RESEARCH ARTICLE

Screening of MicroRNA in Patients with Esophageal Cancer at Same Tumor Node Metastasis Stage with Different Prognoses

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Abstract

Patients at the same pathological stage of esophageal cancer (EC) that received the same surgical therapy by the same surgeon may have distinct prognoses. The current study aimed to explore the possibility of differentially-expressed microRNAs (miRNAs) underlying this phenomenon. Samples were collected from EC patients at the same tumor node metastasis (TNM) stage but with different prognoses. Paracancerous normal tissues were taken as controls. The specimens were histopathologically analyzed. Differentially-expressed miRNAs were analyzed using real-time quantitative reverse transcription polymerase chain reaction. Compared with patients with poor prognosis, those with good prognosis exhibited 88 two-fold or more than two-fold increased miRNA fragments and 4 half-decreased miRNAs. The most noticeably up-regulated miRNAs included hsa-miR-31, hsa-miR-196b, hsa-miR-652, hsa-miR-125a-5p, hsa-miR-146b, hsa-miR-200c, hsa-miR-23b, hsa-miR-29a, hsa-miR-186, hsa-miR-205, hsa-miR-376a, hsa-miR-410, hsa-miR-532-3p, and hsa-miR-598, whereas the most significantly-downregulated miRNAs were hsa-let-7e, hsa-miR-130b, and hsa-miR-103. EC patients at same TNM stage but with different prognoses show differentially-expressed miRNAs.

Keywords: Esophageal cancer - tumor node metastasis (TNM) - prognosis - microRNA

Asian Pacific J Cancer Prev, **14 (1)**, 139-143

Introduction

Esophageal cancer (EC) is a common digestive tract malignancy, ranking second among various death-caused tumors of the digestive system. EC leads to yearly deaths up to 300,000 worldwide, approximately half of which occur in China, making China occupy the first position in EC incidence (Mao et al., 2011; Zhang et al., 2012). Although chemotherapy, radiotherapy and concurrent chemoradiotherapy for EC have got great progress, surgery remains the major treatment method. However, surgery cannot achieve satisfactory curative effect, only with a 5-year survival rate of about 30%. In particular, early lymph node metastasis after early EC surgery greatly destroys both patients' and surgeons' confidence and some patients even become dubious about the curative effect of surgery (Griffin et al., 2011; Zhu et al., 2011; Usami et al., 2012).

Numerous factors can influence the prognosis of EC. The present major method for EC prognosis prediction is still based on traditional tumor node metastasis (TNM) staging (Talsma et al., 2012; Wang et al., 2012). Although the classic TNM staging method plays an important role in the diagnosis and treatment of EC in clinical practice, it fails to accurately reflect the progression of pathogenetic condition due to tumor heterogeneity as well as patients' individual differences. Therefore, finding a new molecular marker for prognosis prediction as a beneficial complement to TNM pathological staging is of great significance. The initiation and development of EC involve the mutation of numerous oncogenes and anti-oncogenes, leading to varied zymological changes, and thus are characterized by the synergetic effect of multiple genes, factors, and steps. Nowadays, seeking the molecular biological markers associated with the diagnosis and prognosis of EC has become a research focus in this field (Zhou et al., 2009; Kim et al., 2010; Zhu et al., 2010). miRNA is a class of endogenous non-coding regulatory RNAs constituted by more than 20 nucleotides. It regulates gene expression through sequence specific translational suppression or mRNA cleavage, thereby participating in a series of important biological processes such as cell development, proliferation, differentiation, and apoptosis. Recent studies have found that miRNA has the effects of both oncogenes and anti-oncogenes and plays an important role in the initiation and development of tumors

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(Zhou et al., 2009; He et al., 2012). Numerous miRNAs directly participate in the initiation and development of tumors, and miRNA spectra are correlated with the diagnosis, staging, progression, and prognosis of EC (Lin et al., 2012; Xu et al., 2012; Yang et al., 2013). Studies using microarray technology on the miRNA expression spectra of 31 EC tissue samples have discovered that 46 miRNAs exhibit abnormal expression, 7 of which exhibit significant differences, compared with those in normal tissues; furthermore, the high expression of has-miR-103/107 is closely correlated with low survival rate. To date, has-miR-335, has-miR-181d, has-miR-25, has-miR-7, and has-miR-495 have been confirmed correlated with histopathological stages of EC. In addition, abnormal miRNA expression spectra are different between esophageal squamous cell carcinoma (ESCC) tissues and esophageal adenocarcinoma (EAC) tissues (Guo et al., 2008; Mathée et al., 2009).

Although some studies on the correlation between miRNA and EC have been reported, a study on whether the expression of miRNAs in EC patients at same pathological stage but with different prognoses is different has not yet been found in literature. EC patients at same pathological stage that receive same surgical therapy by the same surgeon may have distinct prognoses. Whether it is different miRNA expression among patients that leads to different postoperative prognoses remains to be explored. In this study, to explore the mechanisms underlying the metastasis and prognosis of EC, differentially-expressed miRNAs in patients at same TNM stage but with different prognoses were determined.

Materials and Methods

Clinical data

Two paraffin-embedded samples from patients at the same pathological stage but with different prognoses that received treatment at the First Affiliated Hospital of Xinxiang Medical University were collected. Both samples were made from primary lesions after naïve resection. Meanwhile, paracancerous normal esophageal mucous membranes, 8 cm distant to the verge of the tumor tissue, were taken as controls. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Xinxiang Medical University. Written informed consent was obtained from all participants.

Hematoxylin and eosin (HE) staining

The samples were fixed in 10% neutral formalin for 4-6 h, routinely dehydrated, embedded, sectioned, and then HE stained. Histopathological characteristics of each sample were observed under a microscope. The pathological stage was confirmed by more than two pathologists.

Sample treatment and RNA extraction

Total RNA was extracted from the oncocytes in the sections using the Trizol one-step method according to the instructions indicated in the kit (Invitrogen, USA). Total RNA was collected according to the instructions indicated in the Recover All Total Nucleic Acid Isolation Kit TRIZOL REAGENT (AB, USA). Light absorption values were respectively read at 230, 260, and 280 nm using spectrophotometry to determine purity and density. Formaldehyde-agarose gel electrophoresis was performed for the 28 s/18 s ratio to determine the purity and integrity of the total RNA sample.

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

miRNAs were quantitated using RT-qPCR. The Megaplex pool reverse transcription system with a total volume of 4.5 μL contained Megaplex™ RT primers (10 ×) at 0.8 μl, dNTPs with dTTP (100 mM) at 0.2 μl, MultiScribe™ reverse transcriptase (50 U/μL) at 1.5 μL, 10 × RT buffer at 0.8 μL, $MgCl_2$ (25 mM) at 0.9 μL, RNase inhibitor (20 U/μL) at 0.1 μL, and nuclease-free water at 0. μL. The solution was reversely blended six times and then slightly centrifuged. Total RNA at 3 μl (1-350 ng) was added into the reaction tube, reversely blended six times, slightly centrifuged, and then placed on ice for 5 min. The PCR conditions consisted of 40 cycles of 16 °C for 2 min, 42 °C for 1 min, and 50 °C for 1 s, followed by 85 °C for 5 min and a termination step at 4 °C. The obtained reverse transcription products were placed on ice.

The pre-amplification PCR system with a total volume of 25 μL was prepared, including: TaqMan® PreAmp Master Mix (2 ×) at 12.5 μL, Megaplex™ PreAmp primers (10 ×) at 2.5 μL, RT product at 2.5 μL, and nuclease-free water at 7.5μL. The solution was reversely blended six times, slightly centrifuged, and then placed on ice for 5 min. The PCR conditions consisted of 95 °C for 10 min and 12 cycles of 55 °C for 2 min, 72 °C for 2 min, 95 °C for 15 s, and 60 °C for 4 min, followed by 99.9 °C for 10 min and a termination step at 4 °C.

0.1 × TE (pH 8.0) at 75 μL was added into the pre-amplification tube, reversely blended six times, and then stored at -20 °C. Taqman universal PCR master mix was swirled for blending.

Regents with a total volume of 900 μL, including TaqMan® universal PCR master mix, No AmpErase® UNG (2 ×) at 450 μL, diluted PreAmp product at 9μL, and nuclease-free water at 441 μL, were added into a 1.5 ml centrifuge tube, reversely blended six times, and then slightly centrifuged.

The PCR reaction mix at 100 μL was applied to each sampling point and then twice centrifuged at 1200 rpm (1 min each time). The reaction conditions consisted of 94.5 °C for 10 min and 40 cycles of 97 °C for 30 s and 59.7 °C for 1 min.

Statistical analysis

U6 snRNA housekeeping gene was used as internal reference. The relative quantitative method was used. Gene expression was calculated based on the following formula: $F = 2^{-\triangle\triangle ct}$ where $\triangle\triangle ct$ = (ct mean of the target gene in the test sample - that of the housekeeping gene in the test sample) - (ct mean of the target gene in the control sample - that of the housekeeping gene in the control sample). A higher F value indicates higher expression.

Table 1. Comparative Analysis of Clinicopathologic Classifications

Case	Gender	Age	Postoperative treatment	Operation method	Anastomotic methods	Pathology	Prognosis
Prognosis well	Male	56	Chemotherapy 2 times	Radical resection of esophageal carcinoma	Neck manual anastomosis	1	3
Prognosis bad	Female	63	Chemotherapy 2 times	Radical resection of esophageal carcinoma	Neck manual anastomosis	2	4

1, High differentiated squamous cell carcinoma in middle esophageal, ulcer type, invaded the outer membrane, without lymph node metastasis T3N0M0; 2, •High differentiated squamous cell carcinoma in middle esophageal, tumor length was 3.3cm, invaded the shallow muscular layer, without lymph node metastasis T2N0M0; 3, Surviving five years after surgery; 4, The neck lymph node metastasis occurred four months after surgery, died 1 year after surgery

Table 2. Ct Value of the Sample

Detector	U6 snRNA	U6 snRNA	Hsa-miR-16	Hsa-miR-16	Hsa-miR-16	Hsa-miR-92a	Hsa-miR-92a	Hsa-miR-92a
Normal tissue	20.6	20.7	24.8	24.8	24.9	36.0	35.2	36.3
Sample with well prognosis	21.4	21.4	31.8	31.9	31.8	33.9	34.3	34.2
Sample with poor prognosis	20.5	20.6	27.4	27.3	27.2	31.5	31.8	31.4

Table 3. miRNA Expression in Different Samples under Same TNM Stage

miRNA	Prognosis well/ Normal tissue	Prognosis bad/ Normal tissue	Prognosis well / prognosis bad Regulation	Fold
hsa-miR-31	39.07105482	2.569872884	Up	15.20349705
hsa-miR-101	38.52937824	4.696164679	Up	8.204435081
hsa-miR-196b	38.07404644	2.557593531	Up	14.88666826
hsa-miR-652	36.57946323	2.442979911	Up	14.97329678
hsa-miR-125a-5p	19.51518183	1.381954388	Up	14.121437
hsa-miR-146b	9.122692571	0.611685931	Up	14.91401404
hsa-miR-200c	8.808503887	0.615668644	Up	14.30721536
hsa-miR-1274B	7.074892858	0.620543263	Up	11.4011275
hsa-miR-23b	2.380093088	0.158999044	Up	14.96922888
hsa-miR-29a	4.782009513	0.313148853	Up	15.27072338
hsa-miR-186	4.614089537	0.300019879	Up	15.37927938
hsa-miR-205	4.531566952	0.302132021	Up	14.99863184
hsa-miR-361	2.429433597	0.160783916	Up	15.10992925
hsa-miR-376a	2.253674249	0.153700845	Up	14.66273169
hsa-miR-410	4.442026501	0.285279305	Up	15.57079823
hsa-miR-532-3p	2.277040576	0.150266571	Up	15.15334091
hsa-miR-598	2.431908586	0.155646711	Up	15.62454205
hsa-miR-625	2.243425071	0.280411418	Up	8.000476899
hsa-let-7e	0.300872	1.178887	Down	0.255217
hsa-miR-130b	0. 4124613	2.6198309	Down	0.157438138
hsa-miR-103	1.35687297	3.86370304	Down	0.351184590
hsa-miR-144	4.8404750	11.3953810	Down	0.4247751786

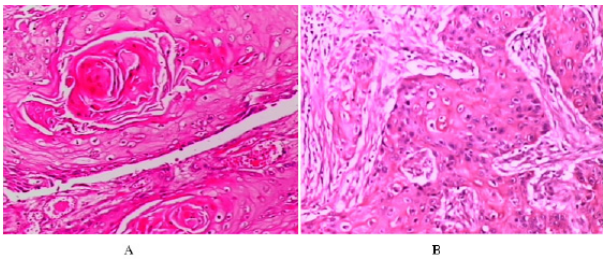

Figure 1. Both Samples were HE Stained. A) the sample of good prognosis. B) the sample of poor prognosis (400×)

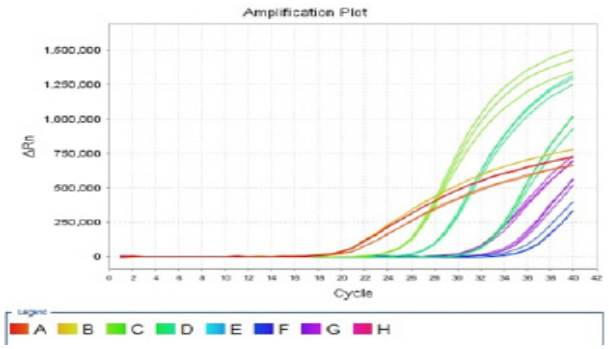

Figure 2. The Gene Amplification Curves of the Samples. A-B) U6 snRNA; C-E) Hsa-miR-16; F-H) Hsa-miR-92a

Results

Pathological data

Both patients were subjected to EC radical operation, manual cervical anastomosis, and twice chemotherapies. Both were diagnosed with ulcerative highly-differentiated esophageal squamous-cell carcinoma (Figure 1) which invaded to the outer membrane but without lymph node metastasis. However, they had different prognoses: One still survived 5 years after operation, whereas the other suffered from cervical lymph node metastasis at 4 months and died at about 1 year (Table 1).

Single-point amplification analysis

Gene amplification curves are shown in Figure 2 and Table 2. The pre-amplified samples required site U6 snRNA amplification ct less than 25. All the samples met the quality requirement of the miRNA array project. Thus, further experiment was carried out.

miRNA array analysis

Total RNA was extracted. miRNA expression in

different tissues was compared using qRT-PCR. A total of 770 human miRNAs were detected, most of which have been known well. Compared with the poor-prognosis sample, the good-prognosis sample exhibited 88 double-upregulated miRNAs and 8 downregulated miRNAs, with the most noticeable upregulation as 15 times as high. A total of 18 miRNAs were upregulated more than 8 times and the most noticeably upregulated were hsa-miR-31, hsa-miR-196b, hsa-miR-652, hsa-miR-125a-5p, hsa-miR-146b, hsa-miR-200c, hsa-miR-23b, hsa-miR-29a, hsa-miR-186, hsa-miR-205, hsa-miR-376a, hsa-miR-410, hsa-miR-532-3p, and hsa-miR-598. A total of 8 miRNA were downregulated, 4 of which were donwregulated below half, with hsa-let-7e, hsa-miR-130b, and hsa-miR-103 and hsa-miR-144 as the most marked ones (Table 3).

Discussion

Tumor markers (TMs) are a class of substances which reflect the existence and growth of tumors; TMs are synthesized and secreted by tumor cells through gene expression or abnormally produced due to the reactions of the organism to tumors (Lumen et al., 2012; Nitsche et al., 2012). EC is a common malignancy. Accurate judgments of the clinical stage and prognosis of EC are important bases for clinicians to take a rational treatment protocol. Although traditional examination methods such as computed tomography, X-ray barium meal fluoroscopy, gastroscopy, and B-ultrasonography can discover large metastases, they lack sufficient sensitivity to micrometastases in blood and tissues (Nakashima et al., 2012).

Although more than one hundred TMs have been found to date, none of them can serve as an ideal marker for the metastasis and prognosis of EC in clinical practice. In addition, single marker detection always has the drawbacks of low specificity and low positive rate. These drawbacks greatly limit its application in the diagnosis of EC as well as in the prediction of the course and prognosis of EC (Koppert et al., 2005; Sarbia et al., 2007). Tumor metastasis is not only influenced by protein coding genes but also regulated by non-coding genes. miRNAs are subject to non-coding genes which principally negatively regulate gene expression at post-transcriptional level. miRNA expression is highly preservative, sequential, and tissue specific. Meanwhile, their expression is regulated by time and space. Abnormalities in miRNA expression can coexist in the same tumor, and some miRNAs can influence expression levels with each other. Studies have shown the abnormal expression of some miRNAs is correlated with the metastasis and poor prognosis of malignancies (Feber et al., 2008; Ohta et al., 2008; Ogawa et al., 2009; Zhang et al., 2010).

In this study, miRNA array technology was utilized to determine the miRNA expression in the samples from patients at the same pathological stage of EC that received the same surgical treatment by the same surgeon but had different prognoses. miRNA spectra related to the prognosis of EC were obtained. The results showed that 58 miRNA were upregulated. Among the screened miRNAs, most exhibited upreguation in the good-prognosis sample, except for the four miRNAs hsa-let-7e, hsa-miR-130b, hsa-miR-103, and hsa-miR-144 that were downregulated. This result is consistent with the conclusion by Guo that the high expression of has-miR-103 is correlated with low survival rate.

Compared with normal esophageal epithelial tissues, EAC tissues exhibit high expression of miR-194, miR-192, and miR-200c; however, these miRNAs are not expressed in ESCC tissues; miR-342 is only abnormally expressed in ESCC tissues (Feber et al., 2008). In this study, miR-200c was upregulated in the EC sample of good prognosis, whereas the expression of miR-192 was basically similar between the two samples. Seven miRNAs (miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a, and miR-127-3p) are abnormally expressed in the serum samples of ESCC patients (Zhang et al., 2010). By contrast, this study showed that among these miRNAs, only miR-100 and miR-133a were abnormally expressed in the EC tissues. The low GNG7 expression group had a higher degree of tumor invasiveness than the high GNG7 group; the expression of GNG7 is regulated by miR-328 (Ohta et al., 2008). miR-129 is closely correlated with the low survival rate of ESCC patients, for which it can be used for the prediction of patient's prognosis and as an indication for surgical treatment (Ogawa et al., 2009).

However, this study had a limitation, that is, only a small sample size was involved. Therefore, the conclusion of this study needs to be verified by more samples. In EC, the abnormal expression of miRNAs is associated with multiple factors, including chromosomal abnormalities (translocation, loss, amplification, and so on), epigenetic changes (such as methylation), mutations and polymorphisms (single nucleotide polymorphisms), and the mechanism defect of miRNA synthesis. However, whether these factors are related to EC, whether they exert actions simultaneously, and which factor among them is the main one influencing miRNA expression remain to be explored.

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