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Original Article

MiR-17 promotes cervical squamous cell tumorigenesis and metastasis by targeting E2F1

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Abstract: MicroRNAs (miRNAs) are critical regulators in tumor genesis and development. The expression of miR-17 altered significantly in various cancers. In this study, the relationship between miR-17 and squamous cervical cancer was investigated. First, by quantitative real-time polymerase chain reaction (qRT-PCR), we detected the expression level of miR-17 in squamous cervical cancer tissue and normal cervical tissue. Then the effect of miR-17 was investigated in vitro by transfected with miR-17-inhibitor in SIHA cell line: proliferation, apoptosis, invasion and metastasis, the potential target gene E2F1 was also proved by western blot. Finally, serum miR-17 was detected and its relation with clinicopathology was analyzed. We found tissue miR-17 was significantly higher in squamous cervical cancer tissue than normal cervical tissue (P<0.01). Inhibition of miR-17 prevented cell proliferation by promoting cell apoptosis, inhibiting migration and invasion, with up-regulated expression of E2F1. Additionally, patients tended to overexpress serum miR-17, which was significantly higher in cervical cancer patients compared to normal controls (P<0.001), the odds ratio was 1.129. When miR-17 was used for differentiation of patients from normal controls, the value of the area under the receiver-operating curve (AUC) was 0.777±0.063, the sensitivity and specificity of serum miR-17 were 95% and 55% respectively, the cut-off point was 0.858. These results indicated that miR-17 acted as an oncogene promoting tumorigenesis in cervical squamous cell cancer, and the circulating miR-17 could be a potential biomarker for checking out early stage cervical squamous cell cancer patients. We propose that miRNAs might be used as therapeutic agent for cervical cancer.

Keywords: Cervical squamous cell cancer, miR-17, proliferation, metastasis, E2F1, serum tumor marker

Introduction

Cervical cancer is the most common malignant tumor in the female reproductive system, and it is still the second most common cause of cancer-related death among women worldwide, with over 500,000 new cases diagnosed annually and 50% mortality rate [1]. In the United States, according to the report in 2013 of the American Cancer Society (ACS), 12,360 new cases of cervical cancer were diagnosed annually, and estimated 4030 deaths occurred from the disease [2]. Although the incidence and mortality of cervical cancer has declined due to the early cervical cytology screening in recent years, the incidence is still relatively high in some ethnic minority areas and medical limited areas. Moreover we found more and more young cases during our clinical work. So, identification of pathogenesis and suitable biomarkers may be a key role for improving the efficiency of diagnosis and therapy for cervical cancer patients.

microRNAs (miRNAs) are small, evolutionarily conserved, non-coding RNAs of approximately 18-25 nucleotides (nt) in length, which are generally involved in post-transcriptional gene regulation. When the complementary matching degree between miRNAs and the target mRNA is high, the mRNA get degradation. When the complementary matching degree is low, the translation of the target mRNA is restrained with the down-regulated protein expression, but no change in mRNA level [3, 4]. Through that, miRNAs participated in a lot of biological processes, such as proliferation, differentiation, motility, apoptosis and so on. Numerous stud-

ies have shown that aberrant miRNAs expressions were found in human tumor samples and normal samples. In our previous study, we compared the miRNAs expression profile between squamous cervical cancer tissue and normal cervical tissue by hybridization arrays, miR-17 was up-regulated significantly. miR-17 was a member of miR-17-92 family, which is being currently accepted as an oncogenic family [5, 6]. The purpose of this study was to investigate the biological functions and molecular mechanisms of miR-17 in human cervical squamous cell cancer in vitro and to find the target gene regulated by miR-17.

Materials and methods

Clinical samples and cell lines

The human clinical samples were collected from the department of gynecologic oncology, the Affiliated Tumor Hospital of Guangxi Medical University between 2010 and 2012. Eighty cervical cancer tissue of international Federation of Gynecology and Obstetrics (FIGO) stage I-IIA were obtained from patients who underwent surgical treatment. All cancer samples were proved as squamous cell carcinoma by pathology. Twenty normal cervical tissues were obtained from the patients who underwent panhysterectomy due to uterine myomas. The median age of cervical cancer patients was 48.5 years with a range from 25 to 69 years. For normal control subjects, the mean age was 44.9 years, ranging from 33 to 57 years. The blood was drawn when patients were admitted in hospital, and then the tubes were kept upright for 30 min at room temperature and then stored in a 4°C refrigerator. After centrifugation at 514×g, 4°C for 10 min, serum was extracted and distributed into aliquots of 0.5 ml per 1.5 ml tube. Then the serum tubes were stored at -80°C freezer. After surgical removal, the tissues were frozen in liquid nitrogen immediately and then stored at -80°C with the serum sample until use. No previous local or systemic treatment had been conducted on these patients before the samples were collected. All protocols were approved by the Ethics Committee of the Affiliated Tumor Hospital of Guangxi Medical University.

The human cervical cancer cell SiHa was kept by central laboratory of the Affiliated Tumor Hospital of Guangxi Medical University. The cells were cultured in RPMI 1640 medium (HyClone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS; HyClone), and 1% penicillin and streptomycin in a humidified 37°C and 5% CO_2 incubator.

Transient transfection

The miR-17 inhibitors and miR-negative control of inhibitors (anti-miR-NC) were synthesized and purified by GenePharma (Shanghai, China): miR-17 inhibitors, 5'-CUACCUGCACUGUAAGCACUUUG-3', anti-miR-NC, 5'-CAGUACUUUUGUGUAGUACAA-3'. miR-17 inhibitors and anti-miR-NC were transfected at a final concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA) following the manufacturer's protocols, 4 hours later, the medium was changed with the complete culture medium. Total RNA were extracted 48 h after transfection, and protein were collected 72 h after transfection.

RNA isolation and real-time PCR

Total RNA was extracted by miRcut miRNA isolation kit (Tiangen, Beijing, China) according to the manufacturer's protocols and then 2 μg RNA was reverse transcribed using MiraMas™ Kit (Bioo scientific, Austin, TX, USA) to generate cDNA. qRT-PCR was performed using a standard SYBR® Premix Ex Taq™ II (Takara, Shiga, Japan). The primers were synthetized by GenePharma (Shanghai, China) as follows: miR-17 forward primer, 5'-CAAAGTGCTTACAGTGCAGGT-3'; U6 forward primer, 5'-GTGCTCACTTCGGCAGC-3'; universal reverse primer: 5'-GTCCTTGGTGCCCGAGTG-3'. The reaction was conducted following the procedure: predegeneration at 95°C for 30 sec, and then 40 amplification cycles of 95°C for 10 sec, and 60°C for 35 sec using StepOne Software v2.1 (Applied Biosystems, USA) quantitative Real-Time PCR instrument. U6 was used as references. Each sample was analyzed in triplicate. Comparative threshold cycle (CT) method-fold change ($2^{-\Delta\Delta CT}$) was used to analyze relative changes.

Cell proliferation assay

The cellular proliferation ability was measured with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 5×10^4 cells were seeded in four 96-well plate and

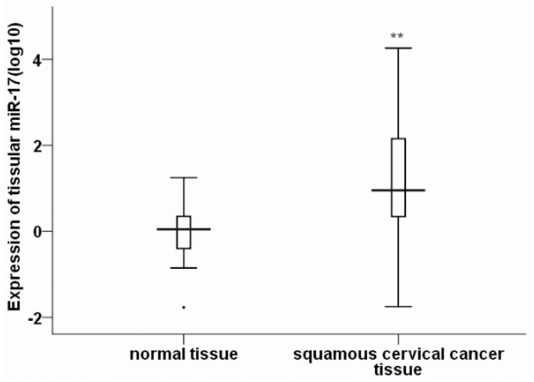

Figure 1. Expression of miR-17 in normal cervical tissues and squamous cervical carcinoma tissues (***P*<0.01), miR-17 was measured by qRT-PCR. Data were presented as log10 of fold-change. The Mann-Whitney test was performed to examine the difference between normal cervical tissues and squamous cervical carcinoma tissues. *P*<0.05 was considered significant.

allowed to adhere overnight in complete RPMI-1640 medium to get a 60-80% confluence. Followed, the cells were transfected with miR-17 inhibitor and anti-miR-NC as described above. At 24, 48, 72 and 96 h after transfection, the medium was removed and cells were incubated with 20 μl MTT (5 mg/ml, PH=7.4) in basic medium for 4 h at 37°C and 150 μl dimethyl sulfoxide (DMSO) was added to solubilize the crystals, then the plate was shocked with low speed for 10 min at room temperature to make sure ample dissolution. The optical density (OD) of all wells were recorded at 490 nm. All samples were run in triplicate independently, and all experiments were performed three times.

Transwell invasion and migration assay

Cell invasion assay was conducted in 24-well plates using a Transwell insert (Corning, Corning, NY, USA) preloaded with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The upper chamber was seeded with 50,000 cells transfected with miR-17 inhibitor or anti-miR-NC in 200 μl medium RPMI1640 free of FBS. The lower chamber was filled with 500 μl medium containing 20% FBS. After 24 hours, the cells remained in the upper chamber was removed by cotton swabs, the transwell membranes were fixed in 95% ethanol and then stained using hematoxylin-eosin staining, cells on the lower surface were counted under the fluorescent microscope (Olympus IX51, Japan). The experiment was performed independently three times in duplicates. For cell migration assay, the procedure is similar as above, except for the transwell insert without Matrigel.

Cell apoptosis analysis

After transfection, cells were harvested and double-stained with Annexin V-PE and 7AAD using the ANNEXIN V-PE Kit (Beckman Coulter, Brea, CA, USA) following the manufacturer's protocols and the analysis was performed by the flow cytometer (Beckman Coulter).

Western blot analysis

The cellular proteins were extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) with phenylmethanesulfonyl fluoride (PMSF, Beyotime). The protein concentration was measured by BCA kit (Beyotime). Equal amounts of protein samples (50 μg) were separated by 10% SDS-PAGE gels and then transferred to a polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked in TBST (TBS and 0.05% Tween-20) containing 5% skimmed milk for 1 h at room temperature, and then incubated with primary antibodies, respectively: anti-E2F1 mouse monoclonal (no.Sc-56661; 1:200; Santa Cruz, USA); anti-ß-actin mouse monoclonal (no.ab88210; 1:10,000; Abcam, England) overnight at 4°C, subsequently incubated with secondary goat anti-mouse antibody (no.926-68020; 1:10,000; LI-COR, USA) for 1 h at room temperature. Protein was detected by Odyssey infrared fluorescence scanning imaging system (LI-COR). β-actin was used to demonstrate equal loading.

Statistical analysis

All data were processed using SPSS 17.0 (SPSS Inc, IL, USA) or GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Data were presented as mean ± standard error of the mean (SEM), for 2-group comparisons using t tests. Non-parametric methods was chose to analyze the data when the results did not display normal distribution (Mann-Whitney U test between two groups and Kruskal-Wallis H test for three or more groups). To estimate the diagnostic accuracy of each parameter, receiver operating characteristic (ROC) curve was generated. The sensitivity and specificity of the optimum cut-off point referred to the values that

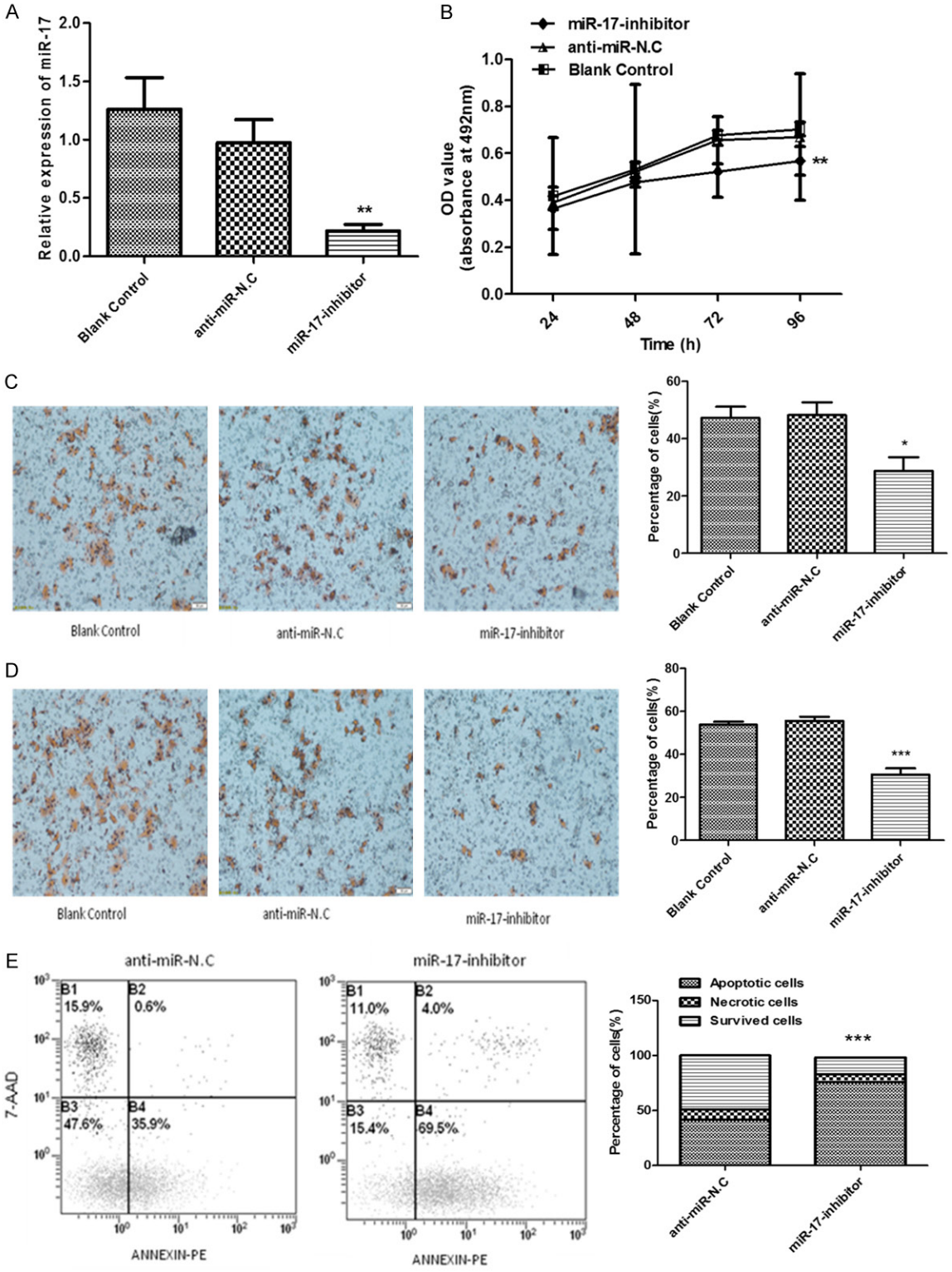

Figure 2. Establish the miR-17 inhibitor cervical cancer cell lines and cell growth characteristics, cell migration, cell invasion and cell apoptosis experiments were performed. A: MiR-17 was lower expressed in SiHa cell line while the expression level of miR-17 was still very high in cells transfected with negative control. B: Down-regulated miR-17 significantly inhibited cell proliferation in SiHa cells by MTT assay. C, D: Represented the results of cell invasion and migration across membrane with or without Matrigel, which showed that miR-17-inhibitor reduced the cell migration and invasion ability. Original magnification ×100. E: Cell apoptosis detected by Annexin V-PE combined labeling flow cytometry; apoptotic rate was increased and reached statistical significant differences in each group. (*P<0.05, **P<0.01, ***P<0.001).

Figure 3. Target validation of miR-17 in cervical cancer. A: Predicted consequential pairing of target region and miRNA by microRNA and Targetscan. B: Western blot analyses showed knockdown of miR-17, through transfection of miR-17-inhibitor in SiHa cells, increased E2F1 protein levels, β-actin served as the loading control.

maximized the area under the ROC curve (AUC). The Youden index (J) was used to identify the optimal cut-off point, which allowed the selection of an optimal cut-off point under the assumption that sensitivity and specificity were equally weighted (J=Maximum (sensitivity+ specificity-1) [7]. One way of interpreting the AUC curve is that a test with an area greater than 0.9 has high accuracy, while 0.7-0.9 indicates moderate accuracy, 0.5-0.7, low accuracy and 0.5 a chance result [8]. All *P* values were two tailed, $P<0.05$ was considered statistically significant.

Results

The expression of miR-17 is upregulated in cervical cancer tissues

Using a qRT-PCR, we compared the endogenous expression of miR-17 in human cervical cancer tissues and normal cervical tissues. The results revealed that miR-17 expression was significantly increased in cervical cancer tissues than the normal cervical tissues (**Figure 1**, $P<0.01$) with a median of 8.80, which suggested that increase of miR-17 was a frequent event in cervical cancer.

MiR-17 promotes cell proliferation, invasion and metastasis, inhibits cell apoptosis

According to the up-regulation of miR-17, we believed that miR-17 could act as a promoter of cell growth. After transfection, the qRT-PCR analysis showed that miR-17 was significantly down-regulated in miR-17 inhibitor group compared to anti-miR-NC group. The data demonstrated that miR-17 was efficiently reduced in SiHa cells (**Figure 2A**). To investigate the effects of miR-17 on growth, migration and invasion abilities of cervical cancer cells, the cells were divided into three groups according to our objectives: a: without transfection; b: transfection of anti-miR-N.C; c: transfection of miR-17 inhibitor. The growth curves showed that when transfected with miR-17 inhibitor, the cells' proliferation ability had become slower significantly (**Figure 2B**). Invasion and metastasis are the two key properties of malignant carcinoma, and cervical cancer is of no exception. Therefore, the migration and invasion abilities of SiHa cells was also detected, miR-17 could facilitate metastasis ($P<0.05$) and invasion ($P<0.001$) (**Figure 2C** and **2D**). Flow cytometry analyzed that the apoptotic rate in the miR-17 inhibitor group was evidently increased compared to the anti-miR-NC group (**Figure 2E**). As concluded, down-regulation of miR-17 significantly suppressed cell proliferation, migration and invasion abilities, and promoted cell apoptosis.

MiR-17 directly targets and inhibits E2F1

To identify the functional target of miR-17 in cervical cancer, we predicted the targets of miR-17 from publicly available databases as microRNA and TargetScan. E2F transcription factor 1 (E2F1) was among the predicted target genes of miR-17, the predicted binding sites was shown in **Figure 3A**. E2F1 was reported to be associated with cell cycle regulation, cell apoptosis and proliferation. E2F1 can act as an oncogene by activating the target gene to promote G0 to S phase transition; whereas, it can be a tumor suppressor gene by inhibiting the target gene to induce apoptosis. To test whether E2F1 expression was regulated by miR-17, we compared the expression of E2F1 in cells among miR-17 inhibitor group, anti-miR-NC group and blank control group by western blot. As shown in **Figure 3B**, down-regulated of miR-

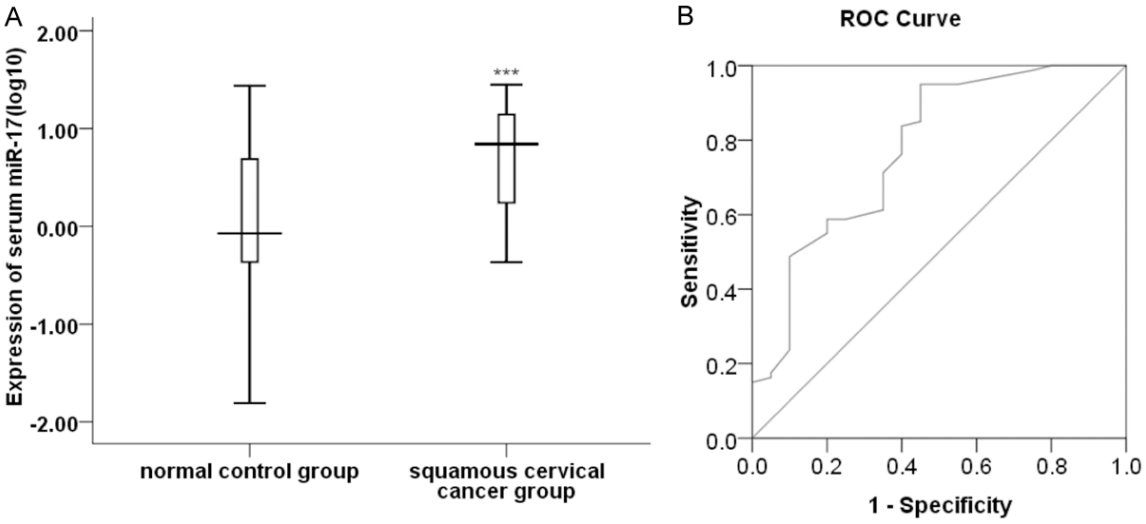

Figure 4. The clinical significance of serum miR-17. A: Expression of serum miR-17 in normal control group and squamous cervical cancer group (***P<0.001), miR-17 was over expressed in patients. Data were presented as log10 of fold-change. The Mann-Whitney test was performed to examine the difference between normal control group and squamous cervical cancer group. P<0.05 was considered significant. B: Receiver operating characteristic analysis (ROC) curve of miR-17 to distinguish cervical cancer patients from normal controls, the area under the receiver-operating curve (AUC) was 0.777±0.063, when the cut-off value was set to the optimal point of 0.858, sensitivity was 95%, and specificity was 55%.

Table 1. miR-17 expression and clinicopathological features

Features	No. (n=80)	miR-17 median
Age (years)		
>50	43	6.94
≤50	37	6.98
Menopause		
Yes	25	13.78
No	55	6.94
Histological grade		
Poorly differentiated	11	27.36***
Moderately differentiated	11	6.94
Well differentiated	58	6.89
FIGO stage		
I	51	3.49
II	29	13.78*
Metastatic lymph node		
Yes	40	10.45
No	40	5.18

*P<0.05, ***P<0.001.

17 led to the overexpression of E2F1. The results indicate that E2F1 is a direct downstream target of miR-17 and E2F1 downregulated in cervical cancer may be attributed to the overexpression of miR-17.

The expression of serum miR-17 is upregulated in cervical cancer patients

In view of the above-mentioned effects of miR-17, we considered whether it could be defined as a noninvasive marker for screening early stage cervical cancer. Using a qRT-PCR, we compared the expression of circulating miR-17 in serum between cervical cancer patients and normal control subjects. The results revealed that serum miR-17 expression was significantly increased in cervical cancer patients than normal control subjects (**Figure 4A**, P<0.001) with a median of 6.888, which suggested that increase of miR-17 was a frequent event in cervical cancer. Then we analyzed the correlation of serum miR-17 and its clinicopathologic features: up-regulation of serum miR-17 had a significantly positive correlation with the International federation of gynecology and obstetrics (FIGO) stage (P<0.001) and pathological grade of early stage squamous cervical cancer (P<0.05), while the expression difference in lymph node metastasis, the patients' age and menopausal status was not apparently (P> 0.05), as shown in **Table 1**.

Logistic regression and ROC analyses were subsequently used to assess the sensitivity and specificity. Using miR-17 to distinguish cer-

vical cancer patients from normal control subjects, the odds ratio was 1.129 (95% confidence interval [CI] 1.024-1.245), the AUC was 0.777±0.063, Youden index was 0.5, when the cut-off value was set to the optimal point of 0.858, sensitivity was 95%, and specificity was 55% (as shown in **Figure 4B**).

Discussion

Increasing studies indicate that miRNAs can act as not only oncogene but also tumor suppressor gene, whose dysregulation is closely related to the development, metastasis and invasion of various malignant tumors [9]. Thus, we shed light on the dysregulated miRNAs in cervical cancer. miR-17-92 cluster, a highly conserved gene sequence, located at 13q31.3, is currently being accepted as tumor promotor. The precursor transcript contains six tandem stem-loop hairpin structures that ultimately yield six mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a [10]. Its expression is increased in a variety of tumors, such as hematopoietic malignancies and solid tumors, which is mainly due to the gene amplification in chromosome 13q31 region and the increased transcription mediated by cMyc [11].

In our study, we focused on the effect of miR-17 on the squamous cervical cancer. Previous study showed that miR-17 was up-regulated in cervical cancer tissues using microarray, and the following study confirmed the result by qRT-PCR in 80 cervical cancer tissues and 20 normal cervical tissues, which was consistent with the other solid tumors: lung, breast, stomach, prostate, colon and pancreatic tumors [12]. According to these finding, we speculated that miR-17 might be a potential tumor promoter in cervical cancer. Therefore, we detected the alteration on proliferation, invasion, metastasis and apoptosis of squamous cervical cancer cell lines SiHa by transfecting with miR-17 inhibitor. As expected, the MTT assay showed down-regulation of miR-17 inhibited proliferation ability. By transwell assay, after transfection, the cells penetrated the membrane became significantly less. The flow cytometry came out that apoptotic cells' ratio increased after transfection, so miR-17 could promote the cells' proliferation, invasion and metastasis, inhibit apoptosis.

Whereas, Wei etc [13] presented that miR-17-5p was down-regulated in cervical cancer tissues compared to the adjacent tissues, and it suppressed cell growth and promoted apoptosis of cervical cancer cells: HeLa and C33A. The results differ from ours, probably due to: Firstly, the number of their tissue samples was twenty-one, too less to represent for the actual expression. Secondly, the control tissue samples they used was adjacent normal tissues, for screening, the normal tissues from benign patients may be of more validity. Thirdly, the pathologic types may contribute a large proportion to the difference [14, 15]. The cervical cancer tissues they used may contain squamous cancer, adenocarcinoma, while the cervical cancer tissues in our study were all squamous cancer. Lastly, HPV infection is of great importance to change the miRNAs expression [16]. The cervical cancer cell line HeLa is adenocarcinoma cell, and HPV18 positive, the C33A cell line is HPV negative, however, the SiHa cell line is squamous cell, and HPV16 positive.

The public available databases widely indicated that E2F transcription factor 1 (E2F1) was a potential target gene of miR-17. Moreover, it had been verified by luciferase assay [17]. E2F1 is not only known to be involved in regulation of cell cycle by promoting G0/G1-S phase transition, but also participates in regulating the cell apoptosis. In our study, the results showed that the regulatory role of miR-17 in cervical cancer cells was dependent on targeting E2F1. According to the flow cytometry results, we speculate that, miR-17 may target on E2F1 to inhibit apoptosis, which promotes the cells proliferation. E2F1 signalling to apoptosis triggers a variety of cell death pathways: 1) E2F1 activates the p53 family; 2) E2F1 strongly induces mitochondria death pathways through a plethora of mediators, such as caspases; 3) E2F1 inhibits the anti-apoptotic signaling, it can downregulate the TRF2 protein level to block the anti-apoptotic NF-κB signals, which sensitizes the cells to apoptosis signal [18]. But the specific pathway that miR-17 may take part in need further experimental study to verify.

MiRNAs may become a powerful new biomarker used in clinical, which fulfills the criteria a biomarker needed, such as structure stability and accessibility in various bodily fluids, sequence conservation between human and clinically important animal models, and available sensitive measurement methodologies

[19]. Serum miR-17-5p was up-regulated in gastric cancer [20] and lung cancer [21] patients, which was negatively correlated with the survival time. Taking consideration of this, we examined the serum miR-17 in cervical cancer patients, and we found serum miR-17 was up-regulated in the early cervical squmous cell cancer patients, in addition, its expression went up with the more poorly differentiation and the more advanced FIGO stages, which was consistent with its carcinogenic properties. However, our study found that using miR-17 to distinguish patients from normal subjects, the odds ratio was 1.129 (95% CI 1.024-1.245), the AUC was 0.777±0.063, when the cut-off value was set to the optimal point of 0.858, the sensitivity was 95%, and specificity was 55%, which indicates moderate accuracy. Consequently, it had not been an independent diagnostic factor used in clinical, but if combined with other indicators, the diagnostic efficiency would be better.

The major prognostic factor of cervical cancer is lymph node metastasis (LNM). According to the statistics, the 5-year survival rate of cervical cancer patients in FIGO Ib-IIb stages without lymph node metastasis was about 95%, whereas the patients with lymph node metastasis was only 64.0% to 68.2% [22]. The current application to judge LNM is imaging examinations, such as CT, MRI and PET-CT, which has limitations to find micro lesions. Supposing miR-17 could be a serum biomarker to detect LNM, it would be of great importance to improve the prognosis of cervical cancer patients. Yet our study revealed that the serum miR-17 expression level had no significantly relation with LNM. The reasons may related to: First, even if the patients had the postoperative pathological data, due to the section quality and method, LNM still could be ruled out in the diagnosis; Second, our study mainly chose the early squamous cervical cancer patients of FIGO Ia-IIa stages, if more stages were enrolled in especially late stages, the results could be different. Last, enlarge the sample size, the results would be more convincing. The conclusion does not exclude the deviation, we need further experiments with a large cohort of patients to validate and exploit miR-17 as a serum biomarker for cervical cancer.

Above all, our findings demonstrate that miR-17 was significantly overexpressed in squamous cervical cancer serum and tissue samples. Down regulated of miR-17 inhibited proliferation, invasion, metastasis, and induced apoptosis of SiHa cells through targeting E2F1, which lay a foundation for understanding the mechanisms of cervical cancer development, and serum miR-17 may be a novel biomarker for clinical use in the future, but we have a long way to go to verify the hypothesis.

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Disclosure of conflict of interest

None.

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