Original Article
Oncogenic function of miR-301 to predicts poor prognosis of endometrial cancer

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Abstract: Background: Endometrial cancer (EC) is a common female malignancy, and dysregulation of microRNAs (miRNAs) is associated with malignant transformation. The present study aimed to identify new molecular markers for predicting the outcomes of EC patients. Methods: Microarray analysis was used to analyze the difference in the expression level of miRNAs between the primary cancer tissue and proliferative endometrium. qRT-PCR was employed to measure the expression level of miR-301 in clinical EC tissues and proliferative endometrium. Survival curves were constructed using the Kaplan-Meier method, and a log rank test was used to assess the differences between clinicopathological characteristics and survival in EC patients. Results: Ten miRNAs showed the highest significant difference after independent examination using qRT-PCR, with 8 up-regulated (miR-21, miR-196a, miR-16, miR-582-5p, miR-15b, miR-301, miR-148b, and miR-128a) and 2 down-regulated (miR-125 and miR-34). As the most highly up-regulated miRNA, miR-301 was further examined. The expression level of miR-301 in EC tissues was significantly higher than that in the proliferative endometrium (1.03 ± 0.18 vs. 3.21 ± 0.76, P<0.0001). The median fold change of miR-301 was used as a cutoff value. High miR-301 expression was observed to be closely correlated with lymph node metastasis (P = 0.073), distant metastasis (P = 0.038), myometrial invasion (P = 0.033), grade (P = 0.007), advanced TNM stage (P = 0.000) and vessel invasiveness (P = 0.026). The 5-year overall survival rate in the high expression group was 45.8%, compared with 69.8% in the low expression group (P = 0.025). Conclusion: Our findings provide convincing evidence that the up-regulation of miR-301 may serve as a novel molecular marker to predict aggressive tumor progression and an unfavorable prognosis in EC patients.

Keywords: Endometrial cancer, miR-301, prognosis

Introduction

Endometrial cancer (EC) is a common female malignancy, and the incidence and death rates of this cancer are increasing [1]. In developed countries, the incidence rate of EC is the highest among all female genital malignancies, and the average age of patients at diagnosis is decreasing. Early stage patients have a good prognosis and a high cure rate. If the diagnosis is made during stage I, the survival rate is approximately 90% [2]. Although the majority of women will be diagnosed with early stage disease and can be cured with surgery alone, patients with high-risk early or advanced endometrial cancer have a significant risk of death despite currently available therapies, with 5-year survival rates ranging from 23 to 72% [3]. However, few biomarkers of EC prognosis have been established. Therefore, there is an urgent need to identify new molecules to more accurately predict the survival of EC patients; moreover, these biomarkers may foster beneficial treatment intensification and act as a potential therapeutic target.

MicroRNAs (miRNAs) belong to the group of small, non-coding, regulatory RNA molecules, ranging between 18 and 25 nucleotides in length, which participate in a variety of biologic processes such as proliferation, apoptosis and migration [4-6]. Increasing evidence has demonstrated that dysregulation of miRNAs can contribute to the progression and metastasis of human tumors. In the present study, we identified specific miRNA profiles associated with EC

Table 1. Relationship between miR-301 expression and clinicopathologic characteristics in EC (n = 67)

Patients Character		Low miR-301 group	High miR-301 group	P value
Age	<50	27	15	0.725
	≥50	16	9	
Lymph node metastasis	Negative	26	15	0.073
	Positive	17	9	
Distant metastasis	Negative	22	6	0.038
	Positive	21	18	
Vessel invasive	Negative	29	2	0.026
	Positive	14	22	
Myometrial invasion	≤1/2	32	11	0.033
	>1/2	11	13	
Pathology	Adenocarcinoma	39	22	0.869
	Non- adenocarcinoma	4	2	
Stage	I	17	2	0.000
	II	20	10	
	III/IV	6	12	
Histological grade	G1	18	3	0.007
	G2	20	14	
	G3	5	7	

on histopathological evaluation. Fresh samples were snap-frozen in liquid nitrogen immediately after surgery and were stored at -80°C until analyzed. For EC, which is classified by differences in histology and molecular characteristics, endometrial carcinoma has been generally distinguished as type I (mainly endometrioid) or II (non-endometrioid) by pathologist. For histologic grading of tumors, the WHO classification was used. All patients were clinically staged according to FIGO guidelines. The clinicopathologic information is shown in **Table 1**.

Detection of differentially expressed miRNAs by miRNA microarray

using a miRNA array screening method. We found that miR-301 was the most up-regulated miRNA in EC tissue compared to proliferative endometrium. The aberrant expression of miR-301 has been reported in several types of human cancers, including prostate cancer, breast cancer, and lung cancer, among others [7-9]. However, the prognostic value of miR-301 in EC has not yet been reported. Herein, we report the findings of our analysis of the significance of miR-301 in EC.

Material and methods

Patients and tissue samples

This study was approved by the Research Ethics Committee of the Nanfang Hospital and the First Affiliated Hospital of Jinan University. Written informed consent was obtained from all patients. All specimens were handled and made anonymous according to ethical and legal standards. EC tissues from 67 cases and proliferative endometrium from 15 cases were obtained from patients who underwent curettage at the Gynecology and Obstetrics Department of Nanfang Hospital and the First Affiliated Hospital of Jinan University between 2008 and 2011 and were subsequently diagnosed based

Pairs of EC tissue and proliferative endometrium were analyzed using a miRNA microarray (Kangcheng Biotech Company, Shanghai, China). Briefly, total RNA was isolated from the cells using TRIzol reagent. The miRNA was separated from 30 to 50 mg of the total RNA, labeled with the miRCURY Hy3TM/Hy5TM Power Labeling Kit (Exiqon), and hybridized to a miRCURY LNA Array (Exiqon, v11.0). Scanning was performed with an Axon GenePix 4000B microarray scanner. GenePix Pro v6.0 was used to read the raw image intensity. Unsupervised hierarchical clustering was performed using the miRNA expression profile results. Each miRNA present in the database was mapped to a precise location in the human genome using a BLAST search with the default parameters and the maps available from the National Center for Biotechnology Information Human Genome Resources (www.ncbi.nlm.nih.gov). Additionally, to validate the data, clones corresponding to each miRNA were identified and mapped to the human genome.

Evaluation of miRNA expression by quantitative RT-PCR

Total RNA was extracted from EC tissues and proliferative endometrium using Trizol accord-
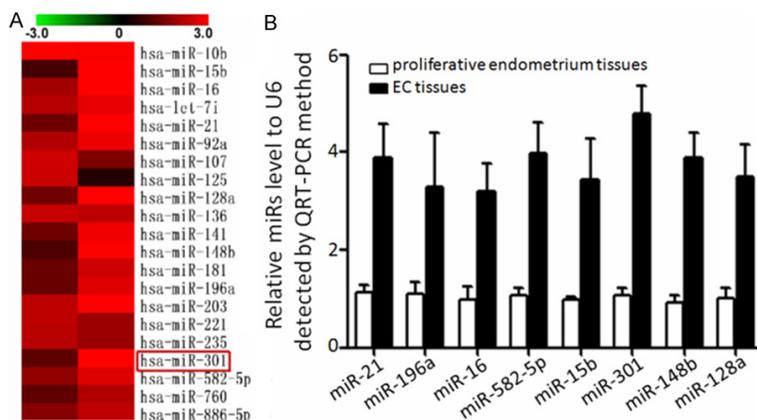
Figure 1. The different miRNA profiles in EC tissue and proliferative endometrium tissue identified by microRNA arrays and qRT-PCR. A. Unsupervised hierarchical clustering based on miRNA expression profiles in 1 pair of EC tissue and proliferative endometrium tissue. B. Total RNA was extracted form 3 pairs of EC tissue and proliferative endometrium tissue. The expression of miR-21, miR-196a, miR-16, miR-582-5p, miR-15b, miR-301, miR-148b, and miR-128a were measured using qRT-PCR. The change in the miR-301 expression level was the most significant among all miRNAs examined.

$2^{-\Delta\Delta Ct}$. All reactions were run in triplicate, and the average threshold cycle and SD values were calculated.

Statistical analysis

All computations were carried out using the software of SPSS version 18.0 for Windows (SPSS, Inc, Chicago, IL). Data were expressed as mean ± SD. We analyzed the difference of the miR-301 level in EC tissue and proliferative endometrium using independent-samples T test. By means of the multivariable correlation analysis were used to evaluate the statistical differences of all the clinicopathological characteristics between the high miR-301 group and low miR-301 group. Differences were considered statistically significant when P was less than 0.05.

Results

Microarray expression profiles

To explore the difference between EC tissues and proliferative endometrium, we screened miRNA expression levels using a miRNA microarray. Differentially expressed miRNAs were selected based on fold change (at least 4-fold change in at least one group comparison). As shown in **Figure 1A**, eight miRNAs were up-regulated (miR-21, miR-196a, miR-16, miR-582-5p, miR-15b, miR-301, miR-148b, and miR-128a), and two miRNAs were down-regulated (miR-125 and miR-34). As shown in **Figure 1B**, we verified all 8 up-regulated miRNAs in 3 pairs of EC tissues and proliferative endometrium tissue using qRT-PCR. MiR-301 was the most highly and stably up-regulated miRNA, which attracted our interest owing to its tumor-promoting role in cancer cells.

The expression of miR-301 in EC tissues

The miR-301 expression level was detected in tissues from 67 cases of EC and 15 cases of proliferative endometrium by qRT-PCR. As shown in **Figure 2**, after normalization to the U6 expression levels, the expression level of miR-

ing to the manufacturer's instructions. Primers for miR-21, miR-196a, miR-16, miR-582-5p, miR-15b, miR-301, miR-148b, miR-128a, miR-125, and miR-34 and the endogenous control U6 snRNA were purchased from Jima Com. The concentration and purity of RNA were determined spectrophotometrically using a Nano-Drop ND-1000 (NanoDrop Technologies, Wilmington, Delaware, USA). Reverse transcription and qRT-PCR amplification were performed in two steps. In the first reverse transcription step, 10 ng of RNA was used in reactions with specific stem-loop RT primers for each miRNA and an endogenous control primer for small nuclear RNA U6 in a 20 µl final reaction volume containing 0.5 µg of RNA, 0.5 µl Prime-Script RT enzyme mix, and 4 µl 5 × PrimeScript buffer, and 1 uL RT primer. The reactions were incubated at 42°C for 60 min and at 85°C for 5 min. In the second step, cDNA samples were amplified using a Real Time PCR instrument 7500 (Applied Biosystems) with the specific miR primers and the endogenous control small nuclear RNA U6. The amplification profile consisted of denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The raw data are presented as the relative quantity of target miRNA, normalized with respect to U6 snRNA and relative to a calibrator sample. The fold changes were calculated through relative quantification using
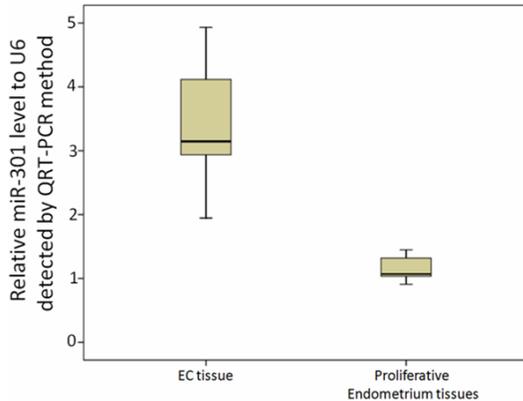
Figure 2. miR-301 is over-expressed in EC patients. Real-time PCR analysis of the miR-301 levels in the 67 cases of EC tissue and 15 cases of proliferative endometrium tissue using qRT-PCR. The difference is P = 0.000.
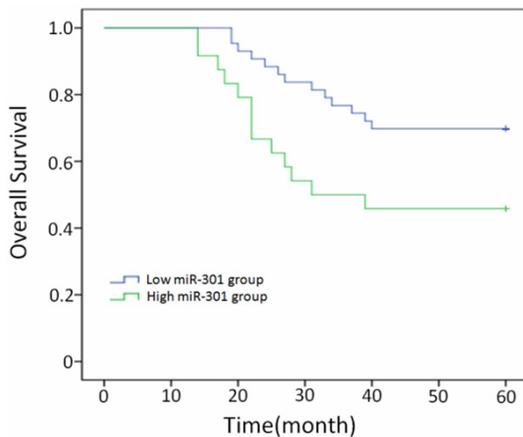

Figure 3. High miR-301 expression is a poor prognostic factor. Kaplan-Meier survival analysis of 67 EC patients, compared based on high and low miR-301 expression groups (P = 0.025).

301 in EC tissues was significantly higher than in proliferative endometrium (1.03 ± 0.18 vs. 3.21 ± 0.76, P<0.0001).

Association of miR-301 expression with clinicopathological characteristics of EC patients

The median fold change of miR-301 was used as a cutoff value to divide all 67 patients into two groups: a low expression group (n = 43) and a high expression group (n = 24). As shown in **Table 1**, high miR-301 expression was observed to be closely correlated with lymph node metastasis (P = 0.073), distant metastasis (P = 0.038), myometrial invasion (P = 0.033), grade (P = 0.007), advanced TNM stage (P = 0.000) and vessel invasiveness (P = 0.026). In contrast, there was no association between miR-301 expression and other clinical factors, including age and pathology (all P>0.05).

High miR-301 expression is associated with poor prognosis in EC patients

To evaluate the prognostic value of miR-301 expression in EC, survival curves were constructed using the Kaplan-Meier method and then compared using the log-rank test. As shown in **Figure 3**, EC patients with high miR-301 expression showed shorter overall survival than those with low miR-301 expression. The 5-year overall survival rate in the high expression group was 45.8%, compared with 69.8% in the low expression group (P = 0.025).

Discussion

EC is the most common gynecological tumor among women in developed countries, and its incidence is increasing. The most frequently occurring histological subtype is endometrioid adenocarcinoma, and patients are often diagnosed when the disease is still confined to the uterus. Standard treatment consists of hysterectomy and bilateral salpingo-oophorectomy, often using minimally invasive approaches (laparoscopic or robotic). Lymph node surgical strategies are contingent on histological factors (subtype, tumor grade, involvement of lymphovascular space), disease stage (including myometrial invasion), patient characteristics (age and comorbidities), and national and international guidelines [10]; adjuvant treatment is tailored according to histology and stage. Various classifications are used to assess the risk of recurrence and to determine optimal postoperative management [11-13]. The 5-year overall survival for EC ranges from 74% to 91% in patients without metastatic disease. Trials are ongoing in patients who have a high risk of recurrence (including chemotherapy, chemoradiation therapy, and molecular targeted therapies) to assess the modalities that best balance optimization of survival with the lowest adverse effects on quality of life. Therefore, clinical indicators that accurately predict EC progression and prognosis are essential for improving patient survival.

Aberrant expression of miRNAs has been demonstrated to have oncogenic properties and to

be involved in cancer initiation, progression, and metastasis. Plasma miRNAs represent a promising source for the development of prognostic and diagnostic tools, owing to their minimally invasive sampling, high stability, and simple quantification by standard techniques such as RT-qPCR. Because early diagnosis is essential for the successful treatment of EC, there is a need for better markers with high sensitivity and specificity to permit early diagnosis and proper management of EC. Aberrant expression of miR-301, which is localized in the first intron of ska2, has been reported in several types of human cancers, including pancreatic cancer, breast cancer, and lung cancer, among others [14, 15]. Previously, Jiang et al. detected more than 200 precursor and mature miRNAs by real-time PCR in 43 and 28 pairs of HCC and adjacent benign liver tissue, respectively, and normal liver specimens. These authors found that the expression of miR-301 was significantly correlated with disease outcome [16]. In addition, Cao proposed a new circuit model in which intronic miR-301 regulates the transcription and function of its host gene MEOX2, which affects the ERK/CREB pathway. CREB directly regulates the expression of the host gene ska2, and this feedback loop is related to lung tumorigenesis [17]. To date, the prognostic value of miR-301 in EC has not yet been reported. In the present study, the miR-301 expression level was detected in 67 cases of EC and 15 cases proliferative endometrium tissues by qRT-PCR. We found that the expression level of miR-301 in EC tissues was significantly higher than that in proliferative endometrium tissues. Furthermore, we divided all 67 patients into two groups according to the median fold change of miR-301. High miR-301 expression was closely correlated with lymph node metastasis (P = 0.073), distant metastasis (P = 0.038), myometrial invasion (P = 0.033), grade (P = 0.007), advanced TNM stage (P = 0.000) and vessel invasion (P = 0.026). To evaluate the prognostic value of miR-301 expression in EC, survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. We found that EC patients with low miR-625 expression levels had shorter overall survival compared to those with high miR-625 expression. Moreover, the 5-year overall survival rate in the low expression group was 38.1%, compared with 68.8% in the high expression group (log-rank test, P = 0.025).

Together, our findings provide novel and convincing evidence that the up-regulation of miR-301 may serve as a novel diagnostic and/or prognostic marker for EC.

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Disclosure of conflict of interest

None.

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