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ORIGINAL RESEARCH

Expression Analysis of *GRHL3* and *PHLDA3* in Head and Neck Squamous Cell Carcinoma

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Background: Head and neck squamous cell carcinoma (HNSCC) includes a group of heterogeneous tumors with generally invasive behavior. The PI3K/AKT pathway plays an important role in the pathogenesis of HNSCC.

Methods: In the current study, we investigated the expression of two negative feedback regulators of the PI3K pathway, namely *PHLDA3* and *GRHL3*, in 45 paired samples of HNSCC and adjacent non-cancerous tissues (ANCTs).

Results: While expression of *GRHL3* was down-regulated in tumoral tissues compared with ANCTs by the factor 4.21, *PHLDA3* expression levels were up-regulated by 5.99-times. Gender-based analysis revealed a significant down-regulation of *GRHL3* gene expression level in male patients compared with the control samples and significant up-regulation of *PHLDA3* gene expression level in both sexes compared with the control samples. Differences in the expressions of both genes were significant in patients aged more than 60 years, but not in the younger patients. Expression of *GRHL3* was only down-regulated in patients with positive smoking history. Expression of *GRHL3* was decreased in grades 2 and 3 samples compared with controls. There was a significant increase in transcript levels of *PHLDA3* in stages II and III HNSCC samples compared with the controls group. ROC curve analysis indicated that the expression level of *PHLDA3* could be a promising marker for the diagnosis of HNSCC patients with a sensitivity and specificity of 0.666 and 0.688, respectively. In addition, sensitivity and specificity of *GRHL3* were 0.755 and 0.577, respectively.

Discussion: The current study indicates dysregulation of regulators of PI3K pathway in HNSCC and their potential application as putative biomarkers for this cancer.

Keywords: *GRHL3*, *PHLDA3*, head and neck squamous cell carcinoma

Introduction

Head and neck squamous cell carcinoma (HNSCC) includes a group of heterogeneous tumors that are generally invasive.[1] Based on the GLOBOCAN statistics, more than 834,000 new cases of HNSCC have been diagnosed in 2018.[2] This kind of cancer accounted for over 380,000 mortalities in 2018.[2] High mortality rates and serious symptoms are the features that make management of HNSCC difficult. Half of patients die in the first 5 years after initial diagnosis and the rate of survival has not been improved significantly during recent decades.[3,4] Regardless of recent advancements in surgery and radiotherapy, up to 50% of advanced local tumors relapse within 2 years after treatment.[1,5] Thus, identification of the underlying mechanism for HNSCC development has practical significance.

The phosphatidylinositol 3-kinase (PI3K) pathway is one of the most common activated signaling pathways in human cancers. PI3K is a member of the family of

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3'-OH phosphatidylinositol phosphorylating enzymes that have important roles in cellular proliferation, differentiation, and survival. PI3Ks are activated by receptor tyrosine kinases such as EGFR and catalyze a number of enzymatic reactions that ultimately lead to phosphorylation of AKT. Activated AKT phosphorylates proteins that are involved in cell proliferation and survival.[6,7] Dysregulation of the PI3K/AKT pathway is a common event in HNSCC.[8] There are many pieces of evidence indicating that negative feedback regulators of the PI3K pathway act as tumor suppressors, and their loss of function leads to the initiation of cancer, proliferation, metastasis, and resistance to chemotherapy. As the PI3K/AKT pathway plays an important role in head and neck cancer, evaluation of transcriptome, proteome and methylation status of negative regulators of this pathway is important in the diagnosis, prognosis, and targeted treatment of HNSCC. Among negative regulators of this pathway are Grainyhead Like Transcription Factor 3 (GRHL3) and Pleckstrin Homology Like Domain Family A Member 3 (PHLDA3). Reduced expression of these negative regulators has been reported in different malignancies.[9]

GRHL3 is one of the very conserved and ancestral transcription factors which is crucial for the development and homeostasis of ectoderm in many species.[10] GRHL3 may play a key role in prevention of HNSCC, as deletion of genomic region 1P36.11 containing this gene has been detected in human HNSCC. Evidence shows that GRHL3 evokes PTEN expression. Deletion of GRHL3 causes reduced expression of PTEN, so the PI3K/AKT/mTOR pathway is activated leading to metastasis of SCC. GHRL3 is a tumor suppressor.[11] Reduced levels of GRHL3 and PTEN are also seen in human SCC and are accompanied by increased expression of miR-21. So the mir21/GRHL3/PTEN axis is defined as a tumor suppressor. Increased levels of miR-21 and reduced levels of PTEN and GRHL3 are seen in some human HNSCCs.[12] GRHL3 also activates AKT and eNOS, two necessary factors for suppression of apoptosis.[13] Loss of GRHL3 causes a loss of anti-apoptotic effect and may lead to faster tumor progression. Studies on GRHL3 expression and its protein in breast cancer have shown that stage I tumors have the highest expression levels and stage III has the lowest expression levels.[14]

PHLDA3 represses AKT activity by its PH domain. PHLDA3 was initially recognized as a DNA-damage-response induced protein by P53 and its expression is directly regulated by P53. PHLDA3 cooperates with P53 in activation of apoptosis. Its overexpression leads to increased apoptosis.[15] P53 activation by PTEN and PHLDA3 suppresses PI3K/AKT and causes apoptosis. Inactivation of PHLDA3 leads to suppression of P53 by AKT.[15,16] Loss of PHLDA3 has been frequently detected in early stages of cancer, so PHLDA3 is regarded as a tumor suppressor. Genomic loss of PHLDA3 locus has been seen in pancreatic neuroendocrine tumors.[17]

In the current study, we assessed expression of *GRHL3* and *PHLDA3* in HNSCC and adjacent non-cancerous tissues (ANCTs) to appraise their role in the pathogenesis of this cancer.

Patients and Methods

Study Participants

The current study was performed on tumor tissues from 45 patients with a definite diagnosis of HNSCC and their corresponding ANCTs as control samples. Tumor and ANCTs were separated from fresh samples by an expert pathologist. Patients were referred to Cancer, Institute Imam Khomeini Hospital during 2018. All the patients were Iranian and none of them received radiotherapy or chemotherapy before surgery. Tissue samples were transferred to the laboratory of the medical Genetics department in liquid nitrogen. The study protocol was approved by the ethical committee of Tehran University of Medical Sciences and all the methods were performed following the relevant guidelines and regulations. Informed written consent forms were signed by all patients.

Table 1 Sequences of Primers Used in This Study

Primer Name	Sequence	Primer Length (nt)	PCR Product Length (nt)
GRHL3-F	CTGCCTCTGAAGCGTACCTG	20	117
GRHL3-R	CTCAGTCTCCCTCCGCACAT	20	
PHLDA3-F	CGCACCATCTTTCCTTCATGCT	22	121
PHLDA3-R	CGTCCATGCCTTCCACCTTG	20	
SDHA-F	CTTGCCAGGACCTAGAGTTTGT	22	86
SDHA-R	CTCTCCACGACATCCTTCCG	20	

Table 2 Demographic and Pathological Characteristics of the Study Participants

Parameters		Number (%)
Age	<60	30 (67.7)
	≥60	15 (33.3)
Gender	Male	33 (73.3)
	Female	12 (26.7)
Stage	I–II	18 (40)
	III–IV	27 (60)
Grade	G1	9 (20)
	G2	15 (33.3)
	G3	21 (46.7)
Smoking	Yes	30 (66.7)
	No	15 (33.6)
Alcohol consumption	Yes	2 (4.4)
	No	43 (95.6)

Expression Study

Total RNA was extracted from cancerous tissues and ANCTs using the TRIzol™ Reagent (Invitrogen, Carlsbad, CA). The quality and quantity of RNA were evaluated using Nanodrop 2000C (Thermo Scientific, USA) and gel electrophoresis. cDNA was synthesized using SuperScript IV Reverse Transcriptase cDNA Synthesis Kit (Roche, Germany). Relative expressions of mRNAs were assessed in cancerous and non-cancerous tissues using SYBR Premix Ex Taq II (Tli RNaseH Plus) (TAKARA, Japan) in a LightCycler 96 Real-Time PCR System (Roche). *SDHA* gene was used as the reference gene. The sequences of primers and PCR product lengths are shown in Table 1.

Statistical Analysis

Expressions of mRNAs in tumoral tissues compared with ANCTs were calculated using the Efficiency ^Ct normalizer Gene-Efficiency ^CT target gene method. To compare the expression level of two or multiple groups, an independent *t*-test or One-Way ANOVA was utilized. Pearson correlation coefficient was calculated to demonstrate the level of correlation between the variables. The receiver operating characteristic (ROC) curves were depicted to determine the diagnostic values of *PHLDA3* and *GRHL3* transcript levels for diagnosis of HNSCC. Two-sided *P*-values <0.05 were considered as statistically significant. The data analysis software used was GraphPad Prism 8.2.1 (CAA).

Results

General Data of Study Participants

The general data of the study participants are shown in Table 2.

Expression Analysis of *GRHL3* and *PHLDA3* in the HNSCC Patients

The results of *GRHL3* and *PHLDA3* gene expression in HNSCC tissues as compared with their ANCTs are

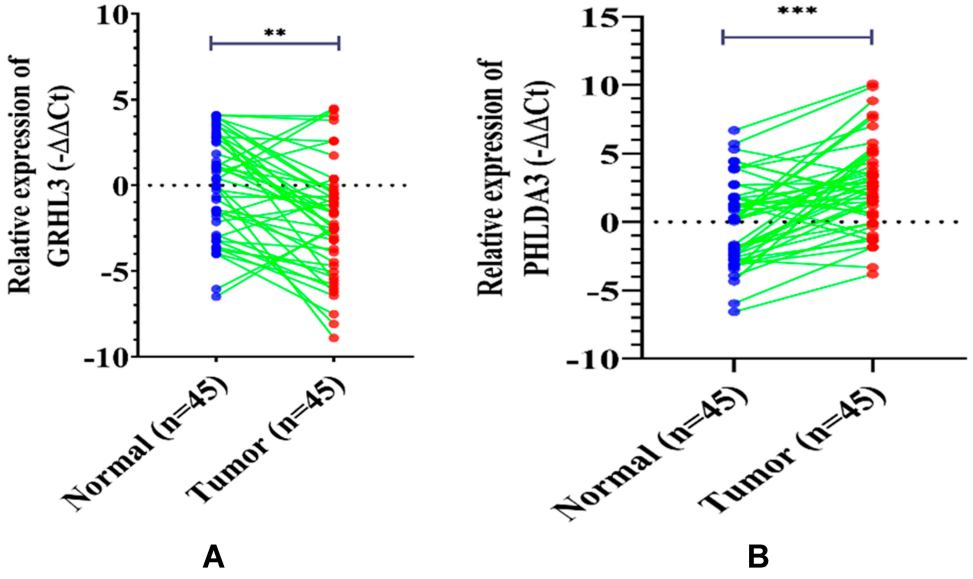

Figure 1 While *GRHL3* expression was downregulated (**A**), the transcript level of *PHLDA3* was increased (**B**) in HNSC tissues (n=45) compared with ANCTs (n=45). Expression was assessed by qRT-PCR and normalized to *SDAH1* expression (shown as the -ΔΔCT method). The data represent the mean±SD of three replicates. **P<0.01; ***P<0.001.

Table 3 Relative Expression of GRHL3 in HNSCC Tissues and Adjacent Tissues Based on Clinicopathological Features

Characteristics	Mean Difference	95.00% CI of Difference	Adjusted *P*-values	Significance
Gender				
Normal (n=45) vs Male (n=33)	2.230	0.514 to 3.945	0.007	**
Normal (n=45) vs Female (n=12)	1.646	−0.785 to 4.078	0.24	ns
Male (n=33) vs Female (n=12)	−0.583	−3.107 to 1.939	0.845	ns
Age				
Normal (n=45) vs >60 years (n=30)	2.06	0.292 to 3.827	0.018	*
Normal (n=45) vs <60 years (n=15)	2.104	−0.131 to 4.34	0.069	ns
>60 years (n=30) vs <60 years (n=15)	0.044	−2.326 to 2.416	0.998	ns
Smoking history				
Normal (n=45) vs Yes (n=30)	2.137	0.370 to 3.904	0.013	*
Normal (n=45) vs No (n=15)	1.949	−0.286 to 4.184	0.100	ns
Yes (n=30) vs No (n=15)	−0.188	−2.559 to 2.182	0.980	ns
Histologic grade				
Normal (n=45) vs Grade 1 (n=9)	−2.089	−5.213 to 1.034	0.303	ns
Normal (n=45) vs Grade 2 (n=15)	−2.561	−5.111 to −0.010	0.048	*
Normal (n=45) vs Grade 3 (n=21)	−2.811	−5.072 to −0.550	0.008	**
Grade 1 (n=9) vs Grade 2 (n=15)	−0.471	−4.079 to 3.135	0.986	ns
Grade 1 (n=9) vs Grade 3 (n=21)	−0.721	−4.130 to 2.686	0.94	ns
Grade 2 (n=15) vs Grade 3 (n=21)	−0.250	−3.142 to 2.642	0.995	ns
Pathologic stage				
Normal (n=45) vs Stage 2 (n=18)	2.502	0.417 to 4.586	0.014	*
Normal (n=45) vs Stage 3 (n=27)	1.789	−0.030 to 3.609	0.054	ns
Stage 2 (n=18) vs Stage 3 (n=27)	−0.712	−2.987 to 1.561	0.736	ns

Notes: *P<0.05; **P< 0.01.
Abbreviation: ns, non-significant.

depicted in Figure 1. While expression of *GRHL3* was down-regulated in tumoral tissues compared with ANCTs by the factor 4.21 (P<0.0022, 95% CI=−10.439 to −1.698), *PHLDA3* expression levels were up-regulated by 5.99-times (P<0.0003, 95% CI= 2.344 to −15.326).

Expression Analysis of *GRHL3* and *PHLDA3* in Association with Clinicopathological Features of Patients

Data were also analyzed according to gender, age, smoking history, histologic grade, and pathologic stage of study participants (Tables 3 and 4). The gender-based analysis revealed significant down-regulation of *GRHL3* gene expression level in male patients compared with the control samples (adjusted P=0.0073) and significant up-regulation of *PHLDA3* gene expression level in both sexes compared with control samples (adjusted P-values of 0.0035 and 0.0223 for males and females, respectively). Moreover, the differences in the expressions of both genes were significant in patients aged more than 60 years but not in the younger patients. Expression of *GRHL3* was only down-regulated in patients with positive smoking history. However, expression of *PHLDA3* was up-regulated in patients with both positive and negative smoking history. Besides, expression of *GRHL3* was decreased in grades 2 and 3 samples compared with controls. There was a significant increase in the transcript levels of *PHLDA3* in stage II and III HNSCC samples when compared with the control group.

Figure 2 shows the relative expression of the mentioned genes in different groups of tissues.

Co-Expression Analysis of *GRHL3* and *PHLDA3* in HNSCC Tissues and ANCTs

Pearson's correlation analysis revealed a positive correlation between *GRHL3* and *PHLDA3* expression levels (Figure 3).

ROC Curve Analysis

We evaluated the diagnostic value of the studied genes for HNSCC using the calculation of the area under the curve

Table 4 Relative Expression of PHLDA3 in HNSCC Tissues and Adjacent Tissues Based on Clinicopathological Features

Characteristics	Mean Difference	95.00% CI of Difference	Adjusted *P*-values	Significance
Gender				
Normal (n=45) vs Male (n=33)	−2.485	−4.261 to −0.708	0.003	**
Normal (n=45) vs Female (n=12)	−2.854	−5.372 to −0.336	0.022	*
Male (n=33) vs Female (n=12)	−0.369	−2.981 to 2.243	0.939	ns
Age				
Normal (n=45) vs >60 years (n=30)	−2.936	−4.753 to −1.120	0.000	***
Normal (n=45) vs <60 years (n=15)	−1.878	−4.175 to 0.420	0.131	ns
>60 years (n=30) vs <60 years (n=15)	1.059	−1.379 to 3.496	0.556	ns
Smoking history				
Normal (n=45) vs Yes (n=30)	−2.494	−4.321 to −0.666	0.004	**
Normal (n=45) vs No (n=15)	−2.762	−5.073 to −0.451	0.014	*
Yes (n=30) vs No (n=15)	−0.268	−2.720 to 2.183	0.963	ns
Histologic grade				
Normal (n=45) vs Grade 1 (n=9)	3.482	0.496 to 6.468	0.015	*
Normal (n=45) vs Grade 2 (n=15)	1.683	−0.755 to 4.121	0.276	ns
Normal (n=45) vs Grade 3 (n=21)	1.751	−0.410 to 3.912	0.154	ns
Grade 1 (n=9) vs Grade 2 (n=15)	−1.800	−5.247 to 1.648	0.523	ns
Grade 1 (n=9) vs Grade 3 (n=21)	−1.731	−4.989 to 1.527	0.507	ns
Grade 2 (n=15) vs Grade 3 (n=21)	0.068	−2.696 to 2.833	>0.999	ns
Pathologic stage				
Normal (n=45) vs Stage 2 (n=18)	−2.272	−4.432 to −0.113	0.0368	*
Normal (n=45) vs Stage 3 (n=27)	−2.791	−4.675 to −0.905	0.001	**
Stage 2 (n=18) vs Stage 3 (n=27)	−0.518	−2.874 to 1.838	0.859	ns

Notes: *P<0.05; **P<0.01; ***P<0.001.
Abbreviation: ns, non-significant.

(AUC) in ROC curves. AUC close to 1.0 signifies that the test has almost perfect diagnostic value. The maximum Youden index was used as a cut-off point. ROC curve analysis indicated that the tissue *PHLDA3* expression level could be considered as a promising marker for the diagnosis of HNSCC patients with a sensitivity and specificity of 0.666 and 0.688, respectively (AUC= 0.710, *P*-value=0.000, 95% CI=0.603–0.816). In addition, the sensitivity and specificity of *GRHL3* were 0.755 and 0.577, respectively (AUC=0.674, *P*-value=0.004, 95% CI=0.562–0.786), demonstrating its ability to discriminate HNSCC patients from healthy controls (Figure 4).

TCGA Dataset Analysis

TCGA dataset analyses were performed to evaluate expressions of *GRHL3* and *PHLDA3* in HNSCC. The results showed that expression of *PHLDA3* gene was significantly up-regulated in HNSCC tissues compared with normal tissues (Figure 5). Further assessment of data based on stages of HNSCC showed significant up-regulation of *PHLDA3* in all stages of HNSCC compared with normal tissues (Figure 6 and Table 5). However, no difference was detected between different stages. We also assessed expression of genes based on their sex. Expression of *PHLDA3* was higher in HNSCC tissues of both sexes compared with normal tissues. However, there was no difference between male and female patients (Figure 7 and Table 6). Assessment of expression of *PHLDA3* in TCGA samples showed different levels of expression in tumors of all age-based subgroups compared with normal samples (Figure 8 and Table 7). Additional evaluation of data based on HNSCC grades showed significant up-regulation of *PHLDA3* in all grades of HNSCC compared with normal tissues (Figure 9 and Table 8). However, no difference was detected between different grades except for comparison between grades 3 and 4.

Expression of *GRHL3* was significantly down-regulated in TCGA tumor samples compared with normal tissues (Figure 10). Additionally, *GRHL3* was down-regulated in all stages of HNSCC compared with normal tissues (Figure 11 and Table 9). However, no difference

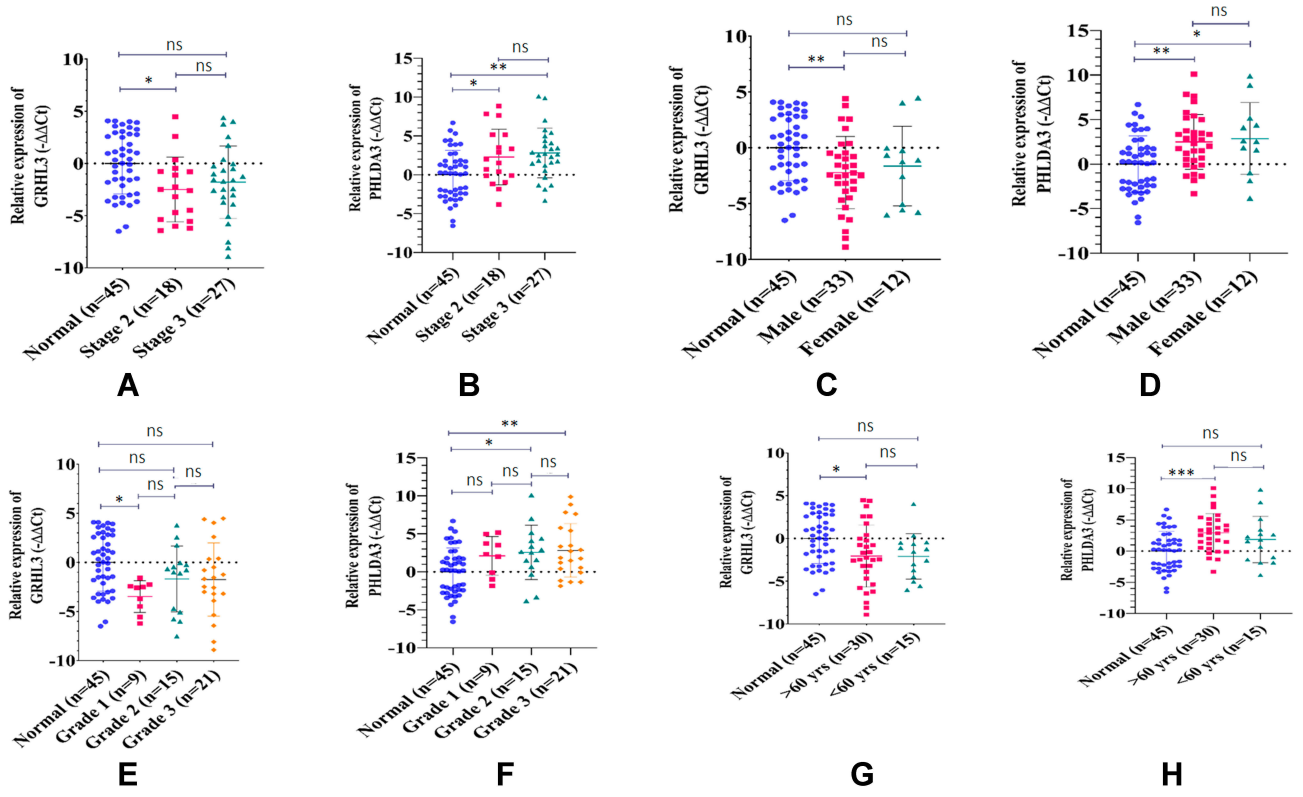

Figure 2 Expression of *GRHL3* and *PHLDA3* in association with clinicopathological features of HNSCC. Expression levels of *GRHL3* (**A**) and *PHLDA3* (**B**) were determined by qRT-PCR in different clinical stages of HNSCC tissue samples. The gender-based analysis showed that the expression levels of *GRHL3* (**C**) and *PHLDA3* (**D**) were significantly different in males and females. High HNSCC grade was associated with significant reduction of *GRHL3* (**E**) and up-regulation of *PHLDA3* (**F**). Patients with age of greater than 60 presented a down-regulation in the expression of *GRHL3* (**G**) and up-regulation of *PHLDA3* (**H**) expression level. The data represent the mean±SD of two replicates. **P*<0.05; **P<0.01; ****P*<0.001, ns, not significant.

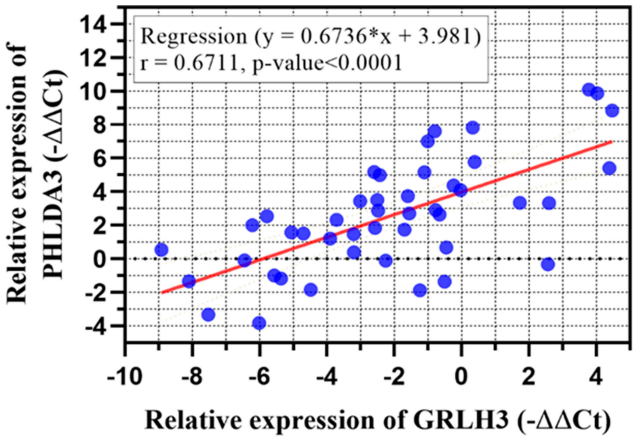

Figure 3 Co-expression analysis between expression of genes in ANCTs and HNSCC tissues demonstrated a significant correlation between *PHLDA3* and *GRHL3* (r=0.6711, *P*<0.0001). Each dot indicates an individual sample.

was detected between different stages. Expression of *GRHL3* was lower in HNSCC tissues of both sexes compared with normal tissues. However, there was no difference between male and female patients (Figure 12 and Table 10). Assessment of expression of *GRHL3* in TCGA samples showed different levels of expression in tumors of all age-based subgroups compared with normal samples (Figure 13 and Table 11). Finally, evaluation of data based on HNSCC grades showed significant down-regulation of *GRHL3* in grades 1–3 compared with normal tissues (Figure 14 and Table 12). However, no difference was detected between grade 4 and normal tissues.

Discussion

In the current study, we appraised the expression of two negative regulators of PI3K/AKT pathway, namely *PHLDA3* and *GRHL3*, in 45 paired samples of HNSCC and ANCTs. We also compared our results with the results of TCGA data. Based on our results, expression of *GRHL3* was down-regulated in tumoral tissues compared with ANCTs. However, expression of *PHLDA3* was up-regulated in tumor tissues compared with ANCTs. Notably, analysis of their expression in TCGA samples verified our results.

GRHL3 has been identified as an important tumor suppressor which directly modulates PTEN and the PI3K/AKT/mTOR signaling pathway.[12] Deletion of this

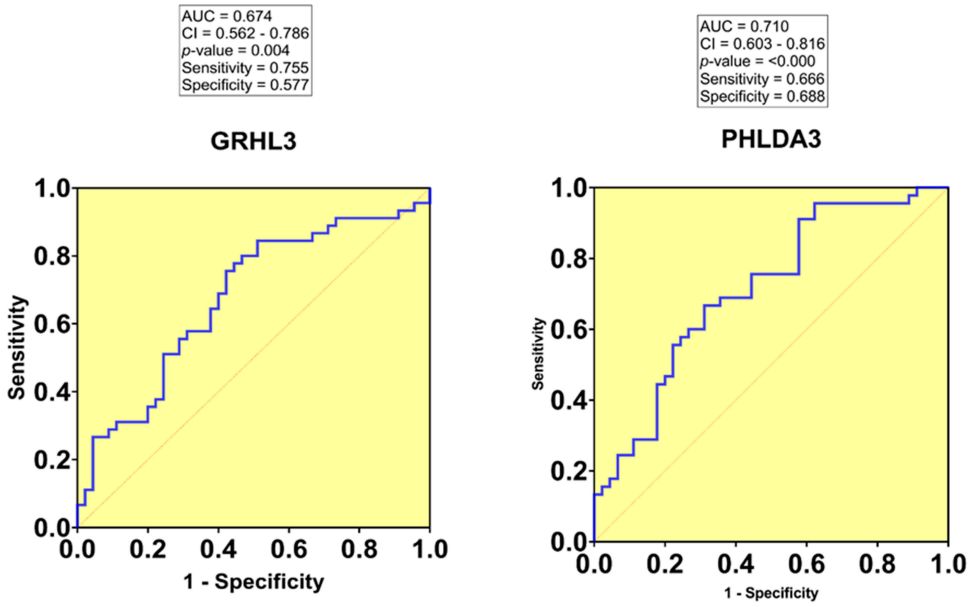

Figure 4 The ROC curve was constructed using GraphPad Prism. The area under the curve (AUC) for GRHL3 and PHLDA3 is 0.674 and 0.710, respectively.

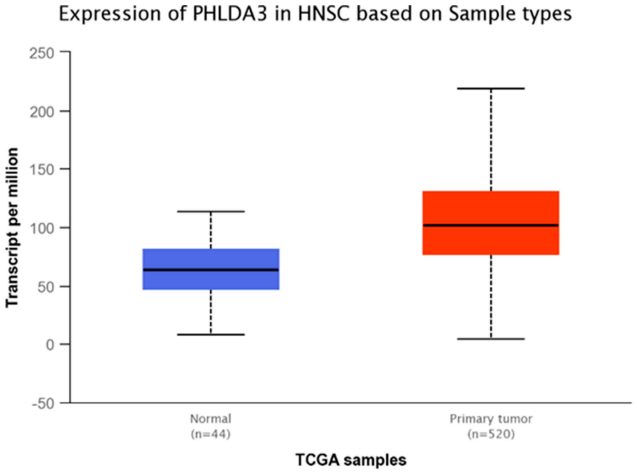

Figure 5 Relative expression of *PHLDA3* in primary HNSCC tumors and normal tissues based on the data provided by TCGA (Statistical significance=1.0325074129014E-14).

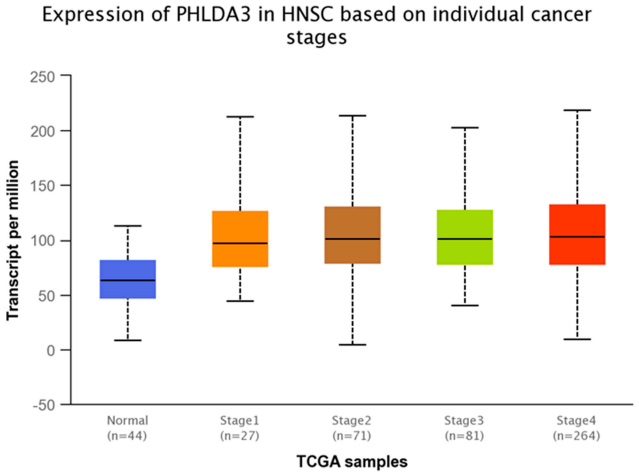

Figure 6 Relative expression of *PHLDA3* in different stages of HNSCC tumors and normal tissues based on the data provided by TCGA.

gene has been commonly detected in HNSCC.[11] Tumor suppressor function of Grhl3 in HNSCC has been verified in a conditional knockout mouse line. Although deletion of Grhl3 in oral epithelial cells did not affect the PTEN/PI3K/AKT/mTOR pathway, it caused significant down-regulation of GSK3B, leading to stabilization and accretion of c-MYC and development of aggressive HNSCC.[11]

PHLDA3 has been regarded as a p53-regulated suppressor of Akt.[18] Assessment of genetic aberrations of the *PHLDA3* locus in a large cohort of lung cancer samples revealed loss of this locus in large-cell neuroendocrine carcinoma and carcinoid samples, though no chromosomal aberration was detected in this locus in other histological types of lung cancer.[18] Notably, Muroi et al[19] have evaluated expression of PHLDA3 protein in esophageal SCC samples and detected a high level of its expression in the majority of samples, which is in accordance with our results in HNSCC. However, they found low PHLDA3 expression as a prognostic factor for tumor recurrence and low survival.[19] Although PHLDA3 has been identified as a tumor suppressor which acts through suppression of Akt,[18] Qiao et al.[20] have shown that PHLDA3 inhibits somatic cell reprogramming through activation of the Akt-GSK3β pathway. This signaling pathway has a prominent

Table 5 Statistical Significance of Relative Expression of PHLDA3 in Different Stages of HNSCC Tumors and Normal Tissues Based on the Data Provided by TCGA

Comparison	Statistical Significance
Normal vs Stage 1	**4.967000E-04**
Normal vs Stage 2	**9.33959998228318E-09**
Normal vs Stage 3	**2.27800001084688E-09**
Normal vs Stage 4	**1.68376423914651E-12**
Stage 1 vs Stage 2	7.347200E-01
Stage 1 vs Stage 3	8.158600E-01
Stage 1 vs Stage 4	9.008800E-01
Stage 2 vs Stage 3	8.705000E-01
Stage 2 vs Stage 4	6.441400E-01
Stage 3 vs Stage 4	7.983600E-01

Note: Significance is shown by bold numbers.

Table 6 Statistical Significance of Relative Expression of *PHLDA3* in HNSCC Samples of Different Sexes and Normal Tissues Based on the Data Provided by TCGA

Comparison	Statistical Significance
Normal vs Male	**6.7612582199672E-14**
Normal vs Female	**5.14033260401447E-14**
Male vs Female	2.862600E-01

Note: Significance is shown by bold numbers.

Table 7 Statistical Significance of Relative Expression of PHLDA3 in HNSCC Patients and Normal Tissues Based on Patients' Age

Comparison	Statistical Significance
Normal vs Age (21–40 years)	**8.019400E-04**
Normal vs Age (41–60 years)	**1.06492592522045E-12**
Normal vs Age (61–80 years)	**1.64968039229052E-12**
Normal vs Age (81–100 years)	**6.767000E-04**
Age (21–40 years) vs Age (41–60 years)	1.880810E-01
Age (21–40 years) vs Age (61–80 years)	3.723800E-01
Age (21–40 years) vs Age (81–100 years)	5.286600E-01
Age (41–60 years) vs Age (61–80 years)	3.376200E-01
Age (41–60 years) vs Age (81–100 years)	7.099800E-01
Age (61–80 years) vs Age (81–100 years)	9.649600E-01

Note: Significance is shown by bold numbers.

Table 8 Statistical Significance of Relative Expression of PHLDA3 in Different Grades of HNSCC Tumors and Normal Tissues Based on the Data Provided by TCGA

Comparison	Statistical Significance
Normal vs Grade 1	**5.96100000471722E-08**
Normal vs Grade 2	**1.96609395430869E-12**
Normal vs Grade 3	**1.91924254266951E-12**
Normal vs Grade 4	**2.179900E-02**
Grade 1 vs Grade 2	4.443600E-01
Grade 1 vs Grade 3	5.715600E-01
Grade 1 vs Grade 4	6.253600E-02
Grade 2 vs Grade 3	8.107700E-02
Grade 2 vs Grade 4	3.607800E-01
Grade 3 vs Grade 4	**1.877650E-02**

Note: Significance is shown by bold numbers.

Table 9 Statistical Significance of Relative Expression of GRHL3 in Different Stages of HNSCC Tumors and Normal Tissues Based on the Data Provided by TCGA

Comparison	Statistical Significance
Normal vs Stage 1	**1.384340E-04**
Normal vs Stage 2	**1.212600E-04**
Normal vs Stage 3	**2.177500E-04**
Normal vs Stage 4	**2.26539999998998E-05**
Stage 1 vs Stage 2	7.566200E-01
Stage 1 s Stage 3	6.730800E-01
Stage 1 vs Stage 4	8.224800E-01
Stage 2 vs Stage 3	8.817200E-01
Stage 2 vs Stage 4	4.218400E-01
Stage 3 vs Stage 4	3.271000E-01

Note: Significance is shown by bold numbers.

Table 10 Statistical Significance of Relative Expression of GRHL3 in HNSCC Samples of Different Sexes and Normal Tissues Based on the Data Provided by TCGA

Comparison	Statistical Significance
Normal vs Male	**5.94999999999901E-05**
Normal vs Female	**3.90430000000164E-05**
Male vs Female	5.796800E-01

Note: Significance is shown by bold numbers.

role in attainment of the epithelial-mesenchymal transition (EMT) phenotype in the gefitinib-resistant HNSCC cells.[21] Thus, it is possible that PHLDA3 exerts dual tumor suppressor and oncogenic roles based on the preferential mode of its regulatory effects on AKT downstream genes.

Based on the different directions of regulatory roles of *PHLDA3* and *GRHL3* on GSK3β, dysregulation of this downstream target of Akt is the possible mechanism for participation of *PHLDA3* and *GRHL3* in the pathogenesis of HNSCC.

The gender-based analysis revealed significant down-regulation of *GRHL3* gene expression level in male patients compared with the control samples. However, the *PHLDA3* gene was up-regulated in both sexes compared with control samples. Thus, one can consider a sex-based difference in the

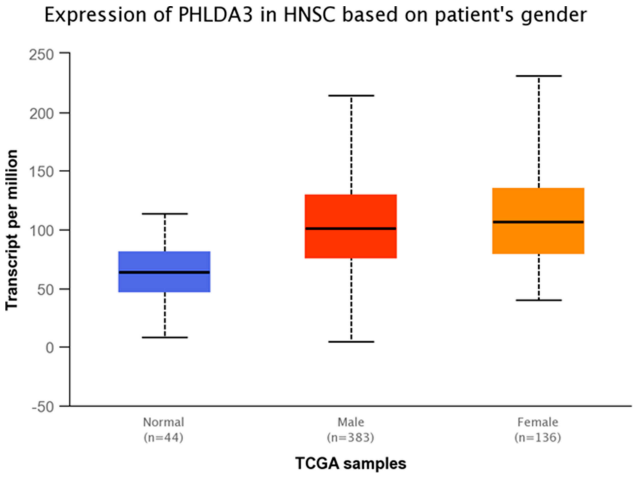

Figure 7 Relative expression of *PHLDA3* in HNSCC tumors obtained from male and female patients and normal tissues based on the data provided by TCGA.

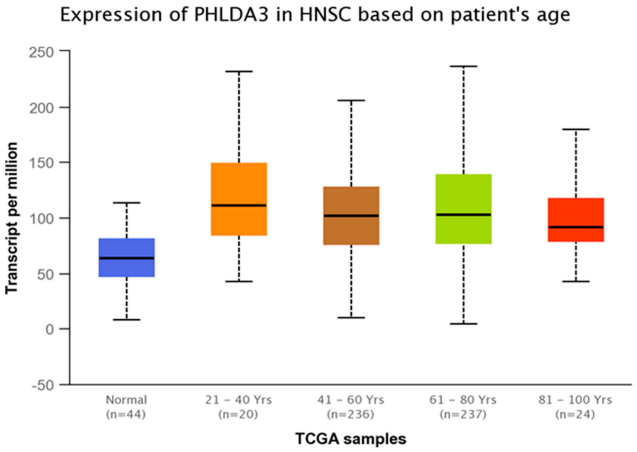

Figure 8 Relative expression of *PHLDA3* in HNSCC patients and normal tissues based on patients' age.

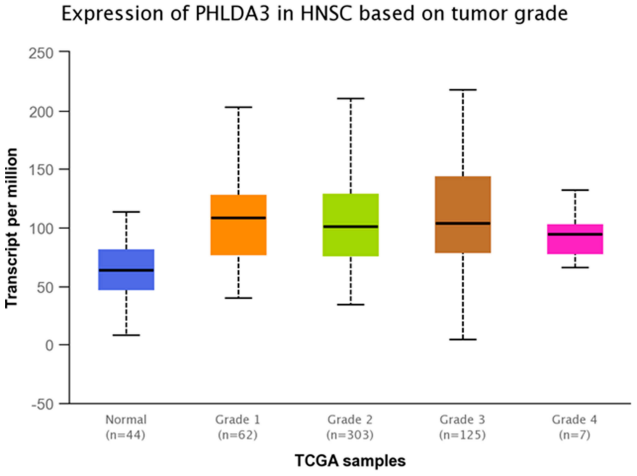

Figure 9 Relative expression of *PHLDA3* in different grades of HNSCC tumors and normal tissues based on the data provided by TCGA.

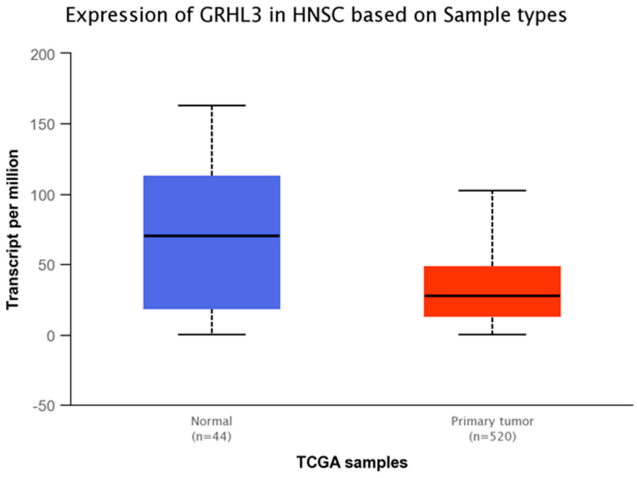

Figure 10 Relative expression of *GRHL3* in primary HNSCC tumors and normal tissues based on the data provided by TCGA (Statistical significance=4.35819999999865E-05).

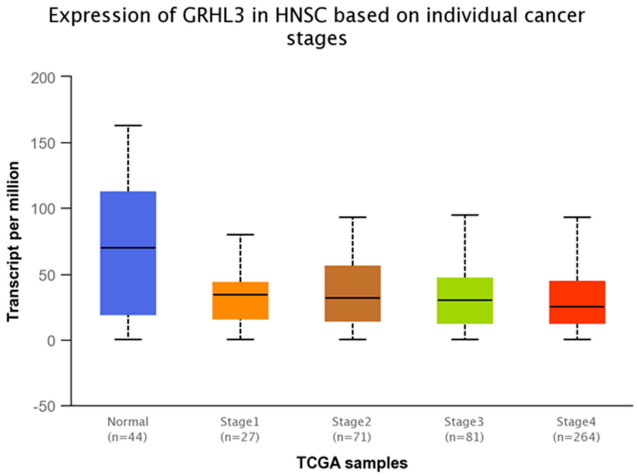

Figure 11 Relative expression of *GRHL3* in different stages of HNSCC tumors and normal tissues based on the data provided by TCGA.

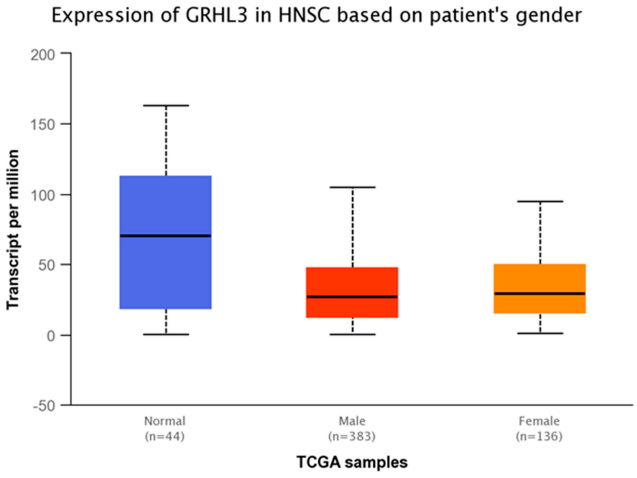

Figure 12 Relative expression of *GRHL3* in HNSCC tumors obtained from male and female patients and normal tissues based on the data provided by TCGA.

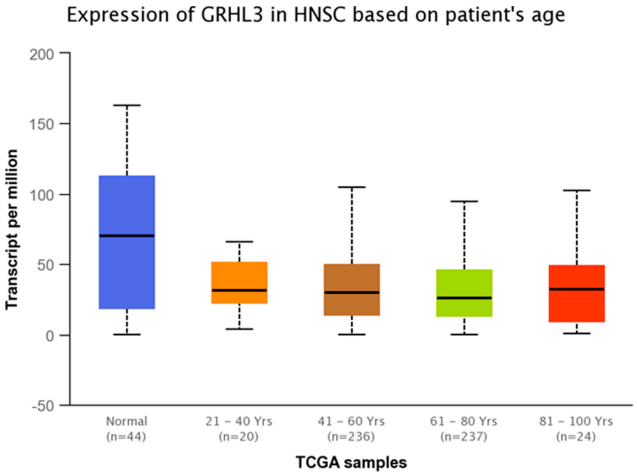

Figure 13 Relative expression of *GRHL3* in HNSCC patients and normal tissues based on patients' age.

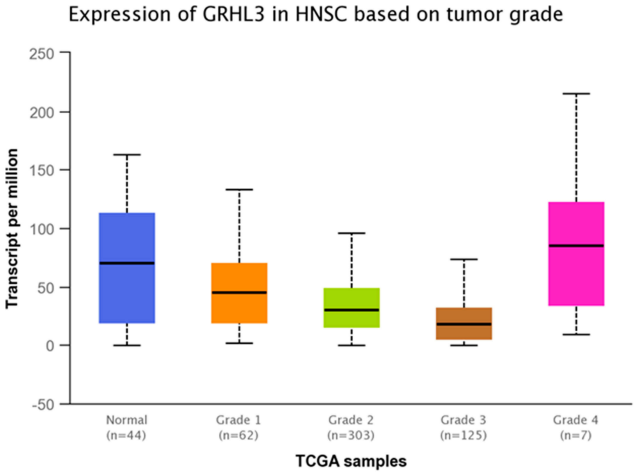

Figure 14 Relative expression of *GRHL3* in different grades of HNSCC tumors and normal tissues based on the data provided by TCGA.

Table 11 Statistical Significance of Relative Expression of GRHL3 in HNSCC Patients and Normal Tissues Based on Patients' Age

Comparison	Statistical Significance
Normal vs Age (21–40 yeas)	**1.606560E-04**
Normal vs Age (41–60 years)	**1.205040E-04**
Normal vs Age (61–80 years)	**2.27739999999477E-05**
Normal vs Age (81–100 years)	**6.153900E-04**
Age (21–40 years) vs Age (41–60 years)	7.301600E-01
Age (21–40 years) vs Age (61–80 years)	6.165200E-01
Age (21–40 years) vs Age (81–100 years)	9.720000E-01
Age (41–60 years) vs Age (61–80 years)	1.568030E-01
Age (41–60 years) vs Age (81–100 years)	7.824200E-01
Age (61–80 years) vs Age (81–100 years)	7.384200E-01

Note: Significance is shown by bold numbers.

Table 12 Statistical Significance of Relative Expression of GRHL3 in Different Grades of HNSCC Tumors and Normal Tissues Based on the Data Provided by TCGA

Comparison	Statistical Significance
Normal vs Grade 1	**9.601700E-03**
Normal vs Grade 2	**6.41519999999984E-05**
Normal vs Grade 3	**4.95859999949388E-07**
Normal vs Grade 4	4.573400E-01
Grade 1 vs Grade 2	**8.861900E-03**
Grade 1 vs Grade 3	**2.76570000001808E-06**
Grade 1 vs Grade 4	1.804570E-01
Grade 2 vs Grade 3	**6.20969999999277E-05**
Grade 2 vs Grade 4	9.618300E-02
Grade 3 vs Grade 4	5.520000E-02

Note: Significance is shown by bold numbers.

role of *GRHL3* in HNSCC. Previous studies have noted sex-based differences in clinical manifestations and risk factors of HNSCC in terms of smoking and drinking habits and primary tumor site distribution.[22] Moreover, the aneuploidy index has been different among HNSCC tumors of males and females.[23] Consistent with these reports, Qadir et al.[24] reported distinct molecular signatures in the HNSCC of males and females. However, assessment of expression of *PHLDA3* and *GRHL3* in TCGA samples revealed no difference in their expression between males and females.

Moreover, the differences in the expressions of both genes were significant in patients aged more than 60 years but not in the younger patients. Age has a remarkable influence on HNSCC prognosis and is associated with a distinctive epidemiology, hazards, and preferences.[25] The distinct expression of these genes in elderly HNSCC patients adds to these unique patterns and might be associated with patients' prognosis. However, assessment of TCGA data showed no difference in expression of genes between age-based subgroups. The inconsistency between TCGA data and our data is explained by the difference in determination of age subgroups.

Expression of *GRHL3* was only down-regulated in patients with a positive smoking history. However, expression of *PHLDA3* was up-regulated in patients with both positive and negative smoking history. Smoking might affect gene expression in HNSCC samples. Irimie et al.[26] have identified a number of differentially expressed genes that distinguish HNSCC patients based on their smoking history. Most of these genes were enriched in cellular metabolism pathways. These observations indicate the prominence of the interaction

between environmental risk factors and genetic elements in the pathogenesis of HNSCC.

Besides, expression of *GRHL3* was decreased in grades 2 and 3 samples compared with controls. There was a significant increase in the transcript levels of *PHLDA3* in stage II and III HNSCC samples when compared with the control group. Differences in the expression levels of these genes between distinct stages and grades of HNSCC have also been detected in TCGA samples. These data further support the participation of these genes in the pathogenesis of HNSCC.

ROC curve analysis indicated that the tissue expression level of *PHLDA3* and *GRHL3* could be considered as putative markers for assessment in larger cohorts of HNSCC patients. Taken together, the current study indicates dysregulation of regulators of the PI3K pathway in HNSCC and their potential application as putative biomarkers for this cancer.

Availability of Data

The data that support the findings of this study are available from the corresponding author (Dr. Abbas Shakoori) upon reasonable request.

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Disclosure

The authors report no conflicts of interest in this work.

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