Microarray analysis reveals altered expression of multiple circular RNAs in the pathogenesis of esophageal squamous cell carcinoma

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Background: Esophageal carcinoma remains one of the most prevalent malignancies worldwide and has a poor prognosis. Esophageal squamous cell carcinoma (ESCC) is the predominant type of esophageal carcinoma in Eastern Asia. The predilection site and biological behavior of ESCC differ from those of esophageal adenocarcinoma among patients in Western countries, indicating that ESCC has a distinctive oncogenesis mechanism. There are only a few studies reporting the role of circular RNAs (circRNAs) in the molecular mechanisms of ESCC. The purpose of this study was to identify the specific circRNAs involved in ESCC pathogenesis.

Methods: Cancerous tissues were acquired from surgical specimens from five ESCC patients, and adjacent non-neoplastic tissues were also collected as controls. All samples were analyzed by Arraystar Human circRNA Arrays. Differentially expressed circRNAs between two groups were identified by bioinformatics analysis, and seven circRNAs were randomly selected for further validation by qRT-PCR. circRNA/microRNA interactions were predicted with an in-house miRNA target prediction software from Arraystar.

Results: Compared with the adjacent non-neoplastic tissues, 267 circRNAs were significantly differentially expressed in ESCC tissues (fold change >1.5, P value <0.05). Among the seven selected circRNAs, the microarray data for five circRNAs were validated by qRT-PCR—two were upregulated (hsa_circRNA_101125 and hsa_circRNA_406826), and three were downregulated (hsa_circRNA_101004, hsa_circRNA_103836, and hsa_circRNA_101839). Finally, potential target miRNAs for the five confirmed circRNAs were predicted.

Conclusions: Our study demonstrates that multiple differentially expressed circRNAs are involved in the process of ESCC pathogenesis. Hsa_circRNA_101125 and hsa_circRNA_406826 may be potential new biomarkers for the diagnosis and treatment of ESCC. However, further studies are needed to clarify their specific regulatory mechanisms.

Keywords: Circular RNA (circRNA); microarray; esophageal squamous cell carcinoma (ESCC); microRNA; pathogenesis

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Introduction

Esophageal carcinoma is the eighth most prevalent and sixth most fatal malignancy worldwide (1). In China, esophageal carcinoma is the fourth most common cancer with a much higher incidence rate than in Western countries (2). Esophageal adenocarcinoma (EAC) and squamous cell carcinoma (ESCC) are the two main types of esophageal carcinoma. Unlike Western countries, where EAC is rapidly increasing in incidence and is the dominating type (3), ESCC is the most common type of esophageal cancer in China (2). It is well known that the predilection site and biological behavior differ between ESCC and EAC, indicating that a distinctive molecular biological mechanism is involved in the development and metastasis of ESCC. Many controversies exist with respect to therapy of ESCC, including the extent of lymphadenectomy (4) and the clinical value of preoperative chemoradiotherapy or chemotherapy alone (5). These unclear issues in clinical practice are the manifestation of an incomplete understanding of the molecular and genetic basis of ESCC and may partially be responsible for the poor prognosis of ESCC at present. The development of new biomarkers that could act as diagnostic indicators, therapeutic targets, or prognosis predictors is eagerly anticipated to improve the diagnosis and treatment of ESCC.

Circular RNAs (circRNAs) are an emerging class of endogenous noncoding RNAs characterized by a particular covalently closed-loop molecular structure resulting from a 3’ to 5’ end joining that is stable and resistant to degradation. circRNAs were first discovered in eukaryotic cells in 1979 by Hsu and Coca-Prados using electron microscopy (6); however, research on circRNA in the following decades has been slow due to limited understanding and lack of specific detection technology. In the past few years, the introduction of high-throughput RNA sequencing technologies has made it possible to directly detect circRNA sequences in the whole genome, which has shed new light on circRNA studies. Recent studies revealed that circRNAs are widely involved in a variety of diseases such as neurodegenerative diseases (7), hematological malignancies (8), and gastric cancer (9) through miRNA sponging or other regulatory roles (10,11).

At present, there are only a small number of studies reporting the function of circRNA in ESCC (12-14), and the specific molecular mechanism by which circRNAs regulate ESCC is still unclear. In this study, we performed a circRNA microarray analysis to identify differentially expressed circRNAs between ESCC and adjacent non-neoplastic tissues and then explored their underlying function by prediction of circRNA/miRNA interactions. Our results suggest that multiple circRNAs may be involved in the pathogenesis of ESCC.

Methods

Patients and sample collection

ESCC specimens were obtained from 5 patients (3 males and 2 females with age ranging from 51 to 58 years) with postoperative pathologic stage III disease (IIIa in 4 and IIIc in 1 patient) who underwent minimally invasive esophagectomy and three-field lymph node dissection between May 2016 and June 2016 at Fujian Union Hospital (Table 1). The control group consisted of adjacent non-neoplastic tissues collected from the same patients. All samples were preserved in liquid nitrogen immediately after resection and then transferred to the Institute of Cardiothoracic Surgery of Fujian Union Hospital for storage at −80 °C.

RNA extraction and sample quality control

Total RNA was extracted from five pairs of tumor and adjacent non-neoplastic tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. The purity and concentration of total RNA samples were measured and quantified by NanoDrop ND-1000 (NanoDrop Technologies/Thermo Scientific, Wilmington, DE, USA). The integrity of RNA was tested by electrophoresis on a denaturing agarose gel.

RNA labeling and hybridization

We completed RNA labeling and array hybridization.
following the manufacturer’s protocol (Arraystar Inc., Rockville, MD, USA). Extracted total RNAs were treated with RNase R (Epicentre, Madison, WI, USA) so that linear RNAs were removed and circular RNAs were enriched. The enriched circular RNAs were then amplified and transcribed into fluorescent cRNA with random primers following the protocol of Arraystar Super RNA Labeling Kit. RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to purify the labeled cRNAs (pmol Cy3/μg cRNA) and NanoDrop ND-1000 was used to determine the concentration and specific activity of the labeled cRNAs in order to assess labeling efficiency. Next, 5 μL of 10x blocking agent and 1 μL of 25x fragmentation buffer were added into 1 μg of each labeled cRNA sample, followed by heating the mixture at 60 °C for 30 min to fragment the labeled cRNA. Finally, the labeled cRNA samples were diluted by adding 25 μL of 2x hybridization buffer. A 50 μL volume of the hybridized solution was then dispensed into a gasket slide, which was assembled with a circRNA expression microarray slide. After incubation at 65 °C for 17 hours in an Agilent hybridization oven, the hybridized arrays were washed and fixed and then scanned by the Agilent Scanner to generate circRNAs expression profiles for microarray data analysis.

Microarray data analysis

Raw data extraction was performed after the scanned images were imported into Agilent Feature Extraction software. Quantile normalization of the raw data and subsequent data processing were performed using the limma package in R software. Low intensity filtering was then performed, and the circRNAs with flags in “Present” or “Marginal” (“All Targets Value”) in at least 5 out of the 10 samples were selected for further analyses. To compare differences in circRNAs expression profile between the ESCC and adjacent non-tumor tissue groups, we calculated the “fold change” (i.e., the ratio of the group averages) between the groups for each circRNA. t-test was used to estimate statistical significance of the differences. Significant differential expression of circRNAs was defined by fold change >1.5 and P value <0.05 in this study. We selected Data/Sort & Filter function in Microsoft Excel to filter the acquired data and rank the differentially expressed circRNAs based on parameters such as fold change and P value.

qRT-PCR validation of candidate circRNAs

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to validate the differentially expressed circRNAs between ESCC tissues and control tissues. The extracted total RNA was reverse transcribed into cDNA using Super Script III Reverse Transcriptase (Invitrogen) following a standard protocol. The relative expression of circRNAs was determined using the ViiA 7 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). circRNA levels were normalized using β-actin as the internal control. The data were calculated by the 2−ΔΔCt method, and quantitative PCR was performed in triplicate. The specific primers used in this study are listed in Table 2.

Prediction of circRNA/miRNA interaction

An in-house miRNA target prediction software from Arraystar, which was based on TargetScan (15) & miRanda (16) applications and databases, was used to facilitate the prediction of circRNA/microRNA interaction, and the differentially expressed circRNAs between groups were annotated in detail using the circRNA/miRNA interaction information.

Statistical analyses

All data are presented as the mean ± standard deviation (SD). Student’s t-test was used or comparisons between groups. A P value of <0.05 was considered statistically significant.

Results

Differentially expressed circRNAs based on microarray analysis

In this study, in total, 12,652 circRNAs were detected by the Arraystar Human circRNA Array. We used a box plot to examine and compare the distributions of expression values for the samples after normalization. As shown in Figure 1, the distribution of circRNAs was similar among all tested samples. Hierarchical clustering was performed to analyze circRNA expression in order to formulate hypotheses about the relationships among samples. The hierarchical clustering showed distinguishable circRNA expression profiles among the samples (Figure 2). A scatter plot was used to facilitate assessment of the variation in circRNA expression between the ESCC and non-neoplastic tissue groups (Figure 3). Volcano plots were then used to determine differential expression between the two
Table 2 Primers used in this study for qRT-PCR validation

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences</th>
<th>Annealing temperature (°C)</th>
<th>Length (bp)</th>
</tr>
</thead>
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<tr>
<td>β-actin (H)</td>
<td>F: 5’ GTGGCCGAGGACTTTGATTG 3’</td>
<td>60</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>R: 5’ CCTGTAACACGACATCTCATT 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa_circRNA_404458</td>
<td>F: 5’ CAACACCAGCCTGGCTTAT 3’</td>
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<td>185</td>
</tr>
<tr>
<td></td>
<td>R: 5’ GTGTGCTGACTTGGCCCTCTGT 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa_circRNA_100980</td>
<td>F: 5’ GCTCCTTGACACGTCTGGTG 3’</td>
<td>60</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>R: 5’ GAGGGTCTGCGTGGTGATT 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa_circRNA_101125</td>
<td>F: 5’ TCCATAACACAGCTTGAGTG 3’</td>
<td>60</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>R: 5’ GATGATGGACTGAGGAGG 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa_circRNA_101004</td>
<td>F: 5’ TCTCCGAGAGGAGGTGTC 3’</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>R: 5’ CCATTATGAAGAGGCTGTTCTG 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa_circRNA_101839</td>
<td>F: 5’ TCCAGCTTGCTCCCTGG 3’</td>
<td>60</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>R: 5’ AACAGGGATCTCCCTGATTA 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa_circRNA_103836</td>
<td>F: 5’ GCCACAGCAATCTCCCTCAT 3’</td>
<td>60</td>
<td>158</td>
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<tr>
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<td>R: 5’ ACTATGATGCTAAACACCT 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa_circRNA_406826</td>
<td>F: 5’ AAATAAGCCCTTAAGAACCCTGAC 3’</td>
<td>60</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>R: 5’ ACTATGATGCTAAACACCT 3’</td>
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</tr>
</tbody>
</table>

qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

Figure 1 Box plot of circRNA distribution in all samples. A box plot was introduced into this study to inspect and compare the distribution of expression values of all samples after normalization. The distribution of circRNAs was similar among all tested samples.

Validation of selected circRNAs using qRT-PCR

We randomly selected seven significantly differentially expressed circRNAs for validation in all samples by qRT-PCR, and among them, five circRNAs exhibited expression patterns consistent with the microarray data. These five circRNAs included two upregulated (hsa_circRNA_101125 and hsa_circRNA_406826) and three downregulated (hsa_circRNA_101004, hsa_circRNA_103836 and hsa_circRNA_101839) circRNAs (Table 3). The results of qRT-PCR validation are shown in Figure 7.
Hierarchical clustering for analysis of circRNA expression among samples. circRNA expression was analyzed by hierarchical clustering in order to formulate hypotheses about the relationships among samples. The results of hierarchical clustering revealed distinguishable circRNA expression profiles among the samples. In this plot, each column represents the expression profile of a sample, where ‘A’ represents tumor tissues and ‘B’ represents the adjacent non-neoplastic tissues. Each row represents a particular circRNA. Red denotes higher expression level, and green denotes a lower expression level.

Prediction of circRNA/microRNA interactions

According to the ceRNA hypothesis and a previous report (10), circRNAs may interact with miRNAs through miRNA response elements (MREs) and thus function as miRNA sponges. Therefore, we used an in-house miRNA target prediction software from Arraystar to predict miRNAs that may bind to the selected circRNAs. The information on circRNA/miRNA interactions for the five confirmed circRNAs including the most likely potential targeted miRNAs is summarized in Figure 8.

Discussion

In the classic gene expression model, known as the central dogma, the expression and adjustment of genetic and functional information within the genome are mediated by linear RNAs. The emergence of circRNAs represented both a challenge and a supplement to the central dogma. Recent studies have shown that circRNAs are highly abundant in the cytoplasm of eukaryotic cells, at levels comparable to or even exceeding those of their canonical linear isoforms (17,18). Moreover, circRNAs are structurally stable, highly evolutionarily conserved, and tissue specific, with multiple regulatory functions (18-22). The mechanism by which circRNAs exert regulatory functions is far from clear. Exonic circRNAs, the most abundant type, have been found to function as miRNA sponges. ciRS-7, one of the best known circRNAs, contains more than 70 MREs. ciRS-7 can specifically bind to miR-7 and strongly suppress miR-
Figure 4 Visualization of differential expression of circRNAs between groups by volcano plots. The red dots in the plot represents circRNAs with statistically significant altered expression (fold change >1.5 and P<0.05).

Figure 5 Chromosomal distribution of the differentially expressed circRNAs.

Figure 6 Classification of the differentially expressed circRNAs.

Table 3 Biological information on the 5 differentially expressed circRNAs validated by qRT-PCR

<table>
<thead>
<tr>
<th>CircRNA</th>
<th>P value</th>
<th>FDR</th>
<th>Fold change</th>
<th>Regulation</th>
<th>Chrom</th>
<th>CircRNA type</th>
<th>Strand</th>
<th>Best transcript</th>
<th>Gene symbol</th>
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<tr>
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<td>0.012689931</td>
<td>0.658963807</td>
<td>1.74054</td>
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<td>chr12</td>
<td>exonic</td>
<td>+</td>
<td>NM_018370</td>
<td>DRAM1</td>
</tr>
<tr>
<td>hsa_circRNA_406826</td>
<td>0.016903098</td>
<td>0.66764039</td>
<td>2.8077028</td>
<td>Up</td>
<td>chr6</td>
<td>exonic</td>
<td>+</td>
<td>NM_014845</td>
<td>FIG4</td>
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<tr>
<td>hsa_circRNA_101004</td>
<td>0.015303904</td>
<td>0.658963807</td>
<td>2.245734</td>
<td>Down</td>
<td>chr12</td>
<td>exonic</td>
<td>−</td>
<td>NM_080730</td>
<td>IFFO1</td>
</tr>
<tr>
<td>hsa_circRNA_101839</td>
<td>0.03912424</td>
<td>0.717378352</td>
<td>1.7768939</td>
<td>Down</td>
<td>chr16</td>
<td>exonic</td>
<td>+</td>
<td>NM_003983</td>
<td>SLC7A6</td>
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<tr>
<td>hsa_circRNA_103836</td>
<td>0.017769049</td>
<td>0.66764039</td>
<td>1.67992</td>
<td>Down</td>
<td>chr5</td>
<td>exonic</td>
<td>+</td>
<td>NM_024615</td>
<td>PARP8</td>
</tr>
</tbody>
</table>

+ represents sense strand; − represents antisense strand. qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

7 activity (10,23). Likewise, the sex-determining region Y (SRY) serves as a miR-138 sponge (10,24). In the past few years, hsa_circ_001569 (25), circPVT1 (26), circTCF25 (27) and cir-ITCH (12) were discovered as new circRNAs with miRNA sponge function. These circRNAs have been shown to be involved in the development of several kinds of carcinoma by sequestering tumor-related miRNAs. In addition, exonic circRNAs can also function by regulating transcription (28) and interacting with RNA binding proteins (29), but elucidation of specific mechanisms requires further investigation. For other circRNA types, such as intronic circRNAs and exon-intron circRNAs...
(EiCIRNAS), there have only been a few relevant studies. Existing reports reveal that these circRNAs regulate transcription by enhancing the expression of their parental gene (11,30). The characteristics mentioned above qualify circRNAs as a promising target for the diagnosis and treatment of diseases.

There are very few studies on circRNAs in ESCC. Li et al. (12) discovered that cir-ITCH was expressed at low levels in ESCC compared with peritumoral tissues. Furthermore, it was shown to function as a sponge for miR-7, miR-17, and miR-214, subsequently increasing the level of ITCH and thus playing an inhibitory role in ESCC by regulating the Wnt pathway. Su et al. (13) performed circRNA microarray and bioinformatics analyses to compare expression profiles of circRNA in radioresistant and non-radioresistant ESCC cells. They reported a comprehensive expression and functional profile of differentially expressed circRNAs in radioresistant esophageal cancer cells, among which circRNA_001059 and circRNA_000167 were identified as the two largest nodes in the circRNA/microRNA co-expression network. Xia et al. (14) found that hsa_circ_0067934 was upregulated in ESCC tumor tissues, and showed that in vitro silencing of hsa_circ_0067934 resulted in the inhibition of proliferation and migration of ESCC cells and cell cycle arrest. This suggested that circ_0067934 has potential as a novel biomarker and therapeutic target of ESCC. Although the specific regulatory mechanism of circRNA in ESCC was unclear, the inspiring results of these studies confirmed that circRNAs play a role in the process of ESCC pathogenesis.

In this study, we compared circRNA expression profiles between ESCC and adjacent non-neoplastic tissues using Arraystar Human Circular RNA Microarrays to investigate the mechanism of ESCC pathogenesis. Multiple differentially expressed circRNAs were observed and validated in the ESCC tissues compared with the adjacent non-neoplastic tissues. Our results revealed that these dysregulated circRNAs might be involved in the process of ESCC pathogenesis, especially in the case of hsa_circRNA_101125 and hsa_circRNA_406826, which showed significantly upregulated expression in the ESCC tissues. According to the prediction results for circRNA/microRNA interactions, hsa_circRNA_101125 has a potential binding site for miR-143-3p and thus might function as a miR-
miR-203a-3p. According to previous reports, both miR-143-3p and miR-203a-3p are downregulated in ESCC tissues (31,32), and further in vivo and in vitro studies have provided strong evidence that both miRNAs act as tumor suppressors in ESCC. Enforced expression of miR-143-3p and miR-203a-3p inhibited growth, proliferation, migration and invasion of ESCC cells through different mechanisms, while their reduced expression was associated with poor patient survival (33-43). Specific binding of hsa_circRNA_101125 and miR-143-3p or that of hsa_circRNA_406826 and miRNA-203a-3p might relieve the suppression of tumor-related

![Figure 8](image-url)
genes by miRNAs, thus promoting the occurrence and development of ESCC. Regarding the three circRNAs with downregulated expression, we believe that miRNA sponging may not be their main function in ESCC pathogenesis, and the mechanism associated with their low expression level remains to be clarified.

Conclusions

In summary, we detected differential circRNA expression profiles between ESCC and adjacent non-neoplastic tissues. Our results demonstrated that multiple circRNAs were aberrantly expressed in ESCC tissues and might be involved in the process of ESCC pathogenesis. In particular, hsa_circRNA_101125 and hsa_circRNA_406826 were significantly upregulated in ESCC, and these circRNAs might serve as potential diagnostic or therapeutic markers in ESCC. Further studies are needed to clarify their specific regulatory mechanisms.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by the ethics committee of Fujian Medical University Union Hospital (No. 2016ky012), and written informed consent was obtained from all patients. The experiments were performed according to the Ethical Guidelines for Human Genome/Gene Research issued by the Chinese Government.

References
