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RESEARCH ARTICLE

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Determination of cytokine profile and associated genes of the signaling pathway in HNSCC

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ABSTRACT

Head and neck squamose cell carcinoma (HNSCC) is an aggressive group of tumors that are generally heterogeneous. Despite treatment advances, disease-free survival has not significantly improved. Therefore, it is of great importance to understand the molecular etiology of HNSCC and genetic alterations in the signal pathways in order to develop new therapeutic approaches. In this study, firstly we used a cytokine array to analyze the secretomes of HNSCC patients and healthy controls. In the next step, the results from the cytokine sequence were validated by qRT-PCR and western blot, including genes in the associated signaling pathway. In array analysis, the levels of EGF, IGF-1, IGFBP-1, and PDGFBB were significantly higher in patients than in the controls. The results of qRT-PCR analyses showed that expression levels of *PDGFRB* gene were significantly up-regulated ($p = 0.006$) and *PTEN* ($p > 0.001$) were significantly down-regulated in tumors compared with normal tissues. When groups (early vs. advanced) were compared, higher expression of *IGFBP-1* was observed in the larynx ($p = 0.045$) and larynx + oral cavity tumors ($p = 0.010$) in an advanced stage. In western blot analysis, pEGFR, pIGF-IR, pIR-β, pPDGFRB, and pAKT levels were upregulated, and pPTEN was downregulated in tumors. Based on our observations, determining the interactions of *EGFR, PDGFRB, IGF-1R* and *PTEN* or the activation of each might represent a promising new and innovative treatment approach in HNSCC patients. It seems clear that, in most cancers, effective targeted therapy may be involved the blockade of each one or multiple targets.

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Introduction

Head and neck cancers are the seventh most common cancers worldwide and account for 3% of all cancer [1]. Each year nearly 890,000 people are affected by head and neck cancers and about 40–50% of them survive 5 years [2,3]. Head and neck squamose cell carcinoma (HNSCC) accounts for about 90% of all head and neck cancers and most commonly arise in the oral cavity and larynx. Despite improved radiotherapy, innovative surgery and chemoradiotherapy, the 5-year survival rate in HNSCC patients has not significantly improved within the last years [4].

Various risk factors have been identified in the development of HNSCC. These risk factors include smoking/alcohol consumption, premalignant lesions, family history, some virus infections (such as the Human Papilloma Virus, Epstein-Barr Virus) and genetic alterations. Although it is known that some genomic rearrangements (gene mutations such as EGFR, p16, p53 and cyclin D1) are important in the pathogenesis of HNSCCs, mechanisms including the aberrant signaling pathways, cytokine, chemokine, and growth factor expressions are incompletely understood [5–7].

Cytokines, chemokines and growth factors are important immunmodulators and prognostic markers which have a complex regulatory effect in the control of cellular processes. They act over short distances and at very low concentrations and are also coordinate intercellular signal transduction pathways [8]. To trigger these signal transduction pathways, they bind to receptors (receptor tyrosine kinases) on the membranes of responsive target cells and induce gene expression in target cells. Many cellular activities associated with survival, activation, proliferation, and differentiation of cells are under the control of intracellular signal transduction pathways. Aberrant activations of these components in the signal transduction pathways play a major role in the malignant transformation of many cancers [6]. Therefore, determining these activators involved in the etiopathogenesis of the HNSCC can be notable molecular targets for novel therapeutic approaches. The study aims to evaluate the cytokine, chemokine and growth factor profiling of HNSCC patients, and the relationship of genes involved in the pathway related to these cytokines.

Materials and methods

Clinical specimens

Tissue and blood samples were obtained from sixteen patients undergoing surgical resection at the Departments of Otolaryngology of the Medical Faculty in Selcuk University.

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The control group consisted of four cases without any other type of cancer or chronic disorder. None of their first-degree relatives had a cancer history. All subjects were questioned for smoking habits and alcohol consumption. Smoking status was defined as the consumption of 100 cigarettes per lifetime for a current cigarette smoker. Alcohol consumption was defined as consuming no more than two units of alcohol a week – the equivalent of a pint of average strength beer or a glass of wine. The investigation has been conducted following the ethical standards according to the Declaration of Helsinki and the ethical committee approval was obtained from Selcuk University with the decision number 2011-08. Written informed consent was obtained from all subjects. All cases were diagnosed histopathologically as HNSCC and staged according to the TNM classification. In addition, tumor specimens and adjacent nonmalignant tissues from each patient were analyzed. Tumor specimens included laryngeal and oral cavity carcinoma with TNM stage [different tumor sizes (T1–T4), lymph node status (N0-2), distant metastasis (M0-M1)] and all patients were HPV-negative. According to the study protocol, patients with local recurrences, second primary tumor, HPV + status, and a previous history of chemo-or radiotherapy were excluded from the study.

Upon resection, tissue samples (tumor and adjacent nonmalignant) were immediately placed on liquid nitrogen. Also, venous serum samples were collected and immediately frozen in aliquots and stored at −80 °C until use. Fresh aliquots were used for each analysis.

Cell lines and cell culture

SCCL-MT1 and HEK-293 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, MA, USA) at 37 °C in a humidified atmosphere of 5% CO_2. Before experiments, cells were cultured in a drug-free medium for at least 7 days. SCCL-MT1 (EGFR+, HPV-) (HNSCC) cell line was supplied by Professor Alan Eppstein Laboratory and HEK-293 (negative control) cell line was supplied by ATCC.

Cytokine antibody array

Human Cytokine Array C5 of RayBioTM (Raybiotech, USA) was used to detect 80 different cytokines, chemokine and growth factors in the serum samples of HNSCC patients and controls. The assays were strictly performed according to the manufacturer's instructions. Briefly, the array membranes were incubated in blocking buffer at 25 °C for 30 min and followed by incubation with the sample at 25 °C for 120 min. Membranes were then washed with Wash Buffer I for 3 times and Wash Buffer II for 2 times at 25 °C for 5 min per wash. After washing, the membranes were incubated with biotin-conjugated antibodies at 25 °C for 120 min. Subsequently, the membranes were washed and incubated with horseradish peroxidase-conjugated streptavidin at 25 °C for 120 min. The ChemiDocTM XRS (Bio-Rad, USA) was used to detect the chemiluminescence and generate an image. The spots were quantified by a computer-assisted system for image analysis (Koadarray 2.6 software); normalized intensities were calculated from each array by first subtracting the local background from each spot and then normalizing by the average intensity of the arrays. The data were then adjusted for the cell protein content of each well. The relative expression level of each protein was calculated according to both spot pixel mean ± standard deviation (SD) by the Koadarray.

cDNA and RT-PCR analysis

Cell-mediated factors important in cytokine analysis, as well as their downstream *PTEN* and *AKT* genes, were selected for mRNA quantification. Total cellular RNA was isolated using RNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. The quantitation of RNAs was determined by NanoDrop ND-1000 (NanoDrop Technologies, Inc. Wilmington, New Jersey). Additionally, qualities of all total RNA samples obtained were separated by electrophoresis in 1% agarose gel, stained with ethidium bromide (EtBr) and observed under UV. One microgram of total RNA was reverse transcribed in a 20-μl reaction volume using the Oligo-DT and the SuperScript First-Strand Synthesis Kit (Thermo Fisher, USA). The primer sequences were designed by the Universal probe library of Roche (Germany) and shown in Table 1. We performed RT- PCR (LightCycler 480, Roche, Germany) using SYBR Green Mix (LightCycler® 480 SYBR Green I Master, Roche, Germany) containing 10 μM of specific forward and reverse primers (Table 1). Furthermore, we carried out the relative quantification of gene expression using the comparative threshold cycle (CT) method, which allowed us to determine the target amount as $2^{-(\Delta CT\ target\ -\Delta CT\ calibrator)}$ or $2^{-\Delta\Delta CT}$. In order to normalize data, the best housekeeping gene selection for qRT-PCR analysis was performed in our previous study [9].

Western blot analysis

For protein level expression analysis, tissues were isolated by crushing in liquid nitrogen. Estimation of protein concentration was quantitated by Bradford assay and 40 micrograms of total protein were then resolved by 9–12% SDS-PAGE gel. After the transfer of proteins onto PVDF membranes (Immobilon-P, Millipore, Billerica, MA, USA), blocking was

Table 1. Sequences of primers used in RT-qPCR assays.

Gene	Sequence
EGFR	F-5′-AGGAAGAAGCTTGCTGGTAGC-3′
	R-5′-CTCTGGAAGACTTGTGGCTTG-3′
PDGFBB	F-5′-CGACGATCGAGTCCAATGCTG-3′
	R-5′-AGGCTACCATGCATCCCTGCTG-3′
IGF1	F-5′-GCACCATCTTCAAGGGCAATTTG-3′
	R-5′-AGGAAGGACAAGGAGACCAAGG-3′
IGFBP1	F-5′-GGGACGCCATCAGTACC-3′
	R-5′-CCATTTTTTGATGTTGGTGAC-3′
PTEN	F-5′-GGGGAAGTAAGGACCAGAGAC-3′
	R-5′-TCCAGATGATTCTTTAACAGGTAGC-3′
AKT	F-5′-TCTATGGCGCTGAGATTGTG-3′
	R-5′-CTTAATGTGCCCGTCCTTGT-3′
GAPDH	F-5′-ATCACCATCTTCCAGGAGCGAGA-3′
	R-5′-GTCTTCTGGGTGGCAGTGATGG-3′

done with 1% BSA. The membranes were probed with antibodies and then followed by incubation with HRP conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA). Membranes were developed *via* an enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA) and the signals were detected using Fusion Fx Imaging System (France) and the emitted light was captured by Bio-1D ++ (Ver.11.11) gel imaging system.

Statistical analysis

All experiments were conducted twice and the results were expressed as the mean ± SD. GraphPad Prism® V.5.00 software (La Jolla, CA, USA) and Bio-1D ++ (Ver.11.11) programs were used for data analysis. The difference between groups was analyzed by using the OneWay Analysis of variance (ANOVA) and the Kruskal-Wallis test. In all statistical tests, $p \leq 0.05$ was considered as statistically significant.

Results

Characteristics of the patients

Clinical and pathological data of the sixteen HNSCC patients enrolled in this study were presented in Table 2. The median age (±SD) of patients was 63.15 ± 1.2 (between 34 and 83) and 75% were males and 25% were females. There was a male dominance in the incidence of the cancers at a male-to-female ratio of 3:1. The larynx was a more common site compared to the oral cavity. Among the risk factors, all patients had a history of cigarette smoking. The majority 10 (62.5%) of cases had the advanced disease; Stages III and IV. No Stage I disease and second primary tumor was recorded (Table 2).

Table 2. Clinical characteristics of patients with HNSCC.

Characteristic	No (%)
Age	
Range	34–83
Mean ± SD	63.15 ± 1.2
Sex	
Male	12 (75%)
Female	4 (25%)
Tumor localization	
Larynx	10 (62.5%)
Oral cavity	6 (37.5%)
Risk factors	
Smoking	16 (100%)
Alcohol	0
Smoking/Alcohol	3 (18.75%)
Tumor size	
T1 + T2	8 (50%)
T3 + T4	8 (50%)
Lymphoid nodal states	
N0-1	13 (81.25%)
N2-3	3 (18.75%)
Metastasis	
Yes	0
No	16 (100%)
Clinical Stage	
Early (1 + 2)	6 (37.5%)
Advanced (3 + 4)	10 (62.5%)

Levels of cytokine expression

According to the results of the cytokine array, EGF, IGF-1, IGFBP-1 and PDGF-BB levels were increased in the serum of HNSCC patients compared with controls (Figure 1). In addition, these cytokine levels were also statistically significant between early and advanced stage tumors ($p \leq 0.05$).

mRNA expression analysis

The mRNA level expression of six selected genes (*EGF*, *IGF-1, PFGF-BB, IGFBP-1, PTEN*, and *AKT*) were analyzed in all HNSCC samples. The results of qRT-PCR analyses showed that expression levels of the *PDGF-BB* were significantly up-regulated ($p = 0.006$) however *PTEN* ($p > 0.001$) was significantly down-regulated in tumors compared with normal tissues. The expression levels of these genes were evaluated depending on cancer localization. *PTEN* expression was significantly down-regulated in the larynx ($p = 0.041$) and oral cavity ($p = 0.006$) tumors compared to normal tissues. *PDFGBB* gene expression was upregulated in the larynx ($p = 0.002$) and larynx + oral cavity ($p = 0.006$) tumors compared to normal tissues. When TNM stages were compared, significantly higher expression of *IGFBP-1* was observed in the larynx ($p = 0.045$) and larynx + oral cavity tumors ($p = 0.010$) in an advanced stage.

In addition, *EGFR* (3.3 fold), *IGF-1* (1.1 fold), *PDGFBB* (0.8 fold), *IGFBP* (3.3 fold) and *AKT* (1.4 fold) genes were up-regulated, however, *PTEN* (15 fold) expression was down-regulated in tumor tissues compared to HEK-293 cell line. When normal tissues compared to SCCL-MT1 cell line, *EGFR* (2.7 fold), *IGF-1* (1.3 fold), *PDGFBB* (1.6 fold), *IGFBP* (1.7 fold) and *AKT* (1.2 fold) expressions were increased but *PTEN* (3.75 fold) was decreased (Data not shown).

Results of protein expressions in tumor tissues, normal tissues, and cell lines by western blot analyses

Western blot analysis was performed for the quantification of non-phosphorylated EGFR, IGF-IR, PDGFRB, AKT, PTEN proteins and phosphorylated EGFR (Tyr1068), IGF-IR (Tyr980), PDGFRB (Tyr751), AKT, and PTEN. The GAPDH was used as an internal control. No expression was observed for some phospho-proteins including phospho-EGF receptor (Tyr992 or Tyr1045), phospho IGF-1 receptor β (Tyr1135, Tyr1131 or Tyr1135/1136), insulin Receptor (Tyr1150/1151), phospho PDGF Receptor β (Tyr740, Tyr771, Tyr1009 or Tyr1021).

pEGFR, pIGF-IR, pIR-β, pPDGFRB, and pAKT levels were upregulated, and pPTEN was downregulated in tumors compared to normal tissues. Levels of pIGF-IR and pPDGFRB were statistically significant ($p = 0.01$ and $p = 0.0172$). In addition, we identified that pEGFR and pIGF-1R were significantly upregulated, and pPTEN was downregulated in SCCL MT1 compared to HEK-293 cell lines (Figure 2).

Phosphorylated protein levels are upregulated in pEGFR, pIGF-IR, pIR-β, pPDGFRB and pAKT but downregulated in pPTEN in tumors compared to normal tissues. However, only

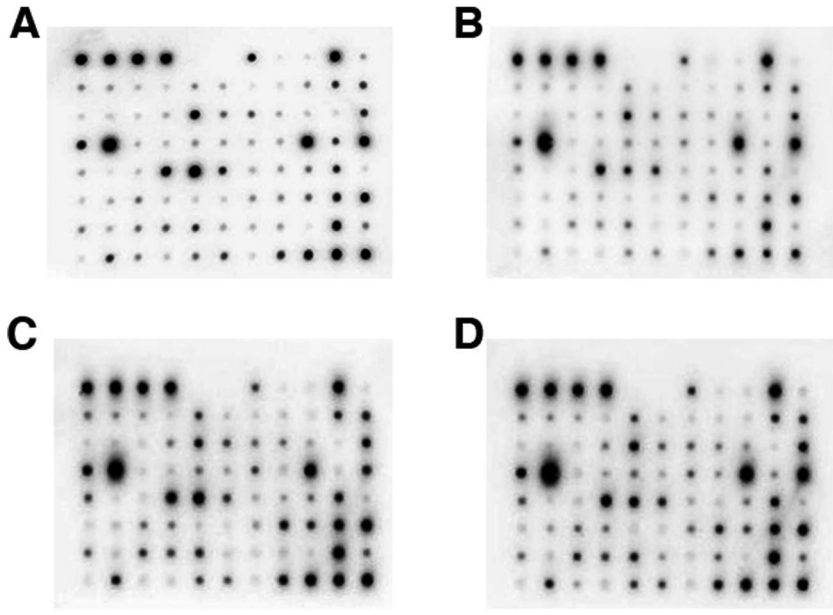

Figure 1. Cytokine profiles in serum of patients with HNSCC and controls. (A) Control, (B) Patient 1 (C) Patient 2 and (D) Patient 3.

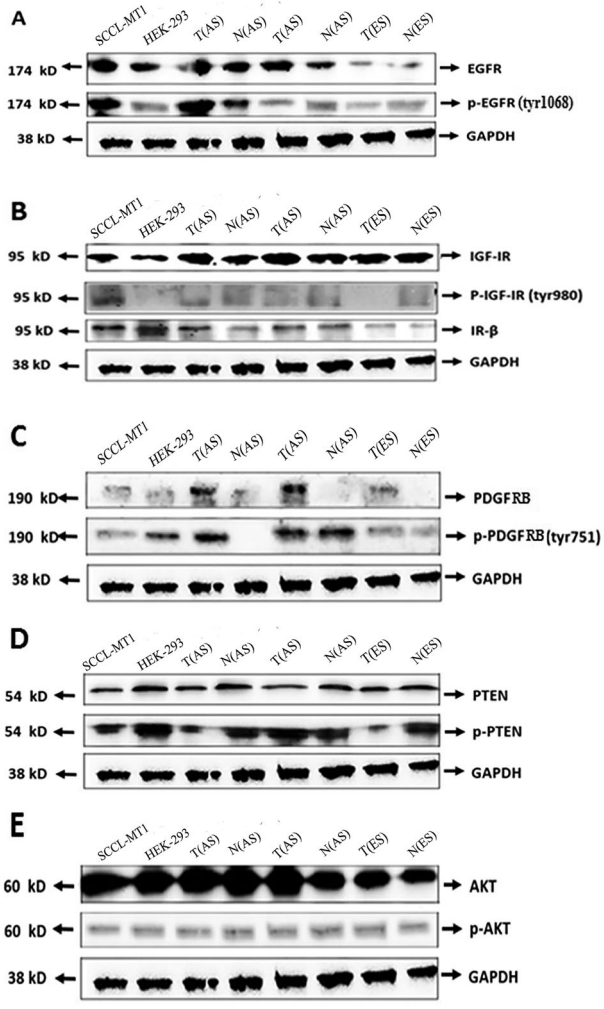

Figure 2. Results of Western-blot analysis (A) EGFR, pEGFR, (B) IGF-IR, pIGF-IR, IR-ß, (C) PDGFRB, pPGDFRB, (D) PTEN, pPTEN, (E) AKT, pAKT (AS: Advanced stage, ES: Early stage, N: Normal, T: Tumor, kD: kilodalton).

pEGFR and pPDGF expressions were statistically significant ($p = 0.027$ and $p = 0.004$, respectively) (Figure 2 and 3).

Discussion

Head and neck cancers are an aggressive group of tumors that are generally heterogeneous. More than 90% of these cancers constitute the squamous cell carcinomas of the head and neck originating from the squamous epithelium (HNSCC) [10,11]. The heterogeneity of HNSCCs and the multiple factors in etiology complicates the diagnosis and treatment. Therefore, only one in three of the patients with HNSCC can be diagnosed at an early stage. Two-thirds of the patients are diagnosed at an advanced stage and their treatment options were surgery, radiotherapy, chemotherapy, or combined therapy (chemoradiotherapy) and immunotherapy. Despite advances in treatment, the disease-free survival of HNSCC has not been significantly improved [12,13]. Therefore, it is of great importance to understand the molecular etiology of HNSCC and genetic alterations in the signal pathways for developing novel therapeutic approaches.

Signal transduction pathways play a vital role in the control of cellular communication, those from the extracellular environment, cytokines, chemokines, immune modulators, growth factors or hormones. Dysregulation of signaling pathways is one of the most frequent events in the malignant transformation of many cancers including HNSCC [14]. Secretion of polypeptides including cytokines, chemokines, growth factors or hormones and also other chemical inducers from the stromal and cancer cells activate a variety of signaling pathways for instance EGFR, phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinases (MAPKs) and mammalian target of rapamycin (mTOR). Most of the

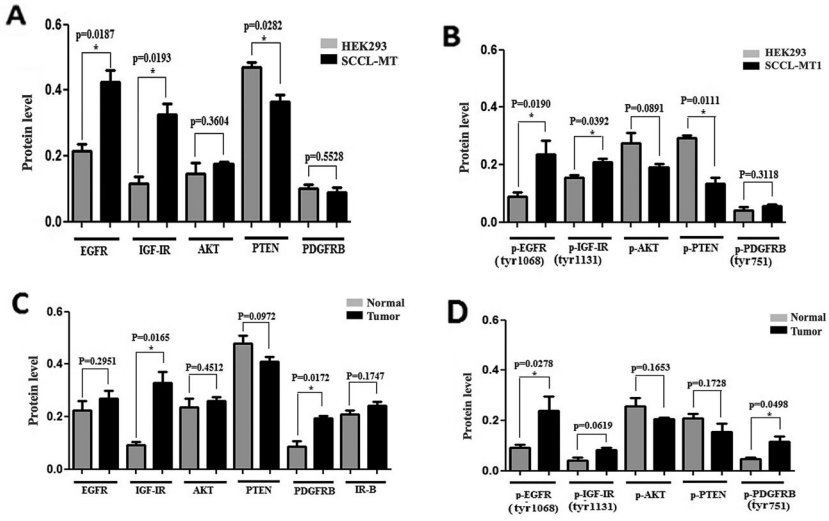

Figure 3. Protein levels of non-phosphorylated (A,C) and phosphorylated (B,D) in HEK-293, SCCL-MT1 cell lines and normal-tumor tissues.

previous studies were mainly focused on a signal pathway and targeting only cancer cells [15,16]. HNSCCs have a high rate of genetic heterogeneity. For this reason, the identification of both the cytokines and growth factors that trigger signal transduction pathways and their associated signal pathways in tumor/adjacent normal tissues and cell lines will be important in determining the molecular pathogenesis of HNSCCs. In this study, we first performed a cytokine array and investigated genes in the associated signal pathway in tumor vs normal tissues of HNSCC.

We found that advanced-stage HNSCC serum samples exhibited significantly elevated levels of EGF, IGF-1, IGFBP-1 and, PDGF-BB compared with those of early-stage ($p < 0.05$). Previous studies also indicated that the expression of these cytokines and growth factors may have implications in the HNSCC [17–19]. However, to the best of our knowledge, this is the first study to evaluate EGF, IGF-1, IGFBP-1 and, PDGF-BB in HPV-negative HNSCC. The EGF, IGF-1, IGFBP-1 and, PDGF-BB signaling pathways regulate critical cellular processes including differentiation, homeostasis and proliferation. Their downstream signaling molecules were PTEN/AKT and have cross-talks with multiple pathways. For this reason, qRT-PCR analysis of *PTEN* and *AKT* genes, which are associated with the results in the cytokine array was performed. We found that the expression level of *PDGFRB* was significantly upregulated ($p = 0.006$) but *PTEN* ($p > 0.001$) was significantly down-regulated in tumors compared with normal tissues.

PDGFRB, a homodimer of PDGFB, is associated with a poor prognosis of many common malignancies especially fibroblast-rich stroma [20], and it is also a crucial factor for tumor growth, angiogenesis and tumor survival. *PDGFRB* has been found to interact with members of several different RTK families, including *EGFR* [21]. *PTEN* is an important tumor suppressor gene, which functions in many cellular processes such as cell proliferation, survival, growth, metabolism, cell migration by inactivating PI3-kinase-dependent signaling. In the literature, associations between *PTEN* and *PDGF* isoforms were reported in human prostate cancer cell lines [22]. Lin *et al.* reported that the expressions of *PDGFA* and *PDGFB* mRNAs were higher in oral squamose cell carcinoma tissue than in the adjacent normal tissues [23]. Taken together, our results suggest a mechanism by which loss of *PTEN* may promote HNSCC progression *via PDGFR* signal transduction in HNSCC.

In this study, *IGFBP-1* was significantly overexpressed ($p = 0.045$) in the groups of advanced stage larynx tumors and larynx + oral cavity ($p = 0.010$). The IGF signaling pathway, such as PTEN, facilitates communication between cells and their physiologic environment and regulates critical cellular processes including differentiation, homeostasis, apoptosis, and aging. IGF system involves a complex network of ligands (IGF-1 and −2), their recognize receptors (IGFR-1 and −2), IGF-binding proteins (IGFBP1-6), and IGFBP proteases. Its downstream signaling molecules were PI3K-PTEN-AKT and RAS-RAF-MAPK pathways. A study showed that a higher IGFBP-1 and lower IGF-1 serum ratio was significantly associated with poor prognosis in nasopharyngeal carcinoma patients [19]. Several other studies have also reported a correlation between circulating levels of IGF-1 coupled with IGFBPs and the risk of various cancer development [24,25]. The IGFBPs bind strongly to IGFs and inhibit the action of IGFs by blocking their access to the receptors and this may be a novel therapeutic option [26]. In our study increased expression of *IGFBP-1* and high IGF-1 serum levels may be associated with advanced stage in HNSCCs.

In this presenting study, the expressions of *EGFR*, *IGF-1*, *PDGFBB*, *IGFB,* and *AKT* genes were increased both in tumor tissues compared to the HEK-293 cell line and normal tissues compared to SCCL-MT1 cell line. Also, the expression of *PTEN* was decreased in the same tissues. The interaction between *EGFR*, *IGF-1*, *PDGFBB*, *IGFBP*, *AKT,* and *PTEN* showed that PTEN/AKT signaling is one of the most critical cancer-promoting pathways in HNSCC. EGFR is also the most common biomarker in HNSCC, but our data showed that it was weakly expressed than PDGFRBB and IGFBP-1. New therapeutic approaches can focuses on the molecules acting on the interaction of different signal pathways.

Eventually, the protein level expressions of EGFR, IGF-IR, PDGFRB, AKT, PTEN and phosphorylated EGFR (Tyr1068), IGF-IR (Tyr980), PDGFRB (Tyr751), AKT and PTEN were analyzed in all tissues and cell lines. But, no signals were observed for other phosphoproteins. The results of both nonphosphorylated and phosphorylated proteins indicated that *EGFR, IGF-IR, IR-β, PDGFRB* and *AKT* protein levels both in tumor tissues and SCCL-MT1 cell line were highly expressed; and, PTEN had lower expression level. Also, nonphosphorylated IGF-IR ($p = 0.01$), PDGFRB ($p = 0.0172$) and phosphorylated EGFR ($p = 0.027$), PDGFRB ($p = 0.043$) protein levels were statistically significant in tumor tissues compared to normal tissues. Interestingly, when cell cultures were compared, both nonphosphorylated and phosphorylated EGFR, IGF1R and PTEN protein levels were statistically significant.

The present study confirmed previous reports of *EGFR* expression in HNSCC [27,28]. However, protein and gene expression of EGFR was the least frequently expressed according to *IGF-IR, IR-β, PDGFRB,* and *AKT* in tumor specimens. Nevertheless, *EGFR* expression in SCCL-MT1 was dramatically higher than that in the HEK-293 cell line. This may be due to the SCCL-MT1 being positive for EGFR.

Activation of the receptor tyrosine kinases (RTKs) especially *EGFR* is an important pathogenetic event in HNSCC [15]. In the studies [29,30] co-activation of multiple RTKs has been described in many cancer types. Receptor tyrosine kinases (RTKs) have revealed unexpected diversity in the mechanisms of their activation by growth factor ligands. Our data showed that co-activation of IGF-1R (Tyr980), PDGFRB (Tyr751), and EGFR (Tyr1068) or separately activation can be described in HNSCC. As a result of overactivation of IGF-1R (Tyr980), PDGFRB (Tyr751), and EGFR (Tyr1068) may be upregulated and overexpression these genes. In this context, the PI3K/AKT pathway seems to be overactivated downstream of the RTKs, alterations of PI3K inhibitory phosphatase, *PTEN*, and *AKT* have been found. Although there have been many reports of expressions of EGFR in HNSCC, to the best of our knowledge, this is the first report detailing differential expressions of the EGFR, PDGFRB, IGF-1R, and PTEN in HPV-negative HNSCC.

Conclusion

Based on our observations, determining the interactions of *EGFR, PDGFRB, IGF-1R* and *PTEN* or the activation of each might represent a promising new and innovative treatment approach in HNSCC patients. It seems clear that, in most cancers, effective targeted therapy may be involved the blockade of each one or multiple targets.

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Author contributions

AKY and TC designed the study, KO provided the tissues, AKY and AA performed the experiments, AKY interpreted the results of the experiments, MS made critical revisions to the manuscript.

Disclosurestatement

The authors declare that they have no competing interests.

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