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Original Article

Array profiling identified dysregulated miRNAs and target genes and pathways in laryngeal squamous cell carcinoma

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Abstract: Background: Laryngeal squamous cell carcinoma (LSCC) is the most common type of head and neck squamous cell carcinoma (HNSCC), and comprehensive post-transcriptional regulation is needed to be clarified. Objective: The purpose of this study was to investigate the miRNA basis of tumorigenesis in LSCC, and miRNA-gene-network and miRNA-pathway-network will be established with microarray analysis to reveal the molecular mechanism regulated by miRNAs. Methods: MicroRNA array and microarray were used to detect differently expressed miRNAs and mRNAs between the LSCC samples and para-carcinoma tissues collected from 2008 to 2013, then miRNA-mRNA network and miRNA-pathway network analysis were performed. Finally, the top dysregulated miRNAs were validated by quantitative real-time polymerase chain reaction (qRT-PCR). Results: 50 miRNAs (47 up- and 3 down-regulated), 2381 mRNAs (1542 up- and 839 down-regulated) were found to be differentially expressed. Bioinformatics analysis show that there were 31 different genes and 71 functional pathways regulated by the screened miRNAs. Hsa-miR-30a-5p expression level was validated by qRT-PCR. Conclusions: The research revealed tumorigenesis-related miRNAs, mRNAs, miRNA-mRNA, miRNA-pathway-network in LSCC. Hsa-miR-30a-5p could be considered as a potential diagnostic and therapeutic marker in LSCC.

Keywords: Microarray, LSCC, tumorigenesis, microRNA array

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer, and laryngeal squamous cell carcinoma (LSCC) is the most common type, accounting for 1% to 2% of all malignancies worldwide [1-3]. Corresponding to ~25% of HNSCC cases, of which the long-term survival rate has remained at 50%, LSCC should be drawn attention [4]. The development and progression of LSCC are multistep processes accompanied by changes of molecular biology. Various studies have revealed numerous molecular abnormalities in LSCC. However, the complete array of molecular changes that occur during oncogenesis remains elusive [5]. Hence, it is a need to acquire deeper understanding of LSCC biology and to develop predictive molecular signatures, which would improve patient selection for appropriate treatment and guide the development and evaluation.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that control gene expression by targeting mRNAs and triggering either translation repression or RNA degradation. Their aberrant expression may be involved in human diseases, including cancer. Among human diseases, it has been shown that miRNAs are aberrantly expressed or mutated in cancer, suggesting that they may play a role as a novel class of oncogenes or tumor suppressor genes [6]. MicroRNAs (miRNAs) represent a class of naturally occurring small noncoding RNA molecules, distinct from but related to small interfering RNAs. Mature miRNAs are 19- to 25-nucleotide long molecules cleaved from 70- to 100-nucleotide hairpin pre-miRNA precursors [7]. The discovery of this class of genes

Table 1. Clinical data of patients for microRNAs array

Tumor tissues	Age	T	N	M	Corresponding non-neoplastic tissues
FX3T	53	2	0	0	FX3Z
FX11T	55	4	3	0	FX11Z
HN3T	56	4	0	0	HN3Z
HN3DT	74	3	0	0	HN3DZ
FX21T	71	4	2a	0	FX21Z

Table 2. Clinical data of patients for microarrays

Tumor tissues	Age	T	N	M	Corresponding non-neoplastic tissues
C1	64	4	1	0	N1
C2	46	4	1	0	N2
C3	47	4	1	0	N3
C4	52	4	0	0	N4
C5	68	4	0	0	N5
C6	58	4	2c	0	N6
C7	71	4	0	0	N7
C8	58	4	0	0	N8
C9	52	4	0	0	N9

Table 3. Clinical data of patients for RT-PCR

TNM	Number	Average age (mean ± SD)
T1N0M0	1	51.00±0.00
T2N0M0	7	49.00±8.40
T3N0M0	8	58.00±5.39
T4N0M0	6	60.00±7.32
T3NxM0 (X≠0)	6	58.00±6.69
T4NxM0 (X≠0)	8	61.00±6.82

has identified a new layer of gene regulation mechanisms. MicroRNAs have also been recognized to play important roles in Head and neck squamous cell carcinomas and are regarded as key regulators of gene expression in biological systems [8-10].

In the present study, we focus on the microRNAs expression about tumorigenesis in LSCC. With Microarray Gene Expression Analysis of Tumorigenesis in LSCC, we demonstrated the gene expression profiles mediated by screened miRNAs. The microarray data were subjected to miRNA-Gene-Network analysis and miRNA-pathway-Network analysis, which revealed the molecular mechanism regulated by miRNAs. Finally, the significant screened miRNAs which regulated important molecular mechanism were validated by qRT-PCR. Our findings contribute to the understanding of the molecular basis of tumorigenesis in LSCC, and provide a set of miRNAs that may be useful for the development of novel diagnostic markers and/or more effective diagnostic and therapeutic strategies.

Materials and methods

Tissue samples and patients

All patients we had selected were treated in Department of Head and Neck Surgery, Beijing Tongren Hospital between 2008 and 2013. All patients provided written informed consent before their participation. The Ethics Committee of Capital Medical University approval was obtained for the use of all samples by using a protocol that conforms to the provisions of the Declaration of Helsinki.

A total number of 5 patients (no females) who underwent surgery for primary laryngeal squamous cell carcinoma were recruited for microRNAs array analysis, and the TNM stage of each patient was determined as **Table 1**. Another total number of 9 patients (no females) who underwent surgery for primary laryngeal squamous cell carcinoma were recruited for Microarray Gene Expression analysis, and the TNM stage of each patient was determined as **Table 2**. A set of 36 patients (no females) who underwent surgery for primary LSCC were also recruited for RT-PCR, and the TNM stage of each patient was determined as **Table 3**. The three patient cohorts used for array analysis and qRT-PCR investigations were separated. The cancer tissues and corresponding adjacent non-neoplastic tissues were collected during surgery. Each specimen was immediately snap-frozen in liquid nitrogen and stored at -80°C for subsequent study. The pathology of all the cancer tissues were squamous cell carcinoma, which were evaluated by pathologists.

RNA extraction and quality assessment

Total RNA was extracted from tissue samples using Trizol Reagent (Invitrogen). Then the RNA quantity was determined using denaturing gel electrophoresis which produced at least 2 distinct bands representing the 28S and 18S ribo-

somal RNA, confirming that the total RNA was not contaminated with DNA and the RNA was not degraded.

MicroRNA array

The Affymetrix GeneChip miRNA Array 3.0 (Beijing Compass Biotechnology Company) was used to investigate miRNA expression. The procedure was carried out using FlashTag Biotin RNA Labeling Kit (Affymetrix) for Affymetrix GeneChip miRNA arrays in accord with the manufacturer's protocol. The labeled RNA samples were processed by Poly A tailing, biotin ligation, hybridization, washing and staining, then the microarrays were scanned and the raw data were extracted. After raw data was processed by the Affymetrix GeneChip Command Console (version 4.0, Affymetrix) software, the differential miRNAs could be screened. The dataset had been submitted to Gene Expression Omnibus, and the accession number was GSE62819.

Microarray assay

Agilent lncRNA Gene Expression 4 × 180K Microarray (Design ID: 042818, Agilent Technologies, USA) was used to test the mRNA expression profiling. The sample labeling, microarray hybridization and washing were performed based on the manufacturer's standard protocols. Briefly, total RNA were transcribed to double strand cDNA, then synthesized into cDNA and labeled with Cyanine-3-CTP. The labeled cDNAs were hybridized onto the microarray. After washing, the arrays were scanned by the Agilent Scanner G2505C (Agilent Technologies). Random Variance Model (RVM) t-test was applied to filter the differentially expressed genes between tumor tissues and adjacent non-cancerous tissues according to the *p*-value threshold. *P* value < 0.05 was considered as significant difference. The Hierarchical Clustering was conducted to analyze the differentially expressed mRNAs. The microarray data have been deposited in Gene Expression Omnibus (GEO) database and are accessible through GEO accession number GSE84957.

Data analysis

MicroRNA-Pathway-network is built according to the relationship of significant PATHWAYs and genes and the relationships among MicroRNA and genes. The adjacency matrix of MicroRNA and genes A= $[a_{i,j}]$ is made by the attribute relationships among PATHWAYs and MicroRNA, and $a_{i,j}$ represents the relation weigh of PATHWAY i and MicroRNA j. In the MicroRNA-Gene-Network, the circle represents gene and the shape of square represents MicroRNA, and their relationship was represented by one edge. The center of the network was represents by degree. Degree means the contribution one MicroRNA to the PATHWAYs around or the contribution one PATHWAY to the MicroRNAs around. The key MicroRNA and gene in the network always have the biggest degrees [11, 12].

To build a miRNA-Gene-Network, the relationship between miRNAs and genes was counted by their differential expression values, and according to their interactions in the Sanger miRNA database. The adjacency matrix of MicroRNA and genes A= $[a_{i,j}]$ was made by the attribute relationships among genes and MicroRNA, where $a_{i,j}$ represents the weight of the relationship between gene i and MicroRNA j. In the miRNA-Gene-Network, the circles represent is represented by one edge. The center of the network was represented by degree. Degree is the contribution of one miRNA to the genes around or the contribution of one gene to the miRNAs around. The key miRNA and gene in the network always have the biggest degrees [13].

Quantitative real-time PCR

The transcriptional level of the target genes were measured by qRT-PCR detection. Trizol was applied to extract total cellular RNA. Prepared Template RNA (5 μl)/primer (1 μl) mixture in microtube. Keep in 70°C for 10 minutes, then rapid quenched in ice no more than 5 minutes. After that, centrifuged for a few seconds so that the template RNA/primer solution of denatured aggregation gathered at the bottom of the microtube. Then added 5 × M-MLV Buffer, RNase Inhibitor and dNTP Mixture preparation called reverse transcription reaction solution in the microtube, 4 μl totally. This solution had to keep in 42°C for 1 hour. Cooled by ice after hold in 95°C for 15 minutes, then we got the cDNA solution. Mixed this 1 μl cDNA solution, Taq DNA Polymerase, 2XSYBR to 20 μl the mixed system. Hold it in 95°C for 5 mins for denaturing, then followed by 45 cycles

Table 4. Differentially expressed miRNAs analyzed by microRNA array

p-value	FDR	Fold change (T/Z)	Style	Accession	miRNA
0.049827	0.234	0.051	Down	MIMAT0000728	miR-375
0.045292	0.234	0.11	Down	MIMAT0017990	miR-3613-5p
0.001363	0.147	0.23	Down	MIMAT0000087	miR-30a-5p
0.011938	0.184	4.07	Up	MIMAT0002872	miR-501-5p
0.02809	0.21	4.37	Up	MIMAT0016872	miR-4317
0.042284	0.234	4.38	Up	MIMAT0000266	miR-205-5p
0.021747	0.21	4.41	Up	MIMAT0004507	miR-92a-1-5p
0.02254	0.21	4.46	Up	MIMAT0004657	miR-200c-5p
0.032786	0.214	4.48	Up	MIMAT0009447	miR-1972
0.032483	0.214	4.57	Up	MIMAT0019073	miR-4534
0.036512	0.227	4.57	Up	MIMAT0005586	miR-1231
0.00396	0.153	4.61	Up	MIMAT0000688	miR-301a-3p
0.028905	0.21	4.84	Up	MIMAT0001536	miR-429
0.008558	0.164	4.87	Up	MIMAT0002891	miR-18a-3p
0.020081	0.21	5	Up	MIMAT0018178	miR-3180
0.020246	0.21	5.02	Up	MIMAT0015058	miR-3180-3p
0.046047	0.234	5.14	Up	MIMAT0002876	miR-505-3p
0.02424	0.21	5.2	Up	MIMAT0001080	miR-196b-5p
0.042981	0.234	5.4	Up	MIMAT0005825	miR-1180-3p
0.008372	0.164	5.87	Up	MIMAT0018000	miR-23c
0.04804	0.234	5.89	Up	MIMAT0018968	miR-4449
0.023286	0.21	6.14	Up	MIMAT0004498	miR-25-5p
0.024156	0.21	6.6	Up	MIMAT0005951	miR-1307-3p
0.031906	0.214	6.64	Up	MIMAT0018929	miR-4417
0.021131	0.21	6.95	Up	MIMAT0003886	miR-769-5p
0.04815	0.234	6.98	Up	MIMAT0005797	miR-1301-3p
0.007944	0.164	7.14	Up	MIMAT0009197	miR-205-3p
0.027598	0.21	7.27	Up	MIMAT0001412	miR-18b-5p
0.006355	0.157	7.38	Up	MIMAT0003150	miR-455-5p
0.0467	0.234	7.68	Up	MIMAT0002821	miR-181d-5p
0.033348	0.216	9.24	Up	MIMAT0004497	miR-24-2-5p
0.013133	0.184	9.5	Up	MIMAT0005880	miR-1290
0.006736	0.157	9.52	Up	MIMAT0004509	miR-93-3p
0.014237	0.184	9.71	Up	MIMAT0004672	miR-106b-3p
0.010201	0.183	10.65	Up	MIMAT0000261	miR-183-5p
0.011403	0.184	11.18	Up	MIMAT0000267	miR-210-3p
0.003852	0.153	11.3	Up	MIMAT0004496	miR-23a-5p
0.004903	0.153	11.32	Up	MIMAT0004560	miR-183-3p
0.032302	0.214	11.46	Up	MIMAT0000424	miR-128-3p
0.015601	0.189	11.52	Up	MIMAT0004494	miR-21-3p
0.034939	0.223	11.53	Up	MIMAT0004588	miR-27b-5p
0.005825	0.157	12.02	Up	MIMAT0004749	miR-424-3p
0.013711	0.184	13.3	Up	MIMAT0003339	miR-421
0.0023	0.153	13.57	Up	MIMAT0004504	miR-31-3p
0.001178	0.147	14.7	Up	MIMAT0002874	miR-503-5p
0.005236	0.153	18.28	Up	MIMAT0004501	miR-27a-5p
0.007751	0.164	18.57	Up	MIMAT0001635	miR-452-5p
0.004997	0.153	23.35	Up	MIMAT0009198	miR-224-3p
0.006671	0.157	24.32	Up	MIMAT0000226	miR-196a-5p
0.002876	0.153	48.16	Up	MIMAT0000281	miR-224-5p

totally which were keeping in 95°C for 30 s, keeping in 65°C for 30 s, and keeping in 72°C for 5 mins. The gene expression levels were determined based on Livak method. The results that $2^{-\Delta\Delta CT}$ values of all samples were analyzed automatically by computer control with β-actin gene as an internal reference.

Statistical analysis for qRT-PCR

All data were imported to SPSS 20.0. The data which did not meet normality would be converted into normality. Pair ed sample t tests were used for analysis involving two samples. Differences were considered statistically significant at *P*-value < 0.05.

Results

MiRNAs expression analysis of tumorigenesis in LSCC

Expression analysis using the miRNA array was initially performed on five laryngeal squamous cell carcinoma tissues and their corresponding adjacent non-neoplastic tissues. We found there were 50 miRNAs showed statistically significant differences in the expression between LSCC tissues and corresponding non-neoplastic tissues. Among these 50 miRNAs, 47 miRNAs showed a higher expression in tumor than in non-tumor tissue, and the other 3 miRNAs presented the contrasting pattern.

Supervised hierarchical clustering analysis revealed that the expression patterns of the selected set of 50 differentially expressed miRNAs were able to perfectly distinguish

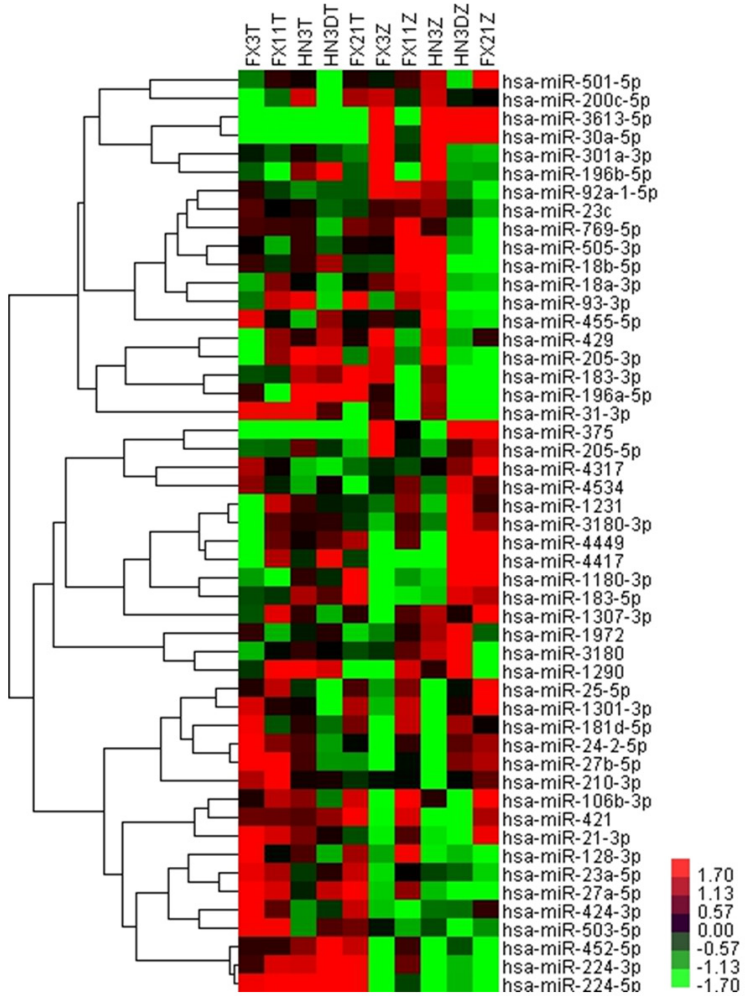

Figure 1. Hierarchical Trees: All differentially expressed miRNAs of tumorigenesis in LSCC For each miRNA (row), red indicated a higher expression and green a lower one relative to the average level of expression of all the 50 miRNAs (columns).

tumors from non-neoplastic tissues in the set of samples. The result was shown below in **Table 4** and **Figure 1**.

Microarray-based integrated analysis

The microRNA array hierarchical clustering analysis showed a clear separation between laryngeal squamous cell carcinoma tissues and their corresponding adjacent non-neoplastic tissues. To determine the potential direct targets or pathways of screened miRNAs in LSCC, we next performed mRNA microarray to detect the gene expression profiles in LSCC. We found that total 2381 genes were differentially expressed. The 2381 genes showed statistically significant differences in the expression between LSCC tissues and corresponding non-neoplastic tissues ($P < 0.05$). Among these 2381 genes, 1542 showed a higher expression in tumor than in non-tumor tissue, and 839 presented the contrasting pattern. Supervised hierarchical clustering analysis revealed that the expression patterns of the selected set of the differentially expressed genes were able to perfectly distinguish tumors from non-neoplastic tissues in the set of samples. The result was shown below in **Figure 2**.

To predict potentially functional target genes and pathways between miRNAs and mRNAs, we performed the two expression profiles of miRNAs and mRNAs to establish functional miRNA-gene-network and miRNA-pathway-network relationships with a high precision. We found that 31 different screened genes and 71 different functional pathways related to the screened miRNAs (**Table 5**, **Figure 3**; **Table 6**, **Figure 4**). Among the screened miRNAs, hsa-miR-30a-5p should be paid attention to. Hsa-miR-30a-5p was down-expressed obviously, and it regulated PI3K/Akt signaling pathway, TGF-beta signaling pathway, Wnt signaling pathway and so on, all of which were considered as important signaling pathway related with tumorigenesis. Hsa-miR-30a-5p could be considered as potential diagnostic and therapeutic markers in LSCC.

Quantitative real-time PCR

As the potential diagnostic and therapeutic marker, hsa-miR-30a-5p was run on qRT-PCR for a subset of 36 cancer tissues and their adjacent non-neoplastic tissues to investigate whether this miRNA was able to distinguish LSCC from non-tumor larynx tissues. The reverse primer was CTCAACTGGTGTCGTGG AGTCGGCAATTCAGTTGAGCTTCCAGT, the quantita-

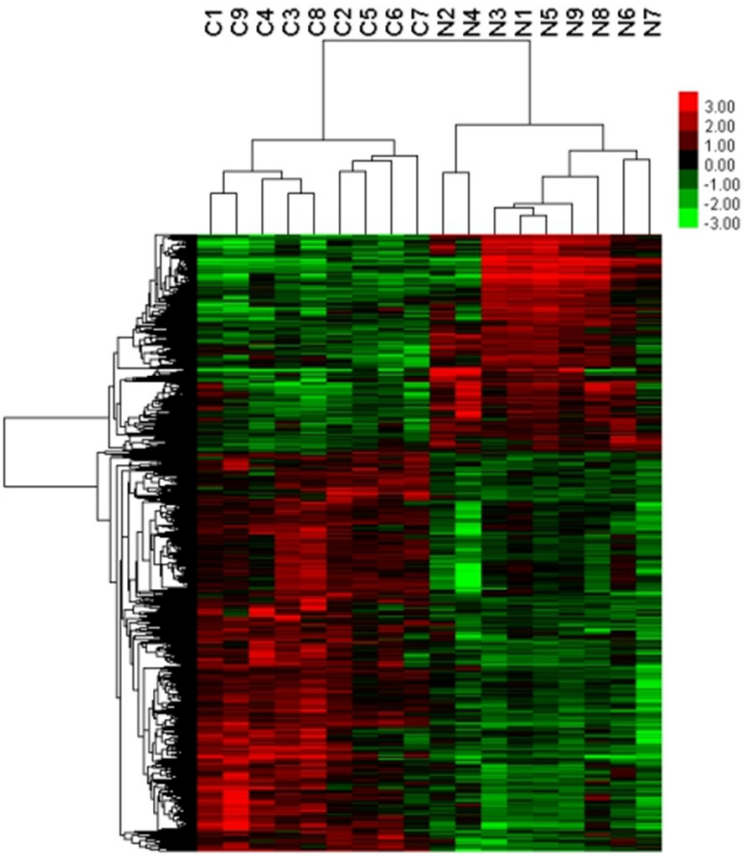

Figure 2. Hierarchical clustering of aberrant expressed mRNAs detected in LSCC. Red color indicates over expression and green color indicates low expression. Every column represents a tissue sample and every row represents an mRNA probe.

Table 5. The result of miRNA-Gene-Network analysis

miRNA	miRNA style	Gene symbol	miRNA	miRNA style	Gene symbol
miR-30a-5p	Down	ACVR1	miR-1972	Up	EPB41L1
miR-30a-5p	Down	CHST1	miR-200c-5p	Up	ASAP3
miR-30a-5p	Down	DDIT4	miR-205-5p	Up	ACACB
miR-30a-5p	Down	EDNRA	miR-205-5p	Up	AMOT
miR-30a-5p	Down	EPHB2	miR-205-5p	Up	MAGI3
miR-30a-5p	Down	FZD2	miR-27a-5p	Up	DLG2
miR-30a-5p	Down	GALNT2	miR-301a-3p	Up	ATP1A2
miR-30a-5p	Down	GJA1	miR-301a-3p	Up	GPT2
miR-30a-5p	Down	GNG10	miR-301a-3p	Up	NR3C2
miR-3613-5p	Down	CDK6	miR-301a-3p	Up	TGFBR2
miR-128-3p	Up	PIK3R1	miR-421	Up	SLC4A4
miR-181d-5p	Up	PBX1	miR-429	Up	LPIN1
miR-181d-5p	Up	TGFBR2	miR-4317	Up	PIK3R1
miR-18a-3p	Up	PPAP2B	miR-452-5p	Up	MEIS1
miR-196a-5p	Up	PBX1	miR-501-5p	Up	LPIN1
miR-196b-5p	Up	PBX1			

tive primer was F: GGGTGTAAACATCCTCG; R: CTCAACTGGTGTCGTGGAGTC, and the quantitative universal primer was CTCAACTGGTGTCGTGGAGTC. We found that hsa-miR-30a-5p expression levels in LSCC tissues were significantly down-regulated ($P < 0.05$).

Hsa-miR-30a-5p could be considered as a potential diagnostic and therapeutic marker in LSCC. The results were shown below in **Table 7**.

Discussion

Since miRNAs were proved to be master regulators of post-transcriptional regulation and participate in diverse cellular physiological processes, it has been a priority to search for their functional roles. Dysregulation of miRNAs is found to be involved in distinct signaling pathways and closely associated with pathoclinical parameters of carcinogenesis, such as apoptosis, proliferation, migration and invasiveness [14].

In this study, both the differentially expressed microRNAs mRNAs between LSCC samples and para-carcinoma tissues were investigated and analyzed. We identified 50 miRNAs (47 up- and 3 down-regulated) and 2381 mRNAs (1542 up- and 839 down-regulated) as differentially regulated in LSCC tissues as compared to corresponding non-neoplastic tissues. Then the two differentially expression profiling between miRNAs and mRNAs were established to identify functional miRNA-gene-network and miRNA-pathway-network relationship. Results show that there were 31 different screened genes and 71 dif-

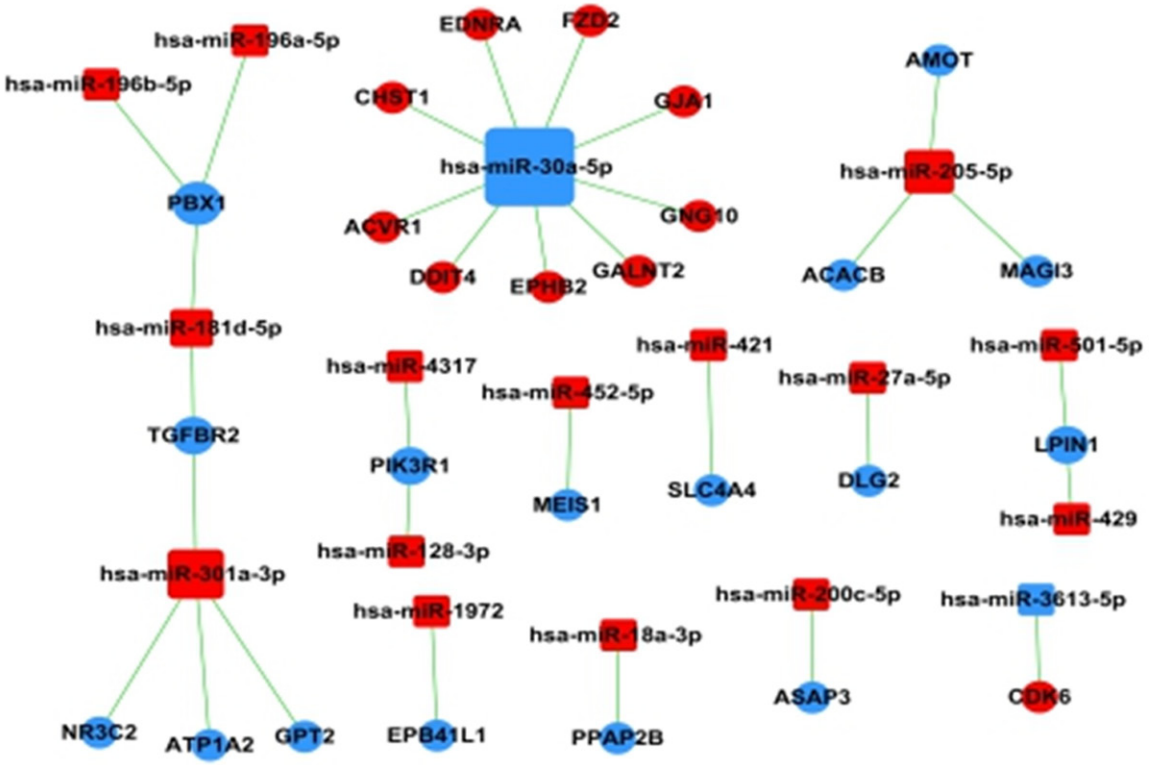

Figure 3. MiRNA-Gene-Network Analysis. The miRNA-mRNA network was generated according to the relationship of significantly functional screened mRNAs and miRNAs. Red squares represent up-regulated miRNAs while blue ones represent down-regulated miRNAs, and cycles represent targeted screened genes. The relationship between miRNAs and genes was represented by lines.

ferent functional pathways regulated by the screened miRNAs potentially. Carcinogenesis relating pathways are found to be regulated a set of microRNAs, such as hsa-miR-3613-5p, hsa-miR-205-5p, and hsa-miR-301a-3p and so on. And a significant decrease of hsa-miR-30a-5p is noticed and validation has been performed using qRT-PCR. Further investigation should be done to elucidate the functional role of hsa-miR-30a-5p. Previous relevant research reported dysregulated microRNAs in head and neck squamous cell carcinoma (HNSCC). In locally advanced HNSCC, miR-21, miR-155, let-7i, and miR-142-3p were overexpressed, miR-125b and miR-375 were down-regulated [8]. The expression level of miR-21, let-7, 18, 29c, 142-3p, 155, 146b (overexpressed) and 494 (underexpressed) was altered in primary HNSCC [15]. In our study, a novel set of discrepantly expressed microRNAs was found in primary LSCC tissues, including miR-30a-5p, 36-13 (up-regulated), and 128-3p, 181-5p, 18a-3p, 196a-5p, 1972, 200c-5p, 205-5p, 27a-5p, 301a-5p, 421, 429, 4317, 452, 501 (down-regulated).

Aberrant expression of hsa-miR-30a-5p has been reported in a wide range of tumors, such as cervical cancer [16], non-small cell lung cancer [17], colon carcinoma [18], gastric cancer [19], nasopharyngeal carcinoma [20], hepatocellular carcinoma [21], and gliomas [22, 23]. It also has been reported that E-cadherin, FOXL2 [24] and PI3K/AKT signaling pathway [25] are targeted by hsa-miR-30a-5p in different solid tumors. From our study, hsa-miR-30a-5p also regulates nine targeted genes and eighteen pathways potentially, such as PI3K/Akt signaling pathway, TGF-beta signaling pathway and Wnt signaling pathway [26-29], which are the important signaling pathways related with tumorigenesis. Besides, hsa-miR-30-5p exhibited as a potential biomarker for diagnosis and prognosis. Hsa-miR-30-5p was discrepantly expressed in the plasma of colorectal cancer patients [30], miR-30-5p of tumor-derived exosomes was established as a candi-

Table 6. The result of miRNA-Pathway-Network analysis

miRNA	miRNA style	Pathname	miRNA	miRNA style	Pathname
miR-30a-5p	Down	Cytokine-cytokine receptor interaction	miR-181d-5p	Up	Hippo signaling pathway
miR-30a-5p	Down	TGF-beta signaling pathway	miR-18a-3p	Up	Metabolic pathways
miR-30a-5p	Down	Glycosaminoglycan biosynthesis - keratan sulfate	miR-18a-3p	Up	Glycerolipid metabolism
miR-30a-5p	Down	PI3K-Akt signaling pathway	miR-18a-3p	Up	Fc gamma R-mediated phagocytosis
miR-30a-5p	Down	MicroRNAs in cancer	miR-196a-5p	Up	Transcriptional misregulation in cancer
miR-30a-5p	Down	Neuroactive ligand-receptor interaction	miR-196b-5p	Up	Transcriptional misregulation in cancer
miR-30a-5p	Down	Axon guidance	miR-1972	Up	Tight junction
miR-30a-5p	Down	Pathways in cancer	miR-200c-5p	Up	Fc gamma R-mediated phagocytosis
miR-30a-5p	Down	Proteoglycans in cancer	miR-205-5p	Up	Metabolic pathways
miR-30a-5p	Down	HTLV-I infection	miR-205-5p	Up	Propanoate metabolism
miR-30a-5p	Down	Hippo signaling pathway	miR-205-5p	Up	Insulin signaling pathway
miR-30a-5p	Down	Wnt signaling pathway	miR-205-5p	Up	Pyruvate metabolism
miR-30a-5p	Down	Basal cell carcinoma	miR-205-5p	Up	Adipocytokine signaling pathway
miR-30a-5p	Down	Metabolic pathways	miR-205-5p	Up	Fatty acid metabolism
miR-30a-5p	Down	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	miR-205-5p	Up	Hippo signaling pathway
miR-30a-5p	Down	Chemokine signaling pathway	miR-205-5p	Up	Tight junction
miR-30a-5p	Down	Alcoholism	miR-27a-5p	Up	Hippo signaling pathway
miR-30a-5p	Down	Ras signaling pathway	miR-301a-3p	Up	Salivary secretion
miR-3613-5p	Down	Cell cycle	miR-301a-3p	Up	Aldosterone-regulated sodium reabsorption
miR-3613-5p	Down	Pathways in cancer	miR-301a-3p	Up	Proximal tubule bicarbonate reclamation
miR-3613-5p	Down	PI3K-Akt signaling pathway	miR-301a-3p	Up	Carbohydrate digestion and absorption
miR-3613-5p	Down	Small cell lung cancer	miR-301a-3p	Up	Metabolic pathways
miR-3613-5p	Down	p53 signaling pathway	miR-301a-3p	Up	Alanine, aspartate and glutamate metabolism
miR-3613-5p	Down	MicroRNAs in cancer	miR-301a-3p	Up	Transcriptional misregulation in cancer
miR-3613-5p	Down	Viral carcinogenesis	miR-301a-3p	Up	Hippo signaling pathway
miR-3613-5p	Down	Hepatitis B	miR-421	Up	Proximal tubule bicarbonate reclamation
miR-3613-5p	Down	Pancreatic cancer	miR-429	Up	Metabolic pathways
miR-3613-5p	Down	Chronic myeloid leukemia	miR-429	Up	Glycerolipid metabolism
miR-3613-5p	Down	Measles	miR-4317	Up	Insulin signaling pathway
miR-3613-5p	Down	Glioma	miR-4317	Up	Aldosterone-regulated sodium reabsorption
miR-3613-5p	Down	Melanoma	miR-4317	Up	Carbohydrate digestion and absorption
miR-128-3p	Up	Insulin signaling pathway	miR-4317	Up	Fc gamma R-mediated phagocytosis
miR-128-3p	Up	Aldosterone-regulated sodium reabsorption	miR-452-5p	Up	Transcriptional misregulation in cancer
miR-128-3p	Up	Carbohydrate digestion and absorption	miR-501-5p	Up	Metabolic pathways
miR-128-3p	Up	Fc gamma R-mediated phagocytosis	miR-501-5p	Up	Glycerolipid metabolism
miR-181d-5p	Up	Transcriptional misregulation in cancer			

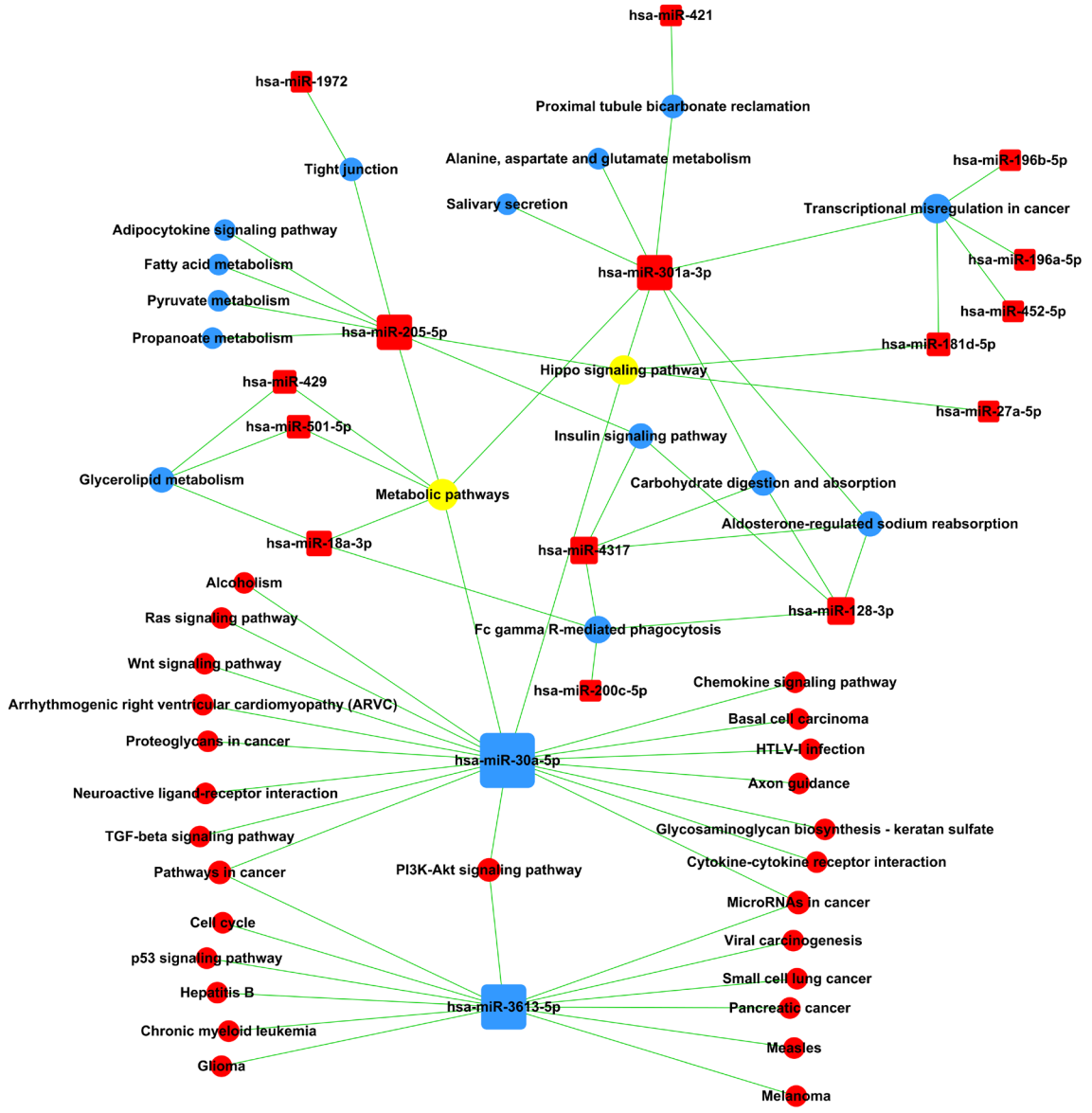

Figure 4. MiRNA-Pathway-Network Analysis. The miRNA-mRNA network was generated according to the relationship of significantly functional screened mRNAs and miRNAs. Red squares represent up-regulated miRNAs while blue ones represent down-regulated miRNAs, and cycles represent pathways. The relationship between miRNAs and pathways was represented by lines.

Table 7. RT-PCR analysis between LSCC tissues and their corresponding adjacent non-neoplastic tissues (paired sample t tests)

miRNAs	Relative mRNA expression levels		P
	Carcinoma tissues	Non-neoplastic tissues	
miR-30a-5p	3.12±1.02	11.42±3.05	0.005

date biomarker for ovarian cancer diagnosis [31]. Up-regulation of hsa-miR-30a-5p was associated with ovarian cancer chemotherapy resistance and promoted carcinogenesis [32]. These findings may provide guidance for clinical prediction, diagnosis and prognosis. And the mechanism of tumorigenesis needs further investigation in LSCC.

In conclusion, results revealed miRNAs expression signature of tumorigenesis in laryngeal squamous cell carcinoma, and the microarray platform was also constructed to screen mRNA. MiRNA-Gene-Network and miRNA-pathway-Network were established to explore the specific

mechanisms and evaluate the clinical application values. Results indicated that the dysregulation of hsa-miR-30a-5p was associated with disease development in LSCC and its down-expression was validated by qRT-PCR. Our findings contribute to the understanding of the molecular basis of laryngeal squamous cell carcinoma, thus helping to improve the diagnosis and treatment.

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Disclosure of conflict of interest

None.

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