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...
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).

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.

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

Figure 1. Survival analyses of hsa-miR-21 and hsa-miR-375 in esophageal squamous-cell carcinoma patients.
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
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-203, and hsa-miR-205) 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-203, and 13 for hsa-miR-205. 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-203 and hsa-miR-205 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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