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Expression Difference of EGFR, Ki67 and p-EPK in Oral Cavity Squamous Cell Carcinoma

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Abstract: The aims of this study were to evaluate the expression of EGFR, Ki67 and p-EPK in the oral cavity and oropharyngeal cancers and to investigate their clinical significance as prognostic markers. The 100 patients who underwent curative surgery for oral cavity or oropharyngeal squamous cell carcinoma in Renmin hospital of Beijing University between March 1999 and October 2010 were evaluated. The level of protein expression of EGFR, Ki67 and p-EPK was assessed by immunohisto chemistry. *In situ* hybridization was used to detect the existence of Human Papillomavirus (HPV). Nineteen of 75 patients with oropharyngeal cancer showed HPV-positive tumors and two of 72 patients with oral cavity cancer showed HPV-positive tumors. EGFR and Ki67 expression was significantly higher in oral cavity cancers than in oropharyngeal cancers. Loss of p-EPK occurred significantly more frequently in oral cavity cancers than in oropharyngeal cancers. Over expression of EGFR and Ki67 and loss of p-EPK were observed more frequently in HPV-negative tumors. Multivariate Cox regression analysis showed that Ki67 expression had a significantly unfavorable impact on relapse-free survival in oropharyngeal cancer. Researchers conclude that the expression levels of EGFR, Ki67 and p-EPK differ between oropharyngeal and oral cavity cancer and it may be attributed to HPV-related molecular pathogenesis. The expression of Ki67 might be an unfavorable prognostic marker for relapse-free survival in oropharyngeal cancer.

Key words: EGFR, Ki67, p-EPK, oral cavity squamous cell carcinoma, expression difference

INTRODUCTION

Mucosal Squamous Cell Carcinoma of the Head and Neck (SCCHN) comprises a heterogeneous group of tumors arising from the epithelial lining of the oral cavity, pharynx and larynx. Despite distinct clinical characteristics, Oral Cavity Cancer (OSCC) and Oropharyngeal Cancer (OPSCC) are frequently described as part of a group of oral cancers and the two disease entities are often confused in the literature. OSCC includes the lips, buccal mucosa, teeth, gums, anterior two-thirds of the tongue, floor of the mouth and hard palate. OPSCC includes the base of the tongue, soft palate, tonsils and the lateral and posterior pharyngeal walls. In recent decades, studies have shown that OSCC and OPSCC have different incidence rate trends, etiology and survival outcome (Auluck *et al*., 2010). For instance, Human Papillomavirus (HPV) is known as an important etiology for OPSCC, especially tonsil cancer. However, there is little research on the differences in molecular pathogenesis between the two diseases. Aber-rant Epidermal Growth Factor Receptor (EGFR) affects cell cycle progression, apoptosis, angiogenesis and metastasis. The EGFR is over expressed in about 90% of all SCCHNs and its over expression correlates with poor prognosis (Machiels and Schmitz, 2011). The Phosphatidylinositol 3-Kinase (PI3K)/Akt pathway is important for cell survival because it promotes cell cycle progression and inhibits apoptosis (Morgensztern and McLeod, 2005). Mammalian Target of Rapamycin (mTOR) a serine/threonine kinase and a downstream target of Akt is important for the oncogenic transformation induced by PI3K and Akt. Signaling through the PI3K/Akt/mTOR pathway is activated by growth factor ligand binding to receptor tyrosine kinases and regulates several cellular functions that are critical for tumorigenesis. p-EPK, a

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tumor suppressor gene, negatively regulates the PI3K/Akt pathway. The loss of p-EPK function causes increased Ki67 activity and continued cell survival and proliferation (Nagata *et al*., 2004). In this study, researchers evaluated the expression of EGFR, Ki67 and p-EPK in OSCC and OPSCC and investigated their clinical significance as a prognostic marker.

MATERIALS AND METHODS

Patients: The 100 patients who underwent curative surgery for OPSCC or OSCC in Renmin Hospital of Beijing University between January 1995 and September 2009 were evaluated. Among the 100 patients, 51 (51.0%) had been diagnosed with OPSCC and 49 (49.0%) with OSCC. Clinical records and pathology reports were reviewed retrospectively. The following clinical data were collected: age, sex, smoking history, tumor staging, surgery type, chemotherapy, radiotherapy, recurrence and survival. Ethical committee approval was obtained from the Institutional Review Board of Beijing University. Informed consent was provided according to the Declaration of Helsinki.

Tissue microarray and immunohistochemical staining: To construct the tissue microarray block, tissue cylinders with a diameter of 2.0 mm were taken from non-necrotic, morphologically representative areas of paraffin-embedded tumor tissues. Duplicate tissue cores from each specimen were assembled on a recipient paraffin block using a manual tissue arrayer. After construction, 4 lm sections were cut and stained with hematoxylin-eosin staining on the initial slide for histological verification. Immunohistochemical staining was done on the 4 lm sections of the tissue microarray blocks. Paraffin sections were deparaffinized in xylene and rehydrated in serial graded ethanol. Endoge-nous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. A ntigen retrieval was performed by boiling the slides in citrate buffer (0.01 mol L^{-1}, pH 6.0) in a pressure cooker at 125°C for 15 min. The following primary antibodies were used: mouse monoclonal anti-EGFR (Ventana Medical Systems, Inc., Tucson, AZ, USA) rabbit polyclonal anti-PIK3CA (Atlas Antibodies, Stock-holm, Sweden) diluted 1:150, rabbit monoclonal anti-phosphor-Akt1 (pS473) (Epitomics, Burlingame, CA, USA) diluted 1:1200, mouse monoclonal anti-p-EPK (DAKO Corporation, Carpinteria, CA, USA) diluted 1:100 and p53 (DAKO Corporation, Carpinteria, CA, USA) diluted 1:100. Sections were then counterstained with Mayer's hematoxylin and dehydrated, cleared and mounted. The results were interpreted by an independent pathologist who was blinded to the specific diagnosis and prognosis for each case. A modified semi-quantitative scoring system adopted from previous studies was applied. The Percentage of Positive tumor cells (PP) was scored as follows: 0: no tumor cells stained; 1: 1-5% of cells stained; 2: 6-20% of cells stained; 3: 21-50% of cells stained and 4: >50% of cells stained. The intensity of Staining (SI) was scored as follows: 0: no staining; 1: weak staining; 2: moderate staining and 3: strong staining. The Immunoreactive Score (IRS) was calculated by multiplying the percentage of positive cells (PP, scored 0-4) by Staining Intensity (SI, scored 0-3). Tumors with an IRS P1 were considered positive for EGFR expression (Fig. 1). The 5 and 6 to quantify the expression of p-EPK, tumors were classified using IRS P9 as the criterion for p-EPK expression according to the Nagata score (Fig. 1). The average PP was scored as follows: 0: no tumor cells stained; 1: 1-50% of cells stained; 2: >50% of cells stained. The Total Score (TS) was calculated by summing the PP and SI. A TS of 3-5 was regarded as positive (Fig. 1).

***In situ* hybridization for HPV:** *In situ* hybridization was processed on the automated Benchmark System from Ventana Medical Systems using INFORM HPV III Family 16 Probe (cocktail of HPV subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 66, Ventana Medical Systems) as per the manufacturer's recommendations. This system removes the paraffin wax from the tissue, subjects it to protease digestion and then hybridizes the tissue with a probe. The probe-target complex is detected because of the action of alkaline phosphatase on the chromogen nitroblue tetrazolium and bromochloroindolyl phosphate which yields a dark-blue color with a pink counterstain for the HPV-negative cells caused by nuclear fast red staining. The signal patterns of HPV in the nuclei were classified as follows: diffuse, signals that were condensed and uniformly packed in the nucleus; punctate, signals that were dot-like and distributed sparsely in the nucleus (Fig. 2).

Statistical analysis: Overall Survival (OS) was defined as the time from the date of diagnosis to the date of death or last follow-up. Relapse-Free Survival (RFS) was defined as the time from surgery to disease recurrence. Continuous and categorical variables were compared using Student's t-test and the χ^2-test. Univariate analysis and survival curves were estimated using the Kaplan-Meier Method and the log-rank test was applied to identify differences. Multivariate analysis was performed with the Cox Hazards Regression Model. All statistical analyses were performed using the SPSS program (Version 18.0) and $p<0.05$ was considered significant.

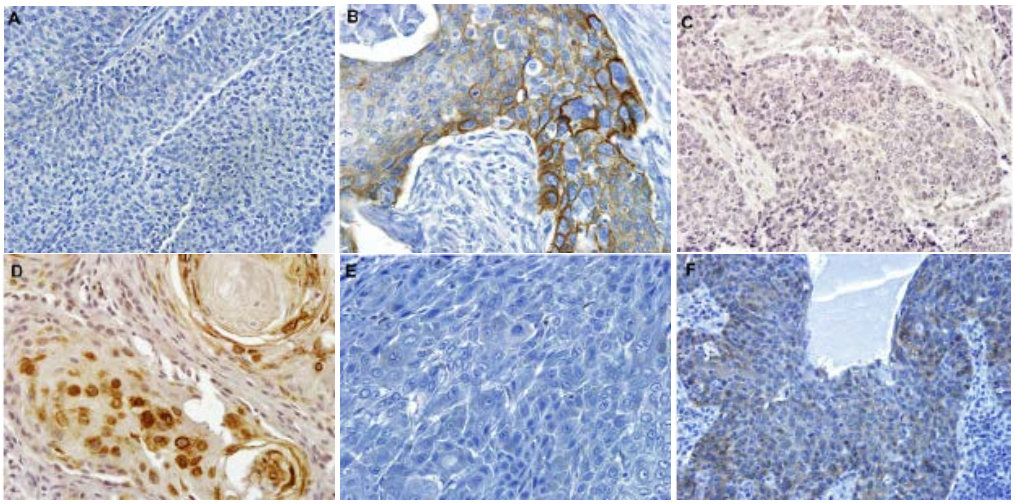

Fig. 1: Immunohistochemical staining for EGFR, Ki67 and p-EPK: A) no staining for EGFR in oropharyngeal squamous cell carcinoma; B) positive membranous staining for EGFR in oral cavity squamous cell carcinoma; C) no staining for Ki67 in oropharyngeal squamous cell carcinoma; D) positive cytoplasmic staining for Ki67 in oral cavity squamous cell carcinoma; E) loss of p-EPK in oral cavity squamous cell carcinoma and F) positive cytoplasmic staining for p-EPK in oropharyngeal squamous cell carcinoma (x400)

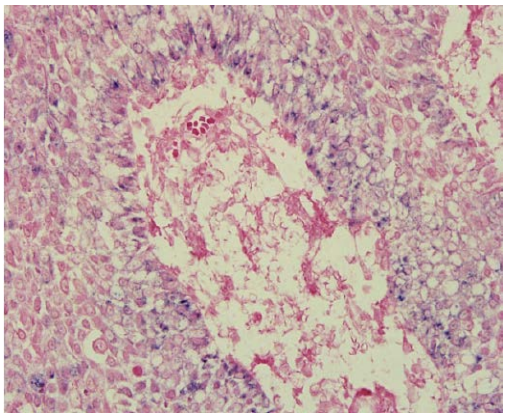

Fig. 2: *In situ* hybridization for human papillomavirus with punctate nuclear staining pattern in oropharyngeal squamous cell carcinoma (x400)

RESULTS

Patient characteristics: The clinicopathological characteristics of the 100 patients are summarized in Table 1. The mean age of the patients was 52.8 (range 25-90) years. Among the 51 OPSCC patients, 48 (78.7%) patients had tonsil cancer, nine (14.7%) patients had cancer of the base of the tongue, two (3.3%) patients had soft palate cancer and two (3.3%) patients had pharyngeal wall cancer. Among the 49 OSCC patients, 51 (85.0%) patients had tongue cancer, five (8.3%) patients had buccal mucosa cancer and four (6.7%) patients had cancer of the floor of the mouth. The

Table 1: Clinicopathological characteristics of oropharyngeal and oral cavity cancer patients

	No. (%)			
Characteristics	All patients (n = 100)	OPSCC (n = 51)	OSCC (n = 49)	p-values
Age (years)				
Mean±SD	52.8±9.4	53.5±8.7	52.4±11.2	0.608
Sex	-	-	-	0.001
Men	72 (72.0)	48 (94.1)	36 (73.5)	-
Women	28 (28.0)	3 (5.9)	13 (26.5)	-
Smoking	-	-	-	0.672
Former or current	71 (71.0)	32 (62.7)	32 (65.3)	-
Non-smoker	29 (29.0)	19 (37.3)	17 (34.7)	-
HPV status	-	-	-	0.001
Positive	25 (25.0)	16 (31.4)	4 (8.3)	-
Negative	75 (75.0)	35 (68.6)	45 (91.8)	-
Histologic grade	-	-	-	0.001
Well	31 (31.0)	10 (20.0)	28 (57.1)	-
Moderate	56 (56.0)	32 (62.7)	15 (30.6)	-
Poor	13 (13.0)	8 (17.3)	7 (14.3)	-
T stage	-	-	-	0.057
<2 cm	32 (32.0)	10 (20)	21 (42.9)	-
»2 cm, <4 cm	59 (59.0)	33 (64.7)	24 (49.0)	-
»4 cm	9 (9.0)	7 (15.3)	5 (10.2)	-
N stage	-	-	-	0.003
N0	51 (51.0)	9 (17.6)	27 (55.1)	-
N1	13 (13.0)	8 (15.7)	8 (16.3)	-
N2a	3 (3.0)	3 (5.9)	2 (4.1)	-
N2b	27 (27.0)	14 (27.5)	9 (18.4)	-
N2c	6 (6.0)	16 (31.4)	4 (8.2)	-
Treatments				
Surgery alone	36 (36.0)	14 (27.5)	24 (49.0)	0.028
Adj RT	31 (31.0)	21 (41.2)	14 (28.6)	0.079
Adj CCRT	22 (22.0)	10 (19.6)	7 (14.3)	0.459
Adj CTx	11 (11.0)	5 (9.8)	5 (10.2)	0.321

Table 2: Immunohistochemical staining for EGFR, Ki67 and p-EPK

	No. (%)			
Expressions	All patients (n = 100)	OPSCC (n = 51)	OSCC (n = 49)	p-values
EGFR	-	-	-	0.006
Positive	63 (63.0)	28 (54.9)	31 (63.3)	-
Negative	37 (37.0)	23 (45.1)	18 (36.7)	-
Ki67	-	-	-	0.004
Positive	12 (8.9)	21 (17.9)	25 (26.6)	-
Negative	85 (91.1)	18 (56.7)	22 (47.5)	-
p-EPK	-	-	-	0.201
Positive	64 (65.6)	32 (74.2)	24 (47.2)	-
Negative	29 (34.4)	18 (20.8)	17 (31.6)	-

percentage of men was higher in those with OPSCC (93.4%) than in those with OSCC (63.3%) (p = 0.001). Nineteen (31.1%) of 61 patients with OPSCC showed HPV-positive tumors and only two (3.3%) of 60 patients with OSCC showed HPV-positive tumors (p = 0.001). Well-differentiated histological type was more frequent in OSCC than in OPSCC (p = 0.001). Cervical lymph node metastasis with advanced N stage was more frequent in OPSCC than in OSCC (p = 0.002). Adjuvant therapy after surgery was followed more frequently in OPSCC than in OSCC (p = 0.031). Age, smoking history and T stage did not differ significantly between patients with OPSCC and OSCC.

Immunohistochemical staining and correlation analysis: The results of immunohistochemical staining are listed in Table 2. EGFR and Ki67 expression was significantly higher in OSCC than in OPSCC (p = 0.005 and p = 0.001, respectively) (Fig. 1). Loss of p-EPK was more frequent in OSCC than in OPSCC (p = 0.004) (Fig. 1). Over expression of EGFR and Ki67 was observed more frequently in HPV-negative tumors than in HPV-positive tumors (p = 0.003 and p = 0.037, respectively). Loss of p-EPK was observed more frequently in HPV-negative tumors than in HPV-positive tumors (p = 0.001). Ki67 over expression and loss of p-EPK occurred more frequently in tumors with well-differentiated histology (p = 0.003 and p = 0.006, respectively). Researchers analyzed the individual relationships between EGFR, Ki67 and p-EPK expression. p-EPK and Ki67 expression correlated inversely (p = 0.032). Other variables did not correlate significantly.

DISCUSSION

Carcinomas of the oropharynx and oral cavity constitute 2-5% of SCCHNs. In the past, OPSCC and OSCC were considered types of oral cancers with the same etiology including smoking and alcohol abuse. However, there is growing evidence to support the idea that OPSCC and OSCC are two distinct diseases with different Etiologies (Marur and Forastiere, 2008). Since, the 1980s, evidence has been emerging that HPV is an etiological factor for a subset of OPSCC (Marur and Forastiere, 2008; Kim *et al*., 2010). Previous studies reported that the rate of HPV positivity is higher in OPSCC, especially tonsil cancer than in OSCC (Laco *et al*., 2011). The result is consistent with these previous results: about 30% of OPSCC patients showed HPV positivity in contrast to 3% of OSCC patients.

Researchers compared the expression of the EGFR and Ki67/p-EPK pathway which is crucial for cell survival and growth in OPSCC and OSCC patients. EGFR and Ki67 expression was significantly higher in OSCC than in OPSCC and loss of p-EPK occurred more frequently in OSCC than in OPSCC. The correlation analysis between the expression of these proteins and HPV status showed that overexpression of EGFR and Ki67 and loss of p-EPK were observed more frequently in HPV-negative tumors. Therefore, these differences in EGFR, Ki67 and p-EPK expression between OPSCC and OSCC may be attributed to HPV-related molecular pathogenesis.

Previous studies showed that Ki67 expression is associated with poor outcome in OPSCC and OSCC. Massarelli *et al*. (2005) reported that tongue cancer patients with Ki67 expression showed significantly shorter disease-free survival. Yu *et al*. (2007) reported that OPSCC patients with a low Ki67 level had a lower 5 years local recurrence rate and better 5 years overall survival rate. However, research on the relationship between Ki67 expression and HPV status is limited. Researchers found that Ki67 over expression was significantly lower in HPV-positive tumors than in HPV-negative tumors. The analysis of RFS showed that T and N stages were only meaningful as a prognostic factor in OSCC patients. Meanwhile, OPSCC patients with Ki67 over expression tended to have a <3 years RFS rate and in patients with tonsil cancer, the overexpression of Ki67 was associated with significantly shorter RFS. These results suggest that Ki67 over expression might be a prognostic marker for RFS in OPSCC patients.

CONCLUSION

The expression levels of EGFR, Ki67 and p-EPK differed significantly between OPSCC and OSCC patients. These results may be associated with HPV-related pathogenesis. The results also suggest that Ki67 may be a prognostic marker for RFS in OPSCC patients, especially in those with tonsil cancer. Further, studies are needed to identify more clearly the role of the EGFR and the Ki67/p-EPK pathway in HPV-related OPSCC.

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