

Original Paper

How Long Non-Coding RNAs and MicroRNAs Mediate the Endogenous RNA Network of Head and Neck Squamous Cell Carcinoma: a Comprehensive Analysis

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lncRNA • miRNA • Endogenous RNA network • Head and neck squamous cell carcinoma

Abstract

Background/Aims: Long non-coding RNAs (lncRNAs) act as competing endogenous RNAs (ceRNAs) to compete for microRNAs (miRNAs) in cancer metastasis. Head and neck squamous cell carcinoma (HNSCC) is one of the most common human cancers and rare biomarkers could predict the clinical prognosis of this disease and its therapeutic effect. **Methods:** Weighted gene co-expression network analysis (WGCNA) was performed to identify differentially expressed mRNAs (DEmRNAs) that might be key genes. GO enrichment and protein–protein interaction (PPI) analyses were performed to identify the principal functions of the DEmRNAs. An lncRNA–miRNA–mRNA network was constructed to understand the regulatory mechanisms in HNSCC. The prognostic signatures of mRNAs, miRNAs, and lncRNAs were determined by Gene Expression Profiling Interactive Analysis (GEPIA) and using Kaplan–Meier survival curves for patients with lung squamous cell carcinoma. **Results:** We identified 2,023 DEmRNAs, 1,048 differentially expressed lncRNAs (DElncRNAs), and 82 differentially expressed miRNAs (DEmiRNAs). We found that eight DEmRNAs, 53 DElncRNAs, and 16 DEmiRNAs interacted in the ceRNA network. Three ceRNAs (HCG22, LINC00460 and STC2) were significantly correlated with survival. STC2 transcript levels were significantly higher in tumour tissues than in normal tissues, and the STC2 expression was slightly upregulated at different stages of HNSCC. **Conclusion:** LINC00460, HCG22 and STC2 exhibited aberrant levels of expression and may participate in the pathogenesis of HNSCC.

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Introduction

Head and neck squamous cell carcinoma (HNSCC), the sixth most common human cancer, is characterised by etiological, phenotypic, biological, and clinical heterogeneity [1]. Many HNSCC patients eventually develop metastatic recurrence [2], and half of all patients die within the 5 years after diagnosis [3]. The survival rate of HNSCC has remained unchanged in the last three decades, partly because rare biomarkers predict the clinical prognosis of this disease and therapeutic effects. As such, there is an urgent need to discover effective regulators associated with the disease stage and clinical prognosis of HNSCC.

Studies over the past two decades have provided much information about large-scale genome sequencing and transcriptome profiling. These studies have identified numerous RNAs, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and short non-coding RNAs (sncRNAs) [4, 5]. lncRNAs serve as transcription factors or enhancers and participate in various critical biological processes such as chromatin remodelling, histone modification, and genomic imprinting [6-8]. With accumulating evidence of its crucial roles in cancer metastasis, the role of lncRNA in cancers has recently gained attention [9]. Studies have revealed that miRNAs repress target gene expression via shared partial complementarity miRNA response elements (MREs) with the mRNA sequence [10], and lncRNAs function as competing endogenous RNAs (ceRNAs) that interact with mRNAs, acting as miRNA sponges, to restrain miRNA function by competing for MREs [11-13]. The aberrant expression of core lncRNAs in the ceRNA network has a significant impact on network crosstalk and disrupts miRNA-mediated lncRNA/mRNA ceRNA crosstalk interactions, contributing to the initialisation and development of cancers [12, 14]. The discovery of these interactions provided an important opportunity to advance our understanding of cancer therapy. Based on this theory, lncRNA-miRNA-mRNA ceRNA networks have been identified in ovarian cancer, pancreatic cancer, and lung adenocarcinoma [15-17]. However, there are few comprehensive analyses of HNSCC-associated lncRNAs and miRNAs based on a ceRNA network in the context of a large sample size.

The Cancer Genome Atlas (TCGA), a large integrated collection of clinicopathological information and gene sequencing data across 33 human cancer types, allows for systematic cancer research to improve the prevention, diagnosis and treatment of cancer. In this study, we analysed and compared RNA sequencing data between 502 tumour tissues and 44 normal tissues, and miRNA sequencing data between 525 tumour tissues and 44 normal tissues of TCGA-HNSCC. The miRDB, miRTarBase, and miRcode databases were used to identify the core DE miRNAs and DE lncRNAs, as well as DE miRNA-DE mRNA interactions. In total, 2,023 mRNAs, 1,048 lncRNAs and 83 DE miRNAs were identified as being aberrantly expressed. To illuminate potential crosstalk in the lncRNA-miRNA-mRNA ceRNA network, we further predicted interactions between miRNAs and lncRNAs. Ultimately, we constructed a ceRNA network that involved 54 lncRNAs, eight miRNAs, and 16 mRNAs.

Materials and Methods

Acquisition and analysis of expression profiles

The miRNA expression profiles (44 normal tissues, 525 tumour tissues) and RNA expression profiles (44 normal tissues, 502 tumour tissues) were downloaded from the TCGA website (<https://portal.gdc.cancer.gov/>). The lncRNA and mRNA expression profiles were then separated from the RNA sequencing data.

After tumour data and normal tissue data were combined, the raw count data were processed with edgeR, an R/Bioconductor package for analysis of differential gene expression profiles with biological replication. Fold-change (tumour/normal) was used to measure the change degree. For *p*-values, the false discovery rate (FDR) was applied to determine the significance threshold for multiple tests. Genes with an absolute $|\log_2\text{FoldChange}| > 2$ and an $\text{FDR} < 0.01$ were considered significant.

Regulatory relationship between DEmiRNAs and DElncRNAs

Putative interactions of miRNA-lncRNA were predicted by the miRcode database (<http://www.mircode.org/>), which includes 1, 329, 071 interactions between miRNAs and lncRNAs, and allows whole transcriptome human miRNA target predictions based on a comprehensive GENCODE lncRNA annotation.

Weighted gene co-expression network analysis (WGCNA)

WGCNA is an algorithm used in gene co-expression network identification. This algorithm is based on high-throughput mRNA gene expression profiles and has been widely used to determine changes in various diseases [18]. To identify the interactions between DEmRNAs and co-expression modules, we applied WGCNA with a power cut-off threshold of 12 and a module size cut-off 20.

Functional annotation and protein-protein interaction (PPI)

GO enrichment analysis is widely used in functional annotation. A functional synergistic relationship is defined as when more than one co-regulated target gene is significantly enriched in GO functional terms. To further understand each term, clusterProfiler (<http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>) with a strict cut-off of FDR<0.05 was applied to depict the biological process enrichment [19]. Based on a projection at a specific level of the GO corpus, the clusterProfiler package offers a classification method to classify genes, and calculates the enrichment test for GO terms and KEGG pathways based on a hypergeometric distribution. Encoded by multiple genes, tightly interacted groups of proteins can execute complex cellular functions. STRING (Search Tool for the Retrieval of Interacting Genes, <http://string-db.org/>) [20] was applied to identify PPIs with a high confidence interaction score of 0.7 (the turquoise module) and 0.4 (other modules). In addition, Cytoscape software was used to visualise the PPI networks (<http://cytoscape.org/>) [21].

Target mRNAs of DEmiRNAs

DEmiRNA-targeted mRNAs were retrieved from miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>), TargetScan (http://www.targetscan.org/vert_71/) and miRDB (<http://www.mirdb.org/>). As a database, miRTarBase has accumulated more than 360, 000 miRNA-target interactions (MTIs), which were collected by manually surveying pertinent literature after systematic, natural language processing of the text to filter research articles related to the functional study of miRNAs [22]. TargetScan predicts the biological targets of miRNAs by searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA, as an option, predictions with only poorly conserved sites are also provided [23]. miRDB is an online database for miRNA target prediction and functional annotations, genome-wide miRNA target prediction was performed with the MirTarget algorithm [24].

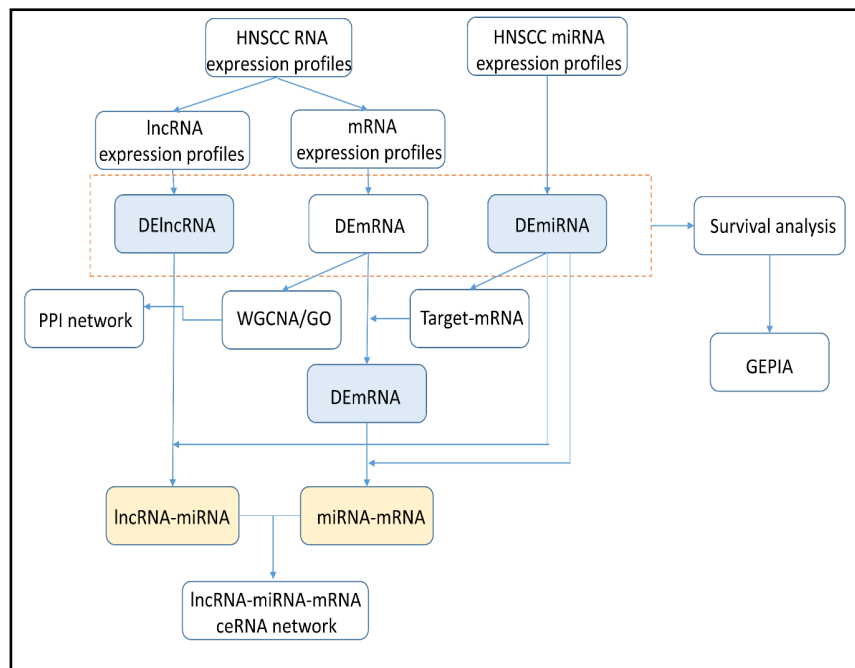
Construction of the ceRNA network

The lncRNA-associated ceRNA network in HNSCC was constructed based on the following procedure: first, miRNA-lncRNA interactions were evaluated using the miRcode database, as mentioned above. Next, miRNA-targeted mRNAs were retrieved from miRTarBase, TargetScan and miRDB, and targets not included in the DEmRNAs were excluded. The results were then visualised using Cytoscape 3.4.0. The main procedure of the method is shown in Fig. 1.

Comparison of gene expression levels

Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancer-pku.cn/>) is a web-based server that provides customisable functions, such as tumour/normal differential expression analysis, profiling according to cancer type or pathological stage, patient survival analysis, detection of similar genes, correlation analysis, and dimensionality reduction analysis [25].

Fig. 1. Main procedure followed when constructing the ceRNA network.



Results

DEmRNAs, DEIncRNAs and DEMiRNAs in HNSCC

The expression profiles of HNSCC patients and their corresponding clinical information were obtained from the TCGA database, and mRNAs and lncRNAs were separated from the RNA expression profiles. Following the differential expression analysis, 2,023 mRNAs and 1,048 lncRNAs were identified as being aberrantly expressed ($|\log_2\text{FoldChange}| > 2$ and $\text{FDR} < 0.01$). Of these, 870 mRNAs and 757 lncRNAs were up-regulated and 1,155 mRNAs and 291 lncRNAs were downregulated in tumour tissues compared with normal tissues.

To construct an lncRNA-miRNA-mRNA ceRNA network, we analysed the miRNA expression profiles of 525 TCGA-HNSCC tumour tissues and 44 normal tissues. A total of 82 DEMiRNAs (44 up- and 38 down-regulated) were differentially expressed.

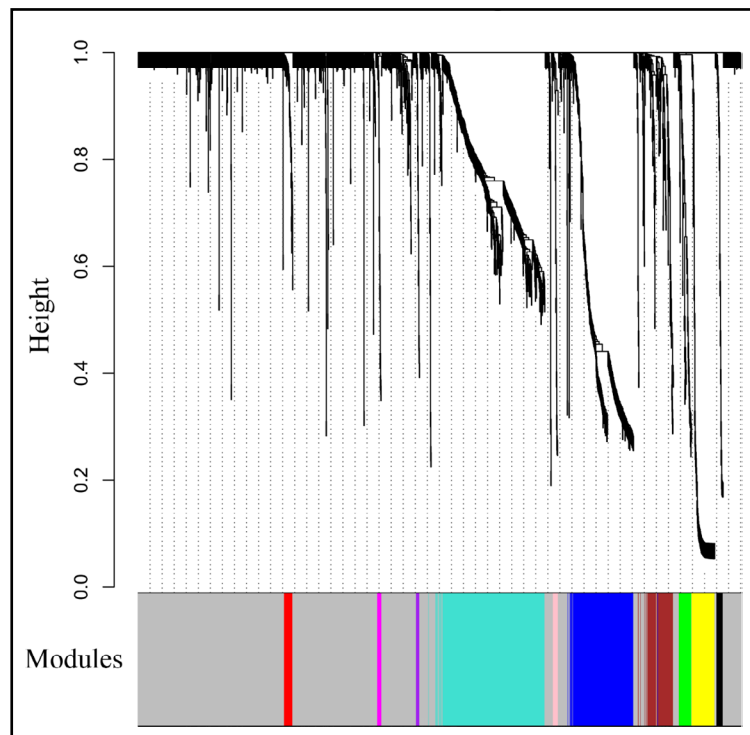
WGCNA analysis, PPI and functional annotation of the modules

To illustrate the functional clusters of DEmRNAs in HNSCC patients, gene co-expression network analysis of 2,023 DEmRNAs was carried out using WGCNA. Ten colour modules (non-clustering DEmRNAs are represented in gray) are shown in Fig. 2. As illustrated in Supp. Fig. 1D, the turquoise module contained 353 nodes and 600 edges. Some genes were central to PPI including TNNI1, TNNI2, TNNC1, TNNC2, and TNNT3. Most proteins in this module are involved in muscle cell development and contraction, myofibrils, contractile fibres, actin binding, and the structural constituents of muscle (Fig. 3D).

The most significant functions of the proteins in the blue module were microtubule-based movement, cilium organisation, and cilium assembly in the ontology of biological processes (BP), ciliary parts, cytoplasmic region and axoneme and motor activity in the ontology of the cellular component (CC), microtubule motor activity, and ATP-dependent microtubule motor activity in the ontology of molecular function (MF) (Fig. 3C). Some genes such as DNALI1 and RSPH4A were at the core of the network (Supp. Fig. 1B - for all supplemental material see www.karger.com/10.1159/000494009/).

The average node degree in the brown module was 1.25; proteins in this module were related to the pathway of salivary secretion and collecting duct acid secretions. GNG7, EDN3, and CST4 were core nodes in the PPI of this module (Supp. Fig. 1C). We found that most of the proteins are related to the negative regulation of endopeptidase and peptidase activity,

Fig. 2. Cluster dendrogram and color display of the co-expression network modules produced by average linkage hierarchical clustering of genes based on topological overlaps in the DEmRNAs. Each branch in the dendrogram is a line that represents a single gene. Its height indicates the Euclidean distance. Each color indicates a single module that contained closely conserved genes. The area occupied by each color is proportional to the number of genes within the respective module.



the detection of chemical stimuli involved in the sensory perception of taste, the sensory perception of taste, enzyme inhibitors, endopeptidase inhibitors, and endopeptidase regulator activity (Fig. 3B).

The yellow module contained 75 nodes and 101 edges (Supp. Fig. 1D). In this module, 77 proteins were enriched and involved in keratinisation, keratinocyte differentiation, epidermal cell differentiation, skin development, epidermis development, intermediate filaments, and the intermediate filament cytoskeleton (Fig. 3A).

Establish and analysis of the ceRNA network in HNSCC

We applied miRcode to search for interactions between miRNAs and lncRNAs. In the network, mRNAs were measured by targets of DEmiRNAs from miRTarBase, TargetScan, and miRDB, and targets not included in DEmRNAs were excluded. Ultimately, the ceRNA network was constructed using eight miRNAs, 53 lncRNAs, and 16 mRNAs (Supp. Fig. 2). Most of the mRNAs in the network have been reported to be cancer-associated genes, such as stanniocalcin 2 (STC2) [26], tropomyosin 2 (TPM2) [27], and transforming growth factor beta receptor 3 (TGFB3) [28]. Some of the miRNAs in the network had no target mRNAs, such as has-mir-310b, has-mir-31, and has-mir-375. Three miRNAs (has-mir-206, has-mir-210, and has-mir-503) targeted a single mRNA. Has-mir-211 had the most target mRNAs. The target mRNAs of has-mir-195 were mostly up-regulated.

Survival and expression level analysis of mRNAs, lncRNAs and miRNAs in the network

Kaplan–Meier curve analysis was used to investigate overall survival for the DElncRNAs, DEmRNAs, and DEmiRNAs as ceRNA counterparts. miRNA has-mir-206, mRNA STC2, and five DElncRNAs (ABCA9-AS1, ANO1-AS2, HOTTIP, LINC00052, and LINC00460) were negatively associated with worse overall survival ($p < 0.05$) for HNSCC patients. By contrast, four DElncRNA (HCG22, MIAT, MUC19, and ZFY-AS1) prolonged patient survival time ($p < 0.05$, Fig. 4).

Next, we applied GEPIA to verify the correlations with overall survival and to detect the difference in expression levels of lncRNAs between tumour and normal tissues. The result indicated that LINC00460 and HCG22 were aberrantly expressed and closely associated

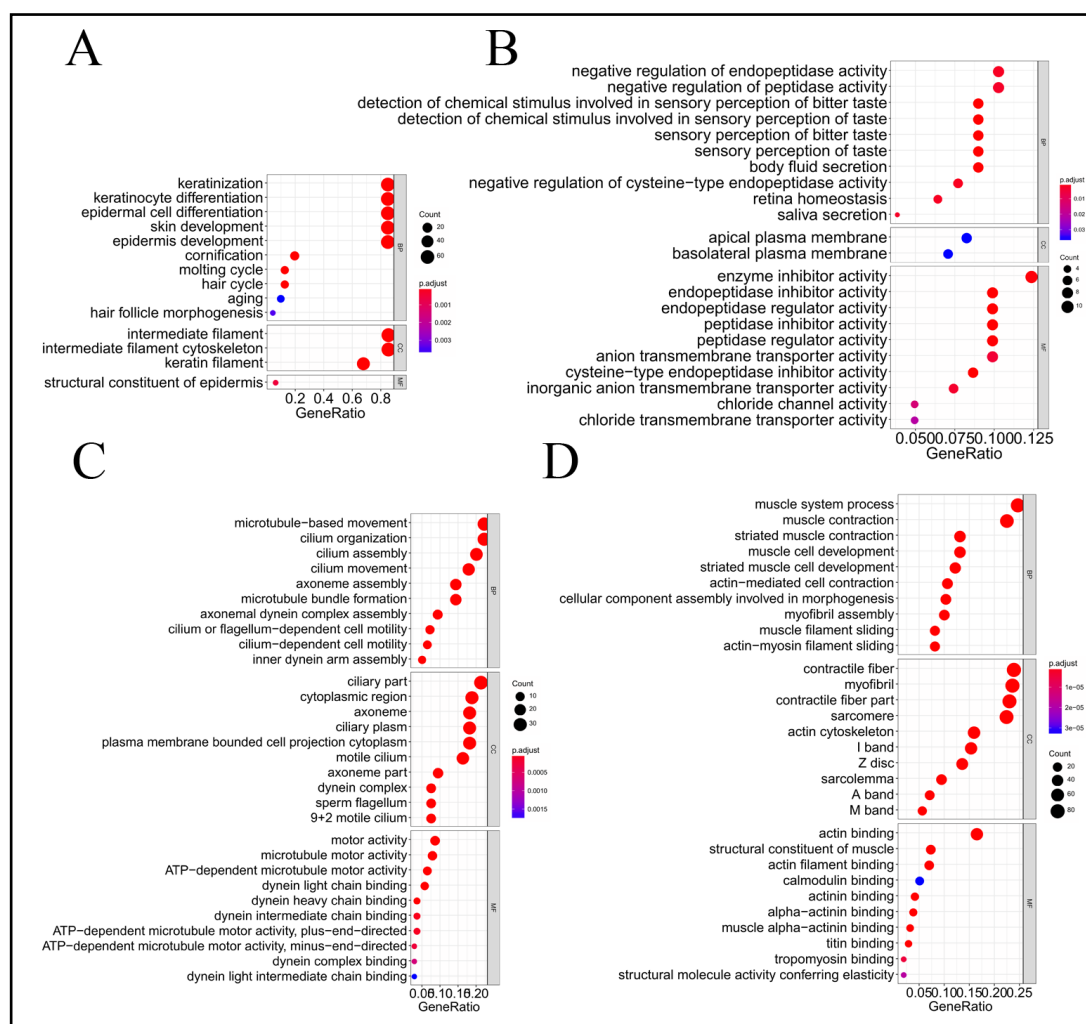


Fig. 3. GO enrichment of gene clusters in the yellow (A), brown (B), blue (C), and turquoise (D) modules. The coloured dots represent term enrichment: blue indicates low enrichment, red indicates high enrichment. The sizes of the dots represent the number of genes in each GO category.

with overall survival (Fig. 4). LINC00460 exhibited higher expression in 519 tumour tissues than in 44 normal tissues, while HCG22 showed the opposite pattern. Kaplan–Meier survival curves indicated that lncRNA HCG22 was positively correlated with overall survival, whereas LINC00460 was negatively associated with overall survival.

Characteristics of STC2 in HNSCC

Next, we evaluated the expression of STC2 in tumour tissues compared to normal tissues. As depicted in Fig. 4A, STC2 transcript levels were significantly higher in tumour tissues than in normal tissues. Kaplan–Meier survival curves indicated that STC2 is positively correlated with overall survival (Fig. 4B). We also examined the expression of STC2 in different major pathological stages of HNSCC; the results indicated that STC2 was slightly upregulated at stages 1, 2, 3, and 4 (Fig. 4C).

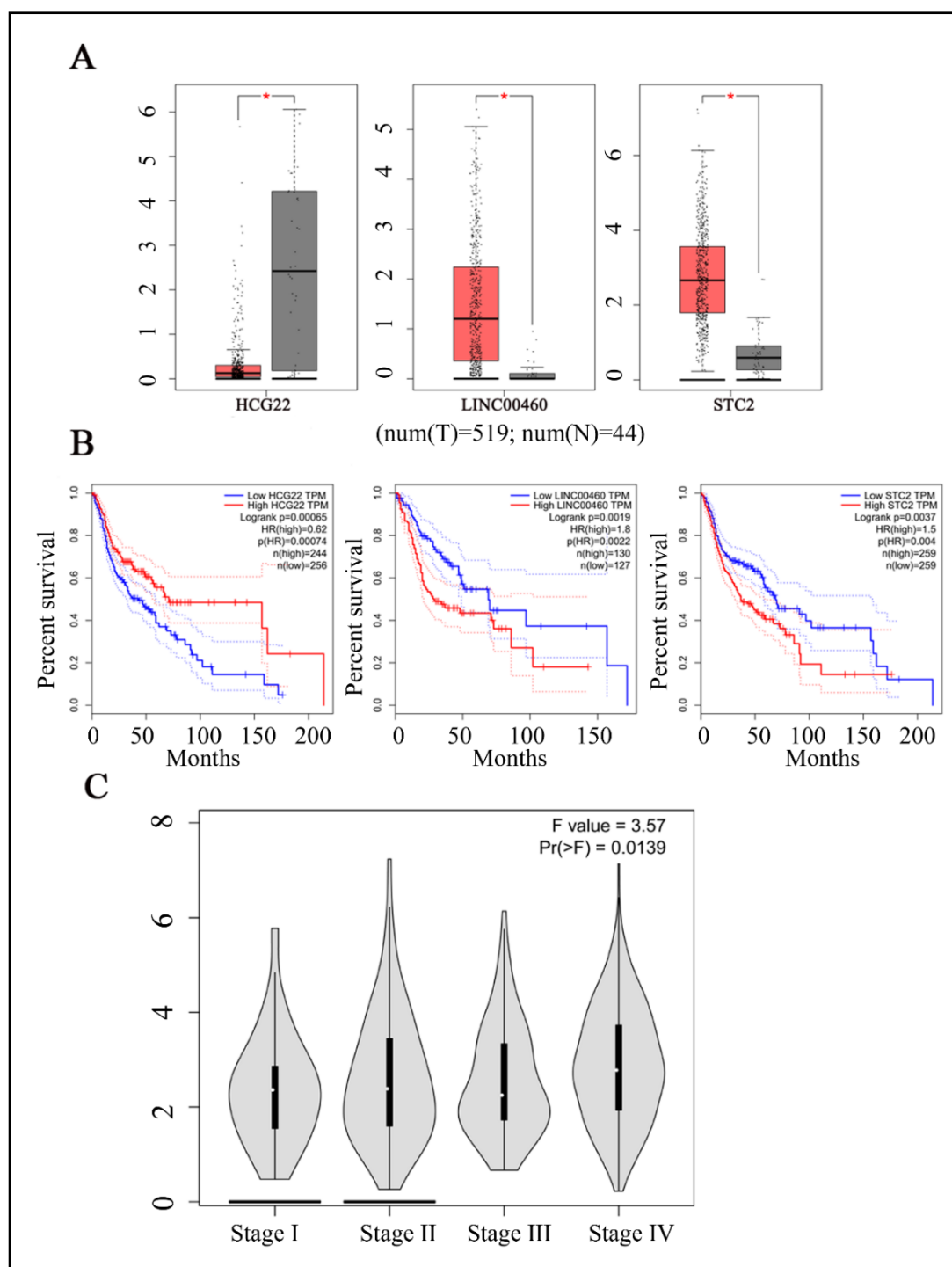


Fig. 4. Expression of HCG22, LINC00460, and STC2 in HNSCC and normal tissues from GEPIA. (A) Expression was examined in 519 HNSCC tissues (T) and 44 normal tissues (N) from GEPIA. The axis units are log2(TPM+1). (B) Kaplan-Meier curve analysis of HCG22, LINC00460, and STC2 for the overall survival in HNSCC patients. (C) Violin plot showing STC2 expression in different major pathological stages of HNSCC.

Discussion

A growing number of studies have lent support to the ceRNA hypothesis, a novel gene regulation layer, in which lncRNAs serve as miRNA sponges and participate in various biological processes and their expression is associated with many complicated disease phenotypes, including cancers [29-32]. Research studies have demonstrated the critical role of lncRNAs [33, 34], and have shown that the aberrant expression of lncRNAs contributes to extensive variation in gene expression via the lncRNA-mediated ceRNA regulatory network. However, the molecular mechanism of the ceRNA regulatory network is unclear in HNSCC. In the current study, we constructed an lncRNA-related ceRNA network by integrating the TCGA-HNSCC expression profiles, which will shed light on how lncRNAs and miRNAs are involved in HNSCC.

The HCG22 transcript, which encodes a novel mucin protein, is stimulated by interleukin (IL)-1, inhibited by triamcinolone acetate and tumour growth factor- β , and is expressed in trabecular meshwork cells. Our analysis suggests a potential mechanism by which HCG22 competes with miR-195 to regulate the expression of TPM2, SH3BGRL2, HOXA10, TGFB3, ALOX12, and TMEM100. Manipulated by the transcriptional co-activator CBP/P300 via histone acetylation, LINC00460 was overexpressed in most tumour tissues. *In vitro* experiments revealed that LINC00460 depletion suppressed oesophageal squamous cell carcinoma (OSCC) growth by regulating cell proliferation, the cell cycle and apoptosis [35]. On reviewing published research, we noticed that LINC00460 exerts its oncogenic function in large part via sponging miR-149-5p and subsequently initiating the IL-6 signalling pathway in nasopharyngeal carcinoma [36]. Our analysis revealed a novel potential mechanism whereby LINC00460 competes with miR-206 to regulate the expression of STC2, or competes with miR-503 to regulate the expression of GREM2. STC2 is correlated with a poor prognosis in OSCC via lymph node metastases [37], and miR-206 is a muscle-specific miRNA that participate in a series of cellular activities including skeletal muscle development and tumourigenesis [38, 39]. lncRNA is conserved in various cancers to an extent, which suggested that lncRNA-related ceRNA regulation is a fundamental gene regulation layer in tumourigenesis.

According to the modules produced by WGCNA, many core genes from the turquoise module, such as CHRDL1, GPD1L, KLHL40, TMEM100, and TPM2, as well as GREM2, HOXA10, and SH3BGRL2 from the grey module, were considered important target proteins. HOXA10, SH3BGRL2, TMEM100, TPM2, and HCG22 are target genes of miR-195. Previous studies have revealed that miR195 promotes colorectal cancer cell (SW620) apoptosis by down-regulating Siah-1S [40]. Moreover, the overexpression of miR-195 suppressed breast cancer cell proliferation and invasion [41]. Therefore, we believe that miR-195 may also be critical to HNSCC.

Conclusion

In conclusion, we performed a comprehensive analysis of lncRNA-mediated ceRNA interplay based on the lncRNA and RNA expression profiles of HNSCC, which for the first time enabled an overview and analysis of the role of a ceRNA regulatory network of HNSCC at a system-wide level. By comprehensively analysing the lncRNA-related ceRNA network, some novel and crucial characters of HNSCC were revealed. These findings shed some light on the regulation mechanism in HNSCC and will be beneficial for mining potential therapeutic targets or candidate diagnostic biomarkers.

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Disclosure Statement

The authors declare that no conflict of interests exist.

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