Integrin α-5 as a potential biomarker of head and neck squamous cell carcinoma

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignant tumors that endanger human health. In recent years, the incidence of HNSCC has been increasing, without any significant improvement in the prognosis. Therefore, increased knowledge on the molecular mechanism underlying HNSCC development will allow the development of new strategies for therapy. The present study attempted to identify key genes involved in HNSCC development. Expression profiles of HNSCC and normal samples were downloaded from The Cancer Genome Atlas database. Differentially expressed genes (DEGs) between the HNSCC and normal samples were identified and subjected to Gene Oncology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis. A protein-protein interaction (PPI) network was constructed, and Cytoscape CentiScape and Gene Expression Profiling Interactive Analysis were used to identify key DEGs. Finally, expression profiles of HNSCCs, including 500 HNSCCs and 44 normal samples, were included in the analysis. A total of 1,181 DEGs were screened, among which 354 genes were upregulated and 827 genes were downregulated in HNSCC compared with normal tissues. The GO enrichment analysis showed that the DEGs were mainly involved in chloride transmembrane transporter, metalloendopeptidase and substrate-specific channel activities. The KEGG pathway analysis revealed that the DEGs were mainly associated with 'protein digestion and absorption', as well as 'extracellular matrix-receptor interaction'. Integrin α-5 (ITGA5) was identified as a hub gene, based on the PPI network complex, and was confirmed to be significantly associated with the overall survival rate. Moreover, ITGA5 was overexpressed specifically in HNSCC. The genes found, notably ITGA5, are potential diagnostic biomarkers and therapeutic targets in HNSCC.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) was reported as the sixth most common type of malignancy in humans, constituting ~4% of all new cases in the United States in 2015 (1,2). According to a recent report (2018), ~600,000 patients were affected worldwide yearly and the incidence rate has significantly increased (3). Improvements in clinical therapy have not led to corresponding improvements in the prognosis of patients with HNSCC. The 5-year survival rate of patients with HNSCC still remains between 40 and 50%. Revealing the underlying mechanism of HNSCC development could provide potential biomarkers or therapeutic targets in HNSCC (4).

Several studies have focused on the mechanism of HNSCC, and the new generation of sequencing technology provides a rich resource for the study of significant genetic changes during tumorigenesis and for the screening of potential diagnostic and prognostic markers of cancer. For instance, it was reported that actin-like protein 8 (ACTL8) was increasingly expressed in HNSCC and regarded as an independent prognostic factor (5). The expression of neutrophil gelatinase-associated lipocalin was lower in HNSCC than in normal tissues, and was correlated with the tumorigenesis of HNSCC (6). Calpain 6 expression was significantly decreased in HNSCC and positively associated with the survival rate of patients with the disease, thereby indicating the role of calpain 6 as a tumor suppressor in HNSCC (7). However, due to the limited sample sizes, previous studies may provide false predictions.

In the present study, integrated analysis was performed to identify the key genes involved in the development of HNSCC. Firstly, the differentially expressed genes (DEGs) between HNSCC and normal tissues were screened, followed by Gene Ontology (GO) enrichment analysis, Kyoto Encyclopedia

of Genes and Genomes (KEGG) enrichment analysis and protein-protein interaction (PPI) network analysis. Finally, the key candidate DEGs were identified according to Centiscape and log-rank survival analysis, and were verified using the Gene Expression Ontology (GEO) datasets. These key DEGs were identified as potential biomarkers for early diagnosis and as therapeutic targets for HNSCC.

Materials and methods

Gene expression profile data and identification of DEGs. The level-3 RNA sequence (RNA Seq) data (fragments per kilobase of transcript per million mapped reads upper quartile data) of HNSCC and corresponding normal tissue samples were downloaded from the TCGA database (dataset no. :544; https://www.cancer.gov/tcga) (8) using the Genomic Data Commons Application Programming Interface (9). A total of 544 samples from 500 patients with HNSCC and 44 normal controls were collected in December 2018. The normal controls included normal tissues from the oral cavity, oral tongue, larynx, floor of the mouth and base of the tongue. The raw data was downloaded and the $\log_2$ fold-change ($\log_2$FC) was calculated using the Limma R package (version 3.2.5; https://www.r-project.org/) to screen DEGs between HNSCC and normal tissues. The following cut-off criteria were applied: $\log_2$FC>2 and P<0.05. The adjustment of P<0.5 was set as the threshold to adjust the P-value for multiple comparisons.

For verification purposes, the microarray expression dataset GSE6631 was downloaded (as minimum information about a microarray experiment notation in mark-up language formatted family files) from the GEO database (10). The GSE6631 was based on the GPL8300 Platforms (Affymetrix Human Genome U95 version 2 array) and included 44 HNCC samples and paired normal samples (submission date, 2007; last updated, 2018) (11). The sample information and expression profile data were extracted by R package (version:3.2.5) from GES6631. Statistical analyses were performed with GraphPad Prism version 8.0 software (GraphPad Software, Inc.). Single comparisons between two groups were performed using Paired Student's t-test.

GO and pathway enrichment analyses of DEGs. GO analysis and KEGG pathway enrichment of DEGs were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (version, 6.7; https://david-d.ncifcrf.gov/) to screen for possible biological processes, cellular components, molecular functions and signaling pathways of the involved DEGs (12). The resulting data were imported into Cytoscape ClueGo software (version: 3.6.0) for visual analysis (13). P<0.05 was considered as statistically significant. The following parameter settings were applied: Identifier, 'official gene symbol.'; list type, 'gene list.'; species, 'homo sapiens.'; count threshold, 2; and ease threshold, 0.05.

PPI network construction and candidate gene identification. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; version, 11.0; http://string-db.org) was used to construct the PPI network (14). The minimum required interaction score was set to a medium confidence of 0.4, and the organism was set to '*Homo sapiens*'. The Cytoscape software was then used to visualize the network. Cytoscape CentiScape (version, 3.6.0; http://apps.cytoscape.org/apps/centiscape) (15) was used to screen candidate key proteins in the network, according to the degree of centrality. The genes with a node degree of ≥15 were considered as the candidate key genes.

Association between candidate key genes and clinicopathological parameters of HNSCC. By using the Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/), the effect of candidate key genes on overall survival (OS) rate was evaluated using log-rank test and the Mantel-Cox test. P<0.05 for log-rank test indicated statistical significance. Genes significantly associated with HNSCC OS rate were considered as key genes. The following parameter settings were applied: Group cut-off, 'median.'; hazards ratio, 'yes.'; 95% confidence interval, 'yes.'; and axis units, 'months.'.

Comparison of key genes in HNSCC and other types of cancer. In order to evaluate the specificity of the key genes screened in the previous step to HNSCC, the expression of these genes was examined in other tumor datasets from the GEPIA database, including adrenocortical carcinoma, bladder urothelial carcinoma (BLCA), breast invasive carcinoma, endocervical adenocarcinoma (CESC), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), liver hepatocellular carcinoma, lung squamous cell carcinoma and lung adenocarcinoma (LUAD). These datasets came from the TCGA and the GTEx projects (16). The unmatched normal and tumor tissues were compared. The raw data were filtered based on the cut-offs $\log_2$FC>2 and P<0.05.

Results

Identification of DEGs in HNSCC. RNAseq data of HNSCC and corresponding normal tissue samples were downloaded from the TCGA database. The data was screened by the Limma package, using P<0.05 and $\log_2$FC>2 as the cut-off criteria, which identified 1,181 DEGs (Fig. 1), including 354 upregulated and 827 downregulated genes.

GO analysis and signaling pathway enrichment of DEGs in HNSCC. The GO analysis of the 1,181 DEGs was performed using the DAVID database, with the criterion set at P<0.05. The DEGs were divided into three groups, namely, biological process, cellular component and molecular function groups. As shown in Fig. 2A, the main DEG-associated biological functions were 'cell adhesion', 'extracellular matrix organization', 'skeletal system development' and 'ion transmembrane transport'. As demonstrated in Fig. 2B, the cellular component analysis revealed that the selected DEGs were mainly located at the 'extracellular exosome', 'extracellular region' and 'extracellular space'. The molecular function of the DEGs was mainly associated with 'actin binding', 'heparin binding' and 'cytokine activity' (Fig. 2C). As demonstrated in Fig. 3, the KEGG pathways enriched by the DEGs were mainly associated with 'protein digestion and absorption', 'extracellular matrix-receptor interaction', 'drug metabolism' and the 'PPAR signaling pathway'.

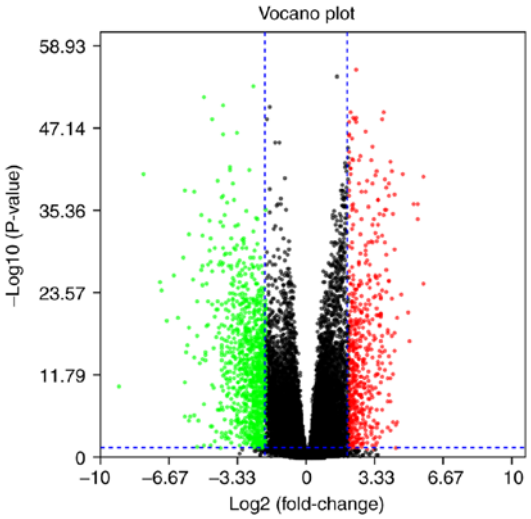

Figure 1. Differential gene expression between head and neck squamous cell carcinoma and normal tissues. The volcano plot presents upregulated (red points) and downregulated genes (green points), screened on the basis of fold-change >2.0 and a correction for P<0.05. The black points represent genes with no significant difference.

Key candidate DEG identification with PPI network analysis. The PPI network of the DEG expression products was constructed using Cytoscape software and the STRING database (http://string-db.org). A total of 1,035 DEGs were incorporated into the PPI network complex. The Cytoscape CentiScape was used to screen candidate key genes in the network, with degree of centrality ≥15 set as the inclusion criterion, which identified 50 genes that were included in the following analysis (Fig. 4).

Log-rank survival analysis by the GEPIA database, found 8 out of the 50 candidate key genes, including integrin α-5 (ITGA5) and serpin family E member 1 (SERPINE1), to be significantly associated with HNSCC OS rate (P<0.05; Table I). If the threshold was set to P<0.01, only ITGA5 and SERPINE1 were found to be associated with the OS rate of HNSCC (Fig. 5). The Cox proportional hazard ratios of ITGA5 and SERPINE1 were both 1.5.

ITGA5 is highly expressed in HNSCC specifically. To evaluate the specificity of the key genes previously screened in HNSCC, their expression levels were determined in other tumor datasets from the GEPIA database. The expression of ITGA5 was only significantly increased in HNSCC and decreased in BLCA, CESC, COAD and LUSC (Fig. 6A). However, SERPINE1 expression was significantly increased in ESCA, as well as in HNSCC, with no other significant changes in other tumors (Fig. 6B).

For verification, ITGA5 expression was evaluated in HNSCC based on the microarray express dataset GSE6631. Compared with ITGA5 expression in normal tissues, the expression in HNSCC tissues was upregulated (P=0.007; Fig. 7).

Discussion

HNSCC is one of the most common types of malignancies in humans; it is characterized by rapid progression, a high

Table I. Log regression analysis of differently expressed genes with node degree of centrality ≥15.

Gene symbol	Degree of centrality	Log-rank, P-value
COL1A1	45	0.30
COL1A2	37	0.84
FN1	34	0.11
COL2A1	34	0.26
COL3A1	33	0.56
MMP9	32	0.93
COL4A2	30	0.61
COL4A1	30	0.62
COL5A2	29	0.29
COL4A5	28	0.85
COL11A1	26	0.09
COL7A1	26	0.10
COL5A1	26	0.28
SPARC	26	0.30
COL6A3	26	0.86
SERPINH1	25	0.02
COL6A1	25	0.22
COL4A6	25	0.36
TIMP1	24	0.04
SPP1	24	0.04
CSF2	23	0.02
LUM	23	0.63
COL10A1	23	0.98
GNGT1	22	0.15
COL27A1	22	0.46
ITGA5	21	<0.01
MMP1	21	0.04
CXCL10	21	0.19
CENPA	21	0.30
COL12A1	21	0.53
P4HA3	20	0.06
MMP13	20	0.09
MMP3	20	0.23
POSTN	20	0.30
PLK1	20	0.41
COL13A1	20	0.44
COL22A1	20	0.62
FOXM1	19	0.55
ACAN	18	0.50
AURKB	18	0.63
KIF2C	17	0.29
CDC45	17	0.90
PTHLH	17	0.92
SERPINE1	16	<0.01
ITGA11	16	0.50
TPX2	16	0.61
OASL	16	0.67
NID1	15	0.04
CXCL9	15	0.18
UBE2C	15	0.58

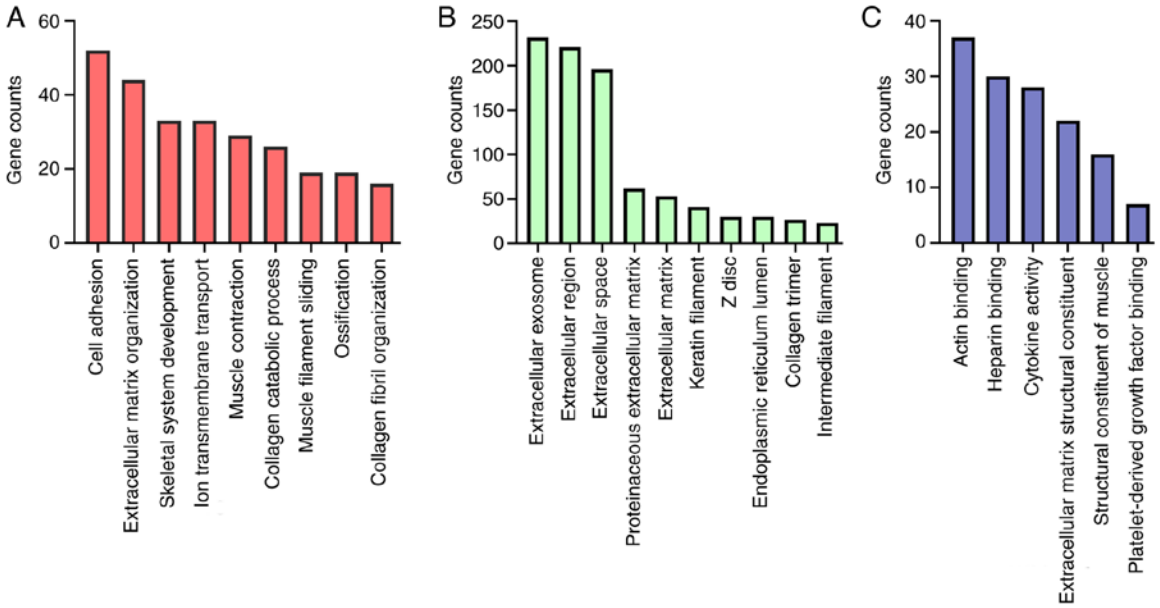

Figure 2. GO analysis of the DEGs in head and neck squamous cell carcinoma. The graphs present the number of DEGs enriched in each GO term: (A) Biological process, (B) cellular component and (C) molecular function. GO, Gene Ontology; DEGs, differentially expressed genes.

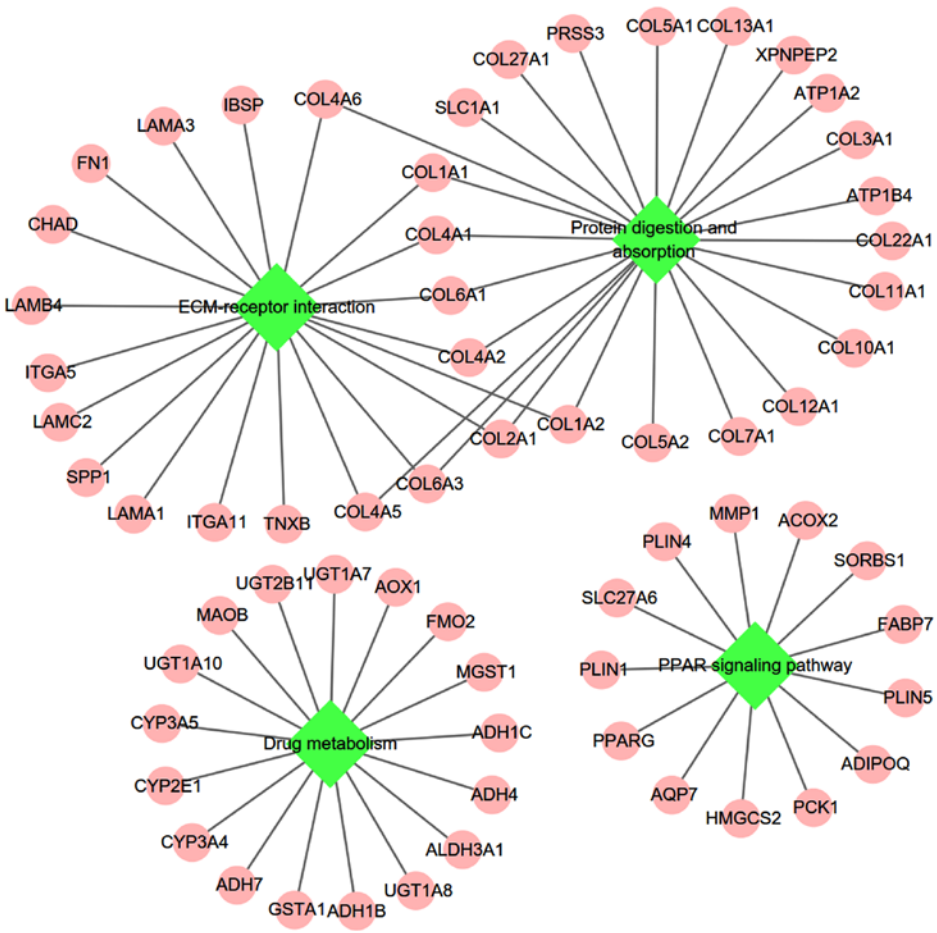

Figure 3. KEGG pathway analysis of the differentially expressed genes in head and neck squamous cell carcinoma. The green diamond in the center represents different KEGG pathways, including 'protein digestion and absorption', 'extracellular matrix-receptor interaction', 'drug metabolism' and the 'PPAR signaling pathway'. The peripheral red nodes represent the genes enriched in the corresponding pathways. KEGG, Kyoto Encyclopedia of Genes and Genomes.

migration capacity and high mortality rate. However, there are almost no biomarkers or targets for the diagnosis and treatment of HNSCC (1). Thus, the identification of genes that are differentially expressed between tumor and normal

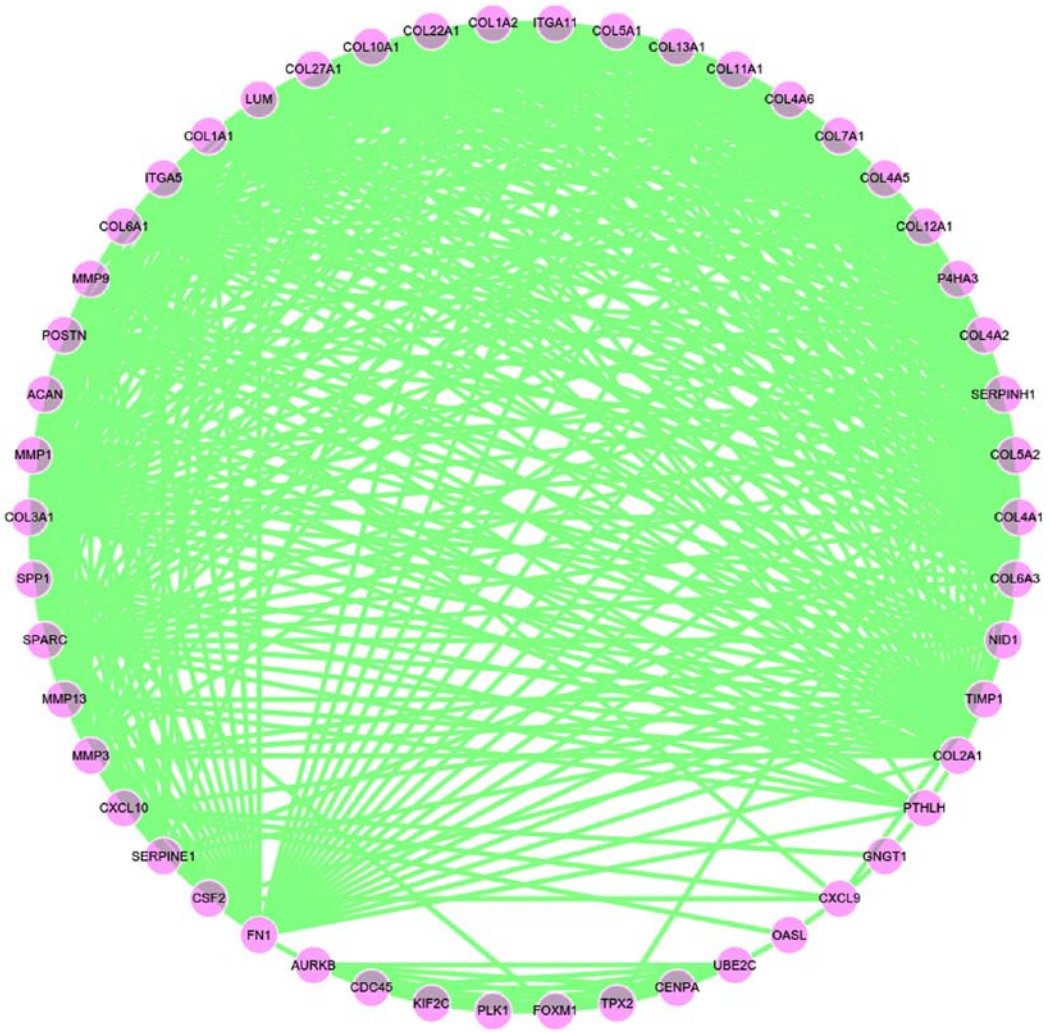

Figure 4. PPI network of the DEGs. A total of 1,035 DEGs were incorporated into the PPI network complex. The peripheral red nodes represent the DEGs with degree of centrality ≥15. The lines represent the interactions between nodes. PPI, protein-protein interaction; DEGs, differentially expressed genes.

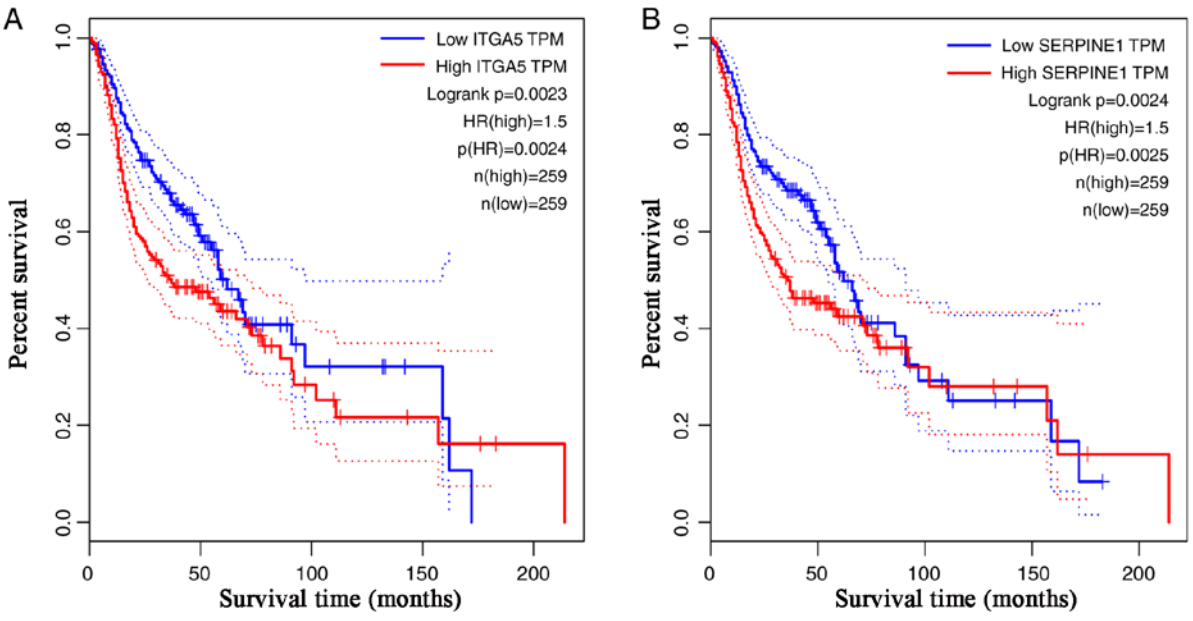

Figure 5. Prognostic relevance of ITGA5 and SERPINE1 in patients with head and neck squamous cell carcinoma. Kaplan-Meier survival analysis of patients with low and high expression of (A) ITGA5 and (B) SERPINE1 was performed using the Gene Expression Profiling Interactive Analysis database. The group cut-off value was based on the median. The dotted lines represent the 95% confidence intervals. ITGA5, integrin α-5; SERPINE1, serpin family E member 1; HR, hazard ratio.

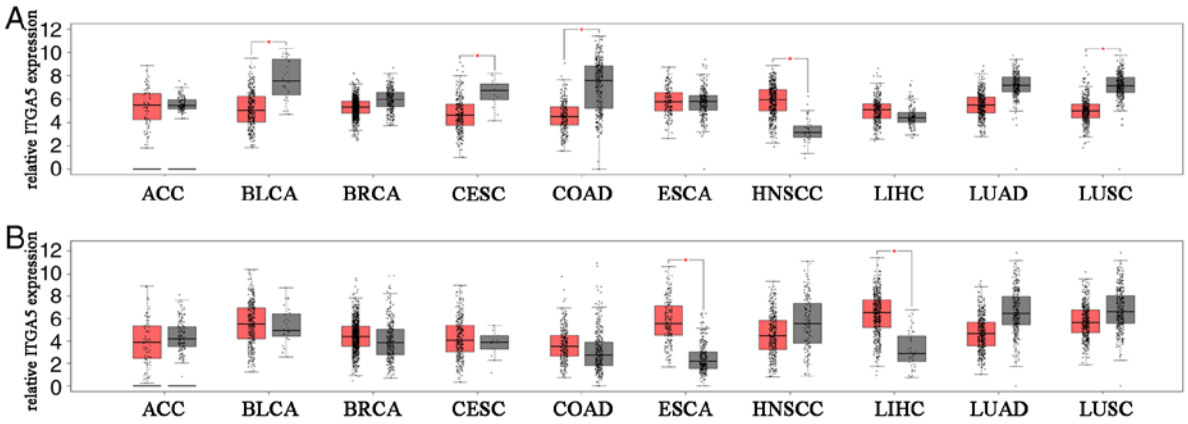

Figure 6. Expression of ITGA5 and SERPINE1 in different types of cancer. The expression levels of (A) ITGA5 and (B) SERPINE1 were compared in different types of cancer in humans. Box plots were drawn using the R software and the raw data from the Gene Expression Profiling Interactive Analysis database. ITGA5 expression was only significantly increased in HNSCC, whereas it was decreased in BLCA, CESC, COAD and LUSC. The expression of SERPINE1 was significantly increased in HNSCC, as well as ESCA. The red boxplots represented tumor samples and the grey boxplots represented normal samples. ITGA5, integrin α-5; SERPINE1, serpin family E member 1; HNSCC, head and neck squamous cell carcinoma; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, endocervical adenocarcinoma; COAD, colon adenocarcinoma; ESCA, esophageal carcinoma; LIHC, liver hepatocellular carcinoma; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma.

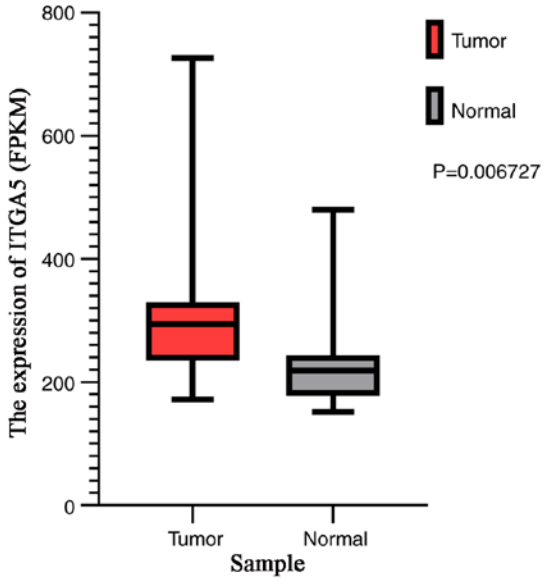

Figure 7. Expression of ITGA5 in HNSCC samples compared with expression in normal samples. The horizontal axis represents the different samples and the vertical axis represents the expression level of ITGA5. ITGA5, integrin α-5.

tissues will contribute to future studies on the pathogenesis of HNSCC and will provide potential diagnostic biomarkers and therapeutic targets.

In the present study, TCGA datasets of HNSCC were integrated and analyzed using bioinformatics. Finally, a total of 1,181 DEGs were identified including 354 upregulated genes and 827 downregulated genes in the initial step. Subsequently, the 1,181 DEGs were analyzed based on GO terms and classified based on KEGG signaling pathways. The GO analysis indicated that the DEGs were mainly involved in 'cell adhesion', 'extracellular matrix organization', 'skeletal system development', 'ion transmembrane transport', 'actin binding', 'heparin binding' and 'cytokine activity'. The KEGG pathway analysis revealed that the DEGs were mainly associated with 'protein digestion and absorption', 'extracellular matrix-receptor interaction', 'drug metabolism' and the 'PPAR signaling pathway'. In addition, the PPI network complex was constructed and eight key genes, including ITGA5, SERPINE1, serpin family H member 1, colony-stimulating factor 2, tissue inhibitor of metalloproteinases 1, nidogen 1, secreted phosphoprotein 1 and matrix metallopeptidase 1, were screened based on centrality and log-rank survival analysis by GEPIA. The GEPIA database contains genotype-tissue expression data, which increases the sample size and accuracy of the analysis, and was therefore was used to identify key DEGs. As a result, ITGA5 and SERPINE1 were identified as key genes.

ITGA5 is an important member of the integrin family, and its coding gene is located at the human chromosome 12q11-q13. Integrin is an extracellular matrix receptor that acts as an adhesive receptor for extracellular matrix proteins, including fibrin, laminin and collagen (17). ITGA5 forms the link between the extracellular matrix and intracellular signal transduction, and also participates in a variety of important physiological processes; it is also associated with tumor occurrence, development, invasion and metastasis (18). A study on hepatocellular carcinoma (HCC) demonstrated a negative correlation between the expression of ITGA5 and miR-128, and the downregulation of ITGA5 leading to the inhibition of HCC cell metastasis and stem cell-like properties (19). However, the expression of ITGA5 played diverse roles in breast cancer cells (20). Expression in highly invasive breast cancer cells was almost absent in comparison with that in less invasive cells, thereby indicating the negative association between ITGA5 and breast cancer metastasis (21,22). In ovarian cancer cells, downregulation of ITGA5 induced by forced expression of miR-17 significantly limited the adhesion, invasion and tumorigenesis ability of cancer cells (23). ITGA5 was reported to be highly expressed in glioblastoma compared with normal brain glial cells, and its downregulation inhibited proliferation, invasion and migration (24). ITGA5 expression was also upregulated in oral squamous cell carcinoma

(OSCC), a common type of HNSCC (25). Knockdown of ITGA5 inhibited the proliferation, migration and invasion of OSCC cells, thereby indicating that ITGA5 could promote OSCC progression.

SERPINE1, also known as plasminogen activator inhibitor type I, is a primary inhibitor of plasminogen activators and a marker of poor prognosis in cancer. In colorectal cancer, SERPINE1 expression was upregulated and was significantly associated with grading and microsatellite instability (26). In esophageal cancer, SERPINE1 expression was upregulated and significantly associated with age range, which was consistent with the analysis of the present study (27). SEPINE1 was highly expressed in oral carcinomas compared with in the matched tumor adjacent normal tissues (28), and its overexpression resulted in increased proliferation and tumor budding. Moreover, SEPINE1 was associated with poor progression-free and cancer-specific survival in patients with head and neck cancer (29,30).

In conclusion, analysis of GEPIA datasets demonstrated the association between high expression of ITGA5 and SERPINE1 and poor OS rate in patients with HNSCC, with identical hazard ratio for both genes. The investigation of these genes was conducted in other tumor datasets, in order to determine their specificity to HNSCC. The expression of ITGA5 was significantly increased in HNSCC only and decreased in BLCA, CESC, COAD and LUSC. On the other hand, the expression of SERPINE1 was significantly increased in ESCA, as well as in HNSCC. A limitation of the present study was that the gene expression in HNSCC was only analyzed by using the public database. In subsequent experiments, molecular biology and cell biology methods will be utilized to further verify these results. Overall, these findings suggest the potential of ITGA5 as a diagnostic or prognostic marker and as a therapeutic target in HNSCC.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

BoZ performed data download, survival analysis and drafted the manuscript. DW and KX performed the survival analysis, GO analysis and pathway enrichment analysis. DY worked on PPI network construction. ZM analyzed the association between the candidate genes and prognosis, and also participated the design of the study. BiZ was the major contributor in designing the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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