IncRNA TMEM51-AS1 and RUSC1-AS1 function as ceRNAs for induction of laryngeal squamous cell carcinoma and prediction of prognosis

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Background: Long non-coding RNAs (lncRNAs) can function as competing endogenous RNAs (ceRNAs) to interact with miRNAs to regulate target genes and promote cancer initiation and progression. The expression of lncRNAs and miRNAs can be epigenetically regulated. The goal of this study was to construct an lncRNA-miRNA-mRNA ceRNA network in laryngeal squamous cell carcinoma (LSCC) and reveal their methylation patterns, which was not investigated previously.

Methods: Microarray datasets available from the Gene Expression Omnibus database were used to identify differentially expressed lncRNAs (DELs), miRNAs (DEM), and genes (DEGs) between LSCC and controls, which were then overlapped with differentially methylated regions (DMRs). The ceRNA network was established by screening the interaction relationships between miRNAs and lncRNAs/mRNAs by corresponding databases. TCGA database was used to identify prognostic biomarkers.

Results: Five DELs (downregulated: TMEM51-AS1, SND1-IT1; upregulated: HCP5, RUSC1-AS1, LINC00324) and no DEMs were overlapped with the DMRs, but only a negative relationship occurred in the expression and methylation level of TMEM51-AS1. Five DELs could interact with 11 DEMs to regulate 242 DEGs, which was used to construct the ceRNA network, including TMEM51-AS1-miR-106b-SNX21/ TRAPPC10, LINC00324/RUSC1-AS1-miR-16-SPRY4/MICAL2/ SLC39A14, RUSC1-AS1-miR-10-SCG5 and RUSC1-AS1-miR-7-ZFP1 ceRNAs axes. Univariate Cox regression analysis showed RUSC1-AS1 and SNX21 were associated with overall survival (OS); LINC00324, miR-7 and ZFP1 correlated with recurrence-free survival (RFS); miR-16, miR-10, SCG5, SPRY4, MICAL2 and SLC39A14 were both OS and RFS-related. Furthermore, TRAPPC10 and SLC39A14 were identified as independent OS prognostic factors by multivariate Cox regression analysis.

Conclusion: DNA methylation-mediated TMEM51-AS1 and non-methylation-mediated RUSC1-AS1 may function as ceRNAs for induction of LSCC. They and their ceRNA axis genes (particularly TMEM51-AS1-miR-106b-TRAPPC10; RUSC1-AS1-miR-16-SLC39A14) may be potentially important prognostic biomarkers for LSCC.
Intronic noncoding RNA (IncRNA) TMEM51-AS1 and RUSC1-AS1 function as ceRNAs for induction of laryngeal squamous cell carcinoma and prediction of prognosis

Running title: ceRNAs for LSCC

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**Introduction**

Laryngeal squamous cell carcinoma (LSCC) is one of the common malignancies of the
upper respiratory tract that has been associated with a deterioration of the environment and an
increase in the occupational stress. It was estimated that 13,360 new cases were diagnosed in
2017 in the United States, of which over 3,660 were fatal (Siegel et al. 2017). In China, an
estimated 26,400 new cases of LSCC and 14,500 cancer-related deaths also occurred in 2015
(Chen W 2016). Although patients with LSCC can be managed by surgical intervention,
radiation therapy and chemotherapy, the overall five-year survival remains poor (approximately
60%) (Rudolph et al. 2011). Therefore, there is an urgent need to deeply understand the
molecular mechanisms underlying LSCC carcinogenesis or progression in order to develop more
effective therapeutic strategies.

Accumulating evidence has suggested that non-coding RNAs (ncRNAs) play crucial roles
in the initiation and development of tumors. ncRNAs are loosely categorized into small ncRNAs
and long non-coding RNAs (lncRNAs), both of which have regulatory functions in various
biological processes. The well-documented small ncRNAs are microRNAs (miRNAs; ~22
nucleotides long) that regulate gene expression by binding to complementary sequences in the 3’
untranslated region (UTR), leading to either inhibition of translation or degradation of the
transcripts (Jean & Mihaela 2014). Although the mechanisms remain unclear, growing evidence
supports that lncRNAs could function as competing endogenous RNAs (ceRNAs) by
competitively binding to miRNAs through their miRNA response elements (MRE) and
subsequently regulate target RNA expression (Salmena et al. 2011). This ceRNA mechanism has
generated much interest to explain tumor development and progression in many malignancies,
such as gastric cancer (Song Z 2018), thyroid carcinoma (Zhao et al. 2018) and hepatoblastoma
Recent studies also have preliminarily revealed several underlying ceRNA regulatory interactions in LSCC. Luciferase reporter assay and Western blotting results suggested that AC026166.2-001 could act as a sponge of miR-24-3p and regulate the expression of p27 and cyclin D1 (Shen et al. 2018). IncRNA H19 was shown to serve as a ceRNA by sponging miR-148a-3p to upregulate the target gene DNA methyltransferase 1 (Wu et al. 2016). NEAT1 was also reported to regulate the expression of cyclin dependent kinase 6 through modulating miR-107 (Wang et al. 2016b). Furthermore, a ceRNA network, including 30 genes, 21 miRNAs and 19 lncRNAs was also built based on microarray analysis of 6-paired clinical samples in LSCC (Zhang et al. 2016). However, analysis of the lncRNA-miRNA-mRNA regulatory network of LSCC with larger sample sizes and confirmation of their clinical associations are still lacking.

In addition, DNA methylation has been identified as an important mechanism to regulate gene expression in cancer cells epigenetically, which not only regulates the expression of protein-encoding genes, but also affects miRNAs and lncRNAs. For example, hyper-methylation of the promoter region was observed to lead to a loss of expression of lncRNA maternally expressed gene 3 (MEG3). Downregulated MEG3 was insufficient to sponge miR-9 and block its inhibition effects on the expressions of E-cadherin and FOXO1, consequentially resulting in poor prognosis in patients with esophageal squamous cell carcinoma (Dong et al. 2017). The study of Guo et al. also suggested lncRNA CTC-276P9.1 was hyper-methylated in esophageal squamous cell carcinoma. Over-expression of CTC-276P9.1 inhibited cancer cell proliferation and invasion in vitro probably by regulating epithelial-mesenchymal transition (Guo W 2018). Liao et al.
identified 761 lncRNA genes with DNA hyper-methylation in colorectal cancer using a free
MethylCap-seq dataset (Liao et al. 2015). Cheung et al. found that the loci of three miRNAs
(namely miR-199a-2, miR-124a-2 and miR-184) were linked to hyper-methylated differentially
methylated regions (DMRs) in human testicular cancer (Cheung & Lee 2010). However, the
DNA methylation regulatory mechanisms of miRNAs and lncRNAs have rarely been reported in
LSCC.

The goal of this study was to establish an lncRNA-miRNA-mRNA ceRNA network in
LSCC using larger samples and to investigate their methylation patterns. Our results may provide
new clues for biologists to further understand the pathogenesis of LSCC.

Materials and methods

Data source

lncRNA, miRNA, mRNA and methylation data were retrieved from Gene Expression
Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) in January 2018 according to the
following inclusion criteria: 1) lncRNA, miRNA, mRNA expression or methylation profiles; 2)
laryngeal tissue samples, not blood, interstitial fluid or cells; 3) inclusion of control; 4) human
samples; and 5) patients with LSCC.

Two lncRNA microarray datasets were obtained under accession number GSE59652 (7
LSCC and 7 paired adjacent normal tissues) (Shen et al. 2014) and GSE84957 (9 LSCC and 9
paired adjacent non-neoplastic tissues) (Feng et al. 2016). The microarray platforms of
GSE59652 and GSE84957 were Agilent-033010 (GPL13825, Arraystar Human LncRNA
microarray V2.0) and Agilent-042818 (GPL17843, Agilent-042818 Human LncRNA Micorarray
Two miRNA microarray datasets were collected under accession number GSE70289 (12 LSCC tissues and 4 adjacent normal tissues) (Karatas et al. 2015) and GSE62819 (5 LSCC carcinoma and 5 paired adjacent non-neoplastic tissues). The microarray platforms of GSE70289 and GSE62819 were Agilent-031181 (GPL15018, Unrestricted_Human_miRNA_V16.0_Microarray 030840) and Affymetrix Multispecies miRNA-3 Array (GPL16384), respectively.

Four mRNA microarray datasets were available under accession number GSE51985 (10 LSCC and 10 paired adjacent normal tissues), GSE84957 (9 LSCC and 9 paired adjacent normal tissues) (Feng et al. 2016), GSE59102 (29 LSCC and 13 normal margin tissues) and GSE58911 (15 LSCC and 15 normal tissue distant to LSCC) (Sharon et al. 2015). The microarray platforms of GSE51985, GSE84957, GSE59102 and GSE58911 were Illumina HumanHT-12 V4.0 (GPL10558), Agilent-042818 (GPL17843, Human lncRNA Micorarray 8_24_v2), Agilent-014850 (GPL6480, Whole Human Genome Microarray 4x44K G4112F) and Affymetrix Human Gene 1.0 ST Array (GPL6244), respectively.

One set of DNA methylation data was acquired under accession number GSE25093 (Poage et al. 2012; Poage et al. 2011) which included 213 blood and 109 tissue samples. Among the 109 tissue samples, 56 were isolated from oral, 16 from pharyngeal, and 22 from laryngeal origin, while 15 were of unclear origin. Thus, only these 22 samples from laryngeal origin (15 LSCC tissues and 7 controls) were used in our study. The microarray platform of GSE25093 was Illumina HumanMethylation27 BeadChip (GPL8490, HumanMethylation27_270596_v.1.2).
The mRNA and miRNA Seq-data of head and neck squamous cell carcinoma (Level 3) were also downloaded from The Cancer Genome Atlas (TCGA; https://tcga-data.nci.nih.gov/). After sample barcode screening, 559 were miRNA-mRNA matched samples, of which 18 were distributed in the alveolar crest, 30 in the root of the tongue, 22 in the buccal mucosa, 67 in the mouth floor, 8 in the hard palate, 9 in the laryngeal pharynx, 138 in the larynx, 3 in the lip, 38 in the oral cavity, 156 in the tongue, 10 in the oropharynx, 45 in the tonsil and 15 from an unclear location. Only the 138 samples from the larynx were used in our study.

**Data preprocessing**

For the data from Affy platform, the raw data in CEL files were preprocessed using the `oligo` package (version 1.41.1; http://www.bioconductor.org/packages/release/bioc/html/oligo.html) in R (version 3.4.1; http://www.R-project.org/), including data transformation, missing value imputation with median, background correction with MAS method and quantile normalization.

For the data from Agilent and Illumina platforms, the raw data in TXT files were preprocessed using the Linear Models for Microarray Data (LIMMA) package (version 3.34.0; https://bioconductor.org/packages/release/bioc/html/lmma.html) in R, including data log2 transformation and median normalization.

The data (FPKM, fragment per kilobase per million mapped reads) from TCGA were quantile normalized using the `preprocessCore` package (version 1.40.0; http://bioconductor.org/packages/release/bioc/html/preprocessCore.html) in R.

**Differential expression analysis**
The differentially expressed lncRNAs (DELs) and miRNAs (DEM) between LSCC and normal controls were identified using the LIMMA method in R from their two included microarray datasets (lncRNA: GSE59652 and GSE84957; miRNA: GSE70289 and GSE62819). The p-value < 0.05 and |logFC(fold change)| > 0.263 were set as the cut-off points. The overlap in the above two datasets was used for the following analysis of lncRNAs and miRNAs, respectively.

The differentially expressed genes (DEGs) between LSCC and normal controls were identified using the MetaDE.ES function in MetaDE package (version 1.0.5, https://cran.r-project.org/web/packages/MetaDE/) of R from its four included microarray datasets (GSE51985, GSE84957, GSE59102 and GSE58911). The p-value < 0.05 and false discovery rate (FDR) < 0.05 were set as the cut-off points. The DEGs with the same expression trend (tau² statistic = 0, p-value of Chi-square based Q-test > 0.05) in the four datasets were selected for the following analysis.

Wilcoxon signed-rank test (http://127.0.0.1:26738/library/stats/html/wilcox.test.html) was used to screen the DMRs between LSCC and normal controls. P < 0.05 was set as the threshold value. Human annotation data were retrieved from GENCODE Release 19 (GRCh37.p13) (http://www.gencodegenes.org/releases/19.html). The sequences of miRNAs, lncRNAs and mRNAs in the corresponding platform GPL8490 were blasted with the GRCh37.p13 to obtain the differentially methylated miRNAs, lncRNAs and mRNAs, which were then overlapped with the DELs, DEMs and DEGs to screen methylated-related DELs, DEMs and DEGs, respectively.

**CeRNA regulatory network construction**
Three reliable online databases, including miRcode (version 11; http://www.mircode.org/), starBase (version 2.0; http://starbase.sysu.edu.cn/index.php) (Li et al. 2014) and DIANA-LncBase (version 2.0; http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2/index-predicted) (Paraskevopoulou et al. 2013) were used to screen the interactions between lncRNAs and miRNAs. The union of these three datasets was used for the following analysis. The target genes of miRNAs that were linked to the lncRNAs were predicted using four frequently used algorithms, including TargetScan (version 7.2; http://www.targetscan.org/vert_71/) (Agarwal V 2015), miRBase (version 22; https://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) (Griffiths-Jones S 2005), miRanda (version 1.9; http://www.microrna.org/microrna/home.do/) (John B 2005) and miRTarBase (version 7.0; http://mirtarbase.mbc.nctu.edu.tw/php/index.php) (Chou et al. 2017). The target genes predicted by at least two databases and a negative association with miRNAs were retained. The lncRNA-miRNA and miRNA-mRNA interactions were integrated to construct the ceRNA network, which was visualized using Cytoscape software (version 3.4; www.cytoscape.org/) (Kohl et al. 2011).

**Function enrichment analysis**

The Database for Annotation, Visualization and Integrated Discovery (DAVID) online tool (version 6.8; http://david.abcc.ncifcrf.gov) (Da et al. 2009) was used for Gene Ontology (GO) terms [including molecular function (MF), biological process (BP) and cellular component (CC) categories] and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analyses of genes in the ceRNA network. P-value < 0.05 was set as the cut-off value.
Clinical associations of lncRNAs, miRNAs and mRNAs in the ceRNA network

The expression levels of lncRNAs, miRNAs and mRNAs in the ceRNA network were downloaded from the TCGA data. Univariate Cox regression analysis was performed to screen for the prognosis-related (including overall survival, OS; and recurrence-free survival, RFS) lncRNAs, miRNAs and mRNAs using the survival package (version 2.40.1; https://cran.r-project.org/package=survival), which was used to construct the prognosis-related ceRNA network. The samples were divided into two groups based on the expression of each lncRNA, miRNA and mRNA: a low expression group (< median) and a high expression (> median) group. The Kaplan–Meier method with the log-rank test was used to estimate the difference in OS and RFS between the high and low expression groups. P < 0.05 was considered statistically significant. Furthermore, multivariate Cox regression analysis was also performed using the survival package (version 2.40.1; https://cran.r-project.org/package=survival) to evaluate the prognostic independence of lncRNAs, miRNAs and mRNAs. The association of nodes in the prognosis-related ceRNA network with other clinical characteristics was also analyzed using the multiple linear regression model (https://stat.ethz.ch/R-manual/R-patched/library/stats/html/lm.html) in R.

Results

Differential expression analysis

The data analysis workflow is displayed in figure 1. After data normalization (Supplemental Information S1-8), the DELs, DEMs and DEGs between LSCC and normal samples were screened according the stated thresholds. The results showed 306 (156 downregulated and 150
upregulated) and 396 (252 downregulated and 144 upregulated) DELs were identified in the datasets of GSE59652 (Figure 2A) and GSE84957 (Figure 2B) (Supplemental Information S9), respectively. After comparison, 40 DELs were found to be shared in these two datasets, including 6 upregulated and 20 downregulated with the consistent expression trend (Figure 3A) (Supplemental Information S9); a total of 1307 (765 downregulated and 542 upregulated) and 491 (126 downregulated and 365 upregulated) DEMs were identified in the datasets GSE62819 (Figure 2C) and GSE70289 (Figure 2D), respectively (Supplemental Information S9). After comparison, 443 DEMs were found to be common in these two datasets, among which 152 upregulated and 63 downregulated DEMs were shown to have a consistent expression trend (Figure 3B) (Supplemental Information S9); 2975 DEGs were found to display the similar expression trend in four mRNA expression profiles GSE51985, GSE84957, GSE59102 and GSE58911 (Figure 2E) (Supplemental Information S9); and 4567 DMRs were identified in the LSCC genome of GSE25093 dataset, including 1616 hypomethylated and 2951 hypermethylated (Figure 2F) (Supplemental Information S9). After GENCODE annotation and blast analysis, 122 lncRNAs, but no miRNAs were found to be located in DMRs. Subsequently, the lncRNAs and mRNAs in DMRs were overlapped with their expression level data above to obtain the methylation-related DELs and DEGs. Consequently, 5 DELs (TMEM51-AS1, HCP5, SND1-IT1, RUSC1-AS1 and LINC00324) were screened (Figure 3C). Among these DELs, only the expression and methylation levels of lncRNA TMEM51-AS1 (Figure 3D-F) were opposite, indicating its expression may be regulated by methylation. These methylation-related genes were used to construct the ceRNA network.
CeRNA network construction

Twenty-four interaction pairs between 5 DELs and 14 DEMs were predicted using miRcode, starBase and DIANA-LncBase databases (Table 1). The expression trends of these DELs and DEMs were opposite. Subsequently, the target genes of these 14 DEMs were predicted using four algorithms, with the resultant interaction pairs of 700 in TargetScan, 486 in miRBase, 341 in miRanda and 268 in miRTarBase. A total of 404 interaction pairs were ultimately left due to prediction by at least two databases and a negative association between them. These interaction pairs between DELs and DEMs, and between DEMs and DEGs were used to construct a ceRNA network, which contained 258 nodes (5 DELs, 11 DEMs and 242 DEGs) (Figure 4). In this network, TMEM51-AS1 functioned as a ceRNA to regulate SNX21 (sorting nexin family member 21) and TRAPPC10 (trafficking protein particle complex 10) by sponging miR-106b; LINC00324 and RUSC1-AS1 acted as ceRNAs to regulate SPRY4 (sprouty RTK signaling antagonist 4), PAWR (pro-apoptotic WT1 regulator), MICAL2 (microtubule associated monooxygenase, calponin and LIM domain containing 2) and SLC39A14 (solute carrier family 39 member 14) by sponging miR-16; RUSC1-AS1 regulated SCG5 (SCG5 secretogranin V) and PRDM5 (PR/SET domain 5) by competitively binding to miR-10; RUSC1-AS1 also served as ceRNAs for ZFP1 (ZFP1 zinc finger protein) by binding to miR-7; HCP5 could interact with miR-143 to regulate RRM2 (ribonucleotide reductase regulatory subunit M2).

Function enrichment analysis

The DEGs in the ceRNA network was subjected to DAVID to predict their potential functions in LSCC. The results showed that 17 significant GO BP terms were enriched, including
GO:0042981~regulation of apoptosis (PAWR), GO:0015031~protein transport (SNX21; SCG5), cell cycle (PRDM5) and GO:0043407~negative regulation of MAP kinase activity 4 (SPRY4). Six KEGG pathways were also enriched, including hsa05210:Colorectal cancer, hsa04210:Apoptosis and hsa05205:Proteoglycans in cancer (Table 2).

Clinical associations of lncRNAs, miRNAs and mRNAs in the ceRNA network

In the 138 miRNA-mRNA matched samples of TCGA data, 114 had OS and 82 had RFS information. Univariate Cox regression analysis in these 138 samples showed that 32 RNAs were significantly associated with OS, including 1 DEL (RUSC1-AS1), 2 DEMs (hsa-miR-16 and hsa-miR-10) and 29 DEGs (i.e., PAWR, SCG5, SPRY4, MICAL2, SNX21, TRAPPC10 and SLC39A14); while 25 RNAs were associated with RFS, including 1 DEL (LINC00324), 3 DEMs (hsa-miR-16, hsa-miR-10 and hsa-miR-7) and 21 DEGs (i.e., PRDM5, SCG5, SPRY4, MICAL2 and ZFP1) (Table 3). The OS and RFS related ceRNA networks were extracted independently as shown in Figure 5A and B.

Subsequently, multivariate Cox regression showed TRAPPC10 and SLC39A14 were independent factors for OS; RRM2 was an independent factor for RFS (Table 4). Although SOD2, SLC44A1 and THEM4 were also screened to be significant, their hazard ratios (HR) were not consistent with the expected according to their expression levels. Combined with the univariate results, we suggested TRAPPC10 and SLC39A14 related ceRNA axes (TMEM51-AS1-miR-106-TRAPPC10; RUSC1-AS1-miR-16-SLC39A14) may be especially important. The Kaplan–Meier curve of these lncRNAs, miRNAs and mRNAs were drawn. As expected, the low expression of miR-16 (Figure 5D) was associated with poor prognosis and the high expression of
RUSC1-AS1 (Figure 5C), SLC39A14 (Figure 5E) and TRAPPC10 (Figure 6A) was associated with shorter OS.

Furthermore, OS- and RFS-related DELs, DEMs and DEGs were also analyzed to investigate their associations with other clinical characteristics of LSCC to further confirm their importance. The results showed that RUSC1-AS1 was significantly associated with Pathologic N; OS- and RFS-related SPRY4 was associated with Pathologic M; OS-related MICAL2 was associated with Pathologic N and Pathologic stage; RFS-related ZFP1 and SLC39A14 were associated with Pathologic N; OS-related SNX21 and RFS-related SCG5 were associated with gender (Table 5). These findings implied SPRY4, MICAL2, ZFP1, SNX21 and SCG5 related ceRNAs (LINC00324/RUSC1-AS1-miR-16-SPRY4/MICAL2, RUSC1-AS1-miR-7-ZFP1, TMEM51-AS1-miR-106-SNX21, RUSC1-AS1-miR-10-SCG5) were also crucial for LSCC. The Kaplan–Meier curve of SNX21 is shown in Figure 6B and the other DEGs are displayed in Figure S1 and Figure S2.

Discussion

Although epigenetics modification has been shown to trigger silencing or overexpression of IncRNAs in cancer (Dong et al. 2017; Zhou et al. 2018), the aberrant methylation-mediated expression changes of IncRNAs remain unclear in LSCC. We, for the first time, found that the downregulation of IncRNA TMEM51-AS1 may be mediated by hyper-methylation. Few studies investigated the roles of TMEM51-AS1 in cancer except one study indicated downregulated TMEM51-AS1 was significantly correlated with poor OS in chromophobe renal cell carcinoma (He et al. 2016). In present study, we predicted that TMEM51-AS1 might function as a ceRNA
to regulate SNX21 and TRAPPCC10 through sponging miR-106b. Evidence demonstrated that miR-106b was up-regulated in LSCC (Lu et al. 2014; Xing et al. 2014), which was also confirmed in our microarray study. miR-106b was reported to promote the proliferation and invasion of LSCC cells by targeting RUNX3 (Ying et al. 2013), while induce cell cycle G0/G1 arrest by inhibiting tumor suppressor RB (Cai et al. 2011). Although no study revealed the roles of SNX21 in cancer, its family genes, such as SNX1 (Zhan et al. 2018), SNX5 (Jitsukawa et al. 2017) and SNX9 (Bendris et al. 2016) were suggested to be tumor suppressor related. Therefore, SNX21 may be theoretically downregulated in LSCC by miR-106b. Consistent with this hypothesis, our study showed that SNX21 was less expressed in LSCC tissues and patients with high expression of SNX21 had a higher OS rate. There was only one study to suggest the roles of TRAPPCC10 until now and showed TRAPPCC10 was an oncogenic driver to predict the poor prognosis for breast cancer patients (Pongor et al. 2015), which seemed to be contrast with our results, implying TRAPPCC10 may be a new tumor suppressor gene for LSCC. The tumor inhibition effects of TRAPPCC10 may be related with its potential to activate GTPase RAB11 (Milev et al. 2018) and then Rab coupling protein, targeted deletion of which led to accelerated tumor onset (Boulay et al. 2016).

Furthermore, we identified several other ceRNA axes, although they were not methylation-related, including LINC00324/RUSC1-AS1-miR-16-SPRY4/MICAL2/SLC39A14, RUSC1-AS1-miR-10-SCG5 and RUSC1-AS1-miR-7-ZFP1. All these lncRNAs, miRNAs and mRNAs were significantly associated with OS and/or RFS, indicating these ceRNA axes may also be underlying therapeutic targets.
Although related report was rare, RUSC1-AS1 (Jian et al. 2015) and LINC00324 (Militello et al. 2017) had been indicated to be highly expressed in cancer cells, which were similarly confirmed in LSCC samples. Accumulating evidence also has proved the roles of miR-16, miR-7 and miR-10 in various types of cancer. miR-16 could be downregulated in tissue samples and cell lines of lung cancer (Ke Y 2013) and osteosarcoma (Jiao et al. 2018). Ectopic expression of miR-16 inhibited cell proliferation, colony formation \textit{in vivo} and, migration and invasion \textit{in vitro} by regulating its target genes RAB23 and Smad3 (Jiao et al. 2018; Zhang et al. 2018). miR-10a was down-regulated in laryngeal epithelial premalignant lesions with increasing grade of dysplasia (Hu et al. 2015). Overexpression of miR-10a inhibited cell metastasis by regulating epithelial-to-mesenchymal transition (EMT) (Liu et al. 2017b). miR-7-5p was lower expressed in brain-metastatic lesions of breast cancer (Hiroshi et al. 2013) and the use of miR-7-5p mimics suppressed cell proliferation and induced apoptosis (Shi et al. 2015) via modulating the expression of Kruppel like factor 4. In agreement with these studies, we also found that these three miRNAs were less expressed (especially miR-10 and miR-7) in LSCC and negatively associated with OS and/or RFS. Although the downstream target genes of these miRNAs have been reported as above, their functions in LSCC remain poorly understood. We predicted that SPRY4/MICAL2/SLC39A14, SCG5 and ZFP1 may be the potential targets of miR-16, miR-10 and miR-7, respectively in LSCC, which had not been validated previously. Nevertheless, the studies on the molecular mechanisms of these DEGs may indirectly explain their potential interactions. The expression of SPRY4 was upregulated in testicular germ cell tumors (Tian et al. 2018). MICAL2 was a recently identified proto-oncogene, which increased cell proliferation to
accelerate tumor growth, and promoted the expression of EMT-related proteins to increase cell metastasis (Mariotti et al. 2016; Wang et al. 2018). Immunohistochemical analysis showed the expression level of SLC39A14 was significantly higher in hepatocellular carcinoma tissues than that in adjacent tissues and negatively correlated with survival time (Gartmann et al. 2018). Also, the upregulation of SLC39A14 in tumor cells may be attributed to the loss of its interactive gene p53, a tumor suppressor (Zhao et al. 2017). Although there were no studies to discuss the roles of SCG5 in cancer, its family member secretogranin II and III had been seen to be overexpressed in prostate cancer (Courel et al. 2014) and small cell lung carcinoma (Togayachi et al. 2017), suggesting SCG5 may also be oncogenic for LSCC. Zinc finger proteins had also been observed to promote cell growth and metastasis in nasopharyngeal carcinoma (Li et al. 2015). In line with these findings, SPRY4, MICAL2, SLC39A14, SCG5 and ZFP1 were all upregulated in LSCC and associated with poor prognosis.

There were some limitations in this study. First, although all the known microarray or sequencing data from the public database had been included, the sample size was still not large which may influence the results. Therefore, additional clinical trials with larger samples may be essential to confirm their expression and prognosis. Second, we only preliminarily predicted that these ceRNA axes may be associated with LSCC development and prognosis. The regulatory relationships between lncRNAs and miRNAs as well as between miRNAs and mRNAs needed further experimental confirmation in vitro and in vivo (i.e., dual luciferase reporter assay or loss-of-function). Third, whether the expression of TMEM51-AS1 was regulated by methylation should be validated by using the methylation inhibitor 5-azacytidine. Fourth, although we have
normalized the data from different platforms, this may still cause some underlying bias.

Conclusion

Our present study identifies several important mechanisms for the development and progression of LSCC: 1) methylation-mediated upregulation of IncRNA TMEM51-AS1 may function as a ceRNA for miR-106b to regulate SNX21 and TRAPPC10; 2) survival-related RUSC1-AS1/LINC00324 may function as a ceRNA to sponge miR-16, miR-10 or miR-7 and then regulate SPRY4/MICAL2/SLC39A14, SCG5/PRDM5 and ZFP1, respectively. Altogether, these IncRNA, miRNAs or mRNAs may be potential prognostic biomarkers and therapeutic targets of LSCC.

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References

Agarwal V, Bell KW, Nam JW, Bartel DP. 2015. Predicting effective microRNA target sites in mammalian mRNAs. Elife 4: e05005.

Bendris N, Stearns CJS, Reis CR, Rodriguezcanales J, Liu H, Witkiewicz AW, and Schmid SL. 2016. Sorting nexin 9 negatively regulates invadopodia formation and function in cancer cells. J Cell Sci 129:2804-2816.

Boulay PL, Mitchell L, Turpin J, Huot-Marchand JÉ, Lavoie C, Sanguin-Gendreau V, Jones L, Mitra S, Livingstone JM, Campbell S, Hallett M, Mills GB, Park M, Chodosh L, Strathdee D, Norman JC, Muller WJ. 2016. Rab11-FIP1C Is a Critical Negative Regulator in ErbB2-Mediated Mammary Tumor Progression. Cancer Res 76:2662-2674.
Cai K, Wang Y, and Bao X. 2011. MiR-106b promotes cell proliferation via targeting RB in laryngeal carcinoma. *J Exp Clin Cancer Res* 30:73.

Chen W ZR, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. 2016. Cancer statistics in China, 2015. *Ca Cancer J Clin* 66:115-132.

Cheung HH, and Lee TA. 2010. Genome-wide DNA methylation profiling reveals novel epigenetically regulated genes and non-coding RNAs in human testicular cancer. *Br J Cancer* 102:419-427.

Chou CH, Shrestha S, Yang CD, Chang NW, Lin YL, Liao KW, Huang WC, Sun TH, Tu SJ, and Lee WH. 2017. miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions. *Nucleic Acids Res* 46: D296-D302.

Courel M, El Yamani FZ, Alexandre D, El FH, Delestre C, Montero-Hadjadje M, Tazi F, Amarti A, Magoul R, Chartrel N. 2014. Secretogranin II is overexpressed in advanced prostate cancer and promotes the neuroendocrine differentiation of prostate cancer cells. *Eur J Cancer* 50:3039-3049.

Da WH, Sherman BT, and Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4:44-57.

Dong Z, Zhang A, Liu S, Lu F, Guo Y, Zhang G, Xu F, Shi Y, Shen S, and Liang J. 2017. Aberrant Methylation-Mediated Silencing of IncRNA MEG3 Functions as a ceRNA in Esophageal Cancer. *Mol Cancer Res* 15:800-810.

Feng L, Wang R, Lian M, Ma H, He N, Liu H, Wang H, and Fang J. 2016. Integrated Analysis of Long Noncoding RNA and mRNA Expression Profile in Advanced Laryngeal
Squamous Cell Carcinoma. *Plos One* 11:e0169232.

Gartmann L, Wex T, Grüngreiff K, Reinhold D, Kalinski T, Malfertheiner P, Schütte K. 2018. Expression of zinc transporters ZIP4, ZIP14 and ZnT9 in hepatic carcinogenesis-An immunohistochemical study. *J Trace Elem Med Biol* 49:35-42.

Gartmann L, Wex T, Grüngeiff K, Reinhold D, Kalinski T, Malfertheiner P, Schütte K. 2018. Expression of zinc transporters ZIP4, ZIP14 and ZnT9 in hepatic carcinogenesis-An immunohistochemical study. *J Trace Elem Med Biol* 49:35-42.

Griffiths Jones S GR, Van DS, Bateman A, Enright AJ. 2005. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 34:140-144.

Guo W, Liu S, Dong Z, Guo Y, Ding C, Shen S, Liang J, Shan B. 2018. Aberrant methylation-mediated silencing of IncRNA CTC-276P9.1 is associated with malignant progression of esophageal squamous cell carcinoma. *Clin Exp Metastasis* 35:53-68.

Guo W, Lv P, Liu S, Xu F, Guo Y, Shen S, Liang J, Kuang G, and Dong Z. 2018. Aberrant methylation-mediated downregulation of long noncoding RNA C5orf66-AS1 promotes the development of gastric cardia adenocarcinoma. *Mol Carcinog* 57:854-865.

He HT, Xu M, Kuang Y, Han XY, Wang MQ, and Yang Q. 2016. Biomarker and competing endogenous RNA potential of tumor-specific long noncoding RNA in chromophobe renal cell carcinoma. *Onco Targets Ther* 9:6399-6406.

Hiroshi O, Fei X, Pandey PR, Sambad S, Misako W, Pai SK, Yin-Yuan M, Megumi IG, Shigeru H, and Yin L. 2013. miR-7 suppresses brain metastasis of breast cancer stem-like cells by modulating KLF4. *Cancer Res* 73:1434-1444.

Hu Y, Jin Y, Li X, Yu J, Wang F, Bu W, and Liu H. 2015. [Expression of microRNA-10a-5p in laryngeal epithelial premalignant lesions]. *Zhonghua Bing Li Xue Za Zhi* 44:184-188.

Jean H, and Mihaela Z. 2014. Identification and consequences of miRNA-target interactions--
beyond repression of gene expression. *Nat Rev Genet* 15:599-612.

Jian Z, Dahua F, Zhixiang J, Chen GG, and Lai PBS. 2015. Cancer Specific Long Noncoding RNAs Show Differential Expression Patterns and Competing Endogenous RNA Potential in Hepatocellular Carcinoma. *Plos One* 10:e0141042.

Jiao ZH, Wang JD, and Wang XJ. 2018. MicroRNA-16 suppressed the invasion and migration of osteosarcoma by directly inhibiting RAB23. *Eur Rev Med Pharmacol Sci* 22:2598-2605.

Jitsukawa S, Kamekura R, Kawata K, Ito F, Sato A, Matsumiya H, Nagaya T, Yamashita K, Kubo T, and Kikuchi T. 2017. Loss of sorting nexin 5 stabilizes internalized growth factor receptors to promote thyroid cancer progression. *J Pathol* 243:342-353.

John B, Enright A, Aravin A, Tuschl T, Sander C. 2005. Human MicroRNA targets. *PLoS Biol* 3:e264.

Karatas OF, Suer I, Yuceturk B, Yilmaz M, Hajiyev Y, Creighton CJ, Ittmann M, and Ozen M. 2015. The role of miR-145 in stem cell characteristics of human laryngeal squamous cell carcinoma Hep-2 cells. *Tumour Biology* 37: 4183-4192.

Ke Y, Zhao W, Xiong J, Cao R. 2013. Downregulation of miR-16 promotes growth and motility by targeting HDGF in non-small cell lung cancer cells. *FEBS Lett* 587:3153-3157.

Kohl M, Wiese S, and Warscheid B. 2011. Cytoscape: software for visualization and analysis of biological networks. *Methods Mol Biol* 696:291-303.

Li JH, Liu S, Zhou H, Qu LH, Yang JH. 2014. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res* 42:D92-97.
Li Y, Yan X, Yan L, Shan Z, Liu S, Chen X, Zou J, Zhang W, and Jin Z. 2015. High expression of Zinc-finger protein X-linked is associated with reduced E-cadherin expression and unfavorable prognosis in nasopharyngeal carcinoma. *International J Clin Exp Pathol* 8:3919-3927.

Liao Q, He W, Liu J, Cen Y, Luo L, Yu C, Li Y, Chen S, and Duan S. 2015. Identification and functional annotation of IncRNA genes with hypermethylation in colorectal cancer. *Gene* 572:259-265.

Liu S, Xie F, Xiang X, Liu S, Dong S, Qu K, and Lin T. 2017a. Identification of differentially expressed genes, IncRNAs and miRNAs which are associated with tumor malignant phenotypes in hepatoblastoma patients. *Oncotarget* 8:97554-97564.

Liu Y, Zhang Y, Wu H, Li Y, Zhang Y, Liu M, Li X, and Tang H. 2017b. miR-10a suppresses colorectal cancer metastasis by modulating the epithelial-to-mesenchymal transition and anoikis. *Cell Death Dis* 8:e2739.

Lu ZM, Lin YF, Jiang L, Chen LS, Luo XN, Song XH, Chen SH, and Zhang SY. 2014. Micro-ribonucleic acid expression profiling and bioinformatic target gene analyses in laryngeal carcinoma. *Onco Targets Ther* 2014:525-533.

Mariotti S, Barravecchia I, Vindigni C, Pucci A, Balsamo M, Libro R, Senchenko V, Dmitriev A, Jacchetti E, and Cecchini M. 2016. MICAL2 is a novel human cancer gene controlling mesenchymal to epithelial transition involved in cancer growth and invasion. *Oncotarget* 7:1808-1825.

Milev MP, Graziano C, Karall D, Kuper WFE, Al-Deri N, Cordelli DM, Haack TB, Danhauser
K, Iuso A, Palombo F, Pippucci T, Proksich H, Saint-Dic D1, Seri M, Stanga D, Cenacchi G, van Gassen KLI, Zschocke J, Fauth C, Mayr JA, Sacher M, van Hasselt PM. 2018. Biallelic mutations in TRAPPC2L result in a neurodevelopmental disorder and have an impact on RAB11 in fibroblasts. J Med Genet 55:753-764.

Militello G, Weirick T, John D, Döring C, Dimmeler S, and Uchida S. 2017. Screening and validation of lncRNAs and circRNAs as miRNA sponges. Brief Bioinform 18:780-788.

Paraskevopoulou MD, Georgios G, Nikos K, Martin R, Manolis M, Dalamagas TM, and Hatzigeorgiou AG. 2013. DIANA-LncBase: experimentally verified and computationally predicted microRNA targets on long non-coding RNAs. Nucleic Acids Res 41:D239-245.

Poage GM, Butler RA, E Andrés H, Mcclean MD, Nelson HH, Christensen BC, Marsit CJ, and Kelsey KT. 2012. Identification of an epigenetic profile classifier that is associated with survival in head and neck cancer. Cancer Res 72:2728-2737.

Poage GM, E Andres H, Christensen BC, Butler RA, Michele AW, Mcclean MD, Tim W, Michael P, Marsit CJ, and Kelsey KT. 2011. Global hypomethylation identifies Loci targeted for hypermethylation in head and neck cancer. Clin Cancer Res 17:3579-3589.

Pongor L, Kormos M, Hatzis C, Pusztai L, Szabó A, Győrffy B. 2015. A genome-wide approach to link genotype to clinical outcome by utilizing next generation sequencing and gene chip data of 6,697 breast cancer patients. Genome Med 7:104.

Rudolph E, Dyckhoff G, Becher H, Dietz A, and Ramroth H. 2011. Effects of tumour stage, comorbidity and therapy on survival of laryngeal cancer patients: a systematic review and a meta-analysis. Eur Arch Otorhinolaryngol 268:165-179.
Salmena L, Poliseno L, Tay Y, Kats L, and Pandolfi PP. 2011. ceRNA hypothesis: The Rosetta Stone of a hidden RNA language? Cell 146:353-358.

Sharon L, Mary E G, Robert D H, Karen T P, Michael R G, Chindo H, and Tejaswi K. 2015. Prognostic biomarkers for HNSCC using quantitative real-time PCR and microarray analysis: β-tubulin isotypes and the p53 interactome. Cytoskeleton 71:628-637.

Shen Z, Hao W, Zhou C, Deng H, Dong Y, Li Q, Lin L, Bing C, and Guo J. 2018. Long non-coding RNA AC026166.2-001 inhibits cell proliferation and migration in laryngeal squamous cell carcinoma by regulating the miR-24-3p/p27 axis. Sci Rep 8:3375.

Shen Z, Li Q, Deng H, Lu D, Song H, and Guo J. 2014. Long Non-Coding RNA Profiling in Laryngeal Squamous Cell Carcinoma and Its Clinical Significance: Potential Biomarkers for LSCC. Plos One 9:e108237.

Shi Y, Luo X, Li P, Tan J, Wang X, Xiang T, and Ren G. 2015. miR-7-5p suppresses cell proliferation and induces apoptosis of breast cancer cells mainly by targeting REGγ. Cancer Lett 358:27-36.

Siegel RL, Miller KD, and Jemal A. 2017. Cancer Statistics, 2017. Ca Cancer J Clin 67:7-30.

Song Z ZW, Cao D, Zhang J, Chen S. 2018. Elementary screening of lymph node metastatic-related genes in gastric cancer based on the co-expression network of messenger RNA, microRNA and long non-coding RNA. Braz J Med Biol Res 51:e6685.

Tian Y, Fu X, Li Q, Wang Y, Fan D, Zhou Q, Kuang W, and Shen L. 2018. MicroRNA-181 serves an oncogenic role in breast cancer via the inhibition of SPRY4. Mol Med Rep 18:5603-5613.
Togayachi A, Iwaki J, Kaji H, Matsuzaki H, and Narimatsu H. 2017. Glycobiomarker, fucosylated short-form secretogranin III levels are increased in the serum of patients with small cell lung carcinoma. *J Proteome Res* 16:4495-4505.

Wang P, Wu T, Zhou H, Jin Q, He G, Yu H, Xuan L, Wang X, Tian L, and Sun Y. 2016. Long noncoding RNA NEAT1 promotes laryngeal squamous cell cancer through regulating miR-107/CDK6 pathway. *J Exp Clin Cancer Res* 35:22.

Wang Y, Deng W, Zhang Y, Sun S, Zhao S, Chen Y, Zhao X, Liu L, and Du J. 2018. MICAL2 promotes breast cancer cell migration by maintaining epidermal growth factor receptor (EGFR) stability and EGFR/P38 signalling activation. *Acta Physiologica* 222:e12920.

Wu T, Qu L, He G, Tian L, Li L, Zhou H, Jin Q, Ren J, Wang Y, and Wang J. 2016. Regulation of laryngeal squamous cell cancer progression by the LncRNA H19/miR-148a-3p/DNMT1 axis. *Oncotarget* 7:11553-11566.

Xing Y, Yibo W, Yang L, Hongxia D, Zhisen S, Bingxiu X, and Junming G. 2014. miR-21, miR-106b and miR-375 as novel potential biomarkers for laryngeal squamous cell carcinoma. *Curr Pharm Biotechnol* 15:503-508.

Ying X, Kai W, Wei G, Zhang C, Huang F, Wen S, and Wang B. 2013. MicroRNA-106b regulates the tumor suppressor RUNX3 in laryngeal carcinoma cells. *FEBS Lett* 587:3166-3174.

Zhan XY, Zhang Y, Zhai E, Zhu QY, and He Y. 2018. Sorting nexin-1 is a candidate tumor suppressor and potential prognostic marker in gastric cancer. *Peer J* 6:e4829.

Zhang C, Gao W, Wen S, Wu Y, Fu R, Zhao D, Chen X, and Wang B. 2016. Potential key...
molecular correlations in laryngeal squamous cell carcinoma revealed by integrated analysis of mRNA, miRNA and lncRNA microarray profiles. *Neoplasma* 63:888-900.

Zhang H, Yang K, Ren T, Huang Y, Tang X, and Guo W. 2018. miR-16-5p inhibits chordoma cell proliferation, invasion and metastasis by targeting Smad3. *Cell Death Dis* 9:680.

Zhao Y, Wang H, Wu C, Yan M, Wu H, Wang J, Yang X, and Shao Q. 2018. Construction and investigation of lncRNA-associated ceRNA regulatory network in papillary thyroid cancer. *Oncol Rep* 39:1197-1206.

Zhao N, Zhang AS, Wortham AM, Jue S, Knutson MD, Enns CA. 2017. The Tumor Suppressor, P53, Decreases the Metal Transporter, ZIP14. *Nutrients* 9: pii: E1335.

Zhou Z, Lin Z, Xin P, Tariq MA, Xiang A, Li P, and Wang J. 2018. Epigenetic regulation of long non-coding RNAs in gastric cancer. *Oncotarget* 9:19443-19458.
Figure legends:

Figure 1 The data analysis workflow.

Figure 2 Hierarchical clustering and heat map analysis. A-B, heat map for differentially expressed lncRNAs identified in GSE59652 (A) and GSE84957 (B) datasets; C-D, heat map for differentially expressed miRNAs identified in GSE62819 (C) and GSE70289 (D) datasets; E, heat map for differentially expressed genes identified by meta-analysis of GSE51985, GSE84957, GSE59102 and GSE58911 datasets; F, heat map for differentially methylated regions identified in GSE25093 dataset. The datasets of laryngeal squamous cell carcinoma collected from Gene Expression Omnibus database. Red, high expression (hyper-methylation); green, low expression (hypo-methylation).

Figure 3 Overlapped genes identification. Venn diagram drawing to display the overlap of differentially expressed lncRNAs (A) and miRNAs (B) in different datasets of laryngeal squamous cell carcinoma collected from Gene Expression Omnibus database and their overlap with differentially methylated regions (C) to screen methylation related lncRNAs and miRNAs. The expression (D-E) and methylated (F) levels of overlapped lncRNAs are displayed in a histogram.* p < 0.05; ** p < 0.01. Contra-regulated: the expression trend of lncRNAs or miRNAs was different in two datasets. Up regulated or down regulated: lncRNAs or miRNAs exhibited the similar expression trend in two datasets, high or down expressed.
Figure 4 Competing endogenous RNAs (ceRNAs) interaction network of lncRNA-miRNA-mRNA in laryngeal squamous cell carcinoma. A, interaction pairs among upregulated lncRNAs, downregulated miRNAs and upregulated mRNAs; B, interaction pairs among downregulated lncRNAs, upregulated miRNAs and downregulated mRNAs. Square nodes represent lncRNAs; triangle nodes represent miRNAs; round nodes represent mRNAs. Edges represent the possible associations between lncRNAs, miRNAs and mRNAs. Red, upregulated; green, downregulated.

Figure 5 Prognosis related competing endogenous RNAs (ceRNAs) interaction axes. A, lncRNA-miRNA-mRNA network for overall survival; B, lncRNA-miRNA-mRNA network for recurrence free survival. Square nodes represent lncRNAs; triangle nodes represent miRNAs; round nodes represent mRNAs. Edges represent the possible associations between lncRNAs, miRNAs and mRNAs. Red, upregulated; green, downregulated.; Kaplan–Meier analysis of the lncRNA (C), miRNA (D) and mRNA (E) of crucial ceRNA axis in which all lncRNA, miRNA and mRNA were prognosis-related and mRNA was an independent prognostic factor.

Figure 6 Kaplan–Meier curve of lncRNA TMEM51-AS1 ceRNA related mRNAs.

A, TRAPPC10, which was an independent prognostic factor; B, SNX21, which was overall survival related in univariate Cox regression analysis.

Supplementary Materials:

Supplemental Information S1: Raw data of GSE59652.

Supplemental Information S2: Raw data of GSE84957.
Supplemental Information S3: Raw data of GSE62819.

Supplemental Information S4: Raw data of GSE70289.

Supplemental Information S5: Raw data of GSE51985.

Supplemental Information S6: Raw data of GSE58911.

Supplemental Information S7: Raw data of GSE59102.

Supplemental Information S8: Raw data of GSE84957.

Supplemental Information S9: Differentially expressed IncRNAs, miRNAs, genes and differentially methylated regions in all datasets.

Figure S1: Kaplan–Meier curve of the IncRNA, miRNAs and mRNAs for overall survival related competing endogenous RNAs interaction axes.

Figure S2: Kaplan–Meier curve of the IncRNA, miRNAs and mRNAs for recurrence free survival related competing endogenous RNAs interaction axes.
Figure 1

The data analysis workflow

Datasets Part B (NCBI GEO)

Set I IncRNA (GSE59652, GSE84957)
Set II mRNA (GSE70289, GSE62819)
Set III mRNA (GSE51985, GSE84957, GSE59102, GSE58911)
Set IV methylation (GSE25093)

Data preprocessing

Identification of DELs, DEMs, DEGs and DMRs

Methylated related DELs, DEMs and DEGs

Univariate Cox regression analysis

Prediction by miRcode, starBase, DIANA-LncBase
Prediction by TargetScan, miRBase, miRanda, miRTarBase

Survival analysis

ceRNA network construction

GO enrichment
KEGG enrichment

OS related prognostic ceRNA network construction
RFS related prognostic ceRNA network construction

Multivariate Cox regression

Independent prognostic factor selection
Figure 2

Hierarchical clustering and heat map analysis.

A-B, heat map for differentially expressed lncRNAs identified in GSE59652 (A) and GSE84957 (B) datasets; C-D, heat map for differentially expressed miRNAs identified in GSE62819 (C) and GSE70289 (D) datasets; E, heat map for differentially expressed genes identified by meta-analysis of GSE51985, GSE84957, GSE59102 and GSE58911 datasets; F, heat map for differentially methylated regions identified in GSE25093 dataset. The datasets of laryngeal squamous cell carcinoma collected from Gene Expression Omnibus database. Red, high expression (hyper-methylation); green, low expression (hypo-methylation).
Figure 3

Overlapped genes identification.

Venn diagram drawing to display the overlap of differentially expressed lncRNAs (A) and miRNAs (B) in different datasets of laryngeal squamous cell carcinoma collected from Gene Expression Omnibus database and their overlap with differentially methylated regions (C) to screen methylation related lncRNAs and miRNAs. The expression (D-E) and methylated (F) levels of overlapped lncRNAs are displayed in a histogram.* p < 0.05; ** p < 0.01. Contra-regulated: the expression trend of lncRNAs or miRNAs was different in two datasets. Up regulated or down regulated: lncRNAs or miRNAs exhibited the similar expression trend in two datasets, high or down expressed.
Figure 4

Competing endogenous RNAs (ceRNAs) interaction network of lncRNA-miRNA-mRNA in laryngeal squamous cell carcinoma.

A, interaction pairs among upregulated lncRNAs, downregulated miRNAs and upregulated mRNAs; B, interaction pairs among downregulated lncRNAs, upregulated miRNAs and downregulated mRNAs. Square nodes represent lncRNAs; triangle nodes represent miRNAs; round nodes represent mRNAs. Edges represent the possible associations between lncRNAs, miRNAs and mRNAs. Red, upregulated; green, downregulated. The red line, the interaction between lncRNAs and miRNAs; the greyish line, the interaction between miRNA and mRNAs.
Figure 5

Prognosis related competing endogenous RNAs (ceRNAs) interaction axes.

A, lncRNA-miRNA-mRNA network for overall survival; B, lncRNA-miRNA-mRNA network for recurrence free survival. Square nodes represent lncRNAs; triangle nodes represent miRNAs; round nodes represent mRNAs. Edges represent the possible associations between lncRNAs, miRNAs and mRNAs. Red, upregulated; green, downregulated.; Kaplan–Meier analysis of the lncRNA (C), miRNA (D) and mRNA (E) of crucial ceRNA axis in which all lncRNA, miRNA and mRNA were prognosis-related and mRNA was an independent prognostic factor.
Figure 6

Kaplan–Meier curve of IncRNA TMEM51-AS1 ceRNA related mRNAs.

A, TRAPPC10, which was an independent prognostic factor; B, SNX21, which was overall survival related in univariate Cox regression analysis.
**Table 1** (on next page)

Interaction relationship between IncRNA and miRNAs
| IncRNA     | miRNA                                      |
|------------|--------------------------------------------|
| HCP5       | hsa-miR-10, hsa-miR-16, hsa-miR-186, hsa-miR-214, hsa-miR-7, hsa-miR-641, hsa-miR-143, hsa-miR-4770, hsa-miR-216b, hsa-miR-876 |
| LINC00324  | hsa-miR-143, hsa-miR-16, hsa-miR-214, hsa-miR-216b, hsa-miR-4770 |
| RUSC1-AS1  | hsa-miR-214, hsa-miR-10, hsa-miR-16, hsa-miR-216b, hsa-miR-7 |
| TMEM51-AS1 | hsa-miR-106b, hsa-miR-765                   |
| SND1-IT1   | hsa-miR-708, hsa-miR-4306                  |
Table 2 (on next page)

Function enrichment analysis for the genes in ceRNA network
### Table 2: Function enrichment analysis for the genes in ceRNA network

| Category          | Term                                | P-value   | Genes                                                                 |
|-------------------|-------------------------------------|-----------|-----------------------------------------------------------------------|
| **Biology process** | GO:0006793–phosphorus metabolic process | 0.00122   | STK38, SLC20A1, ERBB3, NUAK1, MKNK1, ABI1, PIP5K1A, TRIB1, MAP3K3, SRPK2, MINPP1, ADAM10, STK24, MSH2, PRKCI, PKN2, PTPN12, GAK, MAP4K4, MTMR11, MAPK6, GSK3B, Dyrk1A, PTPN1, MAP3K14, ERC1, IKKβ, DUSP7 |
|                   | GO:0006796–phosphate metabolic process | 0.00122   | STK38, SLC20A1, ERBB3, NUAK1, MKNK1, ABI1, PIP5K1A, TRIB1, MAP3K3, SRPK2, MINPP1, ADAM10, STK24, MSH2, PRKCI, PKN2, PTPN12, GAK, MAP4K4, MTMR11, MAPK6, GSK3B, Dyrk1A, PTPN1, MAP3K14, ERC1, IKKβ, DUSP7 |
|                   | GO:0006468–protein amino acid phosphorylation | 0.00498   | SRPK2, ADAM10, STK38, STK24, NUAK1, ERBB3, PRKCI, PKN2, MKNK1, ABI1, TRIB1, GAK, MAP4K4, MAPK6, MAP3K3, GSK3B, Dyrk1A, IKKβ, ERC1, MAP3K14 |
|                   | GO:0016310–phosphorylation          | 0.00801   | SRPK2, ADAM10, STK38, STK24, NUAK1, ERBB3, MSH2, STK24, NUAK1, PRKCI, PKN2, MKNK1, ABI1, PIP5K1A, TRIB1, GAK, MAP4K4, MAPK6, MAP3K3, GSK3B, Dyrk1A, IKKβ, ERC1, MAP3K14 |
|                   | GO:0008104–protein localization     | 0.01176   | STON2, SEC23A, XPO1, AP1M1, NUP160, PRKCI, CENPF, TMSB10, TRAM2, TAP2, GSK3B, NUP210, TAP1, PIKFYVE, SNX21, RAB23, SCG5, SUPT7L, SAR1B, RAB10, ERC1, KPN2, KPNB1 |
|                   | GO:0043407–negative regulation of MAP kinase activity | 0.00991   | STK38, PTPN1, SPRY4, DUSP7                                               |
|                   | GO:0042981–regulation of apoptosis   | 0.0165    | DLC1, DPF2, IER3, ING3, SYVN1, ERBB3, MSH2, KLF10, PRKCI, AKAP13, CD70, PAWR, SOD2, TNFRSF10A, BAG4, TIAM1, GSK3B, GLO1, APBB2, IKKβ, MYC |
|                   | GO:0043067–regulation of programmed cell death | 0.0181    | DLC1, DPF2, IER3, ING3, SYVN1, ERBB3, MSH2, KLF10, PRKCI, AKAP13, CD70, PAWR, SOD2, TNFRSF10A, BAG4, TIAM1, GSK3B, GLO1, APBB2, IKKβ, MYC |
|                   | GO:0015031–protein transport        | 0.0188    | STON2, SEC23A, XPO1, AP1M1, NUP160, PRKCI, CENPF, TRAM2, TAP2, GSK3B, NUP210, TAP1, SNX21, RAB23, SCG5, SAR1B, RAB10, ERC1, KPN2, KPNB1 |
|                   | GO:0010941–regulation of cell death  | 0.0188    | DLC1, DPF2, IER3, ING3, SYVN1, ERBB3, MSH2, KLF10, PRKCI, AKAP13, CD70, PAWR, SOD2, |
| GO:0045184—establishment of protein localization | 0.0204 | TNFRSF10A, BAG4, TIAM1, GSK3B, GLO1, APBB2, IKBKB, MYC |
| GO:0008219—cell death | 0.0211 | FUS, DPF2, DLC1, IER3, MICB, ERBB3, MSH2, AKAP13, RNF216, PAWR, ITPR1, SOD2, TNFRSF10A, BAG4, UNC5B, TIAM1, SIAH1, MYC, SPAST |
| GO:0010033—response to organic substance | 0.0217 | ADAM10, KAT2B, ERBB3, MSH2, KLF10, PRKCI, CALCOCO2, APPL1, TRIB1, B2M, HDAC4, PRKAR2A, SDC1, HDAC2, ADM, TAP2, CTSC, PTPN1, MYC |
| GO:0016265—death | 0.0225 | FUS, DPF2, DLC1, IER3, MICB, ERBB3, MSH2, AKAP13, RNF216, PAWR, ITPR1, SOD2, TNFRSF10A, BAG4, UNC5B, TIAM1, SIAH1, MYC, SPAST |
| GO:0044265—cellular macromolecule catabolic process | 0.0228 | ADAM10, SYVN1, USP1, RNH1, UBE2V2, RNF216, MYLIP, UBE2Q2, ZFP36L2, FBXW7, GMCL1, PSMD3, ZMPSTE24, SIAH1, PCYOX1, USP33, MYC, FBXO11, USP31 |
| GO:0007049—cell cycle | 0.0405 | E2F3, KAT2B, MSH2, PAPD7, CENPF, APPL1, GAK, SASS6, MAPK6, GSK3B, PRDM5, PSMD3, ZNF318, HBP1, SIAH1, APBB2, KPNA2, MYC, SPAST |
| GO:0009057—macromolecule catabolic process | 0.0427 | ADAM10, SYVN1, USP1, RNH1, UBE2V2, RNF216, MYLIP, UBE2Q2, ZFP36L2, FBXW7, GMCL1, PSMD3, ZMPSTE24, SIAH1, PCYOX1, USP33, MYC, FBXO11, USP31 |
| KEGG pathway |  |  |
| hsa05210:Colorectal cancer | 0.0377 | MSH2, GSK3B, APPL1, MYC, FZD7 |
| hsa04210:Apoptosis | 0.0421 | TNFRSF10A, PRKAR2A, EXOG, IKBKB, MAP3K14 |
| hsa00562:Inositol phosphate metabolism | 0.0477 | MINPP1, TPI1, PIKFYVE, PIPI5K1A |
| hsa05169:Epstein-Barr virus infection | 0.00175 | POLR3F, XPO1, HDAC4, GTF2E2, HDAC2, GSK3B, PSMD3, MAP3K14, IKBKB, MYC |
| hsa05166:HTLV-I infection | 0.0122 | WNT5A, XPO1, E2F3, KAT2B, MAP3K3, GSK3B, MAP3K14, IKBKB, MYC, FZD7 |
| hsa05205:Proteoglycans in cancer | 0.0267 | WNT5A, EIF4B, SDC1, TIAM1, ERBB3, MYC, FZD7, ITPR1 |
Table 3 (on next page)

Prognosis related IncRNAs, miRNAs and mRNAs in ceRNA network
### Table 3 Prognosis related lncRNAs, miRNAs and mRNAs in ceRNA network

| RNA         | exp(coef) | p   | RNA         | exp(coef) | p   |
|-------------|-----------|-----|-------------|-----------|-----|
| miRNA       | hsa-miR-16| 0.506| miRNA       | hsa-miR-10| 0.678|
|             | hsa-miR-10| 0.653|             | hsa-miR-16| 0.523|
| lncRNA      | RUSC1-AS1 | 1.09 |             | hsa-miR-7 | 1.75 |
| mRNA        | ADAM10    | 2.01 | lncRNA      | LINC00324 | 1.38 |
|             | AHCYL2    | 0.739|             | AFF4      | 2.43 |
|             | CNPY3     | 0.602|             | CELSR2    | 0.576|
|             | DLC1      | 1.56 |             | ERBB3     | 0.392|
|             | E2F3      | 0.661|             | LRRC8E    | 0.577|
|             | FUS       | 0.531|             | MAP4K4    | 2.12 |
|             | HPN       | 0.878|             | MICAL2    | 1.76 |
|             | ITPR1     | 2.19 |             | NXPH4     | 0.702|
|             | LRRRC40   | 2.56 |             | PCYOX1    | 0.484|
|             | MICAL2    | 1.49 |             | PRDM5     | 1.64 |
|             | NXPH4     | 0.722|             | PTBP1     | 8.8  |
|             | PAWR      | 1.57 |             | PTPN1     | 3.26 |
|             | PRPSAP2   | 0.563|             | PYGO2     | 0.252|
|             | PTPN12    | 2.19 |             | RRM2      | 2.32 |
|             | PUS1      | 0.668|             | SCG5      | 1.84 |
|             | PYGO2     | 0.334|             | SDC1      | 0.459|
|             | RAB10     | 1.59 |             | SLC39A14  | 1.85 |
|             | SAR1B     | 1.62 |             | SLC44A1   | 0.445|
|             | SCG5      | 1.4  |             | SPRY4     | 2.28 |
|             | SLC39A14  | 1.78 |             | ST3GAL2   | 2.34 |
|             | SNX21     | 0.502|             | THEM4     | 0.278|
|             | SOD2      | 0.736|             | ZFP1      | 3.35 |
|             | SPRY4     | 1.98 |             |           | 0.023|
|             | ST3GAL2   | 1.87 |             |           | 0.0345|
|             | TAP2      | 0.68 |             |           | 0.0425|
|             | TCFL5     | 0.613|             |           | 0.033|
|             | TRAPPC10  | 0.324|             |           | 0.037|
|             | TSC22D2   | 1.36 |             |           | 0.0295|
|             | TSENI5    | 1.58 |             |           | 0.0415|
Table 4 (on next page)

Independent prognostic factors for LSCC by multivariate Cox regression
Table 4 Independent prognostic factors for LSCC by multivariate Cox regression

| ID          | P-value | HR     | 95% CI Lower | 95% CI Upper | ID         | P-value | HR     | 95% CI Lower | 95% CI Upper |
|-------------|---------|--------|--------------|--------------|------------|---------|--------|--------------|--------------|
| TRAPP10     | 0.0106  | 0.0941 | 0.01535      | 0.5768       | SLC4A1     | 0.055   | 0.1719 | 0.0496       | 0.5958       |
| Alcohol     | 0.0252  | 0.0391 | 0.00229      | 0.668        | THEM4      | 0.0101  | 0.2215 | 0.07023      | 0.6988       |
| SLC39A14    | 0.0289  | 9.37   | 1.26         | 69.8         | RRM2       | 0.0151  | 34.8562| 1.99009      | 610.503      |
| SOD2        | 0.0456  | 2.748  | 1.01992      | 7.4038       | Age        | 0.0875  | 0.06502| 0.00583      | 1.494        |
| SCG5        | 0.0515  | 8.6    | 0.986        | 75.1         | T          | 0.1882  | 0.09721| 0.00302      | 3.129        |
| Grade       | 0.0536  | 0.0053 | 0.000025     | 1.09         | Stage      | 0.1883  | 14.2194| 0.27247      | 742.056      |
| MICAL2      | 0.0872  | 0.141  | 0.0149       | 1.33         | ZFP1       | 0.2108  | 3.5709 | 0.48649      | 26.2105      |
| RUSC1-AS1   | 0.0894  | 1.1434 | 0.97957      | 1.3347       | LINC00324  | 0.2134  | 1.5429 | 0.77921      | 3.0549       |
| Gender      | 0.1057  | 0.002  | 1.086-06     | 3.72         | PCYOX1     | 0.2206  | 0.16586| 0.009362     | 2.939        |
| CNPY3       | 0.1069  | 0.43   | 0.444        | 4170         | CELSR2     | 0.2233  | 0.12475| 0.004376     | 3.556        |
| PUS1        | 0.1534  | 0.0927 | 0.00354      | 2.43         | NXP4       | 0.2429  | 0.58688 | 0.239935     | 1.435        |
| HPN         | 0.1538  | 0.398  | 0.112        | 1.41         | Gender     | 0.2507  | 0.11327| 0.002754     | 4.658        |
| hsa-mir-16-2| 0.1626  | 0.5574 | 0.24539      | 1.266        | PTPN1      | 0.2567  | 17.1232| 0.126445     | 2318.83      |
| Age         | 0.1641  | 0.0323 | 0.000256     | 4.07         | AFF4       | 0.3093  | 17.52882| 0.070156     | 4379.665     |
| PTPN12      | 0.204   | 0.427  | 0.13         | 14000        | PYGO2      | 0.3218  | 0.06118| 0.000243     | 15.379       |
| ADAM10      | 0.245   | 0.136  | 0.00472      | 3.93         | SLC39A14   | 0.3303  | 1.5919 | 0.62436      | 4.0857       |
| LRRC40      | 0.2467  | 0.0333 | 0.000106     | 10.5         | SPRY4      | 0.3574  | 1.6716 | 0.55973      | 4.9922       |
| Stage       | 0.2589  | 185    | 0.0215       | 158000       | Grade      | 0.4953  | 0.24206| 0.004104     | 14.277       |
| RAB10       | 0.2779  | 0.0066 | 7.71E-07     | 57.1         | MAP4K4     | 0.4973  | 0.21996| 0.002757     | 17.436       |
| T           | 0.2814  | 0.0214 | 0.000020     | 23.3         | hsa-mir-16-2| 0.50105 | 0.7001 | 0.24778      | 1.978        |
| TSC22D2     | 0.3088  | 1.7322 | 0.60129      | 4.99         | ERBB3      | 0.6193  | 2.56113| 0.06268      | 104.645      |
| FUS         | 0.3262  | 0.0128 | 2.11E-06     | 77.1         | PTBP1      | 0.629   | 8.46884| 0.00146      | 49149.03     |
| DLC1        | 0.3279  | 0.168  | 0.00472      | 5.99         | tobacco    | 0.6901  | 0.59073| 0.04442      | 7.856        |
| PRPSAP2     | 0.328   | 0.0598 | 0.000212     | 16.9         | SCG5       | 0.7040  | 1.2036 | 0.46263      | 3.1314       |
| TSEN15      | 0.3295  | 2.1853 | 0.45397      | 10.5197      | PRDM5      | 0.7108  | 1.45392| 0.20106      | 10.514       |
| ST3GAL2     | 0.3643  | 1.4916 | 0.62882      | 3.5381       | N          | 0.7903  | 0.69963| 0.05030      | 9.731        |
| PYGO2       | 0.4608  | 6.86   | 0.0412       | 1140         | LRRC8E     | 0.7914  | 0.70897| 0.05545      | 9.065        |
| E2F3        | 0.4736  | 2.69   | 0.18         | 40.2         | MICAL2     | 0.8244  | 1.2676 | 0.15616      | 10.289       |
| N           | 0.5037  | 0.203  | 0.00188      | 21.8         | SDC1       | 0.8476  | 1.1355 | 0.31079      | 4.1489       |
| SPRY4       | 0.5551  | 1.3026 | 0.54132      | 3.1346       | hsa-mir-7-2| 0.8556  | 1.1305| 0.30175      | 4.2351       |
| TAP2        | 0.574   | 0.7112 | 0.21679      | 2.3332       | hsa-mir-10a| 0.9186  | 0.9692 | 0.53189      | 1.7661       |
| NXP4        | 0.6486  | 0.784  | 0.276        | 2.23         | ST3GAL2    | 0.9385  | 0.9482 | 0.24524      | 3.666        |
| TCFL5       | 0.6954  | 0.7436 | 0.16866      | 3.278        | Alcohol    | 0.9937  | 1.01803| 0.01187      | 87.316       |
| Tobacco     | 0.717   | 1.67   | 0.105        | 26.5         |           |         |       |             |             |
| SNX21       | 0.8253  | 0.8703 | 0.25359      | 2.9871       |           |         |       |             |             |
| Gene     | Value1 | Value2 | Value3 | Value4 |
|----------|--------|--------|--------|--------|
| hsa-mir-10a | 0.8392 | 0.9451 | 0.54739 | 1.6316 |
| PAWR     | 0.842  | 1.7    | 0.00918 | 315    |
| SAR1B    | 0.9106 | 1.25   | 0.0244  | 64.3   |
| ITPR1    | 0.9121 | 1.19   | 0.0539  | 26.3   |
| AHCYL2   | 0.9767 | 1.06   | 0.0236  | 47.5   |
Table 5 (on next page)

Clinical characteristics related IncRNAs, miRNAs and mRNAs in prognostic ceRNA network

Genes with underline were recurrence free survival related; the other genes were overall survival related. Gene in bold was both recurrence free and overall survival related.
| Clinical characteristics          | lncRNA | miRNA | mRNA                      |
|----------------------------------|--------|-------|---------------------------|
| Age(≥60/<60 y)                   | -      | -     | ADAM10, FUS, MICAL2, LRRC8E |
| Gender(Male/Female)              | -      | -     | DLC1, HPN, PTPN12, SNX21, ST3GAL2, LRRC8E, NXPH4, PTPN1, SCG5 |
| Alcohol use(Yes/No)              | -      | -     | CNPY3, PYGO2, SLC39A14, TAP2 |
| Pathologic_M(M0/-)               | -      | -     | ADAM10, CNPY3, E2F3, SPRY4, LRRC8E, MAP4K4, PTBP1, RRM2 |
| Pathologic_N(N0/N1/N2/N3/-)     | RUSC1-AS1 | -     | DLC1, FUS, MICAL2, SOD2, PCYOX1, RRM2, SLC39A14, ZFP1 |
| Pathologic_T(T1/T2/T3/T4/-)     | -      | -     | LRRC40, PRPSAP2, SOD2, TSEN15, CELSR2, PTPN1 |
| Pathologic_stage(I/II/III/IV/-)  | -      | -     | MICAL2, PRPSAP2, SPRY4, CELSR2, MAP4K4, PCYOX1, PTPN1 |
| Grade(G1/G2/G3/G4)              | -      | -     | AHCYL2, DLC1, PCYOX1, PTPN1, RRM2 |
| Tobacco use(Reform/Current/Never)| -      | -     | HPN, RAB10, SAR1B, SOD2, TAP2 |

Genes with underline were recurrence free survival related; the other genes were overall survival related. Gene in bold was both recurrence free and overall survival related.