue which were previously found to be positive for the HER2/neu. The negative controls consisted of duplicated sections of the same specimens in which the primary antibody had been excluded and replaced with PBS.

Immunohistochemical analysis

Representative tumor sections were identified on a light microscope. All slides were observed by two pathologists, separately, who scored the immunostainings twice to decrease intra-observer variability, in a blinded fashion. In unmatched cases, slides were evaluated again by both pathologists using a multiheaded microscope. Randomly ten fields were chosen for each section and the total numbers of positive cells for all 10 examined fields were counted and the percentage of staining was calculated (Seifi et al., 2009). Membranous and/or cytoplasmic staining were regarded as positive. The percentage of membranous and/or cytoplasmic stained tumor cells were calculated separately for each section. Tumors with more than 1% of tumor cells staining for HER2/neu were considered positive .Percentage of positivity ranged from 1% to 100% (Sugano et al., 1992; Albanell et al., 2001). All immune-stainig assessments were blinded to the clinical data.

Statistical analysis

Mann-Whitney test, Independent t-test, Chi-Square and fisher tests were used for analysis the result. Significant level for tests was 0.05.

Results

Clinical data of HNSCC patients of this study for HER2/neu evaluation were presented in Table 1.

All normal oral epithelum were membranous and cytoplasmic positive for HER2/neu. Cytoplasmic staining was limited in basal and parabasal layers in normal epithelium (Figure 1).

All HNSCC specimens were positive (membranous or/and cytoplasmic) for HER2/neu, except one sample (Figure 2 and 3). Only one HNSCC specimen was stained in cytoplasm purely.

The mean percentage of membranous staining in HNSCCs was 63.04±30.77 and in normal epithelium was 66.40±16.36. There was no significant difference in membranous staining percentage between case and control groups. (P-value= 0.494)

The mean percentage of cytoplasmic staining in

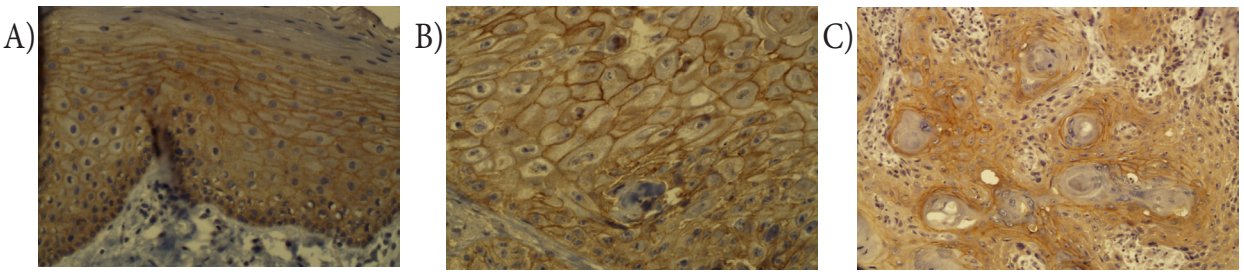

Figure 1. A) Cytoplasmic Staining in Basal and Parabasal Layers of Normal Epithelium B) Membranous Staining in Squamous Cell Carcinoma (×400) C) Cytoplasmic and Membranous Staining in Squamous Cell Carcinoma

Table 1. Clinico-Pathological Profile of 28 Head & Neck SCC Patients

Age (years)			57.96±12.3	
Gender	Male		19	(67.8%)
	Female		9	(32.2%)
Tumor size	T1		0	(0%)
	T2		28	(100%)
	T3		0	(0%)
Regional lymph node involvement		N0	24	(85.7%)
		N1	4	(14.3%)
		N2	0	(0%)
		N3	0	(0%)
Distant metastasis	M0		28	(100%)
	M1		0	(0%)
TNM stage	I		0	(0%)
	II		24	(85.7%)
	III		4	(14.3%)
	IV		0	(0%)
Histological grade	I (well-diff.)		8	(28.6%)
	II (moderately- diff.)		17	(60.7%)
	III (poorly-diff.)		3	(10.7%)
Larynx			22	(78.5%)
Oral cavity			6	(21.5%)

HNSCCs was 48.64±29.12 and in normal epithelium was 13.2±6.7. There was a significant difference in cytoplasmic staining percentage between case and control groups. (P-value<0.001)

There was no correlation between percentage of Her-2 staining and clinicopathologic factors.

There was a high tendency to cytoplasmic staining around keratin pearls in well-differentiated HNSCC samples.

The mean salivary level of HER2/neu in HNSCC patients was 3.12±4.58 ng/ml and for the control group was 13.2± 6.75ng/ml. There was no significant difference in salivary concentrations of HER2 between the case and the control groups (P-value= 0.494).

There was no significant correlation of salivary levels with immune-staining percentage of HNSCC patients, and also there was no significant correlation of salivary levels with clinic-pathological data of patients. (P-value> 0.05) Salivary levels had a reverse relation with cytoplasmic staining percentage in HNSCC patients, but there was no significant correlation. (co.co.= - 0.025, P-value=0.898)

Discussion

We studied HER2/neu expression in HNSCC patients and its correlation with salivary levels of this marker. Although a lack of specificity of tumor markers and lack of sensitivity of testing systems have been noticed which is limited their clinical use, finding biomarkers for cancers could allow physicians to identify individuals who are susceptible to certain types and stages of cancer to tailor preventive and therapeutic modalities based on the genotype and phenotype information. These biomarkers should be cancer specifically, and sensitively detectable in a wide range of specimen(s) containing cancer-derived materials, including body fluids (plasma, serum, urine, saliva, etc.), tissues, and cell lines(Albuquerque et al, 2003).

The amplification of the HER2 gene has been demonstrated in many carcinomas of glandular origin, and its immunohistochemical expression has been proved to be closely associated with its amplification level (Sugano et al., 1992). The amplification and consequently over expression of the HER2 gene as well as its relationship with tumorogenesis was first reported by Schechler et al. (1985) in neuroglioblastomas of rats .

The dysregulation of these receptors is linked to multiple features of malignant tumors, including a loss of cell cycle control, resistance to apoptotic stimuli, invasiveness, chemo-resistance, and the induction of angiogenesis (Woodburn, 1999).

Reports on the role of HER2/neu proto-oncogene product in HNSCC are less conclusive than EGFR(HER1), as HER2/neu has been described to be over expressed in a very few to all the investigated HNSCC specimens and as correlations with clinical parameters are controversial(Kearsley et al., 1991; Reviere et al., 1991; Craven et al., 1992; Field et al., 1992).

In our study, all samples except one expressed HER2/neu and there was no significant difference between staining of the case and the control specimens. All non-tumoral and 96.4% of SCC samples were cytoplasmic and/or membranous positive. One of SCC specimens was pure cytoplasmic positive and another one was negative for marker.

Thus, we found no over expression of HER2/neu in HNSCC tissues. This implies that abnormal expression and over expression of HER2/neu could not play a role in carcinogenesis process of HNSCC. This result is in accordance with some studies that found no HER2/neu over expression in HNSCC cases (Field et al., 1992; Mort et al., 1993; Ekberg et al., 2005; Angiero et al., 2008).

On the other hand, some studies have reported over expression of HER2/neu as a potential useful marker in distinguishing non-cancer from cancer tissue (Lebeau et al., 2001; Fong et al., 2008; Cavalot et al., 2009) suggested, there are dynamic changes in HER2/neu expression in

head and neck carcinogenesis process.

Wilkman et al. (1998)reported an increase in HER2/neu expression during the sequence from normal mucosa to hyperkeratosis and to dysplasia and head and neck squamous cell carcinoma.

In our study there was no difference in membranous staining percentage of HER2/neu in the case and the control groups but there was significant difference of cytoplasmic staining between the case and the control groups. It should be mentioned that cytoplasmic staining in normal epithelial specimens were specified to basal and parabasal layers.

In various studies purely membranous (Craven et al., 1992; Hoffmann et al., 2001; Khademi et al., 2002; Kuropkat, 2002) or cytoplasmic (Craven et al., 1992; Field et al., 1992; Angiero et al., 2008; Khademi et al., 2002), and mixed membranous-cytoplasmic (Kearsley et al., 1991; Craven et al., 1992; Xia et al., 1997; Ibrahim et al., 1997; Xia et al., 1999; Bei et al., 2004; Rautava et al., 2008) expression have been reported.

In squamous cell carcinomas, cytoplasmic staining has been widely reported; however, its interpretation is not clear at present. It has been argued that cytoplasmic staining may be a technical artifact due to cross-reactive antibodies possibly with keratin or antigen retrieval (Cavalot, 2007).

Others, however, propose that it may be represented true protein over expression (37, 40), probably due to incomplete receptor degradation (Ibrahim et al., 1997).

Some of the antibodies such as CB11 has a tendency to cytoplasmic staining, thus some manufacturers suggest that the pure cytoplasmic stained samples should be designated as negative (Hsu et al., 2002).

However, the importance of cytoplasmic staining and whether or not it may be evaluated when determining Her-2 expression in OSCC/ HNSCC is controversial. In our study we evaluated cytoplasmic and membranous staining separately to make it clear and found significant difference between cytoplasmic immune-reactivity between the case and the control groups.

In the literature, the percentage of HER-2 / neu positivity in HNSCC is extremely variable. It is possible that the discrepancies in the results may be attributed to the initial lack of standardization of the assay methods (Cavalot et al., 2007).

Another reason for controversial results in different studies might be due to use of different immunohistochemical methods (direct, indirect), type of antibody (clone CerbB2, CB11,ICR1b, polyclonal DAKO,monoclonal zymed), no specific criteria for positive staining of HER2/neu protein (membrane and/ or cytoplasmic) and /or using different techniques (immunosorbent assay, radioimmunoassay, IHC) or different locations of lesions and sex of patients with HNSCC.

There was no significant correlation between HER2/neu expression and age, gender, tumor size, lymph node and distant metastasis, tumoral stage and histologic differentiation in our study.

Xia et al. (1997) found a strong correlation between cerbb2 over expression and overall survival of OSCC patients .

Quon et al. by a review study in 2001 indicated high expression levels of EGFR and HER2/neu as prognostic markers which correlated with poor clinical outcome in HNSCC patients (Quon et al., 2001). By contrast, in a case review study in 2009 by Tse GM et al. HER2 was associated with longer survival in node-positive patients (Tse et al., 2009).

Some studies had evaluated correlation of clinicopathologic data such as tumoral stage and HER2/neu expression.

In 2008, Fong et al. found that Her2/neu expression was significantly higher in the advanced stage IV cases than the stage I-III cases (Fong et al., 2008).

One of our limitations was the lack of stage IV in HNSCC cases. So we could not judge on level of tumor stage and HER2/neu expression.

In our study, there was a prominent cytoplasmic staining around keratin pearls in well-differentiated samples but there was no significant correlation of the HER2/neu cytoplasmic staining with the tumor grade.

In recent years, increasing interest has developed in the use of saliva as an adjunct test medium to help in conventional medical assessment of serious systemic diseases (Mandel, 1993; Streckfus et al., 2002). Due to its simplicity in collection, saliva may be collected repeatedly with minimal discomfort to the patient. Noninvasive process (i.e., no needle punctures), thereby rendering saliva as a very desirable diagnostic medium. More importantly, saliva contains constituents that are frequently altered in the presence of systemic diseases (Kaufman, 2002; Lawrence et al., 2002). Because of these significant characteristics, detection of serious systemic illnesses, such as cancer, by salivary biomarkers finding is the great interest for most salivary researchers (Mande, 1993).

The first biomarker for cancer found in saliva is HER2/neu, a biomarker for the breast cancer (Streckfus et al., 2007).

Recent studies have exhibited the existence of shed or solubilized forms of HER2/neu (Crombet et al., 2004; Semba et al., 1985). The exact method for the release of soluble truncated HER2/neu and the full-length transmembrane protein is not known (Breuer et al., 1998).

In our study, there was no significant difference of HER2/neu salivary levels between HNSCC and control groups. HER2/neu salivary level in HNSCC patients was lower than control group but there was no significant difference. Also, there was an adverse relation between salivary levels of this marker and cytoplasmic staining percentage.

One of the reasons of lower HER2/neu salivary levels in case group could be high cytoplasmic staining of this marker in HNSCC specimens, which causes decreased membranous transportation of this marker to saliva. This adverse relationship is a proof for this claim.

In this study there was no significant correlation of salivary level of HER2/neu with clinicopathological data of patients. Briefly, we found no higher existence

of HER2/neu protein in saliva in more progressive or aggressive lesions.

In a same study in 2010, Bernardes et al., such as our study , detected no significant association between the salivary levels of the proteins and clinicopathological data, such as patient age, site, histological grading, T status, nodal involvement of the tumor or stage. In addition, there was no difference in Her-2 salivary levels between pre-surgery and healthy control groups, however, both showed increasing levels after surgical removal of the tumor (Bernardes et al., 2010).

In our cases, no overexpression of HER2/neu was observed. Thus, it plays no role to differentiate between normal and squamous cell carcinoma tissues and carcinogenesis process.

Our findings suggested that the use of Her-2 as salivary markers of HNSCC is not recommended because no significant preoperative elevation and no association to clinicopathological features were found.

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