Molecular Analysis of EGFR and PIK3CA Genetic Mutations in Iranian Patients with Head and Neck Squamous Cell Carcinoma

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ABSTRACT

Background: Many studies have demonstrated that mutations in EGFR / PI3K / AKT signaling pathway causes a central effect on the development of many cancers, such as head and neck squamous cell carcinoma (HNSSC). Moreover, these mutations were considered to be a major factor in drug response and prognosis. **Objectives:** In this research, we aimed to identify mutations in exons 18-21 of the EGFR gene and exons 9 and 20 of the PIK3CA gene in patients affected with HNSCC. We also determined the frequency of these mutations in Iranian HNSCC patients. **Materials and Methods:** Reverse hybridization assay (Strip assay) was used to assess the possible mutations in exons 18-21 of the EGFR gene. Concurrently, the presence of point mutations in the exons 9, 20 of the PIK3CA gene was performed by direct Sanger sequencing of PCR products. **Results:** Our study revealed that Iranian HNSCC patients have T790M mutation (2%) in exon 20 of the EGFR gene as the only mutation contributing to the kinase domain of this gene. Our study also indicated that the frequency of mutations in exons 9 and 20 of the PIK3CA gene is 16%. **Conclusion:** In comparison with other populations, the identification of three novel mutations in the PIK3CA gene from 50 patients hypothesizes that Iranian HNSCC patients may have different frequencies of PIK3CA mutations that may affect HNSCC pathogenesis and drug response predictions. In the current study, a mutation frequency of EGFR is similar to previous studies.

Key Words: *HNSCC, EGFR,PIK3CA, Mutation Frequency*

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INTRODUCTION

Head and neck Squamous cell carcinoma (HNSCC) includes malignancies that arise in the soft tissues of the oral cavity, nasopharynx, oropharynx, hypopharynx, larynx, throat, paranasal sinuses and salivary glands [1]. HNSCC is the sixth most prevalent type of malignancies globally and it is mainly prevalent in northern Europe and Asia [2]. Recently, a plethora of studies have been conducted to identify genetic and epigenetic alterations in HNSCC to decipher the molecular mechanisms involved in HNSCC pathogenesis [3]. One of the most common signaling pathways involved in the HNSCC is the EGFR-PI3K-AKT cascade.

Epidermal growth factor receptor (EGFR)

One of the most common signaling pathways involved in the HNSCC is EGFR / PI3K / AKT cascade. Epidermal

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growth factor receptor (EGFR) is a tyrosine kinase receptor, which plays a leading role in tumor progression, cell growth, and differentiation [4]. The EGFR gene located on chromosome 7p12 which encodes for a 185-KDa transmembrane protein tyrosine kinase. EGFR protein is widely expressed in most epithelial cells as well as Head and Neck Squamous cell carcinoma cells [5]. In addition to the normal physiological role of EGFR, it affects processes underlying tumorigenesis through its involvement in tumor development including proliferation, growth, increased angiogenesis, invasion, metastasis, and inhibition of apoptosis [6, 7]. Many epithelial tumors have shown up-regulated expression of EGFR such as head and neck cancer, kidney cancer, lung cancer, breast cancer [8, 9]. High expression of EGFR is linked to invasive disease, decreased survival rate, and poor prognosis [10]. It has been shown that somatic mutations within the tyrosine kinase domain of EGFR (EGFR-TKD), specifically mutations located in exon18-21, affects drug resistance in the cancer treatment process [11]. Hence, identifying genetic mutations in HNSCC could serve as biomarkers capable of predicting poor prognosis or candidates for specific molecularly targeted therapy in cancer treatment.

Phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA):

Phosphatidylinositol 3 kinase (PI3K) / Akt is one of the most important signaling pathways activated by several receptor tyrosine kinases (RTKs) such as EGFR, which stimulates growth, proliferation, cell survival and apoptosis [12, 13]. PI3K, which belongs to a conserved family of lipid kinases, consists of three subclasses of lipid kinases. Among the three previously mentioned subclasses of PI3Ks, class IA PI3K is strongly associated with tumorigenesis. Class IA PI3Ks are heterodimers composed of a regulatory subunit (p85α, p55α, p50α, p85β, p55γ) and a catalytic subunit (p110α, p110β, p110 δ) [14]. The phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA) oncogene is located on chromosome 3q26.32 and encodes for the catalytic subunit p110 alpha of class 1A PI3 Kinases [15, 16]. Activating mutations in PIK3CA gene have already been identified in a wide range of cancers, including breast (26%), endometrial (25%), colorectal (12%), urinary tract (15%), stomach cancer (9%), ovarian (10%), head and neck squamous cell carcinoma (8%), cervical (12%) and lung cancer (4%) [17]. The mutations tend to accumulate in two conserved regions of the PIK3CA gene, which encode the helical and kinase domains of this protein. Abnormalities in the PI3K pathway in HNSCC is probably due to mutations in the PIK3CA gene, or PTEN loss of function. PTEN loss of function acts as a negative feedback loop, which its mutations have been reported in 7% of cases [18]. In HNSCC, PIK3CA mutations are associated with advanced stage, increased vascular invasion, and lymph node metastasis [19, 20]. Investigation of the PI3K pathway genetic mutations may prove to be promising novel biomarkers, as a therapeutic target, for HNSCC treatment.

MATERIALS AND METHODS

Patients and methods

The present study was a hospital-based study conducted on cancer tissue samples from 50 HNSCC patients who referred to Cancer Institute, Imam Khomeini Medical Center, Tehran, Iran for tumor removal. In the current study, none of the enrolled patients underwent chemo/ radiotherapy. According to clinical and pathological examinations, the HNSCC patients were recruited in the current study. Inclusion criteria were appropriateness of the excised tissue for mutation detection assay and precise pathological diagnosis. Positive family history of HNSCC and having tumors in other sites considered as exclusion criteria. The current study was approved by the ethical committee of Tehran University of Medical Sciences. Enrolled patients gave their written informed consent.

Extraction of DNA

DNA was isolated by standard phenol-chloroform extraction and ethanol precipitation procedures. Afterward, it quantified using Nanodrop (Thermo Scientific, Waltham, MA) which were acceptable for conducting tests.

EGFR StripAssay

In the current study, the 16 most frequent EGFR mutations (p.G719A, p.G719C, p.G719S, p.E746_A750del, p.E746_A750del, p.E746_A750del, p.E746_T751delinsA, p.E746_S752delinsV, p.L747_E749del, p.L747_A750delinsP, p.L747_T751delinsP, p.L747_T751del, p.L747_S752del, p.L747_P753delinsS, p.T790M, p.L858R, p.L861Q) were investigated by reverse hybridization assay (FMF strip assay, Vienna lab, Vienna, Austria) according to manufacturer's instructions. The procedure compromised multiplex PCR reaction based on the amplification of exons 18-21 followed by Strip testing. The optimized conditions for PCR program were 33 cycles (94 °C for 15 seconds, 70 °C for 60 seconds and 58 °C for 90 seconds) and a final extension at 60 °C for 3 minutes. The PCR reaction produced six DNA fragments: 90, 107, 133, 149, 158, and 204 bp. PCR products ran on a 3% agarose gel with ethidium bromide DNA Staining. Strip testing compromised selective hybridization of PCR products on a test strip followed by demonstrating a parallel array of allele-specific oligonucleotide probes and visualized by an enzymatic color procedure.

PCR amplification and direct sequencing of PCR products of the PIK3CA gene

Sequence data of the exons 9 and 20 of the PIK3CA gene was obtained from ensembl.org, and then two pairs of primers were designed for either of them with the aid of the online application PRIMER3 (primer3-0.4.0 / primer3 / bioinfo.ut.ee). Specific primers for the PIK3CA gene exons 9 and 20 are shown in Table 1. All PCR reactions were performed in the thermal cycler (Applied Biosystems, GeneAmp 2720, Singapore), using a 200-µl PCR tube (Extra Gene Inc., Taichung City, Taiwan). To investigate mutations in exons 9 and 20 of PIK3CA, PCR was conducted in 50.0 µL volumes containing 1.25 IU Taq DNA polymerase (Ampliqon Co., Denmark), 0.15 mmol/L of each dNTP, 1.5 mmol/L MgCl2, 1x PCR buffer, 20 pM of each primer and 50 ng of DNA. The thermal cycling protocol for PCR was comprised 95 ℃ for 3 min, followed by 33 cycles of 94 ℃ for 1 min, 58 ℃ for 30 s and 72 ℃ for 1 min, with a final extension at 72 ℃ for 5 min. The amplified products were visualized by electrophoresis in 2% agarose gels stained with ethidium bromide. The expected PCR products for exon 9 of 196bp and exon 20 of 387bp. PCR products then purified using the MinElute PCR purification kit (Qiagen). In the next step, PCR products directly sequenced. Finally, the results were analyzed with the assistance of the Mutation Surveyor (version 3.30) and Chromas (version 2.1.1) software to identify possible mutations.

RESULTS

Demographic and clinical data

Among the 50 HNSCC patients recruited for this study, 39 (78%) were men and 11 (22%) were women. The most common tumor location was the larynx (39 cases, 78%), followed by the oral cavity (5 cases, 10%). The basic demographic and clinical characteristics of HNSCC participants are summarized in Table 2. The characteristics include age, gender, site of the primary tumor, tumor grade, tumor size, lymphatic and vascular invasion, and lymph node involvement.

Results of strip assay

In the current study, strip assay results showed at least one missense mutations (2%) in the samples of 50 HNSCC patients.

Sequencing results of exon 9 and 20

A total of 8 genetic mutations (16%) were identified in exons 9 and 20 of the PIK3CA gene in 50 HNSCC patients. Out of 50 HNSCC patients, which had been selected for sequencing of exon 9 and 20 in the PIK3CA gene, 6 different types of missense mutations were shown in table (3). Also, at least one nonsense mutation was observed in exon 9 of PIK3CA in the samples of 50 HNSCC. Three novel mutations including, two missense mutations (c.1598 C>G in exon 9 of PIK3CA and c.3088 A>C in exon 20 of PIK3CA) and at least one nonsense mutation (c.1582 A>T in exon 9 of PIK3CA) were observed in our studied samples. Based on the **COSMIC** database, these three mutations have not previously been reported for HNSCC. Figs. 1 and 2.

DISCUSSION

In this study, Sanger sequencing technology and reverse hybridization strip assay (StripAssay) were used to investigate the EGFR and PIK3CA mutation status in tumor samples of HNSCC patients with squamous cell carcinoma of the various site of head and neck region. In the current study, none of the enrolled patients underwent chemo/ radiotherapy. It must be noted that, in the current study, none of the enrolled patients underwent chemo/ radiotherapy.

EGFR Mutation

EGFR over-expression is the most beneficial indicator for the treatment of numerous epithelial solid tumors such as non-small cell lung cancer (NSCLC), colorectal cancer (CRC), HNSCC, pancreatic cancer, brain cancer and breast cancer [8, 9]. Nonetheless, distinct mechanisms, such as EGFR amplification or activating mutation, existing on these EGFR-overexpressing solid tumors [21, 22]. The identification of activating mutations in the EGFR-TDK in HNSCC subtypes and their association with considerable sensitivity to thymidine kinase inhibitors (TKI) play a major role in the therapy and prognosis of this malignancy [10, 23]. In the current study, mostly investigated mutational hotspots in exon18-21 of EGFR-TKD were examined by StripAssay. StripAssay analysis of the exon18-21 in 50 HNSCC patients demonstrates the presence of only one mutation (p.T790M) which accounts for 2% (1/50) of the total EGFR-TKD studied mutations. To the best of our knowledge, it is the first time that this genetic mutation of EGFR has been investigated in Iranian HNSCC patients. In 2006, Perrone et al. analyzed the EGFR mutations and found that the frequency of EGFR exon 19 mutations was 2.5% in 40 patients with oropharyngeal squamous cell carcinoma from Italy [24]. In concordance with their data, we found a similar frequency (2 %) of EGFR mutations in our study. It must be noted that, in Perrone et al. study, the obtained frequency of EGFR mutation investigated using PCR and sequencing methods. However, in the current study, the mutational hotspots of EGFR were examined by StripAssay. Another study conducted in 2014, by Wang et al. using Multiplex PCR showed that 3/132 (2.3%) patients from China have a similar EGFR mutation frequency rate in comparison to our study [25]. However, in their study, the mutations they investigated were located on exon 20 and 21. In 2017, Christos Perisanidis performed a systematic review and found that the overall prevalence of EGFR-TKD mutations

was 2.8% in HNSCC patients [26]. The result obtained in our study is in line with the Christos Perisanidis and suggests that EGFR mutations are not common in HNSCC patients. In four recent studies in Austria [27], USA [28], Greece [29] and Taiwan [30], EGFR mutation frequency rate was reported to be 2.4%, 2%, 3.3%, and 3.57% respectively and these results are approximately in line with our study.

Ock et al. performed another investigation in 2016, on 71 FFPE blocks of HNSCC patients from Korea, using Targeted NGS and found that the frequency of EGFR exon 18-21 mutations was 26.7% [31]. In 2014, the highest mutation frequency of EGFR (81.39%) was reported by Nagalakshmi K et al. in the southern Indian population [32]. The huge difference between the EGFR mutation frequency rate in our study and Ock et al. and Nagalakshmi K et al. might be explained by using different EGFR mutation detection methods and interethnic genetic variation.

In conclusion, reported differences in the frequency of EGFR mutations in HNSCC can be explained by using non-identical mutation detection methods with different sensitivities, different tumor types, and stage and interethnic genetic variation.

PIK3CA Mutation

PIK3CA is a major downstream signaling regulators of the EGFR-PIK3 cell signaling pathway that could interrupt the regulation of cell proliferation and survival and induces tumorigenesis [33]. Genetic alteration of PIK3CA, including gene amplification or activating mutation, has been reported in several human tumors, such as brain, breast, colon, ovary, liver, lung, and HNSCC [17]. Genetic alteration of PIK3CA is prevalent in HNSCC and is the second-most frequently encountered genetic aberration in HNSCC patients after TP53 [3, 34]. In the current study, genetic mutations in exon 9 and 20 of PIK3CA were investigated by using PCR followed by Sanger sequencing method. In our study, the investigation of activating mutations in exon 9 and 20 of PIK3CA gene demonstrates 16% of the frequency of the total studied patients. While about 80% of PIK3CA mutation hotspots are clustered in the exons 9 (E542K and E545K) and 20 (H1047L and H1047R), none of these mutation hotspots have been detected in the studied patients. This might be explained by small sample size bias or interethnic genetic variation.

In 2016 Ali M. et al. showed that the PIK3CA mutations frequency was 29.2% in 48 Saudi HNSCC patients which is inconsistent with our report [35]. This discrepancy may result from different genetic backgrounds between our studied patients and Ali M. et al. studied patients. Data obtained from The Cancer Genome Atlas (TCGA) reported that the frequency of PIK3CA mutation is 19.3% and 35.3% in HPV negative and positive HNSCC, respectively, which is similar to our results [36]. However, due to the small sample size in our study, we did not divide our sample size into two distinct subgroups (HPV negative and positive). Also, comparing results from different studies reveal that the mutation frequency of the PIK3CA gene in HNSCC patients varies from 6% to 33% among distinct populations, which is in line with our data [19, 20, 34, 37-41]. In 2006, Ken-ichi et al. investigated the PIK3CA mutations and found that the frequency of PIK3CA missense mutations in exons 9 and 20 were 7.4% (8/108) of human oral squamous cell carcinoma (OSCC) by genomic DNA sequencing [38]. While Ken-ichi et al. investigated only one subtype of HNSCC (OSCC), in our study, we examined different subtypes of HNSCC (Larynx, Oral cavity, Pharynx, and others). Different tumor sub-types might be explained the discrepancy between our results and the Ken-ichi et al. data. Another study performed in 2007, by Avaniyapuram et al. using a sequence analysis method showed that 10.5% (2/19) of HNSCC patients from India harbored mutations in exons 9 and 20 [19]. The small sample size used in Avaniyapuram et al. study might be explained the different PIK3CA mutation frequency rates between our results and theirs.

To sum up, several factors such as using non-identical mutation detection methods with different sensitivities, different tumor types, and stage. The interethnic genetic variation might be explained the discrepancy between our results and previous studies.

CONCLUSION

In comparison with other cancers, HNSCC patients have a better prognosis which depends on early diagnosis and better cancer management by adopting molecular targeted therapy. Molecularly targeted therapy works by blocking specific mutations in HNSCC patients. Therefore, determining the different types of genetic mutations can be considered as a new therapeutic goal for the therapy of HNSCC patients. Hence, investigation of the EGFR and PIK3CA genetic mutations not only lay the foundation to develop a new generation of drugs (target-specific drugs) but also could serve as prognostic biomarkers for the risk assessment of HNSCC.

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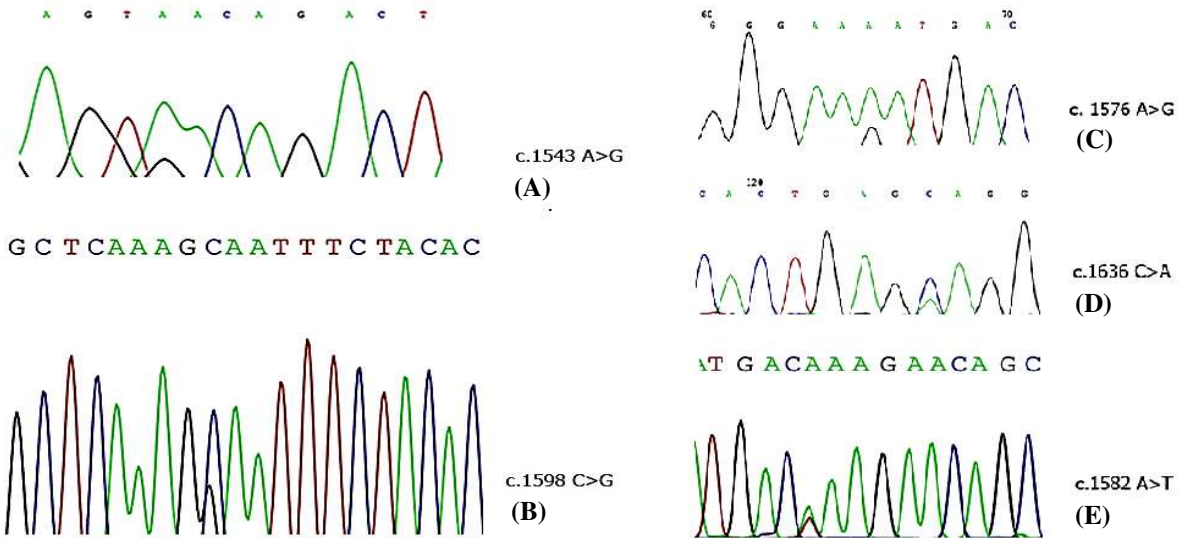

Fig. 1. Electrophorogram of exon 9 of PIK3CA mutations identified in HNSCC patients by targeted DNA sanger sequencing. Nucleotide change (Aminoacid change): (**A**). c.1543 A>G (p.N515D) (**B**). c.1598 C>G (p.A533V) (**C**). c.1576 A>G (p.N526D) (**D**). c.1636 C>A (p.Q546K) (**E**). c.1582 A>T (p.K582X).

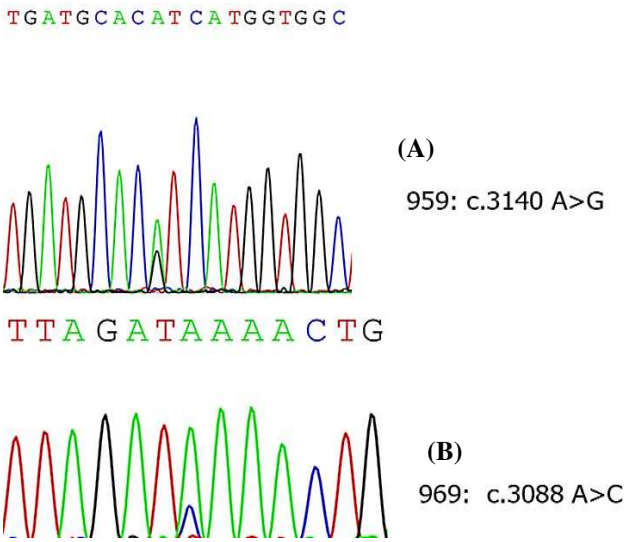

Fig. 2. Electrophorogram of exon 20 of PIK3CA mutations identified in HNSCC patients by targeted DNA sanger sequencing. Nucleotide change (Aminoacid change): (**A**). c.3140 A>G (p.H1047R) (**B**). c.3088 A>C (p.K1030Q).

Table 1. Primer set sequences and the length of products

Gene name	sequence	Product length
PIK3CA (Exon 9)	F CCAGAGGGGAAAAATATGACA R: CATTTTAGCACTTACCTGTGAC	196 bp
PIK3CA (Exon 20)	F: CATTTGCTCCAAACTGACCA R: GGTCTTTGCCTGCTGAGAGT	387 bp

Table 2. patients demographic and clinical data

variables	Values N(%)
Age	
≤40 y	8 (16%)
>40 y	42 (84%)
Gender	
Man	39 (78%)
Woman	11 (22%)

Site of the primary tumor	
Larynx	39 (78%)
Oral cavity	5 (10%)
Pharynx	4 (8%)
others	2 (4%)
Tumor grade	
Well-differentiated	18 (36%)
Moderately differentiated	20 (40%)
Poorly differentiated	12 (24%)
Tumor size	
≥2/5cm	29 (58%)
<2/5cm	21 (42%)
Lymphatic invasion	
Yes	12 (24%)
No	38 (76%)
Vascular invasion	
Yes	15 (30%)
No	35 (70%)
Lymph node involvement	
Yes	13 (26%)
No	37 (74%)

Table 3. Summary of PIK3CA Exon 9 and 20 mutations in head and neck squamous cell carcinoma from Iran

Mutation	**Exon**	**Genotypic change**	**Amino acid change**	**Type of mutation**	**Functional domain**	**Site of the tumor**	**Grade**	**Stage**
Reported	9	c.1543 A>G	p.N515D	Missense	Helical	Larynx	3	I
Novel	9	c.1598 C>G	p.A533V	Missense	Helical	Nasopharynx	2	I
Reported	9	c.1576 A>G	p.N526D	Missense	Helical	Larynx	3	II
Reported	9	c.1576 A>G	p.N526D	Missense	Helical	Larynx	3	III
Reported	9	c.1636 C>A	p.Q546K	Missense	Helical	Oropharynx	2	II
Novel	9	c.1582 A>T	p.K582X	Nonsense	Helical	Larynx	3	III
Reported	20	c.3140 A>G	p.H1047R	Missense	Kinase	Oral Cavity	1	I
Novel	20	c.3088 A>C	p.K1030Q	Missense	Kinase	Larynx	3	IV