# Identification of miR‐145‐5p-centered competing endogenous RNA network in laryngeal squamous cell carcinoma

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## Abbreviations

LSCC- laryngeal squamous cell carcinoma

HNSCC- head and neck squamous cell carcinoma

ceRNA- competing endogenous RNA

miRNA- microRNA

lncRNA- long non-coding RNA

circRNA- circular RNA

GO- Gene Ontology

KEGG- Kyoto Encyclopedia of Genes and Genomes

**Keywords:** laryngeal squamous cell carcinoma, apoptosis, metabolic reprograming, miR-145, competing endogenous RNA

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**Abstract**

This study intended to investigate the transcriptional regulatory role of miR‐145‐5p in laryngeal squamous cell carcinoma (LSCC). LSCC cell line TU-177 was transfected with miR-145-5p mimics, generating miR-145-5p-overexpression LSCC cells. Whole transcriptome microarrays were used to investigate the differentially expressed lncRNAs, circRNAs, mRNAs and miRNAs. The target genes of miRNAs were predicted and performed functional enrichment analysis. Additionally, the circRNAs, lncRNAs, and mRNAs that interacted with miRNAs were predicted, and then the competing endogenous RNAs (ceRNAs) were predicted. Microarray analysis identified 26 miRNAs, 248 mRNAs, 1118 lncRNAs, and 382 circRNAs differentially expressed in miR-145-5p overexpressed LSCC cells. Overall, 675 target genes were identified for the differentially expressed miRNAs, which involved in cell adhesion associated GO terms, and MAPK and FoxO signaling pathways. The up-regulated mRNAs involved in the pathway of ABC transporters, while the down-regulated mRNAs involved in pathway of olfactory transduction. Moreover, 149 ceRNAs were predicted, which were associated with apoptosis, Wnt signaling pathway, and metabolic pathway. Furthermore, qPCR results confirmed that miR-145-5p affects expression of lncRNAs, miRNAs, mRNAs, and circRNAs in LSCC cells. Collectively, miR‐145‐5p may be inhibits LSCC progression via ceRNA-mediated signaling pathways, such as *WNT2B*-miR-145-5p-NONHSAT127539.2, *CASP10*-miR-145-5p-NONHSAT127539.2, *CASP10*-miR-145-5p-circ_0003519, and *TPO*-miR-145-5p-circ_0003519.

**Statement of significance of the study**

LSCC is one of the most frequent forms of head and neck squamous cell carcinoma and is characterized by aggressive local invasion and metastasis. Despite recent advances in diagnosis and treatment, LSCC remains a great challenge in clinical practice, partially because of the insufficient mechanistic knowledge of LSCC tumorigenesis. The non-coding miRNA miR-145-5p has been proved a tumor suppressor in human cancers, and our previous work demonstrated that downregulation of miR-145-5p was associated with poor prognosis of LSCC. In this study, we constructed LSCC cell model with overexpressed miR-145-5p, and then performed microarray analysis to identify coding genes and non-coding genes (lncRNA, circRNA, and miRNA) responsive to miR-145-5p overexpression. Bioinformatics analysis suggested that differentially expressed mRNAs/miRNAs were involved in signaling pathways of cell adhesion, migration, pluripotency regulation, which explains miR-145-5p functions in LSCC cells. Moreover, we constructed the lncRNA-miRNA-mRNA and circRNA-miRNA-mRNA regulatory networks. Overall, this study identified miR-145-5p regulatory network in LSCC cells in the view of competing endogenous RNA, these findings

provide a molecular mechanism for miR-145-5p suppression of malignant progression in cancer.



## 1 Introduction

Head and neck squamous cell carcinoma is one of the most common malignancy worldwide, out of which laryngeal squamous cell carcinoma (LSCC) has the second highest incidence rate (incidence of LSCC is 2.8/100000 - 5.3/100000)[1, 2], besides it is especially prevalent in the northern areas of China [1, 3]. Approximately 13,430 new cases and 3,620 deaths due to LSCC have been estimated in the United States alone [4]. Early-stage LSCC is often curable with surgery or radiotherapy. However, prognosis for the patients with advanced LSCC has not significantly improved during the last 30 years in spite of therapeutic advances in terms of LSCC [5, 6]. Therefore, understanding the molecular mechanisms of LSCC is extremely urgent for its diagnosis, therapy and prognosis.

In the last decades, a group of non-coding, 21–23 nucleotides-long RNAs called microRNAs (miRNAs) gathered a lot of interest as they negatively regulated the target genes expression at the post-transcription level [7, 8]. In recent years, the expression patterns and biological functions of miRNAs in LSCC have been investigated [9, 10]. The miR-145-5p is an important tumor suppressor miRNAs, and is down-regulated in multiple human cancers [11-13]. Importantly, miR-145 has also been reported to be lowly expressed in LSCC, and overexpression of which inhibits the proliferation and metastasis in LSCC cell line, Hep-2, via inducing cell cycle arrest and apoptosis [14, 15]. Recently, we reported that the miR-145-5p plays a critical role in inhibiting LSCC progression via suppressing *FSCN1* [16]. However, its role in the transcriptional regulatory mechanisms of LSCC cells, especially in the regulatory network of lncRNA, circRNA and mRNA remains unclear.

In this study, to explore the transcriptional regulatory role of miR‐145‐5p in LSCC, miR-145-5p mimics were transfected into LSCC cells TU-177, generating miR-145-5p overexpression cells. Then, based on the whole transcriptome microarrays, the differentially expressed lncRNAs, circRNAs, mRNAs and miRNAs between miR-145-5p mimics and NC cells were identified and the competing endogenous RNAs (ceRNAs) were predicted. These findings may be helpful for understanding the transcriptional regulatory role of miR-145-5p in LSCC.

## 2 Materials and methods

### 2.1 Experimental design

miR-145-5p-overexpressed and control (NC) LSCC cells were generated by miRNA mimics transfection, followed by total RNA extraction and integrity examination. LncRNA microarray (including lncRNA, circRNA and mRNA probes) and miRNA microarray profiling were performed using RNA samples of miR-145-5p-overexpressed and NC cells (3 samples each group). Next, differentially expressed lncRNAs, mRNAs, circRNAs, and miRNAs were screened based on the p value $<0.05$ and fold change $>2$. Furthermore, miRNA target prediction, functional annotation, ceRNA network construction were conducted, and expression of some of differentially expressed genes was validated by qPCR.

### 2.2 Cell culture and transfection

Human LSCC cell line, TU-177 obtained from Bioleaf Biotech (Shanghai, China) was maintained in MEM supplemented with 10% FBS (BI, Cromwell, CT). Human LSCC cell line Hep2 was purchased from the China Center for Type Culture Collection and cultured in DMEM supplemented with 10% FBS. All cells were maintained at 37˚C in a humidified incubator supplemented with 5% $CO_2$. For miRNA transfection, cells ($2.0 \times 10^5$ cells/well) were seeded in 6-well plates. miR-145-5p mimics and NC mimics (GenePharma Co., Ltd, Shanghai, China) were transfected (final concentration: 50 nM) using Lipofectamine® 3000

(Invitrogen, Carlsbad, CA). Cells were harvested after 48 h transfection. Expression of miR-145-5p and its representative target FSCN1 [16] was determined by qPCR.

### 2.3 miRNA microarray assay

Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific, Carlsbad, CA) and then quantified using the NanoDrop ND-2000 (Thermo Fisher Scientific). The integrity of RNA was assessed with Agilent Bioanalyzer 2200 (Agilent Technologies, Little Falls, CA). Sample labeling, microarray hybridization and washing were conducted according to the manufacturer's protocols (Agilent Human miRNA, Release 21.0, 8*60K, Design ID: 070156). Total RNA was dephosphorylated, denatured, and labeled with Cyanine-3-CTP. Following purification, the labeled RNAs were hybridized onto the microarray, followed by washing, and scanning with the Agilent Scanner G2505C (Agilent Technologies).

### 2.4 LncRNA microarray assay

Total RNA was quantified by the NanoDrop ND-2000 (Thermo Fisher Scientific) and the integrity of RNA was evaluated using Agilent Bioanalyzer 2100 (Agilent Technologies). Sample was labeled, microarray hybridized and washed according to the manufacturer's protocols (Agilent Human lncRNA Microrray V6, 4*180K, Design ID: 084410). Total RNAs were transcribed to double strand cDNAs, then synthesized into cRNAs followed by labeling

with Cyanine-3-CTP. The labeled cRNAs were hybridized onto the microarray and then washed. The arrays were scanned by the Agilent Scanner G2505C (Agilent Technologies).

**2.5 Data preprocessing and differential expression analysis**

The array images were analyzed using Feature Extraction 10.7.1.1. Based on the obtained raw data, Genespring 13.1 was used to complete the basic analysis. To begin with, the raw data were normalized with the quantile algorithm. For miRNA microarray, the probes with flags in "Detected" in any 1 condition out of 2 conditions for at least 100% of samples were selected for further analysis. For lncRNA microarray, the probes that at least 1 condition out of 2 conditions had flags in “P” were selected for further analysis. Differentially expressed miRNAs or lncRNAs were then identified using t-test. Moreover, multiple tests were performed for p value correction using Benjamini-Hochberg method [17], and false discovery rate (FDR) was provided. The thresholds set for up- and down-regulated miRNA, mRNA, circRNA or lncRNA were fold change ＞ 2.0 and p value ＜ 0.05.

**2.6 Hierarchical clustering**

To show the distinguishable miRNAs and lncRNAs expression pattern among samples, hierarchical clustering was performed. In detail, the distance between two pairs of samples was calculated to form a distance matrix. Then the nearest two clusters were merged into a new cluster, and the distance between the new cluster and the current cluster was calculated

and merged until there was only one cluster. The direct correlation of samples was calculated using the expression of selected differentially expressed miRNAs and lncRNAs. In general, the same kind of samples could appear in the same cluster after clustering, and the genes clustered in the same cluster may have similar biological functions. The results of hierarchical clustering were shown as heatmaps.

### 2.7 Target genes prediction for differentially expressed miRNAs

The target genes of differential miRNAs were predicted from three databases of microRNAorg, Targetscan, and PITA [18-20]. The intersection of target genes obtained from three databases was considered as the targets of miRNAs.

### 2.8 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses

The predicted miRNA target genes and the differentially expressed mRNAs were subjected to GO and KEGG pathway analysis using the DAVID Bioinformatics Resources 6.8 [21]. GO terms includes molecular function (MF), cellular component (CC), and biological process (BP).The significance of genes involved in each GO term or pathway was calculated by statistical test, and top 20 GO terms and pathways were plotted according to p values.

### 2.9 ceRNA network construction

The circRNAs, lncRNAs, and mRNAs that interacted with miRNAs (miRNA-target interaction pairs) were predicted through both base sequence [22] and expression value. Next, the negatively correlated miRNA-target interactions [23]according to the Pearson correlation coefficients were retained (correlation $\geq 0.7$ and p value $\leq 0.05$ [24]). Then, the ceRNA score and p value were calculated based on number of shared miRNAs and miRNA responsive element (MRE) as previous reported [25]. Subsequently, co-expression between ceRNA pair was calculated by Pearson correlation analysis, and ceRNA pair with correlation $<$ 0.7 or p value $>$0.05 was removed. The ceRNA circRNA-miRNA-mRNA and lncRNA-miRNA-mRNA ceRNA networks were constructed based on ceRNA score rank (number of sharing miRNAs $\geq 3$, ceRNA p value $\leq 0.05$) and co-expression relationship. Moreover, the coding genes involved in the ceRNA network were performed functional analysis.

**2.10 RNA extraction and qPCR analysis**

Total RNA was extracted using TRIzol reagent (Invitrogen) following manufacturer's instructions. For lncRNA, mRNA and mRNA, cDNA was synthesized using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). For miRNA, cDNA was synthesized using the All-in-One™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, MD, USA). qPCR was performed on the ABI Stepone Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The procedures for qPCR were 95°C for 30 sec,

followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The 2-ΔΔct method was used to calculate the relative expression level of target genes. 18S rRNA was served as internal control. The primer information was listed in Supplementary Table S1.

### 2.11 Statistical analysis

Statistical analysis was performed using SPSS v19.0 (SPSS Inc., Chicago, IL, USA). qPCR data were analyzed by *t*-test, a two-tailed $P < 0.05$ was considered significant.

## 3 Results

### 3.1 Screening of differentially expressed genes in LSCC cells overexpressing miR-145-5p

Our previous study found that endogenous level of miR-145-5p was very low in LSCC cell lines TU-177 and Hep2 [16, 26]. To investigate genes regulated by miR-145-5p in LSCC cells, we transfected LSCC cell line TU-177 with miR-145-5p mimics. qPCR results showed that miR-145-5p was overexpressed successfully (Figure 1A), and its representative target FSCN1[16] was downregulated in miR-145-5p mimics transfected cells (Figure 1B). After microarray experiments and data preprocessing, 26 differentially expressed miRNAs including 14 up- and 12 down-regulated ones were identified in cancer cells transfected with miR-145-5p mimics when compared to those transfected with control under the thresholds of fold change $>2.0$ and p value $< 0.05$. The results are shown in the volcano plot (Figure

1C). Additionally, 248 differentially expressed mRNAs (53 up- and 195 down-regulated), 1118 differential lncRNAs (203 up- and 915 down-regulated), and 382 differentially expressed circRNAs (46 up- and 336 down-regulated) were obtained between miR-145-5p mimics and control groups (Figure 1D). These genes are shown in Supplementary Table S2. Hierarchical clustering analysis of these RNA showed that they could distinguish the control and miR-145-5p mimics groups obviously (Figure 1E and 1F).

Moreover, expression level of top 5 upregulated and downregulated miRNAs were validated by qPCR in LSCC cell lines TU-177 and Hep2. Results showed that miR-145-5p overexpression upregulated miR-150-3p, miR-2116-3p, miR-1539, miR-550a-5p, and miR-663a, while downregulating miR-8485, miR-3151-3p, miR-4701-5p, miR-4716-3p, and miR-550a-3p (Figure 1G and 1H), which further confirmed the microarray data. To better understand the potential regulatory mechanisms of miR-145-5p on these differentially expressed genes, we predicted miRNA responsive element (MRE) of differentially expressed mRNAs, lncRNAs, and circRNAs. As shown in Figure 1I, 80 mRNAs (66 downregulation and 14 upregulation), 177 lncRNAs (144 downregulation and 33 upregulation), and 102 circRNAs (88 downregulation and 14 upregulation) contain miR-145-5p binding sites. Therefore, these data indicated that miR-145-5p regulates expression of most of the differentially expressed genes indirectly, rather than direct binding to these genes.

### 3.2 Target genes prediction and functional annotation for differentially expressed miRNAs

Using the microRNA.org, PITA and TargetScan databases, a total of 23546, 2446 and 52879 targets were predicted for differentially expressed miRNAs respectively. After VENN analysis, 675 overlapped target genes were identified (Figure 2A). Functional annotation revealed that the target genes were mainly enriched in GO terms associated with homophilic cell adhesion via plasma membrane adhesion molecules (GO: 0007156), cell adhesion (GO:0007399), cytoplasm (GO:0005737), cell junction (GO:0030054), and calcium ion binding (GO:0005509). Additionally, pathway analysis showed that these target genes were significantly involved in MAPK signaling pathway (hsa04010), Endocytosis (hsa04144), FoxO signaling pathway (hsa04068), and Focal adhesion (hsa04510) (Figure 2B, C).

To address whether miR-145-5p affects these signaling pathways, we validated expression of some key genes involved in MAPK and FOXO signaling pathways by qPCR. Compared to that of negative control group, MAP2K1, MAP3K1 (MAPK pathway), FOXO1, TGFB1, AGAP2, SMAD3, and NLK (FoXO pathway) were downregulated significantly in miR-145-5p overexpressed LSCC cells (Figure 2D, E), indicating that miR-145-5p affected these pathways.

### 3.3 Function annotation of differentially expressed mRNA

GO and KEGG pathway analysis revealed that the up-regulated differentially expressed mRNAs were significantly associated with functions of cellular response to fluid shear stress, and bile acid and bile salt transport (Figure 3A), while the down-regulated mRNAs were related to establishment of nucleus localization, and sensory perception of smell (Figure 3B). In addition, pathways of ABC transporters (hsa02010), and Complement and coagulation cascades (hsa04610) were significantly enriched by the up-regulated mRNAs (Figure 3C), while olfactory transduction (hsa04740) and signaling pathways regulating pluripotency of stem cells (path: hsa04550) were involved by down-regulated mRNAs (Figure 3D).

Next, expression of genes involved in the signaling pathways olfactory transduction and signaling pathways regulating pluripotency of stem cells was validated by qPCR. Compared with that in negative control group, OR51S1, OR6Q1, OR4M1, OR2T4, OR13C3 (Olfactory transduction), WNT2B, FZD9, NEUROG1 (Signaling pathways regulating pluripotency of stem cells) were downregulated significantly in miR-145-5p overexpressed LSCC cells (Figure 3E, F), indicating that miR-145-5p affected these pathways.

### 3.4 Construction of miR-145-5p-centered ceRNA network

Based on the differentially expressed miRNAs, mRNAs, and lncRNAs, 245 miRNA-mRNA and 458 miRNA-lncRNA pairs were identified using a ceRNA construction flow (Figure 4A, supplementary Table S3, S4). Finally, 149 ceRNAs, such as *TBX3*-miR-1539-NONHSAT185919.1, and *TNIK*-miR-150-3p-lnc-KLK7-1:1, were obtained and the ceRNA network is shown in Figure 4B. Additionally, the miR-145-5p-involved ceRNA network was extracted, such as *WNT2B*-miR-145-5p-NONHSAT127539.2, and *CASP10*-miR-145-5p-NONHSAT127539.2 (Figure 4C). NONHSAT127539.2 had the highest degree among lncRNAs involved in the network.

Moreover, on the basis of the differentially expressed miRNAs, mRNAs, and circRNAs, total 245 miRNA-mRNA and 212 miRNA-circRNA pairs were identified (supplementary Table S5), which involved in 137 ceRNAs, such as *CASP10*-miR-550a-5p-circ_ 0040331 and GGT6-miR-550a-5p-circ_0077485, were obtained and the ceRNA network is shown in Figure 4D. Additionally, the miR-145-5p-involved ceRNA network was extracted, such as *CASP10*-miR-145-5p-circ_0003519, and *TPO*-miR-145-5p-circ_0003519 (Figure 4E). circ_0003519 had the highest degree among circRNAs involved in the network. The ceRNA pairs are shown in Supplementary Table S6. Additionally, the base-pair plot of WNT2B/CASP10-miR-145-5p-NONHSAT127539.2 and

CASP10/TPO-miR-145-5p-hsa_circ_0003519 showed that hsa_circ_0003519 contains 5 potential miR-145-5p binding sites (Figure 4F, G).

Moreover, we validated expression of a set of key ceRNAs in miR-145-5p overexpressed TU-177 and Hep2 cells. Results revealed that miR-145-5p overexpression resulted in significant reduction of lncRNAs NONHSAT152788.1, NONHSAT197704.1, and FOXD3-AS1:1, and circRNAs hsa_circ_0083752, hsa_circ_0022951 (Figure 4H, I). Meanwhile, the coding genes TBX3, TBX2, POLDIP3, and KLHDC7B levels decreased significantly in miR-145-5p overexpressed cells (Figure 4H, I). Collectively, these data indicates that miR-145-5p plays an important role in this ceRNA network.

### 3.5 Function annotation of miR-145-5p-centered ceRNA network

In the ceRNAs, the mRNAs involved GO functions and KEGG pathways were analyzed. As shown in Figure 5, these mRNAs were enriched in GO terms related to palate development, activation of JNKK activity, axoneme, and transcription corepressor activity, as well as signaling pathways regulating pluripotency of stem cells, Taurine and hypotaurine metabolism, Tyrosine metabolism, Metabolic pathway, Apoptosis, and Wnt signaling pathway.

## 4 Discussion

miR-145-5p is a well-known tumor suppressor miRNAs which expression is downregulated in various human cancers, including LSCC [14, 27]. To investigate the underlying regulatory mechanisms of miR-145-5p downregulation in LSCC pathogenesis, we established the miR‐145‐5p overexpressed LSCC cell model. Moreover, microarray analysis was performed to identify miR-145-5p regulated lncRNAs, circRNAs, mRNAs, and miRNAs. After data analyses, a total of 26 miRNAs, 248 mRNAs, 1118 lncRNAs, and 382 circRNAs were differential between miR-145-5p mimics and NC groups. We noted both tumor suppressive and oncogenic miRNAs were included in the differentially expressed miRNAs, among which miR-148b-3p, miR-150-3p, miR-2116-3p, miR-3940-5p, miR-4653-3p, miR-4701-5p, miR-4741, miR-4749-3p, miR-4769-3p, and miR-663a are tumor suppressive miRNAs [28-35]. Moreover, miR-1539, miR-1825, miR-3151-3p, miR-550a-3p, miR-550a-5p, and miR-575 are oncogenic miRNAs [36-41]. Therefore, miR-145-5p affected miRNAs are involved in both tumor suppressive and oncogenic pathways. It has been reported a single miRNA can amplify its function by regulating other miRNAs [42]. Based on previous study and our microarray data, we speculated that miR-145-5p may targeting transcription factor or epigenetic genes, regulating expression of other miRNAs indirectly, thus amplifying its function in regulation of cancer.

Changes in the adhesion of tumor cells have been recognized to be important in cancer development [43]. The malignant tumor cells leaving the primary tumor to disseminate to distant organs present marked changes in their interactions with extracellular-matrix components, which leads to the sight that change in cell–matrix and cell–cell adhesion coincides with tumor progression [44]. It has been reported that the function or expression changes of cell-adhesion molecules promote tumor progression by influencing cell signaling and altering the cell adhesion status [45]. The present study showed that the target genes of miRNAs, such as protocadherin alpha 4 (*PCDHA4*) and *PCDHA13* were significantly enriched in cell adhesion associated functions. Interestingly, *PCDHA4* and *PCDHA13* were also downregulated differentially expressed genes. These results suggested that overexpressed miR-145-5p may play functions as a tumor suppressor by targeting cell adhesion associated genes.

KEGG pathway analysis showed that the miRNAs targets involved in MAPK and FoxO signaling pathways. The MAPK/ERK pathway is related to cell proliferation, apoptosis, differentiation, and migration. It also plays a role in the regulation of autophagy establishment in cancer cells [46]. A previous study reported that MAPK/ERK pathway suppression led to the activation of FoxO transcription factor, resulting in apoptosis in pancreatic cancer [47]. Our qPCR data confirmed that miR-145-5p affects expression of genes

of MAPK and FoXO pathways. Therefore, we speculated that miR-145-5p may be implicated in LSCC pathogenesis via the MAPK and FoxO signaling pathways.

Functional analysis showed that the up-regulated mRNAs involved in the pathway of ABC transporters, while the down-regulated mRNAs involved in pathway of Olfactory transduction. Expression of ABC transporters reduces the intracellular accumulation of chemotherapy drugs in cancer treatment [48]. The up-regulated mRNAs after miR-145-5p overexpression were involved in ABC transporters pathway, which suggested that miR-145-5p may have inhibitory effect on chemoresistance in cancer therapy. Olfactory receptors can sense small organic molecules, transduce a survival signal and a migratory stimulus, which render them functional in tumor cells [49]. Ranzani et al. [50] have reported that olfactory receptors may be associated with tumorigenesis, we speculated that miR-145-5p may inhibited the expression of olfactory transduction-associated genes to act as a tumor suppressor in LSCC.

The RNAs that share miRNA recognition elements, recognized by the miRNAs, are identified as ceRNAs. The ceRNAs act as important regulators of gene expression by sponging miRNAs [51-53]. Given the important tumor suppressive roles of miR-145-5p in

various kinds of cancer, including LSCC [16, 54]. We extracted the ceRNAs involved by miR-145-5p to explore the potential regulatory mechanism of miR-145-5p in LSCC. Since NONHSAT127539.2 and circ_0003519 had the highest degrees in their respective networks, we focused on the ceRNAs associated with the two RNAs, such as *CASP10*-miR-145-5p-NONHSAT127539.2, *WNT2B*-miR-145-5p-NONHSAT127539.2, and *TPO*-miR-145-5p-circ_0003519.

In the ceRNA pathways of *CASP10*-miR-145-5p-NONHSAT127539.2, and *WNT2B*-miR-145-5p-NONHSAT127539.2, Caspase 10 (*CASP10*) was enriched in apoptosis pathway and Wnt family member 2B (*WNT2B*) involved in Wnt signaling pathway. Dysfunction of Wnt signaling play a key role in cancer initiation, via influencing the cancer stem cells [56]. *WNT2B* has been found to stimulate the canonical Wnt/β-catenin pathway and affect a variety of tumor progressions [57]. Study has demonstrated that apoptosis is orchestrated by the activating caspases, including CASP10 [58]. Cancer involves the abnormal accumulation of cells resulting from the imbalance of apoptosis and proliferation. NONHSAT127539.2 is a long intergenic RNA (lincRNA) located at chr8:85764690-85792352, function of this lincRNA remains unclear. Nevertheless, these data indicates that miR-145-5p may involve in regulation of cancer stem cell and apoptosis via

ceRNA pathways of *CASP10*-miR-145-5p-NONHSAT127539.2, and *WNT2B*-miR-145-5p-NONHSAT127539.2.

Metabolic reprogramming is commonly found during the cancer development, conferring cancer cells the ability of survival and proliferation [59]. In the present study, metabolism associated pathways were enriched by the genes involved in ceRNA networks. Thrombopoietin (*TPO*) (involved in *TPO*-miR-145-5p-hsa_circ_0003519) was enriched in tyrosine metabolism, and metabolic pathway. Wu et al. [60] have indicated that TPO-induced metabolic reprogramming could drive the liver metastasis of colorectal cancer CD110+ tumor-initiating cells. A recent study reported that metabolic pathways promoted cancer cell growth [61]. hsa_circ_0003519 is a circRNA consists of exon and intron of ZEB1. Based on the role of miR-145-5p and TPO in cancer, hsa_circ_0003519 may be involved in metabolic regulation. Thus, we speculated that miR-145-5p may be associated with the metabolic reprogramming in LSCC through *TPO*-miR-145-5p-circ_0003519 ceRNA pathway.

In conclusion, our study elaborated the transcriptional regulatory role of miR‐145‐5p in LSCC in terms of ceRNA. A set of ceRNA regulatory axis were predicted, in which *WNT2B*-miR-145-5p-NONHSAT127539.2, *CASP10*-miR-145-5p-NONHSAT127539.2,

*CASP10*-miR-145-5p-circ_0003519, and *TPO*-miR-145-5p-circ_0003519 are involved in pathways regulating apoptosis, stem cell features, and metabolism. Thus, miR‐145‐5p may be implicated in LSCC pathogenesis via these ceRNA pathways. It is noteworthy that some ceRNAs can bind and sequester their target miRNA, rather than degrade them [62, 63], and these ceRNAs should not have significant expression changes in microarray data. Therefore, in this study, we constructed miR-145-5p affecting ceRNA network where miRNA binded and reduced expression of targets. In the future, combination of RNA-pulldown and RNA-seq technologies to comprehensively explore miR-145-5p involved ceRNA network will benefit for elucidating related mechanisms.

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**Conflict of interest statement**

The authors have declared no conflict of interest.

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## Figure legends

**Figure 1. Screening of differentially expressed genes by microarray profiling**. **(A)** Validation of miR-145-5p overexpression. TU-177 cells were transfected with miR-145-5p mimics or negative (NC) mimics for 48 h, then expression level of miR-145-5p was determined by qPCR. **(B)** Expression of FSCN1 was determined by qPCR using same samples of **(A)**. **(C)** Volcano plots of differentially expressed miRNAs. **(D)** Volcano plots of

differentially expressed mRNAs, lncRNAs and circRNAs. **(E)** Hierarchical clustering heatmap of differentially expressed miRNAs. **(F)** Hierarchical clustering heatmap of differentially expressed mRNAs, lncRNAs and circRNAs. **(G)** and **(H)** Validation of expression of miR-145-5p affected miRNAs**.** LSCC cells TU-177 **(G)** and Hep2 **(H)** were transfected with miR-145-5p mimics or NC mimics for 48 h, then expression level of miRNAs was determined by qPCR. **(I)** miRNA responsive element of differentially expressed mRNAs, lncRNAs and circRNAs was predicted using miRanda, and number of differentially expressed genes which contain miR-145-5p binding site was plotted. In qPCR experiment, data are mean ± s.d. of three independent experiments. *$p < 0.05$; *$p < 0.001$.

**Figure 2. miRNA target prediction and functional annotation.** **(A)** The overlapped target genes predicted from microRNAorg, PITA and TargetScan databases analyzed by VENN analysis. **(B)** The differentially expressed miRNAs target involved Gene Ontology biological process terms. **(C)** Kyoto Encyclopedia of Genes and Genomes pathways enriched by the target genes of differentially expressed miRNAs. **(D)** and **(E)** Validation of miR-145-5p affected signaling pathway molecules. LSCC cells TU-177 **(D)** and Hep2 **(E)** were transfected with miR-145-5p mimics or NC mimics for 48 h, then expression level of genes involved in MAPK and FoXO signaling pathways was determined by qPCR. In qPCR experiment, data are mean ± s.d. of three independent experiments. *$p < 0.05$; *$p < 0.001$.

**Figure 3. Functional annotation of differentially expressed mRNAs.** The Gene Ontology biological process terms enriched by miR-145-5p upregulated **(A) and downregulated (B)** mRNAs. Kyoto Encyclopedia of Genes and Genomes pathways enriched by miR-145-5p upregulated **(C)** and downregulated **(D)** mRNAs. **(E)** and **(F)** Validation of miR-145-5p regulated mRNAs that involved in olfactory transduction and signaling pathways regulating pluripotency of stem cells. LSCC cells TU-177 **(E)** and Hep2 **(F)** were transfected with miR-145-5p mimics or NC mimics for 48 h, then expression level of genes involved in olfactory transduction and signaling pathways regulating pluripotency of stem cells was determined by qPCR. In qPCR experiment, data are mean ± s.d. of three independent experiments. *$p < 0.05$; *$p < 0.001$.

**Figure 4. Competing endogenous network of miR-145-5p-regulated genes. (A)** The flow chart of constructing ceRNA network of differentially expressed miRNAs, lncRNAs, circRNAs, and mRNAs. **(B)** The constructed ceRNA network based on the differentially expressed miRNAs, mRNAs, and lncRNAs. **(C)** miR-145-5p-centered lncRNA-miRNA-mRNA network extracted from the ceRNA network (differentially expressed miRNAs, mRNAs, and lncRNAs). **(D)** The constructed ceRNA network based on

the differentially expressed miRNAs, mRNAs, and circRNAs. **(E)** miR-145-5p-centered ceRNA network extracted from the ceRNA network (differentially expressed miRNAs, mRNAs, and circRNAs). **(F)** Base-pair plot of mRNA-miR-145-5p-lncRNA from ceRNA network. **(G)** Base-pair plot of mRNA-miR-145-5p-circRNA from ceRNA network. **(H)** and **(I)** Validation of differentially expressed genes involved in ceRNA network. LSCC cells TU-177 **(H)** and Hep2 **(I)** were transfected with miR-145-5p mimics or NC mimics for 48 h, then expression level of indicated lncRNAs, circRNAs, and mRNAs was determined by qPCR. In qPCR experiment, data are mean ± s.d. of three independent experiments. *$p < 0.05$; *$p < 0.001$.

**Figure 5. Functional annotation of mRNAs involved in ceRNA network.** The Gene Ontology terms enriched by the mRNAs in ceRNA network, including biological process **(A)**, cellular component **(B)**, and molecular function **(C)**. **(D)** Kyoto Encyclopedia of Genes and Genomes pathways enriched by the mRNAs in ceRNA network.