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A combination of mRNA expression profile and miRNA expression profile identifies detection biomarkers in different tumor stages of laryngeal squamous cell carcinoma

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Abstract. – OBJECTIVE: This study was conducted to investigate microRNA (miRNA) target regulations during the disease progression in laryngeal squamous cell carcinoma (LSCC) and identify biomarkers in different tumor stages.

MATERIALS AND METHODS: The mRNA dataset GSE59102 and miRNA dataset GSE70289 were used in this study. After pretreatment, differentially expressed genes/miRNAs (DEGs/DEMs) in different tumor stages (beginning vs. margin, advanced vs. margin, and beginning vs. advanced) were selected on the basis of their limma package. Then, the enrichment analysis for these DEGs was conducted using ClueGO. Protein-protein interaction (PPI) network analysis was performed on the basis of the BioGRID database. After prediction of target genes of DEMs according to three validated miRNA databases, an integrated miRNA target network and its pathways were drawn using the multiMiR package.

RESULTS: Numerous DEGs were identified in different tumor stages of LSCC (beginning vs. margin, advanced vs. margin, and beginning vs. advanced), and a set of 18 DEMs was identified. Cell cycle was the most significantly enriched pathway of the DEGs. Four hub nodes (MCM2, EGFR, CDK2, and CDK1) were highlighted in the PPI network. In the integrated miRNA target network, 2 miRNAs were predominant: hsa-miR-331-3p (2 predicted targets, E2F1 and TNFRSF10B) and has-miR-375 (1 predicted target, TNNI3). These genes were tied up with cell cycle or apoptosis pathway.

CONCLUSIONS: Several genes and miRNAs might be used as markers for LSCC in different tumor stages (e.g., MCM2, EGFR, CDK1, CDK2, hsa-miR-331-3p, hsa-miR-375). They might function through the involvement of the cell cycle pathway.

Key Words:
Laryngeal squamous cell carcinoma, MicroRNA, Target, Tumor stage, Protein-protein interaction, Pathway.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is an aggressive disease and the second most common head and neck tumor worldwide1. Each year, about 20,000 people in the United States have LSCC2. Although advanced techniques have been developed for LSCC in oncological and surgical treatments, this malignancy still has a low survival rate and poor prognosis[3]. Currently, the tumor, node, metastasis (TNM) staging system is an important method to predict LSCC outcome[4]. However, we need to better understand the molecular mechanisms on LSCC progression, especially during different tumor stages. Reportedly, several homeobox gene family (HOX) genes are increased in LSCC patients, and they are implicated in numerous cellular activities during tumor development (e.g., cell proliferation, migration)[5]. Among the HOX genes, expressions of HOXC8 and HOXD11 are associated with tumor differentiation degree and regional lymph node metastasis, but none relate to tumor stages[5]. A few candidate genes are suspected to be molecular

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therapeutic targets for LSCC in the early stages (e.g., tumor suppressor TSLC1, which functions by regulating the Akt signaling pathway)[6] and CHFR, whose aberrant methylation is strongly correlated with stage I and stage II LSCC[7]. In addition, several genes (e.g., BCL7A, RECQL4) are differentially expressed between the early and late LSCC tumor stages[8]. Hui et al[9] predicted some tumor stage-dependent gene markers for LSCC (e.g., MMPs, PLAU, ADHs). However, the pathogenesis of LSCC in its different stages remains misunderstood. MicroRNAs (miRNAs) are small non-coding RNAs critical for gene expression regulation in various biological processes. Many miRNAs have inhibitory functions during LSCC development. For instance, miR-203 is decreased in LSCC, while its overexpression could inhibit proliferation and induce apoptosis in LSCC[10]. The miR-145 is also down-regulated in LSCC, and its elevated expression inhibits proliferation and migration in LSCC Hep-2 cells[11]. Moreover, SOX2, the expression of which is negatively correlated with miR-145, is thought to be a target of miR-145[11]. However, the role of miRNAs in different stages of LSCC remains obscure. Therefore, we combined the mRNA expression profile GSE59102[12] with the miRNA expression profile GSE70289[13] to extract potential miRNA target interactions in LSCC progression to uncover the underlying regulatory mechanisms and provide novel biomarkers in different tumor stages.

Materials and Methods

Data source of mRNA and miRNA Expression Profilin

The mRNA expression profile GSE5910212 was downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) database, which is based on the platform of the Agilent-014850 Whole Human Genome Microarray (Agilent Technologies, Palo Alto, CA, USA). In the dataset, there were 13 LSCC samples with a surgical safety border (control group), 14 early-stage LSCC samples (beginning group), and 15 advanced LSCC samples (advanced group). The expression matrix that had been pretreated with background correction and normalization was downloaded. The miRNA expression profile GSE7028913 was also downloaded from the GEO database. The dataset contained 4 LSCC samples and 4 pairwise adjacent normal samples. Among them, expression profiling of 4 samples was detected on the platform of the Agilent-031181 microarray, while the remaining 4 were on the Agilent-053955 microarray platform.

Differential Analysis of mRNAs and miRNAs

Differentially expressed genes (DEGs) were identified via pairwise comparison in the 3 groups (beginning vs. margin, advanced vs. margin, advanced vs. beginning) using the Linear Models for Microarray Analysis (limma, http://www.bioconductor.org/packages/release/bioc/html/limma.html) package implemented in the R language of Bioconductor. For significance analysis, the p-value was adjusted as the false discovery rate (FDR) by the Benjamini-Hochberg method[14]. Genes that met the criteria of |log2 fold change (FC)| $\geq$1 and FDR < 0.05 were considered DEGs. For a differentially expressed miRNA (DEM), it had to meet the following 2 criteria: |log 2 FC| $\geq$1 and be differentially expressed in at least 2 of the 4 pairwise tumor-control samples.

Construction of PPI Network

The Biological General Repository for Interaction Datasets (BioGRID: http://thebiogrid.org) is an important public dataset for protein interaction analysis[15]. By mapping the identified DEGs into the BioGRID database (version, 3.4.141), the PPI network of these DEGs was established under the condition of a combined score (which reflects the connection degree of a DEG) > 4 and was then visualized by Cytoscape (http://cytoscape.org/) software.

Biological Function and Pathway Enrichment Analysis

The ClueGO, a Cytoscape plugin, was used to decipher gene ontology (GO, http://www.geneontology.org/) and pathway enrichment analysis, based on the right-sided hypergeometric test[16]. The cut-off value for significant pathway selection was FDR < 0.05. Following pathway enrichment analysis, the pathways were delineated using the pathview package[17], a novel tool for pathway integration and visualization, to further explore the upstream or downstream regulation of the DEGs.

Prediction of Target Genes of miRNAs

We used the multiMiR (http://multimir.ucdenver.edu/) package in R to predict the target genes of DEMs[18]. The multiMiR contains miRNA-target regulation information in 14 miRNA databases: 3

validated miRNA-target databases, as miRecords (version 4, http://mirecords.umn.edu/miRecords/prediction_query.php)[19], miRTarBase (version 4.5, http://mirtarbase.mbc.nctu.edu.tw/)[20], and TarBase[21]; 8 predicted miRNA target interaction databases or algorithms (i.e., DIANA-microT-CDS (version 5)[22], E1MMo (version 5), MicroCosm (version 5, http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/)[23], miRanda (http://www.microrna.org), miRDB (version 4, http://mirdb.org)[24], PicTar (version 2, http://pictar.mdc-berlin.de/)[25], PITA and Target Scan algorithms[26]; and 3 miRNA disease/drug association databases such as miR2Disease (www.mir2disease.org)[27], Pharmaco-miR[28], and PhenomiR (http://mips.helmholtz-muenchen.de/phenomir/)[29].

In the current study, the miRNA was extracted to predict the targets of the identified DEMs. Thereafter, the regulation network was delineated using Cytoscape software.

Results

DEGs and DEMs Between Different Comparisons

Based on the aforementioned criteria, a total of 2652 DEGs (1211 were up-regulated, and 1441 were down-regulated) beginning and margin samples were selected, and 2751 (1364 were up-regulated, and 1387 were down-regulated) were screened out between advanced and margin samples. In addition, 19 DEGs were identified in the comparison of advanced and beginning groups. A set of 18 DEMs was identified in LSCC samples, including 6 up-regulated DEMs and 12 down-regulated DEMs.

Enrichment Result of the DEGs

As a result, the DEGs were significantly enriched in pathways such as ECM-receptor interaction, cell cycle, cytokine-cytokine receptor interaction, DNA replication, tyrosine metabolism, retinol metabolism, and drug metabolism (Figure 1). Genes in a specific pathway of the cell cycle are shown in Figure 2.

PPI Network of DEGs

Integrating protein information in the BioGRID database with the DEGs, a PPI network of DEGs, was established under the condition of combined score > 4. As shown in Figure 3, some proteins obeyed the scale-free network (SFN) distribution. The nodes with multiple degrees had an important connection with others and were defined as hub nodes. Top 10 hub nodes in the PPI network were MCM2 (degree = 74), EGFR (degree = 59), CDK2 (degree = 53), BRCA1 (degree = 42), CDK1 (degree = 35), AURKA (degree = 28), FLNA (degree = 26), MEOKX2 (degree = 24), AURKB (degree = 24), and TUBA1A (degree = 23). Almost all of them were up-regulated, except MEOX2, which was down-regulated (Figure 3).

Integrated miRNA Target Network and Involved Pathways

According to miRNA target interaction information in the 3 validated databases, targets of the DEMs were predicted. Moreover, the potential function of the DEMs was predicted based on their targeted pathways. Thus, a DEM-DEM pathway network was constructed. In this integrated network, down-regulated hsa-miR-375 and hsa-miR-331-3p were 2 central DEMs with numerous up-regulated targets. Of importance, hsa-miR-331-3p was predicted to target genes of E2F1 and TNFRSF10B, which were involved in multiple cellular pathways such as "cell cycle" (E2F1),"cytokine-cytokine receptor interaction" (TNFRSF10B), "p53 signaling pathway" (TNFRSF10B), and "apoptosis" (TNFRSF10B). Hsa-miR-375 targeted the TNNI3 gene, which was enriched in pathways such as "hsa04260", "hsa05414" and "hsa05410". In addition, LAMC2

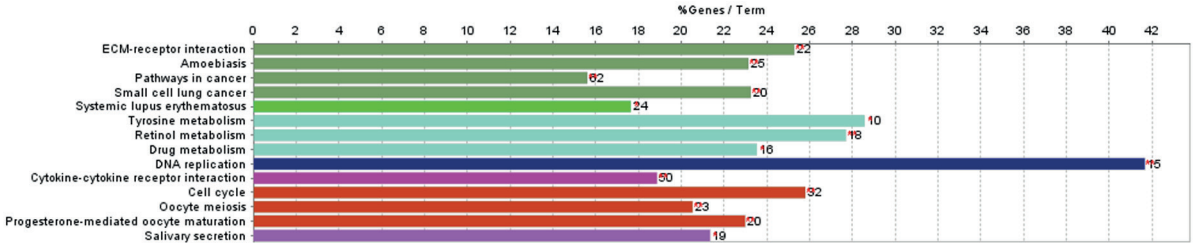

Figure 1. Significantly enriched pathways by differentially expressed genes.

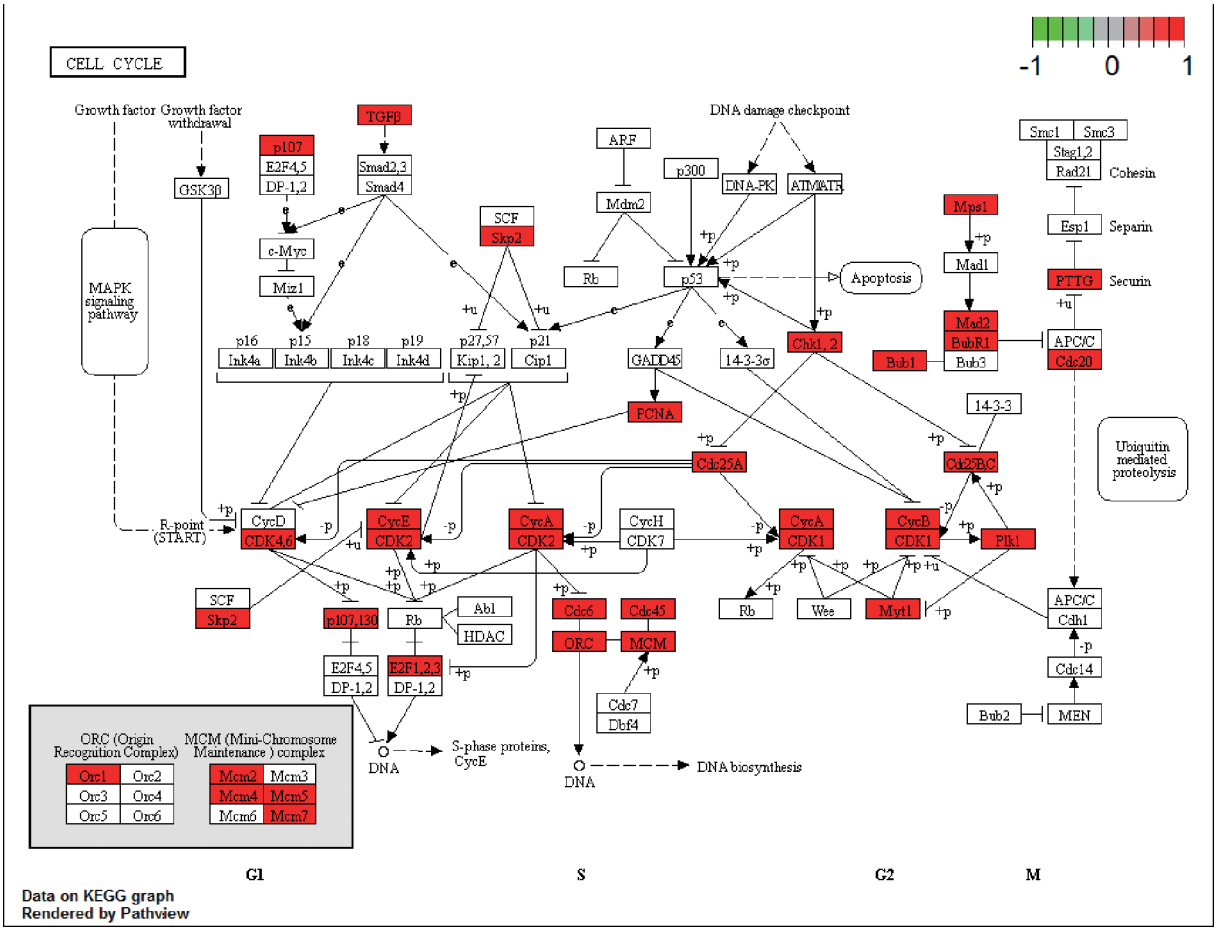

Figure 2. Genes enriched in the cell cycle pathway. Red represents up-regulated genes.

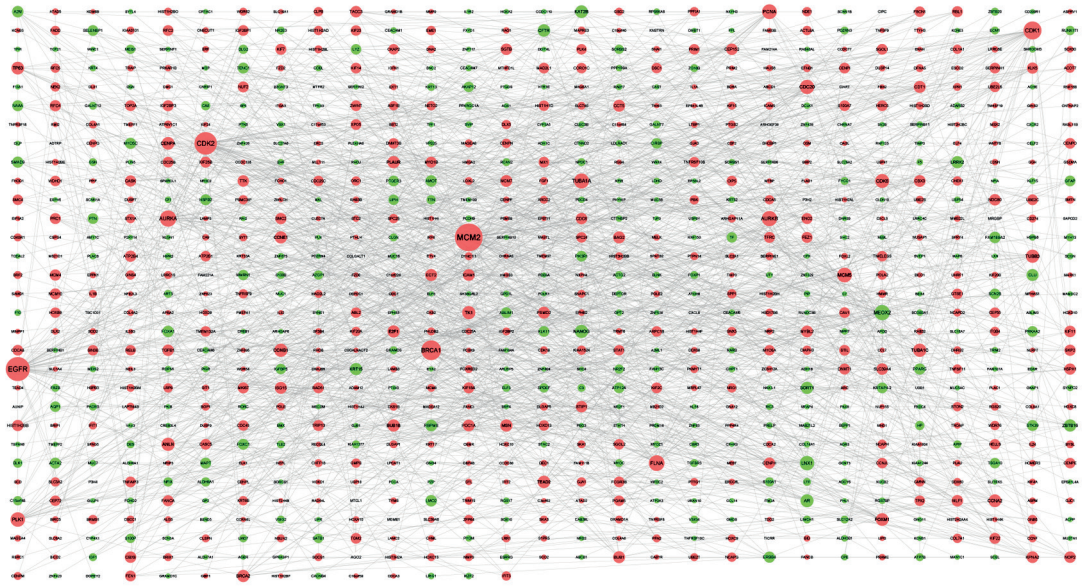

Figure 3. Protein-protein interaction network of the differentially expressed genes. Red denotes up-regulated genes and green denotes down-regulated genes.

was predicted as the target of hsa-miR-199b-5p and was enriched in 6 cancer-related signaling pathways, while CAV1 was targeted by has-miR-199b-5p and associated with 4 signaling pathways such as "focal adhesion" (hsa04510), "bacterial invasion of epithelial cells" (hsa05100), "endocytosis" (hsa04144), and "viral myocarditis" (hsa05416) (Figure 4).

Discussion

In this study, through the analysis of mRNA and miRNA expression profiles, we identified several crucial genes and miRNAs for the detection of LSCC in different stages, which were mainly associated with cell cycle, ECM-receptor interaction, apoptosis, and p53 signaling pathway. MCM2, EGFR, CDK1, and CDK2 were the 4 predominant nodes in the PPI network. In addition, central miRNAs were identified, such as hsa-miR-331-3p (predicted targets: E2F1, TNFRSF10B) and hsa-miR-375 (predicted target: TNNI3). Mini-chromosome maintenance proteins (MCMs) are highly conserved genes involved in cell division and DNA synthesis[30]. The typical cell cycle entry marker MCM2 is expressed in LSCC, along with increased Ki67[31], and the aberrant expression of MCM2 is implicated in the occurrence and progression of LSCC[32]. Cyclin-dependent kinases (CDKs) are important proteins related to the cell cycle. CDK1 has an important role in the transition from the G1 to S and G2 to M phases, while CDK2 is essential during the G1 to S phase. Overexpressed CDK1 at both mRNA and protein levels is detected in LSCC at an early stage

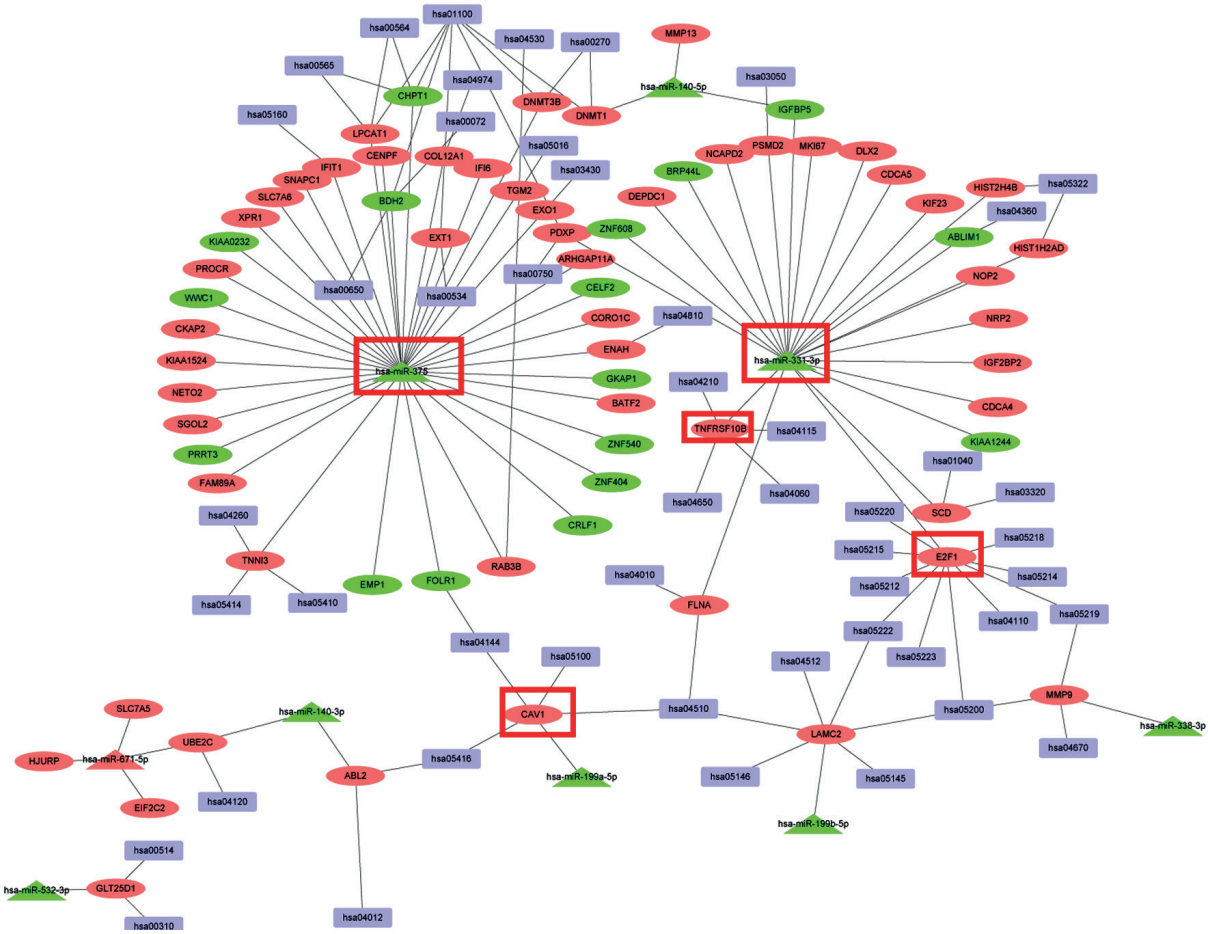

Figure 4. Integrated DEM-DEG-pathway network. The triangle represents DEMs; the oval represents DEGs and the rectangle represents pathway IDs. Red stands for up-regulated DEMs/DEGs, and green stands for down-regulated DEMs/DEGs. DEM: differentially expressed miRNAs; DEGs: differentially expressed genes.

of carcinogenesis[33]. Increased gene expression of CDK2 is also observed in the early stages of LSCC[34]. Notably, a recent study demonstrated that MCM genes (e.g., MCM2, MCM3, MCM4) and CDK genes (e.g., CDK1, CDK2, CDK4) are dysregulated in tumorigenesis and regional lymph node metastasis in LSCC[35]. Of more importance, their expressions can be validated through qRT-PCR[35]. Similar to this finding, in our study, MCM2, CDK1, and CDK2 were critical DEGs in LSCC at different tumor stages, and all were enriched in the cell cycle pathway. These results collectively suggest that the expression of MCM2, CDK1, and CDK2 are associated with different LSCC tumor stages and could be used as therapeutic biomarkers for the disease. The transmembrane glycoprotein encoded by epidermal growth factor receptor (EGFR) is an important factor in the activation of signaling cascades in several cellular responses. Reportedly, elevated expression of LSCC is observed in most LSCC cases[36]. Also, a combination of the tumor suppressor p14ARF and the antisense complementary DNA of EGFR dramatically improves its therapeutic effectiveness in LSCCHep-2 cells[37]. Notably, EGFR has been implicated in overall survival in early-stage LSCC[38]. In many cancer types, EGFR inhibitors suppress tumor growth by ending cell cycle arrest[39,40]. In our study, EGFR was another predominant gene in the PPI network and was significantly enriched in the cell cycle pathway, suggesting that the EGFR-mediated cell cycle pathway might be a pivotal early detection biomarker for LSCC. Several miRNAs (e.g., miR-331-3p) are reportedly down-regulated in LSCC compared with those in healthy individuals[41]. However, the function of this miRNA in LSCC remains misunderstood. In other cancers, miR-331-3p inhibits tumor development. In prostate cancer (PC), for instance, miR-331-3p is down-regulated, but its overexpression through the transfection of PC cell lines could decrease ERBB2 expression and block both the Akt signaling and androgen receptor signaling pathways[42], the activation of which could decrease androgen-independent PC progression[42]. In colorectal cancer (CRC), miR-331-3p is also decreased compared with that in healthy controls; as a result, it is thought to be of potential use in the early detection of CRC[43]. These observations indicate that miR-331-3p might also serve as a suppressor during LSCC progression. The transcription factor E2F Transcription Factor 1 (E2F1) is an essential regulator of the cell cycle during tumor growth. In gastric cancer, E2F1 impairs TGFβ-dependent cell-cycle arrest[44] and miR-331-3p is reported to directly target E2F1 and induce tumor cell cycle arrest[45]. Although the target regulation relationship between miR-331-3p and E2F1 has not yet been reported, down-regulated miR-331-3p was predicted to target up-regulated E2F1 in our study. Moreover, E2F1 was highly associated with the cell cycle pathway. Based on these findings, miR-331-3p might also play an inhibitory role in LSCC progression at different tumor stages through targeting expression of E2F1. In addition, the TNF receptor superfamily member 10b (TNFRSF10B) is a member of the TNF receptor superfamily that mediates tumor apoptosis. TNFRSF10B is identified as a therapeutic target for oral squamous cell carcinoma (OSCC)[46]. Additionally, TNFRSF10B was confirmed as the target of miR-133a/miR-133b in human abdominal aortic aneurysms[47]. However, this targeting relationship has not yet been reported in LSCC. Therefore, this gene might be a novel target of LSCC and should be a focus of future studies.

MiR-375 is another crucial miRNA down-regulated in LSCC. This miRNA may act as a suppressor of the progression of LSCC through the inhibition of IGF1R expression[48]. Due to the tight correlation of cardiac diseases with tumor cancers, several genes altered in cardiac diseases are also dysregulated in some cancer types. For instance, homeodomain only protein X (HOPX) is a key regulator of cardiac hypertrophy[49], and its expression is stage-dependent and does not occur in head and neck squamous cell carcinoma[50]. Mutation of troponin I3, cardiac type (TNNI3), is a causative factor for hypertrophic cardiomyopathy[51]. In our study, TNNI3 was predicted as a target of hsa-miR-375 in different tumor stages of LSCC, suggesting that this targeting regulation might play important roles in the different stages of LSCC.

Conclusions

Several crucial genes and miRNAs were identified as early detection markers for LSCC, such as the genes MCM2, EGFR, CDK1, and CDK2, and the miRNAs hsa-miR-331-3p and hsa-miR-375. TNFRSF10B and TNNI3 might be novel targets of hsa-miR-331-3p and hsa-miR-375, respectively. However, more experiments are needed to validate their expressions and target regulatory relationships.

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Conflict of Interest
The Authors declare that they have no conflict of interest.

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