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Expression analysis of miRNA and target mRNAs in esophageal cancer

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Abstract

We aimed to investigate miRNAs and related mRNAs through a network-based approach in order to learn the crucial role that they play in the biological processes of esophageal cancer. Esophageal squamous-cell carcinoma (ESCC) and adenocarcinoma (EAC)-related miRNA and gene expression data were downloaded from the Gene Expression Omnibus database, and differentially expressed miRNAs and genes were selected. Target genes of differentially expressed miRNAs were predicted and their regulatory networks were constructed. Differentially expressed miRNA analysis selected four miRNAs associated with EAC and ESCC, among which *hsa-miR-21* and *hsa-miR-202* were shared by both diseases. *hsa-miR-202* was reported for the first time to be associated with esophageal cancer in the present study. Differentially expressed miRNA target genes were mainly involved in cancer-related and signal-transduction pathways. Functional categories of these target genes were related to transcriptional regulation. The results may indicate potential target miRNAs and genes for future investigations of esophageal cancer.

Key words: Esophageal adenocarcinoma; Esophageal squamous-cell carcinoma; miRNA expression network; Pathway; Gene ontology

Introduction

Esophageal cancer, with squamous-cell carcinoma (ESCC) and adenocarcinoma (EAC) as the predominant histological types, is the sixth leading cause of cancer-related mortality and the eighth most common cancer worldwide. The overall 5-year survival ranges from 15% to 25%, and the best outcomes are associated with disease diagnosed in the early stages (1). MicroRNAs (miRNAs) are an abundant class of small nonprotein-coding RNAs that function as negative gene regulators. miRNAs have gained significant attention because of their ability to regulate multiple oncogene and tumor suppressor signaling pathways (2). Evidence that alterations in the expression of certain miRNAs (e.g., *hsa-miR-21*, *hsa-miR-223* and *hsa-miR-75*) might be associated with the development, prognosis and survival rates of esophageal cancer is increasing (3). However, most previous studies have focused mainly on differences in expression of single miRNAs instead of focusing on the miRNAs and the specifically regulated mRNAs through a view of the network that plays a crucial role in the whole biological process (4).

This study was designed to investigate the pathogenesis of ESCC and EAC by *1*) screening existing EAC- and ESCC-related miRNA expression microarray data to identify differentially expressed miRNAs and analyze the correlations between miRNA expression and the risk factors, treatment methods and survival rates of patients; *2*) screening the EAC- and ESCC-related gene expression microarray data for differentially expressed genes; *3*) predicting the target genes of differentially expressed miRNAs and constructing regulatory networks depending on the differentially expressed target genes. Their effects on biological processes of the target genes were also investigated with pathway and gene ontology (GO) enrichment analysis.

Material and Methods

Databases

We downloaded EAC- and ESCC-related miRNA microarray data and gene expression microarray data

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Table 1. Differentially expressed miRNAs in esophageal adenocarcinoma (EAC) and squamous-cell carcinoma (ESCC).

miRNAs	FC	P	FDR
EAC			
hsa-miR-21	1.12	1.47E-04	0.023
hsa-miR-202	-1.81	1.02E-04	0.023
hsa-miR-203	-2.71	1.35E-04	0.023
hsa-miR-205	-2.45	3.12E-04	0.041
ESCC			
hsa-miR-21	1.41	2.07E-10	2.37E-07
hsa-miR-202	-1.64	1.81E-04	5.57E-03
hsa-miR-223	1.25	3.74E-06	4.06E-04
hsa-miR-375	-1.40	1.01E-06	1.42E-04

FC: fold change; FDR: false discovery rate. The *t*-test was used for statistical analyses.

from the Gene Expression Omnibus (GEO) database. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Gene and Genome (KEGG) pathway enrichment analysis were used to define functions and pathways in ESCC and EAC. A protein-protein interaction network was constructed from the Biomolecular Interaction Network Database (BIND) to identify modules with close interactions.

Study samples

miRNA analysis. We performed a comprehensive miRNA analysis to identify differential miRNA using the miRNA profile (GSE13937), which was carried out using the OSU-CCC Human MicroRNA Microarray Version 3.0 Array (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL8835), and included data from 76 esophageal cancer cases (44 ESCC and 32 EAC) and 76 adjacent noncancerous tissues.

Gene expression analysis. The gene expression microarray data was based on the profile via the Affymetrix Human Genome U133 Plus 2.0 Array (HG-U133_Plus_2). The profile included four collections: GSE42363 (14 EAC tissue samples), GSE17351 (5 ESCC cases and 5 adjacent normal esophageal tissues), GSE26886 (21 EAC tissues, 9 ESCC tissues, and 19 normal tissues from healthy subjects), and GSE3701 (40 ESCC tissue samples).

Statistical analyses

Differential expression analysis. Raw data including Affymetrix CEL files and simple omnibus format in text files for all samples as described above were obtained from the GEO database. Raw intensity values generated from the CEL files were normalized by robust multiarray analysis (RMA) (5) as follows. Firstly, background noise and the processing artifacts were neutralized using a model-based background correction. Secondly, expression values were normalized by aligning to a common scale. Thirdly, an expression value was generated for each probe using an iterative median polishing method. The resulting $\log_2$-transformed RMA expression values were then used to further identify significantly differentially expressed genes and miRNAs. The *t*-test was used to identify differentially expressed genes, and the Benjamini and Hochberg procedure (6) was carried out for multiple test corrections. The genes or miRNAs with a false discovery rate (FDR) <0.05 were selected as differentially expressed. Differentially expressed genes or miRNAs were identified as up- or down-regulated according to the fold-change value. All the above procedures were performed using the R software (v3.03, http://www.r-project.org/) with BioConductor (http://www.bioconductor.org/), linear models for microarray (limma) data packages (3.12.1) and libraries (7), and differentially co-expressed genes and links packages (8).

miRNA target prediction. miRNA target sites in 3′ UTR gene regions were identified by bioinformatics analysis using the Miranda (microRNA.org), microcode (http://www.mircode.org/mircode/), MirTarget2 (9), Targetscan (http://

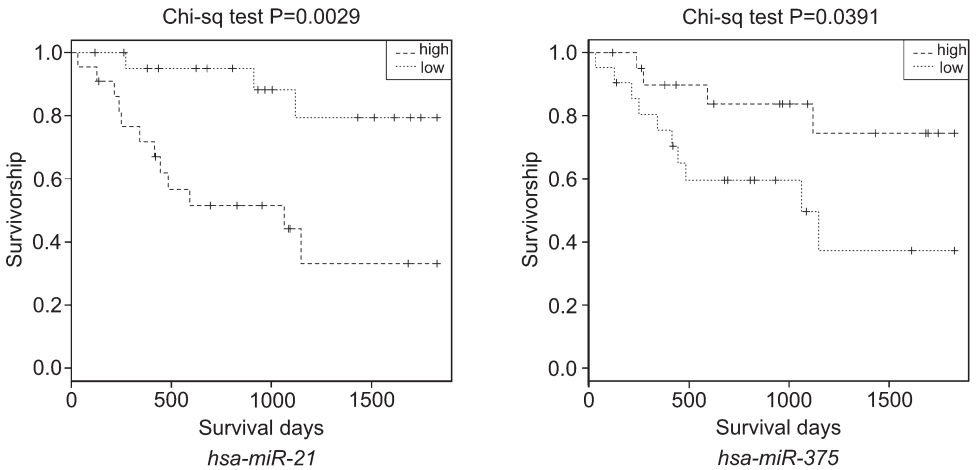

Figure 1. Survival analyses of *hsa-miR-21* and *hsa-miR-375* in esophageal squamous-cell carcinoma patients.

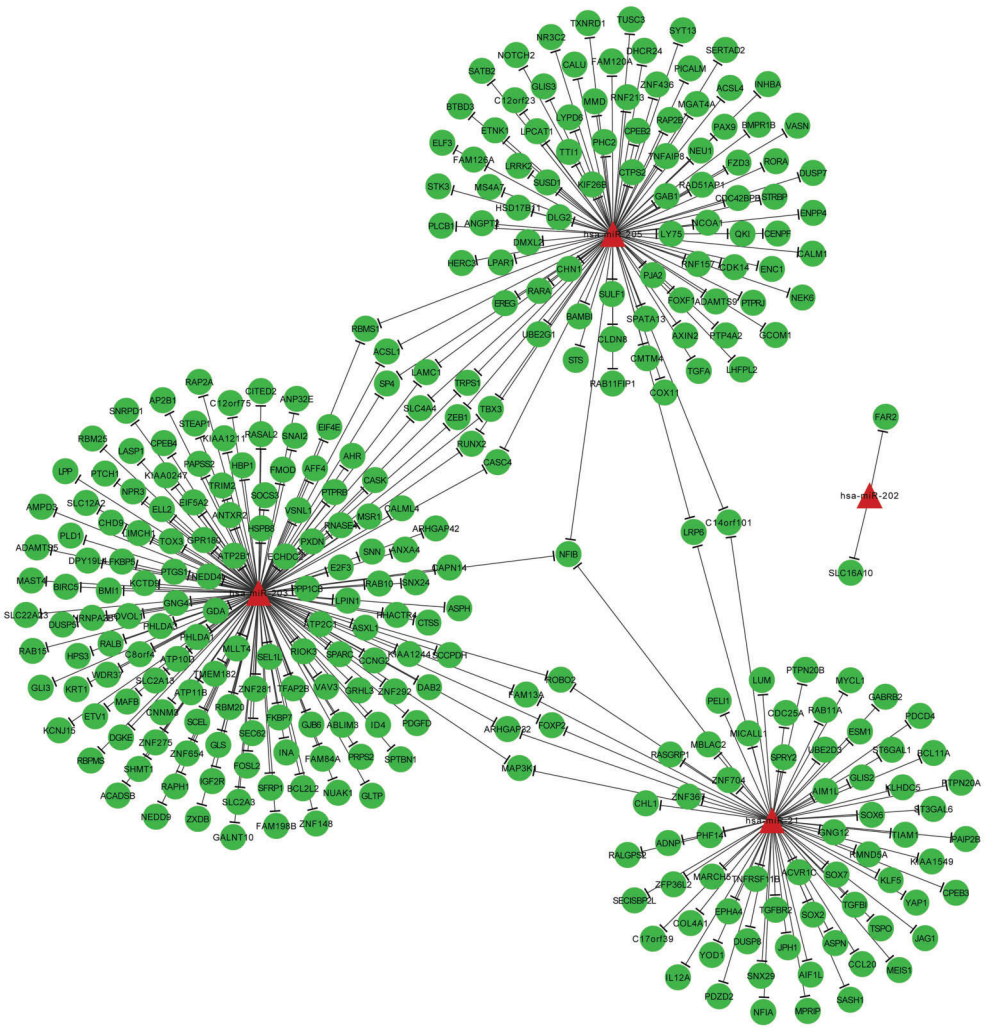

Figure 2. Regulatory network of selected miRNAs and their differentially expressed target genes in esophageal adenocarcinoma.

www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and microT (http://diana.cslab.ece.ntua.gr/microT/) databases. Only those putative miRNA target sites resulting from at least three databases were considered positive.

Enrichment analysis. To capture biologically relevant signatures of the differentially expressed target genes, we carried out enrichment analyses. All dysregulated target genes were mapped to the KEGG pathways (http://www.genome.jp/kegg/) database (10) and the GO database (11). The hypergeometric distribution test was used to identify biological processes significantly enriched with differentially expressed target genes.

Results

Differentially expressed miRNAs in esophageal cancer tissues

Compared with the normal tissues, EAC tissues exhibited 4 differentially expressed miRNAs. One, *hsa-miR-21*, was upregulated and the other three (*hsa-miR-202*, *hsa-miR-203*, and *hsa-miR-205*) were downregulated. In ESCC, the expression levels of *hsa-miR-21* and *hsa-miR-223* were elevated compared with the normal tissue, while those of *hsa-miR-202* and *hsa-miR-375* were decreased (Table 1).

In EAC, the expression levels of the 4 differentially expressed miRNAs had no significant correlation with drinking, smoking, treatment methods or survival time of the patients ($P>0.05$). However, in ESCC, the expression level of *hsa-miR-21* showed a significant negative correlation with the survival time of the patients ($P=0.0029$, Figure 1). In ESCC patients, *hsa-miR-375* was positively associated with survival time ($P=0.0391$, Figure 1).

Differentially expressed genes in esophageal cancer tissues

Compared with the normal tissues, 641 downregulated genes and 628 upregulated genes were detected in

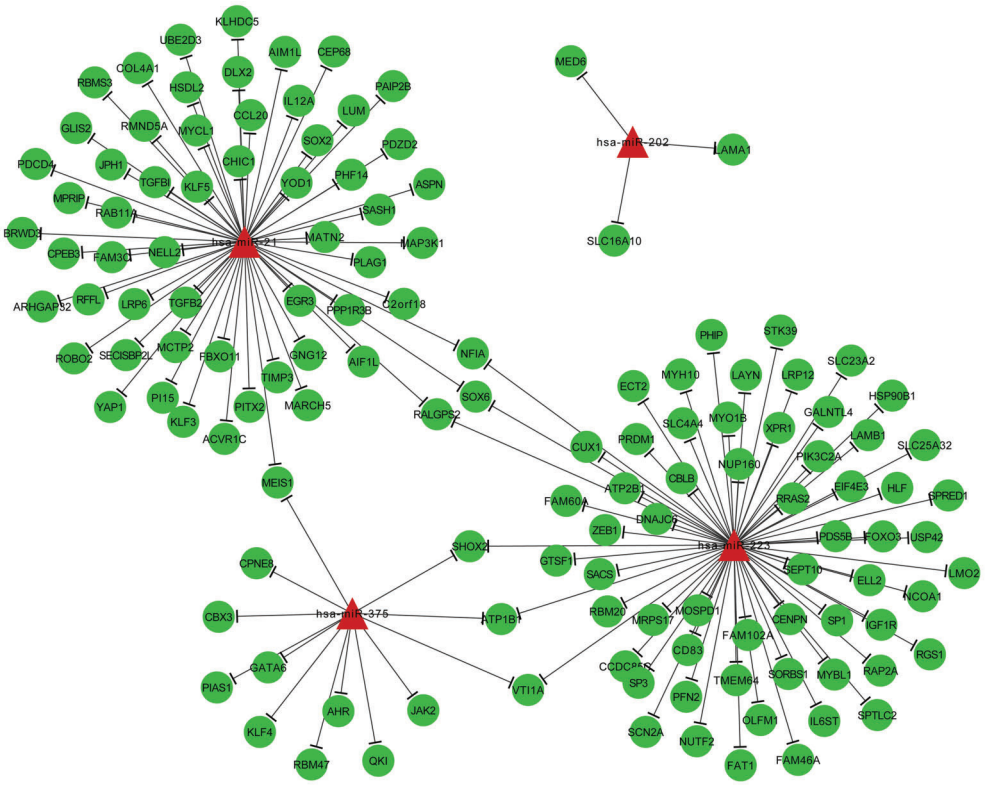

Figure 3. Regulatory network of selected miRNAs and their differentially expressed target genes in esophageal squamous-cell carcinoma.

the EAC samples. There were 1110 differentially expressed genes (516 with decreased expression and 594 with increased expression) detected in ESCC samples compared with their normal counterparts.

Differentially expressed miRNAs and their target genes prediction

As noted, there were 4 differentially expressed miRNAs (*hsa-miR-21*, *hsa-miR-202*, *hsa-miR-203* and *hsa-miR-205*) in EAC samples, and 4 (*hsa-miR-21*, *hsa-miR-202*, *hsa-miR-223* and *hsa-miR-375*) in ESCC samples. We tried to predict the target genes of each differentially expressed miRNA and construct their networks (Figures 2 and 3). For EAC, we screened 71 target genes for *hsa-miR-21*, 2 for *hsa-miR-202*, 152 for *hsa-miR-203* and 98 for *hsa-miR-205*. In ESCC, 59 genes were screened as the target genes for *hsa-miR-21*, 3 for *hsa-miR-202*, 65 for *hsa-miR-223* and 13 for *hsa-miR-375*. Furthermore, we found some genes that were the target genes of more than one differentially expressed miRNA. For example, *NFIB* was the target gene of *hsa-miR-21*, *hsa-miR-203* and *hsa-miR-205* in EAC, and *ATP1B* for *hsa-miR-223* and *hsa-miR-375* in ESCC.

We carried out KEGG pathway and GO enrichment analysis of the differentially expressed target genes in both EAC and ESCC. In EAC samples, 11 pathways were enriched in differentially expressed target genes, with the Hippo signaling pathway (hsa04290) as the most significant one (Table 2). Five of the remaining pathways, microRNAs in cancer (hsa05206), pancreatic cancer (hsa05212), pathways in cancer (hsa05200), basal cell carcinoma (hsa05217) and colorectal cancer (hsa05210), were reported as correlated with cancer. GO enrichment analysis revealed 3 GO items overrepresented with dysregulated target genes of the selected miRNAs, and these were items mainly involved in the transcription process (Table 3).

For ESCC, there were also 11 pathways (Table 2) that demonstrated enrichment of differentially expressed target genes, with the Jak-STAT signaling pathway (hsa04630) related to signal transduction as the most remarkable one. Two cancer-related pathways, proteoglycans in cancer (hsa05205) and transcriptional misregulation in cancers (hsa05202), were also indicated. GO enrichment analysis revealed 7 items enriched with dysregulated target genes (Table 3). Five of the seven items were also related to the transcription process.

Discussion

The results of differentially expressed miRNA indicated

Table 2. KEGG pathway enrichment analysis for the differentially expressed target genes of selected miRNAs in esophageal adenocarcinoma (EAC) and squamous-cell carcinoma (ESCC).

KEGG_id	Pathway description	Pathway subclass	P
EAC			
hsa04390	Hippo signaling pathway	Signal transduction	4.84E-04
hsa05206	MicroRNAs in cancer	Cancers	3.63E-03
hsa04350	TGF-beta signaling pathway	Signal transduction	7.15E-03
hsa05212	Pancreatic cancer	Cancers	1.48E-02
hsa04961	Endocrine and other factor-regulated calcium reabsorption	Excretory system	1.66E-02
hsa05200	Pathways in cancer	Cancers	2.49E-02
hsa04120	Ubiquitin mediated proteolysis	Folding, sorting and degradation	3.43E-02
hsa00564	Glycerophospholipid metabolism	Lipid metabolism	3.81E-02
hsa05217	Basal cell carcinoma	Cancers	3.99E-02
hsa04972	Pancreatic secretion	Digestive system	4.62E-02
hsa05210	Colorectal cancer	Cancers	4.91E-02
ESCC			
hsa04630	Jak-STAT signaling pathway	Signal transduction	4.28E-04
hsa04961	Endocrine and other factor-regulated calcium reabsorption	Excretory system	5.28E-03
hsa05145	Toxoplasmosis	Infectious diseases	8.36E-03
hsa05205	Proteoglycans in cancer	Cancers	1.11E-02
hsa04964	Proximal tubule bicarbonate reclamation	Excretory system	1.34E-02
hsa04972	Pancreatic secretion	Digestive system	1.40E-02
hsa04350	TGF-beta signaling pathway	Signal transduction	2.30E-02
hsa04120	Ubiquitin mediated proteolysis	Folding, sorting and degradation	2.66E-02
hsa04976	Bile secretion	Digestive system	3.14E-02
hsa04151	PI3K-Akt signaling pathway	Signal transduction	3.18E-02
hsa05202	Transcriptional misregulation in cancers	Cancers	4.07E-02

The hypergeometric distribution test was used for statistical analyses.

Table 3. Gene ontology (GO) enrichment analysis for the differentially expressed target genes of selected miRNAs in esophageal adenocarcinoma (EAC) and squamous-cell carcinoma (ESCC).

GO_id	GO_description	GO_class	P
EAC			
GO:0045893	Positive regulation of transcription, DNA-dependent	Process	1.07E-02
GO:0045892	Negative regulation of transcription, DNA-dependent	Process	1.94E-02
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	Process	3.02E-02
ESCC			
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	Process	2.35E-03
GO:0003700	Sequence-specific DNA binding transcription factor activity	Function	6.03E-03
GO:0005667	Transcription factor complex	Component	2.23E-02
GO:0045893	Positive regulation of transcription, DNA-dependent	Process	3.49E-02
GO:0044212	Transcription regulatory region DNA binding	Function	4.62E-02
GO:0003682	Chromatin binding	Function	4.99E-02
GO:0005606	Laminin-1 complex	Component	4.99E-02

The hypergeometric distribution test was used for statistical analyses.

elevated expression of *hsa-miR-21* and decreased expression of *hsa-miR-202* in both EAC and ESCC sample tissues. Saad and colleagues reported increased *hsa-miR-21* expression in EAC in a sample of 34 EAC and 46 normal tissues, which was subsequently confirmed by RT-PCR (12). At the same time, they also found reduced *hsa-miR-203* and *hsa-miR-205* expression in EAC tissues, which is consistent with the present study, and has also been confirmed by RT-PCR. Moreover, the clinical pathological characteristics analysis of this study showed that the low expression of *hsa-miR-203* was significantly associated with process and stage of EAC (12). *Hsa-miR-205* is thought to play a role in inhibiting cancer in the process of EAC formation (13); therefore the decreased expression may promote tumor occurrence. Up to now, no previous study has reported the differential expression of *hsa-miR-202* in correlation with esophageal cancer. However, *hsa-miR-202* has been confirmed related to other cancers, such as breast cancer (14) and gastric cancer (15). *Hsa-miR-21* was also found to be excessively expressed in ESCC tissues in other studies (16,17). It was reported that the increased expression of *hsa-miR-223* in ESCC might influence the expression of gene *FBXW7* (18), which affected the prognosis of patients. Therefore *hsa-miR-223* has also been regarded as a reliable diagnostic biomarker of ESCC (19). *MiR-375* has been seen as a tumor suppressor molecule, modulating *LDHB* action to curb the occurrence of tumor development (20), which is consistent with the *hsa-miR-375* result in this study. The miRNA result revealed a significant negative correlation of *hsa-miR-21* expression and positive correlation with ESCC survival time, which is consistent with a previous study (21) and indicates that *hsa-miR-21* and *hsa-miR-375* might be reliable prognostic markers of ESCC.

We predicted the target genes of the differentially expressed EAC and ESCC miRNAs, constructed their networks, and carried out enrichment pathway analysis of target genes. Among the differentially expressed miRNAs, *hsa-miR-202* was for the first time reported to be correlated with both EAC and ESCC. The Network diagram indicated that the differentially expressed target genes of *hsa-miR-202* were *FAR2* and *SLC16A10* for EAC, and *MED6*, *SLC16A10* and *LAMA1* for ESCC. None of these genes had been previously mentioned in relation with EAC or ESCC.

Five of the 11 pathways enriched for EAC are known to be cancer-related, and two, the Hippo signaling pathway (hsa04390) and the transforming growth factor (TGF)-beta-signaling pathway (hsa04350), are signal-transduction pathways. The Hippo signaling pathway is associated with other key signaling pathways such as the TGF-beta and Wnt mediated pathways, and has been reported to be associated with cancer (22). Previous studies have confirmed that the TGF-beta-signaling pathway promotes the development of EAC by activating Notch signaling and *SOX9* gene function (23). The most significantly target gene-enriched ESCC pathways were also signal transduction pathways: the Jak-STAT signaling pathway (hsa04630), TGF-beta signaling pathway (hsa04350), and PI3K-Akt signaling pathway (hsa04151). All three are known to be correlated with the development of ESCC (24). GO enrichment analysis revealed abnormal regulation of the transcription process in both EAC and ESCC, which may explain the clinical similarity of the two diseases.

Differentially expressed miRNA analysis selected 4 miRNAs associated with EAC and ESCC, among which *hsa-miR-21* and *hsa-miR-202* were shared by both diseases. *hsa-miR-202* was reported for the first time to be correlated with esophageal cancer in the present study. The pathway analysis of miRNA target genes suggested that differentially expressed miRNA target genes were mainly involved in cancer-related and signal-transduction pathways. Functional categories of these target genes were related to transcriptional regulation. Our results indicated potential target miRNAs for future therapeutic investigations.

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