Original Article

Molecular mechanisms and prognostic markers in head and neck squamous cell carcinoma: a bioinformatic analysis

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Abstract: The purpose of the present study was to enhance understanding of the molecular mechanisms underpinning head and neck squamous cell carcinoma (HNSCC). Microarray datasets were obtained from the gene expression omnibus database. By a bioinformatics method, 109 differentially expressed genes were identified between the two mRNA datasets, and these genes were classified primarily into biological process, molecular function, or cellular component. In the protein-protein interaction network analysis, top 20 hub genes were identified, and five (SERPINE1, SERPINH1, SPP1, PLAU and MMP1) of them were associated with the prognosis of HNSCC patients. Immunohistochemistry result also showed that the expression of the proteins encoded by these five genes were significantly upregulated in HNSCC, matching the bioinformatics analysis. Moreover, 28 differentially expressed miRNAs were also identified, with miR-196a and miR-1 being most upregulated and downregulated respectively. Our results provide potential biomarkers for HNSCC and may improve understanding of the molecular mechanisms underlying HNSCC.

Keywords: Head and neck squamous cell carcinoma, gene expression omnibus database, biomarker, overall survival

Introduction

Head and neck squamous cell carcinoma (HNSCC), originating from the epithelium of the mucous membranes of the upper respiratory tract and esophagus, is one of the most common carcinomas in the world, and it is estimated that approximately 600,000 new cases are diagnosed annually [1, 2]. The majority of cases of HNSCC are not diagnosed until they are at an advanced stage (stages III to IVB) with poor prognoses and 5-year survival rates owing to relapse following treatment [3]. As novel effective therapeutics are urgently needed and may arise from an improved understanding of the molecular mechanisms underlying HNSCC, it is important to investigate the specific pathways and molecular interactions involved in HNSCC [4]. Recently, microarray and bioinformatics analyses have become widely used in studies of many diseases [5]. Through such analyses, studies aim to understand the potential molecular mechanisms and pathogenesis associated with malignant neoplasms and to investigate novel diagnostic and prognostic tests as well as effective treatments. In the present study, gene microarray data and bioinformatic analyses were used to identify target genes in HNSCC. Initially, two mRNA microarray datasets and one microRNA (miRNA/miR) dataset were acquired from the gene expression omnibus (GEO) database. The interactive web tool GEO2R was applied to identify genes and miRNAs that were differentially expressed between HNSCC and normal head and neck tissues. Functional annotation, pathway enrichment, protein-protein
interaction (PPI) network construction, and survival analyses were conducted to identify target genes. Immunohistochemistry (IHC) was performed to verify the expression of proteins encoded by key genes involved in the pathogenic mechanisms and which might serve as diagnostic and prognostic biomarkers in HNSCC.

Materials and methods

Microarray data

Gene expression datasets (GSE6631 and GSE107591) and a miRNA expression dataset (GSE31277) were collected from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). GSE6631 includes 22 HNSCC tissue samples and 22 matched normal tissue samples [6]; GSE107591 is composed of 24 HNSCC tissue samples and 23 normal samples [7]; and the miRNA set (GSE31277) includes 15 HNSCC tissue samples and 15 matched normal epithelial samples [8].

Identification of DEGs and DEMs

GEO2R (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html) is an online tool used to identify differentially expressed genes (DEGs) and differentially expressed miRNAs (DEMs) between two or more sets of samples [9]. GEO2R uses the GEOquery and Limma R packages of the Bioconductor tools to compare the processing data tables provided by the original submitter. The cutoff criteria for accepting genes and miRNAs as being differentially expressed as compared with the respective normal tissue were an adjusted P-value < 0.01 and a log fold change (log FC) threshold of |log FC| > 1. The adjusted P-value from the Benjamini-Hochberg method was used to adjust the false-positive results. The differentially expressed genes between HNSCC tissue and normal tissue were also estimated by t-test in the Limma package [10].

Functional and pathway enrichment analyses

The gene ontology (GO) analysis system describes the characteristics of genes and gene products from three aspects: Biologic processes, molecular functions, and cellular components [11]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database for the analysis and linkage of genes to higher-level systemic functions of a given cell, organism, or ecosystem [12]. GO and KEGG analyses were used in concert with the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/). The cutoff criterion was set at P < 0.05.

PPI network establishment and cluster identification

PPI networks may indicate molecular complexes. The Search Tool for the Retrieval of Interacting Genes (STRING) (https://string-db.org/) was used for building PPI networks of the identified DEGs to elucidate the underlying interactions between proteins and the indirectly functional relationships among proteins. STRING applied bioinformatics methods and experimental data originating from PubMed abstracts and other database data to predict the results [13]. Nodes and edges represented proteins and the potential functional associations between proteins, respectively, in the PPI networks. The results of STRING were exported into Cytoscape software, an open source software platform for visualizing complex networks (http://cytoscape.org/). The confidence score threshold was set at > 0.7. In addition, molecular complexes were acquired using cytoHubba (Version 0.1), an automated method used for determining hub genes ranked by maximal clique centrality (MCC) in protein interaction networks.

miRNA target genes

The software miRecords (http://c1.accurascience.com/miRecords/) is an online tool for predicting miRNA target genes. It uses 11 established miRNA target prediction programs (DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA 22, RNAhybrid, TargetScan/TargetScanS) to predict miRNA targets [14]. Potential targets of miRNAs were identified by at least six programs (miRanda, MirTarget2, NBmiRTar, PicTar, PITA, RNA 22 and RNAhybrid).

Survival analysis

Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/index.html) is an online tool used to predict patient survival based on gene status [15]. The genes of interest were entered into the tool. The patients were divided into two groups based on their
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gene expression levels, and their survival rates (Kaplan Meier) were analyzed. The 95% confidence intervals and log-rank P-values of the hazard ratios were calculated.

Immunohistochemical analysis

Human laryngeal carcinoma tissues were acquired by clinical surgery. Samples were collected with prior consent from every participating patient and permission by Ethics Committee of Anhui Medical University. The surgical procedures were in accord with the Good Clinical Practice and Declaration of Helsinki. The tissue samples were fixed with 4% paraformaldehyde and then sliced into 5-μm thick sections. The sections were dehydrated using a graded series of ethanols and washed for 5 min. Subsequently, heat-mediated antigen retrieval was completed with citrate buffer in a microwave oven. After 10-min 3% H$_2$O$_2$ incubation, endogenous peroxidase activity was destroyed. Next, the tissue sections were incubated with an anti-MMP1, anti-PLAU, anti-SPP1, anti-SERPINH1 or anti-SERPINE1 (all antibodies diluted 1:200; all purchased from Sangon Company, Shanghai, China) antibody overnight at 4°C and on the following day, the sections were incubated with anti-rabbit IgG secondary antibodies for 30 min at 37°C. The specimens were processed with horseradish peroxidase and then with 3,3'-diaminobenzidine tetrahydrochloride. Subsequently, the sections were counterstained with hematoxylin. The primary antibody was omitted as a negative control. At last, a light microscope was used to capture all images. Integrated optical density of the images was analyzed by Image Pro Plus 5.1 (Media Cybernetics, USA) software.

Statistical analysis

Collected data are presented as mean ± SE. The Mann-Whitney U test (two-tailed) was used to compare results across different groups. The difference between groups was considered significant at a value of $P < 0.05$.

Results

DEG identification and HNSCC enrichment analysis

Between the two gene expression datasets (GSE6631 and GSE107591), 109 DEGs were identified using GEO2R (Figure 1). DAVID was used to perform pathway and GO enrichment analyses on these DEGs. The results revealed that DEGs were mainly enriched in the following terms: biological processes, molecular functions, and cellular components (Figure 2). In addition, 25 KEGG pathways were identified, especially extracellular matrix-receptor interaction, amoebiasis and focal adhesion (Figure 3).

Establishment of PPI networks and identification of clusters

The STRING human PPI dataset was used to examine protein interactions. After the analysis for 109 DEGs, a total of 91 nodes and 338 edges were present in the PPI network (Figure 4). As shown in Figure 5, top 20 hub genes ranked by maximal clique centrality (MCC) were identified. Thus, 20 proteins and the interactions between them were elucidated. Specifically, the genes serpin family E member 1 (SERPINE1), matrix metalloproteinase (MMP9), MMP13, MMP10, lumican (LUM), plasminogen activator (PLAU), MMP1, secreted phosphoprotein 1 (SPP1), MMP3, periostin (POSTN), c-x-c motif chemokine ligand 8 (CXCL8), actin alpha 1 (ACTA1), collagen (COL) type III alpha 1 chain (COL3A1), COL type I alpha 1 chain (COL1A1), serpin family H member 1 (SERPINH1), fibronectin 1 (FN1), COL type V alpha 2 chain (COL5A2), COL type VI alpha 3 chain (COL6A3), COL type IV alpha 2 chain (COL4A2) and COL type IV alpha 1 chain (COL4A1) were identified as positive controls.
in Figure 5. Thus, the PPI network and analysis of the hub genes revealed the potential interactions among the DEGs involved in HNSCC development.

Survival analysis

The online tool GEPIA was used to assess the prognostic value of the 20 hub genes identified by cytoHubba. The expression levels of the hub genes were analyzed against the survival rates of patients with HNSCC. The results indicated that up-regulated expression of MMP1, PLAU, SPP1, SERPINH1, or SERPINE1 was associated with poor overall survival of patients with HNSCC (P < 0.05; Figure 6). By contrast, expression of MMP9, MMP10, MMP13, FN1, COL6A3, COL1A1, CXCL8, LUM, ACTA1, MMP3, POSTN, COL3A1, COL5A2, COL4A2, or COL4A1 was not associated with overall survival of HNSCC patients (P > 0.05; data not shown).

IHC validation of key genes

The expression of key genes including MMP1, PLAU, SPP1, SERPINH1 and SERPINE1 were examined by IHC in HNSCC and control samples to validate the bioinformatics analysis.
results. As shown in Figure 7, compared with the normal tissues, the expression of MMP1, PLA2, SPP1, SERPINH1 and SERPINE1 were significantly upregulated in the cancer cells from tumor tissues (P < 0.05). The IHC results were matched with the bioinformatic analysis.

**DEM identification and target gene prediction**

A total of 28 DEMs were identified using GEO2R (Table 1). Among these DEMs, miR-1 was significantly and the most markedly downregulated miRNA (adj. P-value = 8.00E-03, log FC = -2.73892), whereas miR-196a was the most significantly up-regulated miRNA (adj. P-value = 2.00E-04, log FC = 2.98987). The predicted target genes for these miRNAs determined using the miRecords database are presented in Table 1.

**Discussion**

In the present study, bioinformatics analysis was used to further elucidate the molecular
mechanisms underlying HNSCC. Although other two previous studies had found some hub genes including PSMA7, ITGA6, ITGB4, APP, FN1, EGFR, COL1A1, and MMP-9, each of these two studies only contained one gene expression dataset respectively that was already included in our study [16, 17]. In addition, a miRNA expression dataset (GSE31277) and its target genes were also analyzed to identify DEMs. Therefore, the identified DEGs and DEMs in our study might be more accurate. The major findings in our study were as follows: i) HNSCC and normal tissues differentially expressed 109 genes, and these 109 DEGs were mainly associated with biological process, molecular function, or cellular components; ii) high expression of any of SERPINE1, SPP1, SERPINH1, PLAU and MMP1 was associated with poor prognosis among patients with HNSCC. The IHC results confirmed that the expression of the proteins encoded by these five genes were significantly increased in the tissues of HNSCC; iii) 28 DEMs were identified, with miR-196a being the most markedly upregulated miRNA, and miR-1 the most markedly downregulated. Biomarkers for diagnostic or prognostic use can often be determined by identifying several of the most substantially altered DEGs in a high-throughput case-controlled trial of a disease. Certain DEGs identified in the current study, including myosin IB (MYO1B), MMP1, MMP3 and parathyroid hormone like hormone (PTHLH), have been previ-
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Yamada et al. [18] reported that MYO1B was overexpressed in HNSCC and that high expression of this gene was associated with poor prognosis among patients with HNSCC. Stokes et al. [19] reported that expression of MMP1 and MMP3 was increased in head and neck tumors, and that the increased expression of these genes may be regarded as a molecular marker for HNSCC diagnosis and patient prognosis. Another previous study demonstrated that the expression level of PTHLH was an indicator for poor prognosis among patients with HNSCC and that the Runt-related transcription factor 2-PTHLH axis contributed to the growth of HNSCC [20]. The current results are consistent with these aforementioned studies and seemingly provide further insight on the molecular mechanisms underpinning HNSCC.

Extracellular matrix organization, extracellular matrix, and extracellular matrix structural constituent were among the main GO term enrichments for the DEGs identified presently. In addition, 20 protein products from these DEGs (including MMP13, SPP1 and COL3A1) were highly connected with one another, as determined in the PPI network analysis. A previous study suggested that up-regulated expression of MMP-13 was associated with decreased overall survival in patients with invasive breast cancer [21]. It has also been previously reported that SPP1 promoted the growth and metastasis of gastric cancer cells [22]. Other previous study had demonstrated that miR-29a/b might enhance cell migration and invasion in the progression of nasopharyngeal carcinoma by regulating secreted protein acidic and rich in cysteine and COL3A1 gene expression [23]. In the present study, up-regulation of any of SERPINE1, SPP1, SERPINH1, PLAU and MMP1 appeared associated with poor prognosis in patients with HNSCC. Taken together, these results are consistent with our findings that 20 hub genes (MMP13, SPP1, FN1, COL3A1, COL6A3, COL1A1, COL4A1, COL4A2, COL5A2, CXCL8, POSTN, LUM, SERPINE1, PLAU, MMP1, MMP3, MMP9, MMP10, ACTA1 and SERPINH1) served crucial roles in tumor development, and thus may be targets in the treatment of HNSCC.

The survival analysis using GEPIA revealed that high expression of any of SERPINE1, SPP1, SERPINH1, PLAU and MMP1 was associated with poor prognosis among patients with HNSCC. Pavón et al. [24] similarly found that overexpression of SERPINE1 in HNSCC cells
was associated with poor prognosis among patients with head and neck cancer. Furthermore, it has been reported that high expression of SPP1 was associated with poor prognosis among patients with renal cell carcinoma [25]. In this study, the IHC results also confirmed that the expression of SERPINE1, SPP1, SERPINH1, PLAU, and MMP1 were significantly upregulated in the tissues of HNSCC. Therefore, the bioinformatics analysis was consistent with the IHC results. Taken together, these results suggest that downregulating SERPINE1, SPP1, SERPINH1, PLAU, and/or MMP1 is a potential approach to improve prognosis among patients with HNSCC.

MiRNAs may base pair with the 3’ untranslated regions of target mRNAs to serve important roles in regulating gene expression [26]. In the present study, 28 DEMs were identified in HNSCC. Among these DEMs, miR-196a was the most markedly upregulated miRNA and miR-1 the most markedly downregulated. A previous report also indicated that miR-1 expression was downregulated in HNSCC compared with normal head and neck tissues, and miR-1 has been reported to significantly inhibit the invasion of HNSCC [27]. Darda et al. [28] observed that compared with normal oral keratinocytes, miR-196a was more highly expressed in HNSCC cells, and that knockdown of miR-196a reduced the migration, invasion, and adhesion of HNSCC cells. Overall, based on the present indications of expression change of previous studies mentioned above, miRs -1, -135a, -30a-3p, -139, -375, -486, -204, -499, -379, -10b, -99a, -100, -95 and -30a-5p may be classified as tumor suppressor genes, while miRs -301,
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Disclosure of conflict of interest

None.

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