



Basic Study

Downregulation of microRNA-382 is associated with poor outcome of esophageal squamous cell carcinoma

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Abstract

AIM: To study the potential prognostic role of microRNA-382 (miR-382) in esophageal squamous cell carcinoma (ESCC).

METHODS: Forty six patients were divided into 2 groups according to postoperative survival time: the poor outcome group (28 patients), who showed early metastasis but no recurrence, and died within 1 year after surgery, 12 patients of the group received postoperative chemotherapy treatment that was given after early metastasis happening; the good outcome group (18 patients), who had no clinical metastasis and recurrence, and survived 5 years or more after surgery, all patients did not receive any postoperative treatment. Total RNA was extracted from the patients' formalin-fixed and paraffin-embedded esophageal cancer tissues. miR-382 level was evaluated using high-throughput real-time quantitative polymerase chain reaction analysis. The correlation between miR-382 level and clinicopathologic features was analyzed through COX regression model, and Kaplan-Meier analysis was used to analyze the relationship between

miR-382 level and patient survival time.

RESULTS: miR-382 was differentially expressed in the two groups. Overall the average miR-382 level in the ESCC patients with good outcome was 9.8 ± 3.8 , while miR-382 level in the ESCC patients with poor outcome was 3.0 ± 0.8 . The differences of miR-382 levels between two groups were significant ($P < 0.05$). Kaplan-Meier analysis results showed that miR-382 expression level generally had a significant reverse-correlation with ESCC patient survival time ($P < 0.001$), in which the patients with higher expressions of miR-382 had a longer survival time either among individuals with the same tumor stage or among the overall patients.

CONCLUSION: miR-382 levels are reverse-correlated with ESCC poor outcomes, suggesting that miR-382 could be a potential predictive biomarker for both prognosis and treatment of ESCC.

Key words: Esophageal squamous cell carcinoma; miR-382; Metastasis; Outcome; Prognosis

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Core tip: Esophageal squamous cell carcinoma (ESCC) patients often have significantly different outcomes due to early metastasis happening or not, although the patients are at the same pathological stage and receive the similar surgical therapy. Exploring novel biomarkers related with ESCC metastasis is required for monitoring the progression of the disease, and predicting the outcome of the patient after clinical intervention. Current research addressed a potential prognostic role of microRNA-382 in ESCC.

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INTRODUCTION

Esophageal cancer (EC) is one of the most common gastrointestinal cancers. Up to 300 thousand people worldwide die from this disease each year^[1]. More than 50% of the global incidence of EC is in China^[2]. Histologically, EC is divided into two main types: squamous cell carcinoma and adenocarcinoma. Esophageal squamous cell carcinoma (ESCC) is the major histological type of EC in China. The global 5-year survival rate of EC is only about 15% with most patients dying within one year of diagnosis^[3].

This is mainly based on EC's highly invasive characteristic, which often leads to early metastasis and reduces treatment efficacy^[4,5]. Although Tumor Node Metastasis (TNM) staging system is still a major criteria for EC prognosis, our clinical observations show that ESCC patients receiving similar therapy (*e.g.*, surgical therapy by the same surgeon) at the same pathological stage had significantly different outcomes. Moreover, metastasis in the early postoperative stage also occurred in early-stage ESCC patients. These observations suggested the hypothesis that metastasis-related molecules may be heterogeneously present in ESCC individuals. Therefore, exploring novel biomarkers related to ESCC metastasis is required for monitoring the progression of the disease, and predicting the prognosis of the patient after clinical intervention.

MicroRNA (miRNA) is a small non-coding RNA with 22-25 nucleotides in length, and controls gene expression *via* the regulation of translation efficiency and mRNA stability by binding to the complementary site in 3'-untranslated region (UTR) of the mRNA^[6,7]. miRNAs are abundantly expressed and play an essential role in the regulation of a large number of biological processes, including cancer^[8,9]. The expression patterns and biological functions of miRNAs in ESCC have been investigated in recent years^[10-12].

Human miR-382 (has-mir-382, MIMAT0000737, 5'-GAAGUUGUUCGUGGUGGAUUCG-3') resides in a miRNA cluster in the imprinted DLK1-DIO3 region on the 14q32 locus which hosts one of the largest miRNA clusters in the genome. Many of these miRNAs are differentially expressed in several pathologic processes and various cancers. Recent studies reported that miR-382 was decreased or increased in several types of human malignancy^[13,14], suggesting that the role of miR-382 contributing to tumor development and metastasis is tissue specific.

Using high-throughput real-time quantitative polymerase chain reaction, we initially evaluated the expression profiles of 754 miRNAs in paraffin-embedded tumor specimens from two ESCC patients with TNM IIa stage. These patients had different outcomes although they both received same postoperative treatment (chemotherapy twice)^[15]. Our results showed that the levels of multiple miRNAs were significantly different between the two patients. One of the findings was that the ESCC patient who had neck lymph node metastasis which occurred four months after surgery and died 1 year after surgery had significantly lower miR-382 level compared to another patient who did not have metastasis and survived five years after surgery.

The present study was to further validate a potential role for miR-382 as an ESCC prognostic biomarker. For this purpose, miR-382 levels were examined from 46 ESCC patients with different outcomes; afterwards the relationship between miR-382 level and clinicopathological characteristics of

the patients was assessed. We found that expression of miR-382 was weaker in the specimens from the patients with poor outcome compared to those with good outcome ($P < 0.05$) and miR-382 level was inversely correlated with ESCC patient survival time ($P < 0.001$).

MATERIALS AND METHODS

Patients and esophageal cancer tissue collection

Our aim was to examine miR-382 levels in cancer tissues from ESCC patients who were at the same TNM stage but had different outcomes. Forty-six patients with ESCC diagnosed by histopathological examination between 2006 and 2009 were enrolled in this study. All patients' clinicopathological information had been recorded and the specimens had been collected before this work started. Additionally, all patients had not received any radiotherapy or chemotherapy prior to the surgical procedure performed in the Department of Thoracic Surgery, the First Affiliated Hospital of Xinxiang Medical University. TNM classifications after surgery were made according to International Union Against Cancer (UICC) staging criteria for esophageal cancer, sixth edition (2002). The 46 patients were divided into 2 groups according to postoperative survival time: the poor outcome group, who showed early metastasis but no recurrence, and died within 1 year after surgery; the good outcome group, who had no clinical metastasis and recurrence, and survived 5 years or more after surgery. This study did not include the patients surviving between 1-5 years after surgery. In addition, postoperative survival time of all stage IV patients undergoing surgical procedure in our department was less than 5 years when we started this work. Thus, patients with stage IV disease were excluded from this study. The postoperative follow-up was a standardized process that included routine computed tomography scan and upper gastrointestinal endoscopy. All patients ($n = 18$) with good outcome did not receive any postoperative treatment, and 12 out of 28 patients with poor outcome received postoperative chemotherapy treatment that was given after early metastasis happening.

Ethics

The present study was conducted in accordance with the declaration of Helsinki, and approved by the Institutional Review Board for Human Research of the First Affiliated Hospital of Xinxiang Medical University. Written informed consent form was obtained from all patients.

Tissue sample collection and RNA extraction

Formalin-fixed and paraffin-embedded esophageal cancer tissues from the patients were used for RNA extraction. Using a microtome, 10- μ m slices of tissue from each patient were captured and placed into 1.5

mL microcentrifuge tubes. Paracancerous normal esophageal mucous membranes (8 cm distant to the verge of the tumor tissue) from 4 ESCC patients were taken as controls. Total RNA (including miRNAs) was extracted using TRI Reagent (Applied Biosystems, Foster City, United States) according to manufacturer's instructions. The RNA yield was determined using a UV spectrophotometer, and then stored at -80°C for further processing.

Reverse transcription reaction

Total RNA samples were reverse-transcribed using Taqman MicroRNA reverse transcription kit in combination with Megaplex reverse transcription (RT) primer Human pool set v3.0 (Applied Biosystems, Foster City, United States). Briefly, 3 μ L of total RNA was supplemented with Megaplex RT primer mix ($\times 10$), RT buffer ($\times 10$), Multiscribe reverse transcriptase (50 U/ μ L), dNTPs with dTTP (100 mmol/L), MgCl₂ (25 mmol/L), and RNase inhibitor (20 U/ μ L) in a total reaction volume of 8 μ L. RT reaction was performed for 40 cycles of 16°C for 2 min, 42°C for 1 min and 50°C for 1 s, followed by a final reverse transcriptase inactivation at 85°C for 5 min. cDNA samples were kept at -80°C until PCR analysis.

Pre-amplification of cDNA

2.5 μ L of cDNA samples was pre-amplified using Applied Biosystems' Taqman preamp master mix ($\times 2$) and Megaplex preamp primers ($\times 5$) in a 25 μ L PCR. Megaplex™ PreAmp Primers (Applied Biosystems, Foster City, United States) that contained forward primers specific to miR-382 were used. The pre-amplification cycling conditions were as follows: 95°C for 10 min, 55°C for 2 min and 75°C for 2 min, followed by 12 cycles of 95°C for 15 s and 60°C for 4 min.

Real-time quantitative polymerase chain reaction analysis

qPCR was performed through 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, United States). Pre-amplified cDNA samples were diluted with low-EDTA (0.1 mmol/L) TE buffer (1:50) and qPCR reaction included TaqMan® 2X Universal PCR Master Mix (No AmpErase® UNG) 10 μ L, TaqMan® MicroRNA Assays, 20X TaqMan® Assay 1 μ L, PreAmp Product 1 μ L and nuclease free water 8 μ L. qPCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 97°C for 30 s and 59.7°C for 1 min. The small rRNA U6 (Assay ID: 001093) was used as an endogenous control in RT-qPCR. We run PCR in triplicate for each sample and all samples' Ct values are less 30, which is in a reasonable range. The relative quantitative method was used. Gene expression was quantitated based on the following formula: $F = 2^{-\Delta\Delta\text{Ct}}$ where $\Delta\Delta\text{Ct} = (\text{mean CT of has-mir-382 in the test sample} - \text{mean Ct of the housekeeping gene in the test sample}) - (\text{mean Ct of has-mir-382 in the control sample} - \text{mean$

Table 1 Characteristics of patients with esophageal squamous cell carcinoma

Characteristic	n (%)
All patients	46 (100)
Sex	
Male	23 (50.0)
Female	23 (50.0)
Age	
< 60	25 (54.3)
≥ 60	21 (45.7)
Size of tumor	
≤ 5 cm	27 (58.7)
> 5 cm	19 (41.3)
Site of tumor	
Upper thoracic	4 (8.7)
Middle thoracic	35 (76.1)
Lower thoracic	7 (15.2)
Differentiation	
Good	11 (23.9)
Moderate	19 (41.3)
Poor	16 (34.8)
Depth of invasion	
Tis, T1	8 (17.4)
T2	6 (13.0)
T3	32 (69.6)
Stage (TNM)	
0- I	7 (15.2)
II a- II b	21 (45.7)
III	18 (39.1)
Lymph node metastasis	
Negative	27 (58.7)
Positive	19 (41.3)
Metastasis	
Early metastasis	28 (60.9)
Non metastasis	18 (39.1)

Ct of the housekeeping gene in the control sample). A high *F*-value indicates a relatively high expression of miR-382.

Statistical analysis

Two-tailed student *t* test was used to determine the expression levels of miR-382, and results were expressed as mean ± SE. *P* < 0.05 was considered statistically significant. The χ^2 test was used to determine the relationship between miR-382 expression level and clinicopathological features, and Kaplan-Meier was used to analyze the relationship between miRNA expression and survival time. COX regression model was used to analyze the influence of the related factor on the survival time of patients with ESCC. SPSS 17.0 software (SPSS Inc., Chicago, United States) was used for data analysis.

RESULTS

Demographic and clinicopathological characteristics

The study included 46 patients with ESCC. The patients were followed up for 3 to 84 mo, and were subsequently divided into two groups - good outcome and poor outcome. The group with good outcome

included 18 (39.1%) patients who showed neither clinical metastasis nor recurrence, and survived 5 years or more after surgery; the group with poor outcome included 28 (60.9%) patients who showed early metastasis but no recurrence, and died within 1 year after surgery.

Demographic variables of the patients are listed in Table 1. In this study, there were 23 (50%) males and 23 (50%) females with ages ranging from 45 to 71 years (median, 59 years). The tumor size in 19 (41.3%) cases was more than 5 cm and in 27 (58.7%) cases was less than 5 cm. Regarding the histological differentiation, 11 (23.9%) patients had well differentiated ESCC, 19 (41.3%) patients had moderately differentiated ESCC, and 16 (34.8%) had poorly differentiated ESCC. Lymph node metastasis occurred in 19 (41.3%) patients, and did not occur in 27 (58.7%) cases. 7 (15.2%) cases were at stage 0 and I (1 case at stage 0 and 6 cases at stage I), 21 (45.7%) cases were at stage II (II a and II b), and 18 (39.1%) cases were at stage III. This study did not include the patients at stage IV and the patients living between 1-5 years.

Differential expression of miR-382 in ESCC patients and their significance in prognosis

RT-qPCR was performed to examine the expression level of miR-382. qPCR amplification plot with Δ Ct values of miR-382 is shown as Figure 1. All Ct values from each sample were less than 30 (data not shown). miR-382 levels shown in Figure 2 were differentially expressed in ESCC patients. Overall the miR-382 level (Figure 2A) in the patients with good outcome was 9.8 ± 3.8 (mean ± SE), while miR-382 level in the patients with poor outcome was 3.0 ± 0.8 (mean ± SE). Therefore, miR-382 average level from all ESCC patients with poor outcome was lower than that from all ESCC patients with good outcome. The differences of miR-382 levels between two groups were significant (*P* < 0.05). Furthermore, in each TNM stage shown in Figure 2B-D, miR-382 levels were decreased in the patients with poor outcome when compared with the patients with good outcome.

miR-382 expression was associated with ESCC patient survival time

Kaplan-Meier analysis results showed that miR-382 expression level generally had a significant reverse-correlation with ESCC patient survival time (Figure 3A, *P* < 0.001). Similarly, at individual stage as showed in Figure 3B-D, expression levels of miR-382 were reversely correlated with patient postoperative survival time. Cox single factor related risk analysis results showed that patient TNM stage (*P* = 0.023), tumor size (*P* = 0.006), postoperative time (*P* = 0.010), miR-382 level (*P* = 0.007), and patient survival time had a significant correlation.

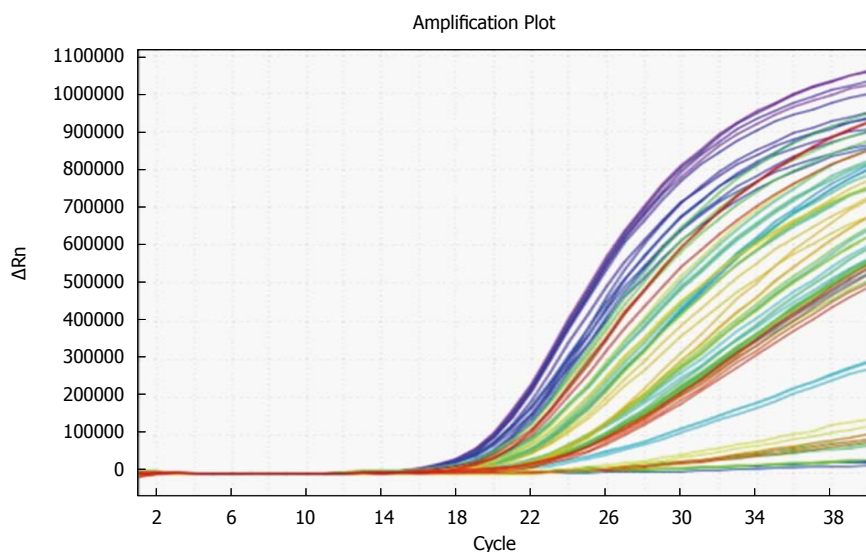


Figure 1 Amplification plot with ΔC_t values of miR-382 in all patients.

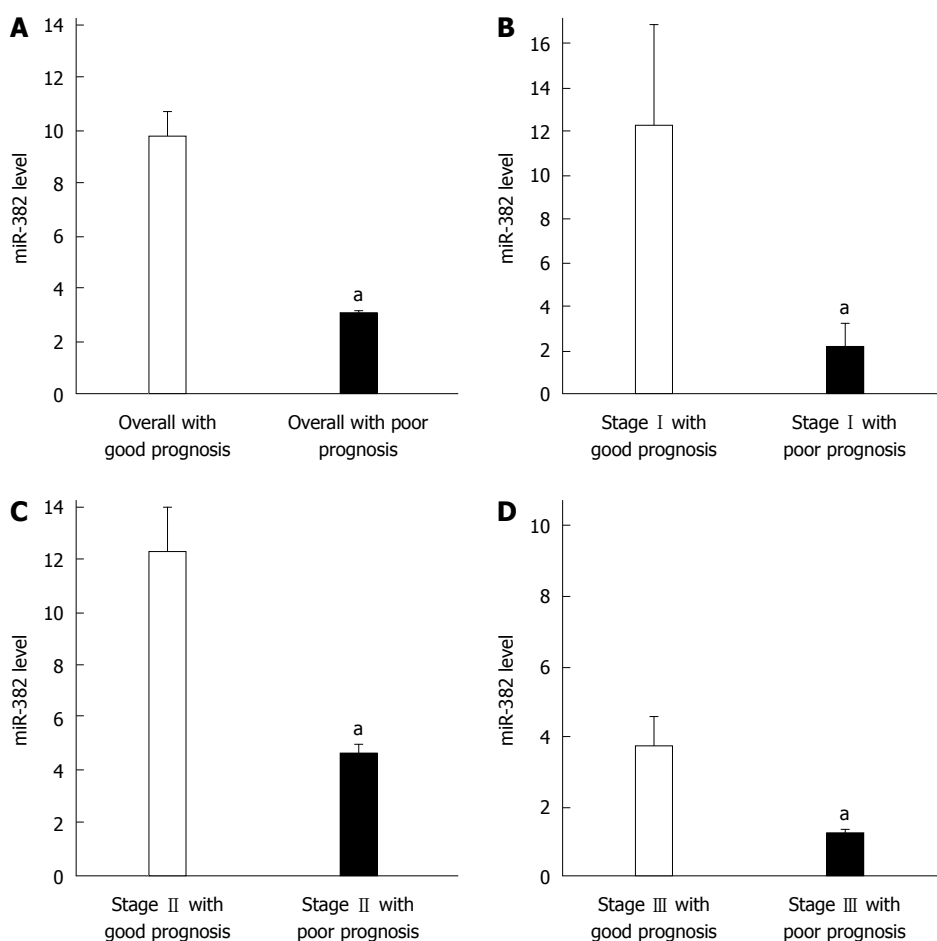


Figure 2 miR-382 expression levels were associated with outcomes of esophageal squamous cell carcinoma patients. A: The esophageal squamous cell carcinoma (ESCC) patients with good outcome generally exhibited higher miR-382 expression than those with poor outcome ($^aP < 0.05$, good outcome vs poor outcome); B: Stage I ESCC patients with good outcome exhibited higher levels of miR-382 than those with poor prognosis ($^aP < 0.05$, good outcome vs poor outcome); C: Stage II ESCC with good outcome exhibited higher levels of miR-382 than those with poor outcome ($^aP < 0.05$, good outcome vs poor outcome); D: Stage III ESCC with good outcome exhibited higher levels of miR-382 than those with poor outcome ($^aP < 0.05$, good outcome vs poor outcome).

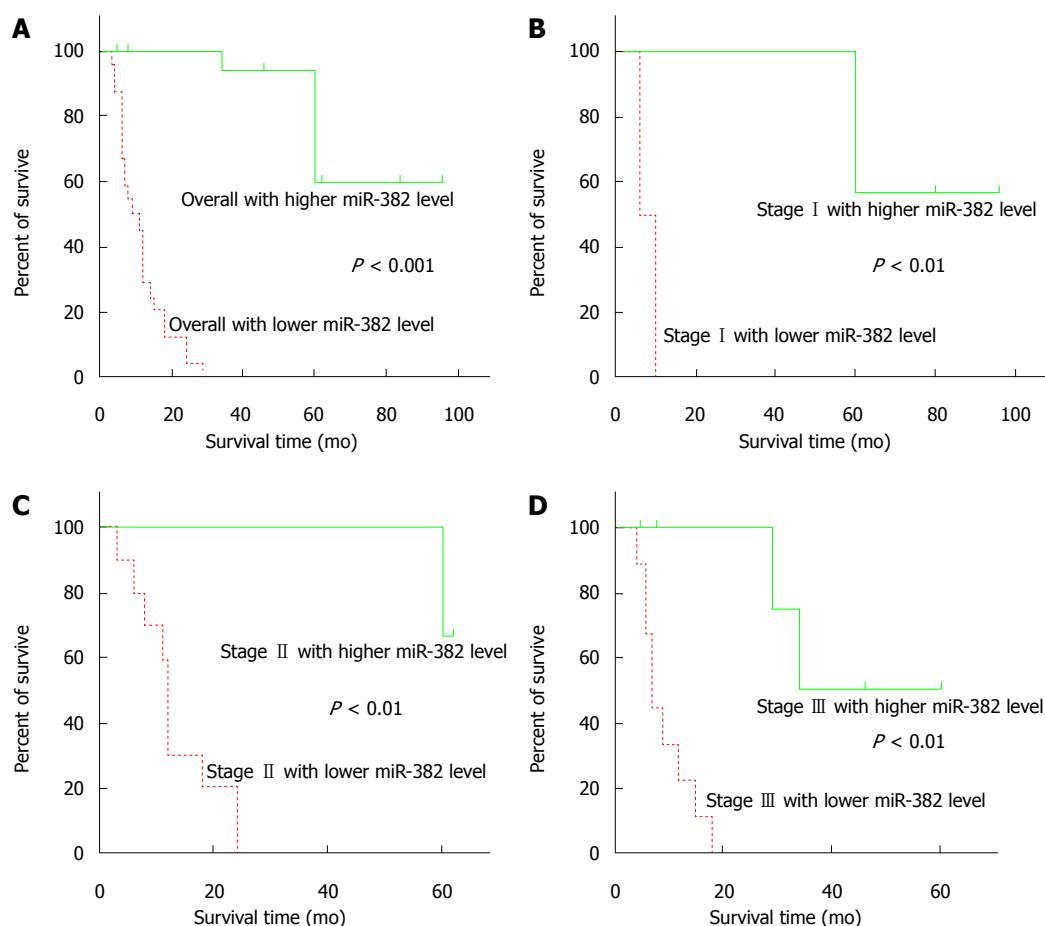


Figure 3 miR-382 expression levels were reverse-correlated with esophageal squamous cell carcinoma patient survival times. A: The esophageal squamous cell carcinoma (ESCC) with higher miR-382 expression generally exhibited longer survival times, compared to those with lower expression levels ($P < 0.001$, higher vs lower miR-382 level); B: Stage I ESCC patients with higher levels of miR-382 exhibited longer survival times, compared to those with lower expression levels ($P < 0.01$, higher vs lower miR-382 level); C: Stage II ESCC patients with higher levels of miR-382 exhibited longer survival times, compared to those with lower expression levels ($P < 0.01$, higher vs lower miR-382 level); D: Stage III ESCC patients with higher levels of miR-382 exhibited longer survival times, compared to those with lower expression levels ($P < 0.01$, higher vs lower miR-382 level).

DISCUSSION

In the current study, we investigated the prognostic impact of miR-382 level in 46 patients with ESCC. We discovered that miR-382 was differentially expressed in cancer specimens from ESCC patients with different outcomes, although the patients were at the same pathological stage and received similar surgical treatment. We showed that miR-382 expression was significantly decreased in specimens from ESCC patients with poor outcomes compared to the patients with good outcomes. Moreover, our clinical results revealed that miR-382 level was inversely correlated with ESCC patient survival time, and significantly linked to the patients' outcomes. These data suggest a potential role for miR-382 as a biomarker for identifying patients who will experience an unfavorable clinical outcome.

ESCC is a common malignancy^[2]. Accurate judgments of the clinical stage and prognosis of ESCC are important bases for clinicians to take a rational approach to the treatment of this disease. Although

TNM staging is traditionally considered the single most important factor to guide treatment decisions and prognosis for ESCC, clinical observations showed that same TNM stage ESCC patients who received the same surgical treatment by the same surgeon had different outcomes, indicating that ESCC metastasis susceptibility is somehow individually specific. Other traditional examinations based on medical equipment such as CT, X-ray barium meal fluoroscopy, gastroscopy, and B-ultrasonography, can be utilized to discover cancer metastases, whereas all these medical examinations are unable to predict distinct metastasis susceptibility. New research methods in molecular techniques have facilitated the biomarker discoveries of cancer metastasis susceptibility^[16]. The current clinical biomarkers for ESCC prognosis are not ideal, as there remains a lack of reliable biomarkers that can specifically distinguish between ESCC patients who are susceptible to metastasis and those who are not.

Numerous studies have established miRNAs as broad and powerful regulators of protein expression in physiology and diseases^[17]. Correlation analyses

between miRNAs and cancers have shown miRNA as potential prognostic biomarkers in variant cancers^[18-20]. miRNAs that are upregulated in cancers are proposed to be oncogenes, whereas those that are downregulated are considered tumor suppressors^[21]. Some miRNAs, such as has-miR-335, has-miR-181d, has-miR-25, has-miR-7, and has-miR-495, have been reported to directly participate in the initiation and development of ESCC^[22-24].

The relationship between miR-382 levels and prognoses has been examined in several types of human malignancy. Decreased miR-382 levels were associated with poor survival in osteosarcoma patients^[14], indicating that the role of miR-382 in osteosarcoma as a tumor suppressor. Conversely increased miR-382 levels were found in acute myeloid leukemia tumor tissue^[15], indicating that miR-382 plays the role of an oncogene in this disease. Consequently the role of miR-382 in tumor development and metastasis is a heterogeneous one for which the physical, cellular and molecular determinants adapt and react throughout the progression of the disease in a cell-driven and tissue-driven manner.

Our study revealed that the average level of miR-382 was significantly lower in specimens from ESCC patients who showed a poor outcome than those in the patients who showed a good outcome, and miR-382 level was reverse-correlated with patient's survival time and linked to a poor outcome. Metastasis had occurred in all ESCC patients with poor outcome but not in the patients with good outcome in our study. Thus, our results indicate that miR-382 is involved in the ESCC metastasis process and is a potential biomarker for ESCC patients who are individual susceptible to metastasis. This could at least partially explain why the ESCC patients at similar clinicopathological stages and receiving similar surgical treatment had completely different outcomes.

The mechanism by which miR-382 affects ESCC behavior is not yet clear. One study reported that miR-382 as a tumor suppressor in osteosarcoma negatively regulated the expression of KLF12 and HIPK3 by directly targeting their 3' UTR sequences to inhibit tumor cell growth *in vivo* and *in vitro*^[25]. The downstream signals of miRNA contributing to tumor development and metastasis are tissue heterogeneous. Thus our future work will be aimed at determining the specific downstream molecules by which miR-382 affects ESCC behavior^[26].

The specimens we used in this study were formalin-fixed and paraffin-embedded esophageal cancer tissues. The expression level of miR-382 in the cancer tissue is dependent on the individual ESCC patient situation rather than the specimen type used for examination. Hence, our results should be applicable to other specimen types such as fresh surgical specimen and forceps biopsies obtained during upper gastrointestinal endoscopy.

In this study, the level of miR-382 we mentioned

just means the relative quantification when comparing the results from two groups. The total number of patients assessed in our study was relatively small, especially for the number of individual pathological stage patients. Accordingly, it is difficult to determine the cutoff level of miR-382 as a biomarker for clinical utility from our current results. A large size cohort study must therefore be an objective of future projects to determine the miR-382 cutoff level, which can be used not only for predicting ESCC outcomes but also for being a supplemental criterion to TNM staging or postoperative treatment decision.

In conclusion, it is the first study to show that miR-382 was downregulated in ESCC patients with early metastasis, and that miR-382 levels were significantly reverse-correlated with ESCC patient outcomes. Therefore, miR-382 could be a potential predictive biomarker for both outcome prognosis and treatment of ESCC. Further studies are needed to define the detailed mechanisms and determine the cutoff level of miR-382.

COMMENTS

Background

Different outcomes often happen to esophageal squamous cell carcinoma (ESCC) patients with the same pathological stage and receiving similar therapy due to early metastasis happening or not. It is important to explore reliable biomarkers that can specifically distinguish between ESCC patients who are susceptible to metastasis and those who are not. microRNAs (miRNAs) as broad and powerful regulators of protein expression are potential prognostic biomarkers of cancers. microRNA-382 (miR-382) has been shown to relate with tumor development and metastasis in variant cancers.

Research frontiers

It was reported that miR-382 was reduced and associated with poor survival in osteosarcoma patients. Conversely increased miR-382 levels were found in acute myeloid leukemia tumor tissue. Consequently the role of miR-382 in tumor development and metastasis is a heterogeneous one in a cell-driven and tissue-driven manner.

Innovations and breakthroughs

This study revealed that miR-382 was downregulated in ESCC patients with early metastasis comparing with the patients without metastasis. This is the first study to show that miR-382 levels were significantly reverse-correlated with ESCC patient outcomes. Therefore, the results indicate that miR-382 is involved in ESCC metastasis process and is a potential biomarker for ESCC patients who are individual susceptible to metastasis.

Applications

By understanding the differential expression of miR-382 in the specimen of ESCC patient with or without early metastasis, and its relationship with clinicopathological characteristics and patient outcomes, this study may provide a future strategy in the development of a novel biomarker for both metastasis predictor and treatment of ESCC.

Terminology

Metastasis is a complex process that involves the spread of a tumor or cancer from its original site to other places in the body. miRNA is a small non-coding RNA with about 22 nucleotides in length, and controls gene expression via the regulation of translation efficiency and mRNA stability. miRNAs have been shown as potential prognostic biomarkers in variant cancers. miR-382 is one of these, the role of miR-382 to tumor development and metastasis is tissue specific.

Peer-review

This is an interested topic aiming to advance management for this difficult disease. The authors first provide the evidence of the divergent expression of microRNA-382 in tumor tissues from ESCC patients with different outcomes,

which indicate that it is associated with prognosis and may develop a novel biomarker for both diagnosis and treatment of ESCC.

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