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Unaltered mRNA Levels of EGFR and PIK3CA in Head and Neck Squamous Cell Carcinoma

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ABSTRACT

Background: Since its identification, the Epidermal Growth Factor Receptor (EGFR) signaling pathway through PIK3CA has been known to be involved in the pathogenesis of several solid tumors, especially Head and Neck Squamous Cell Carcinoma (HNSCC). Impaired signal transduction through EGFR-PIK3CA may give rise to oncogenic processes because of its key role in the regulation of major cellular functions. Several oncogenic mechanisms mediated by unregulated EGFR signaling pathways have been suggested by previous studies including mutations in genes encoding EGFR and PIK3CA and increased ligands of EGFR. Here, we aimed to compare the total gene expression of EGFR and PIK3CA at the mRNA level between HNSCC tissues and normal squamous cell tissues.

Methods: In this pilot study, we examined 31 samples of tumor-infected squamous cell tissues as well as 31 samples of healthy tissues around the tumor as controls for the expression of EGFR and PIK3CA genes using quantitative polymerase chain reactions (Q-PCR).

Results: both EGFR and PIK3CA mRNA levels were slightly altered in HNSCC tissues compared with the control group (3% decrease and 9% increase, respectively), but these differences were not statistically significant.

Conclusion: our results indicate that total EGRF and PIK3CA expression at the mRNA level is not altered in Head and Neck Squamous Cell Carcinomas compared with that of normal squamous cell tissues.

Keywords: HNSCC; EGFR; PIK3CA; Carcinoma

INTRODUCTION:

Head and neck carcinoma is the eighth leading cause of cancer death among the human population, and more than 90% of them arise from squamous cells [1] which are called Head and Neck Squamous Cell Carcinoma (HNSCC). HNSCC can originate from mucous membranes of the mouth, nose, and throat, and is responsible for a mortality rate is 3.7 and 2.3 per 100,000 for men and women, respectively [2]. Alcohol and tobacco are known risk factors for head and neck cancer, especially oral cavity cancer. Also, HPV (Human Papillomavirus) is another risk factor for certain cancers of the head and neck [3, 4].

In the last decades, by identification of signal transduction pathways, epidermal growth factor receptor (EGFR) signaling has been introduced as a key factor in the development of several solid tumors [5]. As a membrane-spanning glycoprotein receptor, EGFR regulates many cellular functions, including cell proliferation, cell survival, and metabolism, through activation of a phosphorylation network mediated by several downstream molecules such as PI3-K, Akt, and PTEN [6]. Several mechanisms have been suggested to be involved in the pathogenesis of HNSCC, and among them, unregulated signal transduction through the EGFR-PIK3CA pathway is studied broadly, and some therapeutic agents have emerged based on the context of EGFR biology [7]. Regarding the critical role of EGFR-PIK3CA tyrosine kinase cascade in the regulation of cellular processes, impaired EGFR activity can give rise to tumorigenic events and malignant alterations such as resistance to apoptosis, and cell invasion leading to metastasis. Various molecular mechanisms have been suggested by which impaired activity of the EGFR tyrosine kinase cascade results in tumorigenic responses. These oncogenic mechanisms for EGFR-PIK3CA signaling include PIK3CA mutations, EGFR mutations resulting in constitutive receptor activity, increased EGFR ligands, and increased EGFR expression due to either enhanced transcription or increased copy number of the gene [8]. Also, impaired EGFR signaling is associated with down-regulation of common tumor suppressors in HNSCC, such as the TP53/P73 family [9]. Mechanisms leading to oncogenic alterations are often specific to the type and origin of the tumors, but since tumorigenesis is a multi-step and multi-factorial process, more than one oncogenic mechanism could be involved in the pathogenesis of cancer. In the present study, our main question was whether the expression of EGFR and PIK3CA in mRNA levels is different in HNSCC tissues compared with normal squamous cells. Therefore, we aimed to compare the gene expression of the mentioned proteins in HNSCC and normal squamous cell tissues.

Materials and methods:

Patients and controls

31 samples of tumor-infected squamous cell tissues and 31 control samples from healthy tissues were included in the present study. The age, sex, and stage of the disease were identified for each cancer case. Accordingly, the number of male and female samples was 20 and 11, respectively. Within all collected specimens, 11 out of 31 samples were in Grade I, 16 samples were in Grade II, and 4 of them were in Grade III. Also, the average age of the selected patients was 62 years. More complete information and the characteristics of the patients are shown in Table 1.

Sample collection

At least 50 mg of fresh samples were obtained from 31 patients who were previously diagnosed as HNSCC subjects by the Department of Pathology. Each patient donated two samples of head and neck squamous cell tissue: cancerous and normal. So, 62 samples were collected in total. Preparation of tissues was done by the Surgery Center and Cancer Institute of Imam Khomeini Hospital (Tehran, Iran) and the Surgery Department of Valiasr Hospital (Tehran, Iran). The samples were received freshly in 2 ml microtubes and were kept at -70◦c.

RNA extraction and complementary DNA synthesis

RNA extraction from all specimens was performed using a “guanidine isothiocyanate-phenol-chloroform” method [10]. The quantity and quality of the extracted RNA were examined by NanoDrop UV-Spectrophotometry (Thermo Fisher Scientific, Inc.), and the integrity of the extracted RNA was assayed by 1% gel electrophoresis. Afterwards, the first strand of cDNA was generated from 500ng of extracted total RNA using the Takara PrimeScript reagent kit (TaKaRa, Cat#: RR037Q) according to the protocol provided by the manufacturer.

Quantitative PCR

The expression of EGFR and PIK3CA was measured at the mRNA level using the Q-PCR method. A single amplification curve was drawn to check the quality of Real-time PCR. PCR efficiency for amplification of EGFR and PIK3CA was 1.00 and 1.04, respectively.

Table 1. Characteristics of patients. 31 patients were included in the present study, and two head and neck squamous cell tissue samples (cancerous and normal) were obtained from each patient. The median and average of the ages were 65 and 66.22 years within the study population, respectively (SD: ±10.17).

Patient No.	Sex	Age	Grade of tumor
1	M	75	I
2	F	66	III
3	M	71	II
4	M	64	III
5	M	84	I
6	M	59	I
7	M	78	II
8	F	81	II
9	F	63	I
10	M	64	II
11	M	59	I
12	M	55	III
13	F	43	I
14	M	81	II
15	F	70	II
16	M	57	II
17	M	82	II
18	F	83	II
19	M	60	II
20	M	73	II
21	M	69	II
22	F	58	I
23	M	68	I
24	M	58	II
25	M	65	II
26	F	59	III
27	F	70	I
28	M	64	I
29	F	71	II
30	F	56	I
31	M	47	II

The SDHA housekeeping gene was used as an internal control so that the relative amount of mRNA for each target was normalized to SDHA expression. Real-time PCR was performed in triplicate using 2X Real Q Plus Master Mix Green High ROX (Ampliqon Denmark, Cat #: A 325402). SYBER green and ROX were used as the reporter and reference dyes, respectively. NRT (No Reverse Transcriptase) and NTC (No Template Control) tests were also used to control internal and external contaminations. Primer sequences for each target are shown in Table 2.

Statistical Analysis

Using the quartile method, outliers were detected in the datasets and excluded from data analysis. The distribution of datasets was analyzed in terms of normality (Shapiro-Wilk normality test: $P>0.05$ is considered as normal distribution). Since all datasets had normal distribution, unpaired T-test was used for comparing means between two groups and $P<0.05$ considered as statistically significant. All tests were repeated in both biological and technical replicates and data analysis was performed using, Microsoft Excel 2013, SPSS 22.0, LinReg PCR, and Rest 2009.

Results:

The total mRNA level of EGFR and PIK3CA was measured in both HNSCC tumor tissues and normal squamous cells. Real-time PCR analysis showed a slight alteration in gene expression of both EGFR and PIK3CA (3% decrease and 9% increase respectively) in HNSCC tissues compared with the control group. However, these alterations in gene expression were not significant for EGFR nor PIK3CA (Fig. 1 and 2).

Discussion:

Impaired tyrosine kinase cascade may give rise to uncontrolled cell proliferation and resistance to apoptosis in some squamous cell carcinomas. As a well-known tyrosine kinase receptor, activation of EGFR triggers a broad phosphorylation network leading to various cellular responses. The kinase cascade initiated by EGFR is mediated by several proteins, including PIK3CA, Akt, mTOR, and PTEN which whose outcomes depend on the activity of these upstream molecules [11-13]. Several molecular mechanisms are suggested to be involved in the tumorigenesis of HNSCC, and regarding the results of previous studies, impaired EGFR signaling through PIK3CA is suspected of triggering tumorigenic processes such as cancer-cell proliferation, cell survival, and metastasis in squamous cell carcinomas, especially HNSCC [7, 14].

The main question of our study was whether the gene expression of EGFR and PIK3CA proteins is different in HNSCC tissues compared with the normal squamous cells in patients with HNSCC. Our results showed no significant differences in gene expression of EGFR and PIK3CA in HNSCC tissues compared with that of normal squamous cells. While Analysis of existing data has revealed that increased expression of EGFR is correlated with poor clinical outcomes in patients suffering from HNSCC [15, 16].

However, increased gene expression is not the only possible mechanism underlying EGFR-PIK3CA-mediated tumorigenesis. Huang et al. have shown that uncontrolled signal transduction through a mutant EGFR is involved in oncogenesis because their results indicate that constitutive tyrosine kinase activity of EGFR may arise from a deletion of the ectodomain of the receptor [17]. Mutant EGFRs are known to be expressed frequently in human cancers, and these mutations are divided into three main groups: those mutations affecting the intracellular domain, those mutations that especially result in changes in the tyrosine kinase domain, and those mutations leading

Table 2. CPrimer sequences

Primer	Sequence
PIK3CAA-Forward	GGAAAATATCTGGCAAAATCAAGGT
PIK3CAA-Reverse	TAAGTCCCACACAGTCACCG
EGFR-Forward	CGTCCGCAAGTGTAAGAAGT
EGFR-Reverse	CTCTCCACGACATCCTTCCG
SDHA-Forward	CTTGCCAGGACCTAGATTTGT
SDHA-Reverse	CTCTCCACGACATCCTTCCG

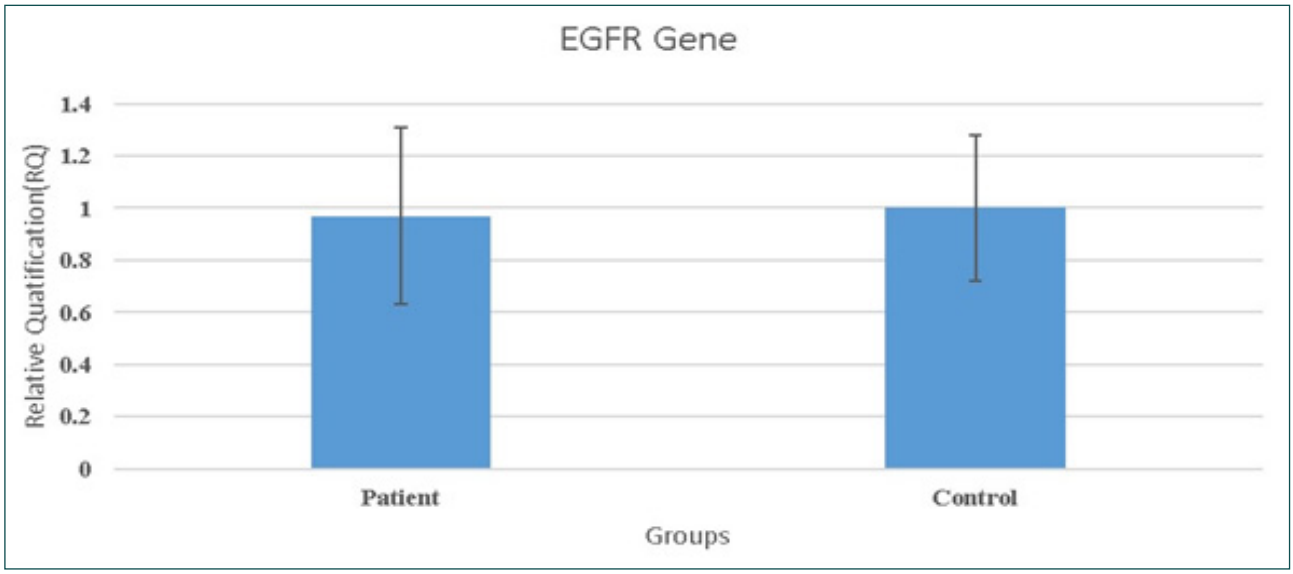

Figure 1. Relative mRNA level of EGFR

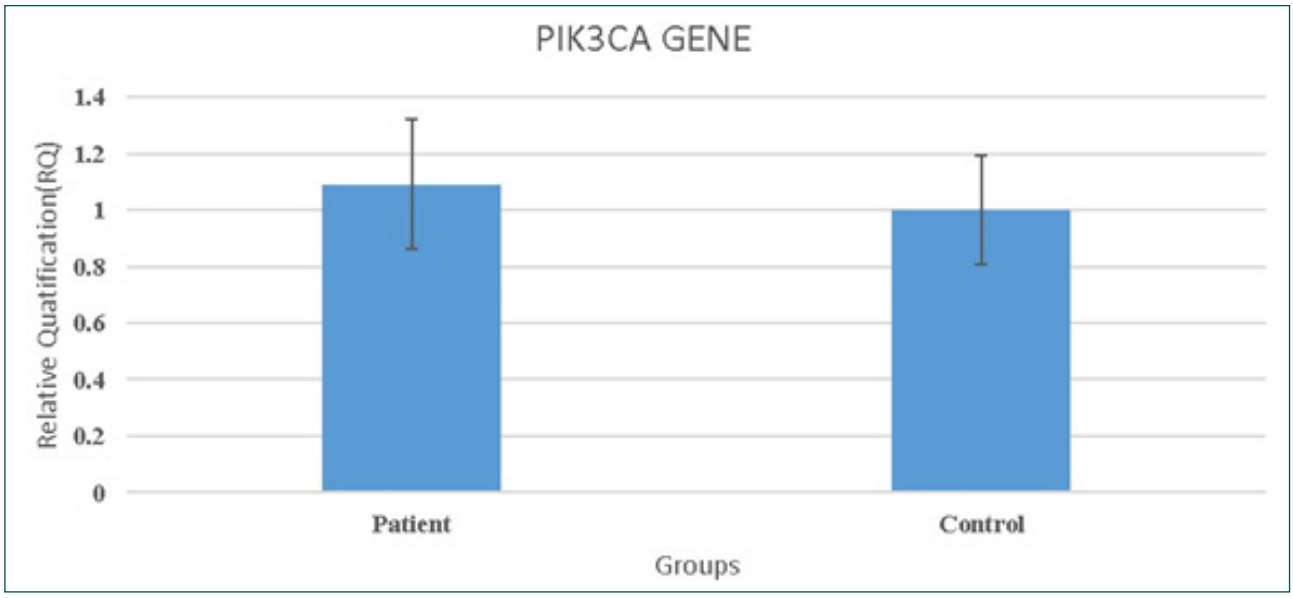

Figure 2. Relative mRNA level of PIK3CA

to a lack of the extracellular domain of EGFR [8, 18]. As well, mutations in the gene encoding PIK3CA may be involved in the uncontrolled activity of the EGFR-PIK3CA signaling pathway [19]. In this regard, Luc et al. have shown that 6% of head and neck tumor samples harbor mutations in PIK3CA [20]. Also, unregulated EGFR activity caused by overexpressed EGF and α-TGF may be a reason for uncontrolled signal transduction. Currently, anti-EGFR signaling agents such as Cetuximab and Gefitinib have been established for the treatment of squamous cell carcinomas. Lee et al. have researched somatic mutations of EGFR in HNSCC patients. Their results showed three mutations in the gene encoding EGFR in HNSCC tumor samples, giving reason for the applicability of Gefitinib in these patients [18].

Conclusion:
In the present study, Analysis of EGFR and PIK3CA gene expression at the mRNA level showed no significant difference between HNSCC tumor cells and normal squamous cells. Nonetheless, it should be noted that we compared only the total gene expression of EGFR and PIK3CA between the two groups, and the expression of mutant and activated forms of proteins was not been assessed in our study. Thus, it is important to stress that the efficiency of anti-EGFR agents or the prognostic significance of EGFR for HNSCC patients could not be criticized with our results. Rather, we showed here that assessing EGFR expression may draw no significant conclusion when only the total cellular EGFR is compared between HNSCC tumor cells and normal squamous cells.

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Conflict of Interest:
The authors declare that no conflict of interest exists.

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