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Original Article

microRNA-21 is a potential indicator for the pathological progression of cervical cancer

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Abstract: Cervical cancer is a disease of high incidence, affecting numerous female patients' health and life. miR-21 is reported to play oncogenic roles in various cancers including cervical cancer, but its correlation with cervical cancer pathological progression is not fully understood. This study aims at revealing the expression pattern of miR-21 in the tissues of cervical intraepithelial neoplasia (CIN) and cervical cancer patients of different pathological grades. miR-21 levels in 35 CIN and 35 cervical cancer specimens were detected by qRT-PCR and compared to 40 normal specimens. Then in the 35 cervical cancer specimens, miR-21 levels were compared in different cancer variables. Its roles in cervical cancer cell migration and invasion were again verified by Transwell experiments after vector transfection to overexpress pre-miR-21 or miR-21 sponge in HeLa cells. miR-21 was promoted in CIN tissues compared to normal tissues ($P < 0.05$), and elevated in cervical cancer tissues compared to CIN tissues ($P < 0.05$). In cervical cancer tissues, miR-21 expression did not vary significantly with different patient ages or tumor sizes ($P > 0.05$), but it was significantly up-regulated in cancer tissues with higher pathology grades, clinical stages and lymphatic or distant metastasis ($P < 0.05$), which was supported by Transwell results that miR-21 promoted HeLa cell migration and invasion. These results indicated that the up-regulation of miR-21 might be associated with severer cervical cancer grades, implying the role of miR-21 as a possible indicator for the pathological progression of cervical cancer.

Keywords: Cervical cancer, miR-21, pathological progression, pathology grade, clinical stage

Introduction

Cervical cancer is a disease occurring in the cervix, thus being a female-attributed cancer that affects the normal life of tens of thousands of women [1]. Cervical cancer consists of two main types, squamous cell carcinoma and adenocarcinoma, with the former causing health problems in the majority of patients. Human papillomavirus (HPV) infection appears to be a leading risk factor of cervical cancer, and most patients are detected with HPV DNAs [2]. In addition, hereditary factors, as well as unhealthy habits like smoking and unhealthy sex life, are also potential causes of cervical cancer. Invasive cervical cancer has become the third most common cancer for women worldwide, adding great burdens to low- and middle-income countries, especially. Though the introduction of screening programs has effectively reduced the incidence and mortality of cervical cancer in developed countries [3], greater efforts are still being paid to acquire more knowledge, diagnostic and therapeutic methods for fighting this disease.

Cervical cancer is developed from a series of precancerous lesions, cervical intraepithelial neoplasia (CIN) for instance, which is a pathological process from quantitative to qualitative changes with the accumulation of various genetic and phenotypic disorders. Continuous infection with high risk HPV may eventually lead grade III CIN to cervical cancer [4]. Modern genome and transcriptome sequencing technology has revealed the specific portraits of different CIN and cervical cancer grades and stages, allowing high diagnostic and staging accuracy and appropriate therapeutic methods [5]. Moreover, as key regulators of gene expression,

microRNAs are coming into focus in the diagnosis and treatment of cervical cancer. For example, miR-124 represses vasculogenic mimicry, migration and invasion via interacting with angiomotin like 1 [6]. miR-125b level is changed with different cervical lesions, thus showing the potential as a predictive biomarker for cervical cancer [7]. Similarly, let-7a and miR-21 are confirmed to be possible targets for management of cervical cancer [8].

miR-21 is one of the first identified microRNAs in human and is overexpressed in various cancers [9]. Its association with cervical cancer, including its influence on radiosensitivity [10] and its oncogenic role of promoting cell proliferation [11], lymph node metastasis [12], is attracting increasing attention. Still, little is known about miR-21 and its relationship with cervical cancer malignancy. The aim of this study is to investigate miR-21 expression pattern in the pathological progression of cervical cancer. We collect tissue samples from normal cervical and different degrees of CIN and cervical cancer, and detect miR-21 expression, which is compared in various cancer variables. miR-21 is found to be discriminately expressed in CIN and cervical cancer, and is related to different pathology grades, clinical stages and metastasis of cervical cancer. These findings imply that miR-21 may be a valuable indicator for pathological progression and malignancy of cervical cancer.

Materials and methods

Tissue sampling

All the cervical tissues used in this study were sampled from patients of cervical cancer or CIN and patients with no cervical disease that were clinically and histopathologically diagnosed and hospitalized from January to December in 2014. Patients with no cervical disease (n = 35) aged from 29 to 66 (48.5 ± 15.8). CIN patients (n = 35) aging from 30 to 65 (47.9 ± 16.1) were fell into three CIN degrees: degree I (11 individuals), degree II (12 individuals) and degree III (12 individuals). The pathological degrees and clinical stages were identified according to Diagnosis Criteria for Cervical Cancer proposed by Ministry of Health (December, 2011, China). The individual numbers of G1, G2 and G3 pathological degrees were 23, 7 and 5, respectively, and those of I, II, III and IV clinical stages were 10, 11, 8 and 6, respectively. Normal tissues, CIN tissues and cervical cancer tissues were sampled during surgical procedures, immediately frozen in liquid nitrogen for RNA extraction and tissue section preparation. The diagnosis of CIN and cervical cancer was performed by two pathologists in a double-blind manner. The sampling procedures were approved by all the patients and a local ethics committee.

miR-21 level detection by qRT-PCR

The tissue samples were ground in liquid nitrogen and miRNAs were extracted using miRNeasy Mini Kit (Qiagen, Shenzhen, China) according to the manufacturer's instructions. Reverse transcription was performed using the miRNAs samples, the specific primer for has-miR-21 (5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG CTA CAA CT-3') and PrimeScript Reverse Transcriptase (TaKaRa, Dalian, China). qRT-PCR was performed to detect the expression level of miR-21 using the specific primers (Fw: 5'-ACA CTC CAG CTG GGT AGC TTA TCA GAC TGA-3' and Rv: 5'-TGG TGT CGT GGA GTC G-3') on QuantStudio 6 Flex platform (Applied Biosystems, Carlsbad, CA). U6 (Fw: 5'-CTC GCT TCG GCA GCA CA-3' and Rv: 5'-AAC GCT TCA CGA ATT TGC GT-3') was used as an internal control. Experiments on each tissue sample were repeated for three times, and data were analyzed with $2^{-\Delta\Delta Ct}$ methods compared to normal tissues.

HeLa cell culture and transfection

Human cervical adenocarcinoma HeLa cell line (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA), and incubated in humidified atmosphere with 5% CO_2 at 37°C. The cells were passaged every two days. HeLa cells were transfected with vectors expressing pre-miR-21 or miR-21 sponge (QuantoBio, Beijing, China) and the corresponding blank vectors as controls using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, at one day before transfection, the cells were seeded in 24-well plates at a density of 1×10^5/mL. Transfected vectors (2 μg/mL) were used to prepare the transfection complex, which was then added to the culture medium.

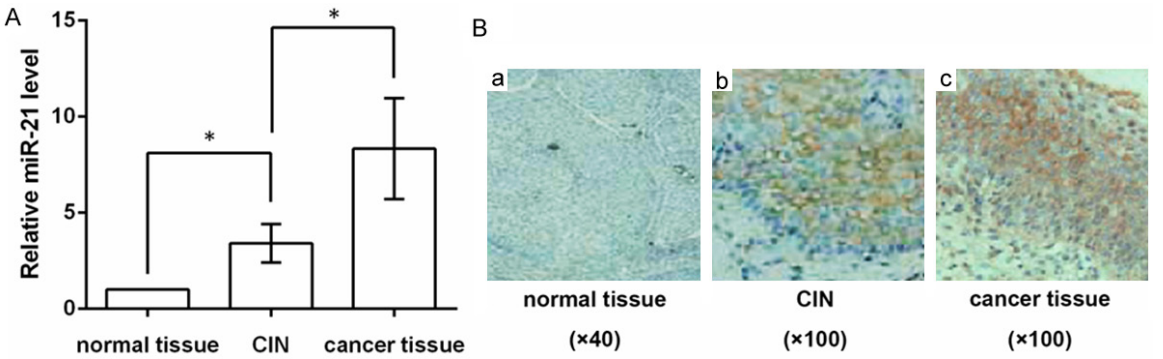

Figure 1. miR-21 level is associated with the pathological progression of cervical cancer. (A) miR-21 expression is detected in normal tissues, all cervical intraepithelial neoplasia (CIN) tissues and all cervical cancer tissues by qRT-PCR (triplicate detection). U6 is used as an internal control. The expression level of CIN and cancer tissue groups was compared to that of normal tissue group. Significant differences are found between CIN and normal tissues ($P < 0.01$) or cancer tissues ($P < 0.01$). (B) Pathological section observation indicates different pathology grades of normal tissue (a), degree II of CIN (b) and G2 of cancer tissue (c). More P16-possitive cells (brown) are observed in CIN sections and even more in cancer tissue sections, indicating the increasing severity of pathological progression.

The cells were incubated in humidified atmosphere with 5% CO_2 at 37°C. Medium was changed at 6 h post transfection. After incubation for 48 h, cells were collected for further migration and invasion assays.

Cell migration and invasion assays by transwell

Cell migration and invasion were detected using Transwell (BD Biosciences, San Jose, CA). For cell migration assay, the cells were starved in serum-free medium for 24 h, and adjusted to a density of 5×10^5/mL, after which the cells were added to the upper chamber of Transwell sets. The lower chamber contained DMEM with 20% FBS, and then the chambers were incubated for 24 h. After the incubation, the medium in the upper chamber was removed, and the cells on the lower side of the membrane were washed in phosphate buffered saline (PBS), fixed in methanol for 30 min and stained by crystal violet for 20 min. The cells left on the upper side of the membrane were wiped out with a cotton swab, and the membrane was washed in PBS and mounted. The migrated cells were observed using an optical microscope (Olympus, Tokyo, Japan) and counted in five random visual fields.

For cell invasion assay, the upper chamber was pre-coated with Matrigel (BD Biosciences) and incubated at 37°C for 4 h for gel formation. Before the experiment, the gel was hydrated in serum-free medium for 2 h, after which the same procedures as the cell migration assay were carried out.

Statistical analysis

Results of observed cell numbers and qRT-PCR were indicated as the mean ± standard deviation. Data were analyzed by *F* test for homogeneity of variance and then *t* test for significant difference. $P < 0.05$ indicated the significant difference between groups. For analysis of qRT-PCR results, in particular, the tissue samples were first divided into different values according to the parameters of age, tumor diameter, pathology grade, clinical stage, lymphatic metastasis and distant metastasis, and then data of the same parameter value were normalized to the corresponding U6 data and compared to normal tissue. Also, data of all CIN samples or all cervical cancer samples were analyzed together compared to those of normal tissues for a general expression pattern.

Results

miR-21 level is associated with the pathological progression of cervical cancer

The expression level of miR-21 was analyzed in all of the CIN tissues and cervical cancer tissues compared to normal tissues for a general understanding of the expression pattern. As was shown in qRT-PCR results (**Figure 1A**), miR-21 expression in CIN tissues was significantly increased than normal tissues ($P < 0.05$), and

Table 1. Relative miR-21 expression in 35 cervical cancer patients divided according to different cervical cancer variables[a]

Variable		n	miR-21 level	*P* value
Age	≤ 45	21	7.027 ± 0.791	0.805
	> 45	14	6.926 ± 0.721	
Tumor diameter	≤ 2.5 cm	17	8.422 ± 0.659	0.503
	> 2.5 cm	18	7.828 ± 0.797	
Pathology grade	G1	23	5.988 ± 0.537	0.006**
	G2/G3	12	8.421 ± 0.782	
Clinical stage	I/II	21	6.882 ± 0.991	0.031*
	III/IV	14	9.232 ± 0.662	
Lymphatic metastasis	Yes	12	9.223 ± 0.922	0.082*
	No	23	5.775 ± 0.792	
Distant metastasis	Yes	6	10.355 ± 0.522	0.043*
	No	29	7.425 ± 0.962	

*$P < 0.05$. **$P < 0.01$. [a]miR-21 level was indicated as the mean ± standard deviation compared to that of normal tissues.

significantly higher in cervical cancer tissues than CIN tissues ($P < 0.05$). It seemed that miR-21 level was higher in the tissue with a severer pathological degree, which implied that the expression pattern of miR-21 was likely to be related to the pathological progression of cervical cancer.

The tissue sections were then checked and results confirmed the increasing severity of cervical pathology degrees from degree I of CIN to G3 of cervical cancer, as indicated by the density of P16-positive cells. For example, P16-positive cells were barely found in normal tissues, but were observed in degree II of CIN and G2 of cervical cancer tissues (**Figure 1B**). Moreover, the signal density of P16 was higher in cervical cancer tissues than CIN tissues. Together with the increased miR-21 level in cervical cancer tissues, miR-21 expression was thought to be associated with the progression of cervical cancer pathology.

Then more detailed analyses were performed to reveal miR-21 expression patterns in various cancer variables including age of patients, tumor diameter, pathology grade, clinical stage, lymphatic metastasis, and distant metastasis (**Table 1**). No significant difference in miR-21 expression was found between the two age groups (≤ 45 and > 45) or different tumor diameters (≤ 2.5 cm and > 2.5 cm) ($P > 0.05$). However, miR-21 expression was obviously up-regulated in G2/G3 tissues compared G1 ($P < 0.01$), and up-regulated in more severe clinical stages (III/IV) and tumor with lymphatic metastasis or distant metastasis ($P < 0.05$). These data indicated that the up-regulation of miR-21 in cervical cancer tissues might correlate with severer pathological grades rather than patient age or tumor size, further confirming that miR-21 expression level was associated with the pathological progression of cervical cancer.

miR-21 promotes HeLa cell migration and invasion

In order to verify the relationship between miR-21 and cervical cancer progression, we performed miR-21 overexpression and inhibition in HeLa cells to detect changes in cell migration and invasion by Transwell experiments. miR-21 was promoted and inhibited by the specific vectors expressing its precursor and sponge, respectively, and results were compared to the control groups transfected with corresponding blank vectors. Cell migration assay showed that the migrated HeLa cells were increased by miR-21 overexpression ($P < 0.01$, **Figure 2A** and **2B**) and decreased by miR-21 inhibition ($P < 0.01$). Similarly, the invasive ability of HeLa cells were promoted by miR-21 overexpression ($P < 0.05$, **Figure 2C** and **2D**) and inhibited by miR-21 inhibition ($P < 0.01$). These results indicated that miR-21 overexpression could promote HeLa cell migration and invasion, which might facilitate the progression of cervical cancer.

Discussion

miR-21 has been studied in cervical cancer before, being up-regulated in cervical cancer tissues and cells. In addition to its roles in cervical cancer cell radiosensitivity, proliferation and metastasis, its underlying relationship with the pathological progression of cervical cancer was investigated in this study. We detect miR-21 levels in CIN and cervical cancer tissues of altogether 75 patients to reveal its expression pattern in different pathological grades. miR-21 is expressed in lower level in normal cervical tissues and moderately expressed in CIN tissues. Its higher expression level is found in cervical cancer tissues with higher pathology

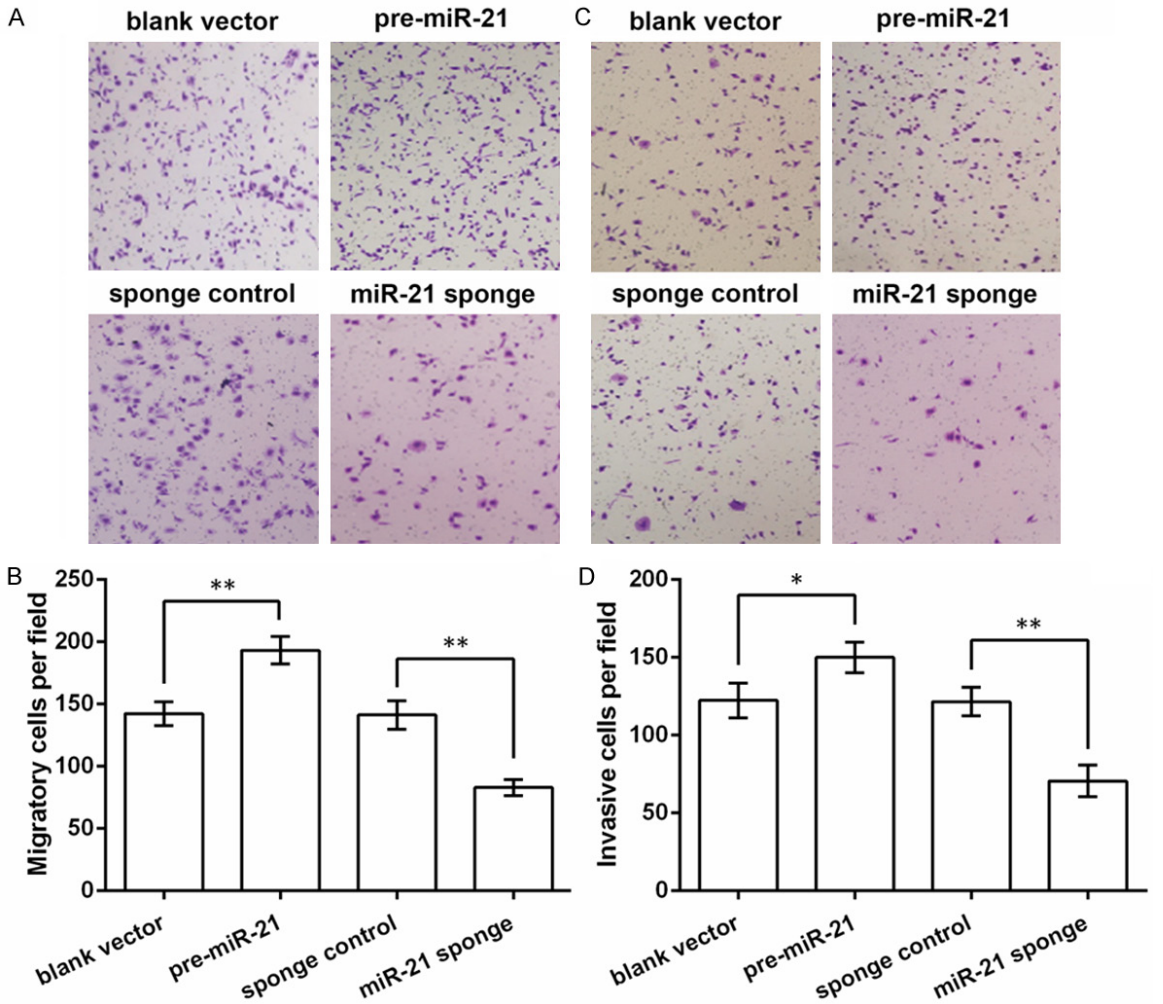

Figure 2. miR-21 promotes HeLa cell migration and invasion. HeLa cells are transfected with the vector expressing miR-21 precursor (pre-miR-21), the vector expressing miR-21 sponge (miR-21 sponge), and the corresponding blank vectors (blank vector, sponge control) as controls. Transwell experiments are performed to detect cell migration and invasion at 48 h post transfection (n = 5). HeLa cell migration and invasion are promoted by miR-21 overexpression and inhibited by miR-21 suppression (A and C). Repeated experiments show significant changes in cell migration and invasion (B and D). *$P < 0.05$. **$P < 0.01$.

grade, clinical stage and lymphatic and distant metastasis.

Expression and functions of miR-21 have been reported in various diseases. Its expression is generally up-regulated in cancers and other diseases such as glioma, breast cancer, colorectal cancer and lung cancer [13]. Specifically, it is up-regulated in breast cancer, and anti-miR-21 oligonucleotides suppress cell growth and tumor growth [14]. It regulates key genes related to cell apoptosis, migration and invasion, and affects metalloproteinases, thus promoting invasiveness of glioma cells [15]. In colorectal cancer, miR-21 inhibits the expression of tumor suppressor *PDCD4* and results in promoted cell invasion and metastasis [16]. Besides the up-regulated miR-21 level found in solid cancers like prostate and pancreatic tumors [17], it is also aberrantly expressed in leukemia [18]. All these findings show a relatively conserved role of miR-21 in cancer cell proliferation and invasion, allowing potential application of miR-21 in diagnosis and treatment of cancers. Consistently, results of this study showed an up-regulated miR-21 expression in cervical cancer tissues of patients. Moreover, HeLa cell migration and invasion were promoted by miR-21 overexpression and inhibited by miR-21 sponge. So miR-21 is a

facilitator of cervical cancer that is capable of promoting cervical cancer cell migration and invasion.

miR-21 has been revealed to possess higher expression in invasive cervical cancer tissues than CIN tissues and normal tissues [19, 20], and a recent study also revealed the promotive function of miR-21 in cervical cancer cells, where the researchers used miR-21 mimics and inhibitors to manipulate its expression in HeLa cells and found miR-21 could promote HeLa cell proliferation, migration and invasion via inhibiting phosphatase and tensin homolog [21]. On the basis of these findings, this study further divided the cervical cancer tissues from 35 patients into different categories according to the cancer variables, which allowed us to discover miR-21 expression patterns in different grades of cervical cancer.

The expression pattern of miR-21 sorted by different cancer variables showed that miR-21 expression did not vary with patient age or tumor diameter. Instead, its expression possessed significant disparities between pathology grades, clinical stages, and cancer with lymphatic/distant metastasis or not, which implied the possible relationship between miR-21 level and these cancer variables. Similar functions of miR-21 have been reported in colorectal cancer, where miR-21 expression is close related to a prognostic value disease-free interval [22], lymph node positivity and development of distant metastases [23]. In breast cancer, high level of miR-21 is significantly associated with advanced clinical stage, lymph node metastasis and shortened survival of patients [24]. Besides, miR-21 may be a prognostic marker for squamous cell lung carcinoma patients [25]. However, it seems that miR-21 expression pattern cannot reflect clinical prognosis in gastric cancer, albeit it promotes gastric cancer cell proliferation and invasion and is a biomarker for this disease [26, 27]. Taken together, the association between miR-21 and cancer pathological progression may vary according to specific diseases, and in cervical cancer, miR-21 is capable of indicating pathological progression, as suggested in this study.

miR-21 has been proved to be a potential tissue biomarker, rather than a good serum marker, for diagnosing cervical cancer [28, 29]. High level of miR-21 exists in the cervical cancer-derived exosomes in cervicovaginal lavage specimens [30]. Results of this study further propose the possibility of using miR-21 as an indicator of cervical cancer pathological progression, but research on more detailed pathological stages performed in more specimens is necessary to verify this microRNA indicator.

In summary, this study shows miR-21 level is elevated in cervical cancer tissues with high pathological grades, revealing the potential of miR-21 to be an indicator of cervical cancer pathological progression. These findings uncover a new function of miR-21 for cervical cancer diagnosis that may facilitate traditional diagnostic methods.

Disclosure of conflict of interest

None.

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