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Dysregulated microRNAs involved in the progression of cervical neoplasm

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Abstract

Purpose MicroRNAs (miRNAs) exhibit dysregulated expression in human cancer and play an important role in carcinogenesis. The aim of this study was to identify a distinct miRNA expression signature for cervical cancer and cervical intraepithelial neoplasia (CIN) and to investigate the function of deregulated miRNAs in cervical carcinoma.

Methods A miRNA microarray was used to compare miRNA expression profiles in cervical cancer, CIN and normal cervical tissues. Real-time RT-PCR was used to validate the expression of 9 miRNAs in 103 cervical tissues. Bioinformatics programs were used to predict potential target genes and their function. Functional studies were performed to characterize the effect on cervical cancer cells by overexpression of miR-218 and miR-21.

Results We identified deregulated miRNAs in cervical cancer and high-grade squamous intraepithelial lesions (HSIL). MiR-218 was the most downregulated (0.175-fold decrease) miRNA, and miR-21 was the most upregulated (5.67-fold increase) miRNA. In addition, the expression patterns of 9 miRNAs were validated by real-time RT-PCR. Bioinformatics analyses and functional studies indicated that miR-218 and miR-21 may be involved in cancer invasion and metastasis.

Conclusion Our study demonstrated that miRNAs are aberrantly expressed in cervical cancer and cervical preneoplastic lesions. These miRNAs may be involved in the progression of cervical neoplasm as potential tumor suppressor genes or oncogenes.

Keywords Cervical cancer · Cervical intraepithelial neoplasia · MicroRNA

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Introduction

Cervical cancer is the third leading cancer in women worldwide. The occurrence of cervical cancer is a multifactorial, multi-step process that involves the transformation of normal cervical epithelium into cervical intraepithelial neoplasia (CIN) that subsequently transforms into invasive cervical cancer. CIN1, CIN2 and CIN3 correspond to mild, moderate and severe dysplasia, respectively. Low-grade squamous intraepithelial lesions (LSIL) represent CIN1, and high-grade squamous intraepithelial lesions (HSIL) represent a combination of CIN2 and CIN3 [1]. Only a few CIN cases are expected to progress to more severe forms of dysplasia or invasive cancer; therefore, it would be highly desirable to have a marker with a higher predictive value for progression. Cervical carcinoma is

almost always related to persistent infection with high-risk types of human papillomavirus (HPV). HPV-16 and -18 account for 70 % and the seven most common types (HPV-16, -18, -45, -31, -33, -52, and-58) account for 87.4 % of all cervical cancer cases worldwide [2]. However, evidence suggests that HPV infection alone is insufficient to induce malignant changes, and other genetic events are required for cancer development. The identification of such genetic alterations would be of considerable importance for the screening and treatment of cervical cancer [3].

MicroRNAs (miRNAs) are a class of endogenous and small noncoding regulatory RNAs that regulate genes at the posttranscriptional level. Many reports have demonstrated that miRNAs play key roles in various biological processes, including cell differentiation, proliferation, apoptosis, tumorigenesis and tumor metastasis [4]. Bioinformation and experimental research has revealed that hundreds of thousands of protein-coding genes are regulated by miRNA [5]. More than half of all miRNA genes are located in fragile sites and genomic regions that frequently exhibit dysregulated expression in human cancer [6]. These dysregulation of miRNAs may contribute to the development of various cancers and those involved in carcinogenesis are classified into oncogenic miRNAs and tumor suppressor miRNAs [7]. It has been proposed that a unique miRNA expression profile could be used to classify human cancers with high accuracy and may serve as a useful biomarker for cancer diagnosis and prognosis for a particular cancer [7–10]. Studies have also shown aberrant miRNA expression in cervical cancer; however, the results are inconsistent across different studies. Moreover, only a few of these studies included precancerous cervical specimens (CIN 1–3). We therefore identified the miRNA expression profile of uterine cervix carcinomas and preneoplastic lesions. We also predicted possible gene targets and explored the function of relevant candidate miRNAs.

Materials and methods

Cervical tissue samples

Clinical specimens were collected from patients treated at People's Hospital of Guangxi Zhuang Autonomous Region. Twelve cervical tissue samples were used for microarray analysis, which including 3 cases of squamous cell cervical carcinoma (SCC), 3 HSIL (CIN2 or CIN3), 3 LSIL (CIN1) and 3 normal cervices. One hundred and three cervical tissue samples were used for real-time RT-PCR analysis, which including 40 cases of SCC, 35 HSIL, 15 LSIL and 13 normal cervices. The clinicopathological data of the patients are listed in Table 1. All of the specimens were collected before undertaking any treatment. The specimens were sectioned and stained with H&E for a pathologic review conducted by a pathologist. Normal cervical tissues were obtained from patients undergoing a hysterectomy for benign gynecological disease. Informed consent was obtained from all participating subjects, and the study was approved by the Clinical Research Ethics Committee of the hospital.

HPV-DNA detection and HPV status

Genomic DNA was extracted by TIANamp FFPE DNA Kit (Tiangen, Beijing, China). Six most common HPV types (HPV-16 and/or -18, -31, -45, -52, -58) were detected by using E6 Nested Multiplex PCR (NMPCR) as described previously [11, 12]. In brief, nested PCRs were performed using MY09-MY11 consensus primers and the PCR product served as template for the nested PCRs using E6 gene type-specific primers for HPV-16, -18, -31, -45, -52, -58. The amplification products were analyzed by electrophoresis on 2 % agarose gels and ethidium bromide staining. PCR products were sequenced. The HPV infection rates in SCC, HSIL, LSIL and normal tissues were 87.5, 80, 53.3 and 23 %, respectively. The most common types detected among the cases (either alone or in coinfection with other types) were HPV16 (56.7 %) and HPV18 (23 %).

RNA extraction and purification from tissues

The cervical lesions tissues in paraffin section were selected and the lesion cells reach more than 90 % by using H&E staining. Total RNA was extracted and purified from formalin-fixed paraffin-embedded (FFPE) tissue using a

Table 1 Clinicopathologic description of patients

Pathological evaluation	Age (years)	HPV infections (%)	Lymph node metastasis	FIGO stage (I, II, III)	Differentiation (low, medium, high)
SCC ($n = 40$)	50.08 ± 8.47	35 (87.5 %)	9	28, 10, 2	14, 20, 6
HSIL ($n = 35$)	45.65 ± 10.22	28 (80 %)			
LSIL ($n = 15$)	42.80 ± 10.03	8 (53.3 %)			
Normal ($n = 13$)	46.65 ± 4.46	3 (23 %)			

FIGO International Federation of Gynecology and Obstetrics

RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, US) (for microarray assays) or a miRNeasy FFPE Kit (Qiagen, CA, US) (for real-time RT-PCR) according to the manufacturer's instructions.

MiRNA microarray assay

The microRNA expression profiles of the cervical tissues were determined using the Agilent Human MicroRNA Array Kit (8*15 K V12.0) (Agilent Technologies, Santa Clara, CA, US), which contains probes for 866 human and 89 viral microRNAs from the Sanger database. The assay and data analyses were performed at Shanghai Biochip Company according to the protocols of the Agilent microRNA microarray system. The data were normalized using a quantile algorithm (Gene Spring Software 11.0, Agilent Technologies, Santa Clara, CA, US). Differentially expressed miRNAs from each sample type were identified using the Significance Analysis of Microarrays (SAM) software. Between samples, miRNAs with at least a two-fold increase or a 0.5-fold decrease in expression with a Q-value (false discovery rate) of 5 % were considered to have significant changes in their expression.

Reverse transcription and real-time quantitative polymerase chain reaction (real-time RT-PCR)

The expression levels of the 9 selected miRNAs were validated by qRT-PCR. All of the RT and PCR primers were synthesized by Integrated DNA Technologies. The RT reactions were performed using Quant reverse transcriptase (Tiangen, China). Each RT reaction included 150 ng of RNA as the template and a pool of RT primers. Real-time PCR was performed with SuperReal PreMix Plus (SYBR Green) (TianGen Biotech Co. China) on an ABI 7500 system. Small RNA U6 was used as an endogenous control, and the relative expression levels of the miRNAs were calculated using the following equation: $2^{-\triangle\triangle Ct}$, $\triangle Ct = (Ct_{miRNA} - Ct_{U6})$.

Cell culture and miRNA transfection

Human cervical cancer (HeLa) cells were cultured in RPMI 1640 medium with 10 % fetal bovine serum and 1 % penicillin/streptomycin at 37 °C in a humidified incubator with 5 % CO_2. HeLa cells were seeded 24 h before transfection. Transfection experiments were performed using 60 nmol/L miRNA mimics (miR-218 or miR-21) (GenePharma, Shanghai, China) and Lipofectamine 2000 (Invitrogen). The negative controls included both the negative control miRNA (GenePharma) and a mock transfection (no RNA added).

Predicted gene targets of selected miRNAs and gene ontology analysis

TargetScan 4.2, PicTar and miRanda were used to predict the gene targets of miR-218 and miR-21, which were differentially expressed miRNAs in cervical cancer tissues. The Gene Ontology (GO) annotation database (http://www.geneontology.org) was used for GO annotations of predictive target genes.

Transwell migration and invasion assay

Twenty-four hours after the miRNA mimic and negative control transfection, the transfected cells were resuspended in serum-free RPMI 1640 medium, and 1.5×10^5 cells were added to the upper chamber of 24-well plates (8-µm pore size, Corning, Corning, NY) for a migration assay. For the invasion assay, the upper chamber was coated with 50 µg of Matrigel (BD Biosciences, USA) before the cells were loaded and then incubated for 4 h at 37 °C and 5 % CO_2. The lower compartment was filled with RPMI 1640 medium containing 10 % FBS. After 8 h (migration assay) or 24 h (invasion assay), the cells that had not migrated or invaded were removed from the upper side of the filters using cotton swabs, and the cells that had migrated or invaded to the lower surface of the filters were fixed in methanol and stained with crystal violet solution. The number of cells migrating or invading to the lower surface was determined microscopically by counting 4 areas of constant size per well. The percentage of migrated or invaded cells with overexpressed miRNA was normalized to that of the mock cells.

Results

MiRNA expression profile in cervical SCC, HSIL, LSIL and normal cervical tissues

To investigate whether miRNAs are differentially expressed in cervical tissues, we used a miRNA microarray to analyze miRNA expression in cervical SCC, HSIL, LSIL and normal cervical tissues. SAM analysis of the cervical SCC tissues relative to the normal cervical tissues showed that 16 miRNAs were significantly upregulated and that 10 miRNAs were significantly downregulated. miR-21, miR-21*, miR-15b and miR-16 were the most overexpressed miRNAs, and miR-218 and miR-376 were the most downregulated miRNAs, with at least a fourfold change (Fig. 1a; Table 2). SAM analysis of the HSIL tissues showed that 13 miRNAs were upregulated and that 31 miRNAs were downregulated compared to normal cervical tissue. miR-663, miR-188-5p, miR-765, miR-1300, miR-

Fig. 1 Significance analysis of microarrays (SAM) of differentially expressed miRNAs in **a** cervical SCC and normal tissues, **b** HSIL and normal tissues, **c** LSIL and normal tissues. The *red dots* represent the significantly upregulated miRNAs (twofold increase or more); the *green dots* represent the significantly downregulated miRNAs (0.5-fold decrease or less); and the *black dots* represent miRNAs with no significant difference in expression
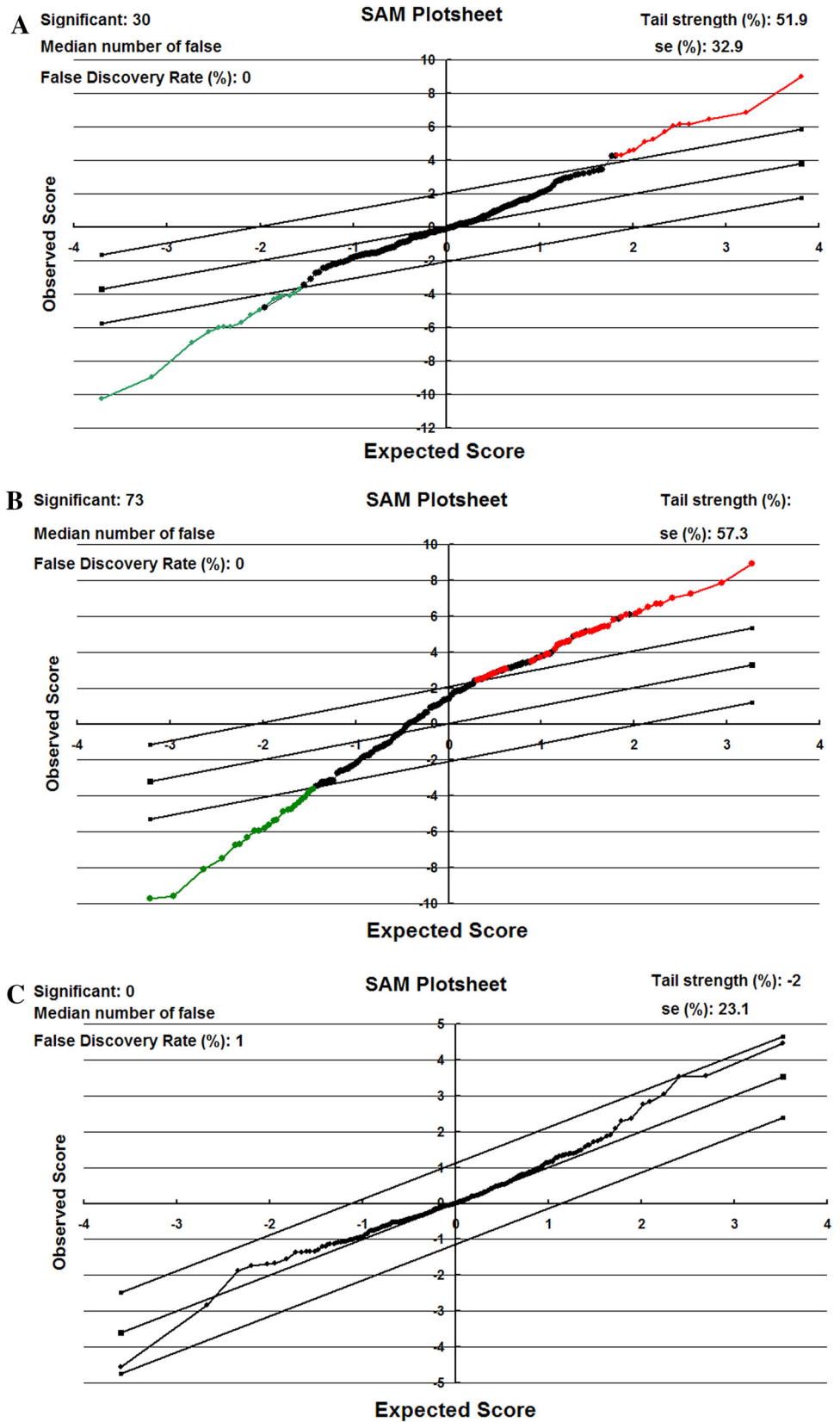
Table 2 Significant expression of miRNAs in cervical cancer tissues relative to normal tissues

miRNAs	Fold change[a]	Q-values[b]	miRNAs	Fold change	Q-values
hsa-miR-218	0.175	0.002	hsa-miR-93	2.674	0.003
hsa-miR-376a	0.234	0.007	hsa-miR-17	2.703	0.002
hsa-miR-127-3p	0.259	0.002	hsa-miR-1280	2.805	0.008
hsa-miR-100	0.261	0.007	hsa-miR-141	2.839	0.006
hsa-miR-195	0.315	0.001	hsa-miR-20a	2.951	0.004
hsa-miR-199b-5p	0.337	0.009	hsa-miR-15a	3.223	0.002
hsa-miR-497	0.357	0.001	hsa-miR-106b	3.39	0.004
hsa-miR-10b	0.372	0.002	hsa-miR-20b	3.668	0.007
hsa-let-7c	0.439	0	hsa-miR-9	3.714	0.021
hsa-miR-214	0.44	0	hsa-miR-16	4.107	0
hsa-miR-185	2.107	0.007	hsa-miR-15b	4.258	0
hsa-miR-130b	2.531	0.001	hsa-miR-21*	5.539	0.009
hsa-miR-31	2.551	0.003	hsa-miR-21	5.677	0.001

[a] Mean fold changes of miRNA expressions in cervical cancer tissues compared with those in normal cervical tissues

[b] The lowest false discovery rate of differential expression with statistically significance

Table 3 Significant expression miRNAs in HSIL relative to normal tissues

| miRNAs	Fold change[a]	Q-values[b]	miRNAs	Fold change	Q-values
hsa-miR-142-3p	0.143	0.001	hsa-miR-20a	0.424	0
hsa-miR-152	0.16	0.005	hsa-miR-29a	0.436	0.009
hsa-miR-149	0.19	0.007	hsa-miR-193b	0.439	0.002
hsa-miR-374b	0.22	0	hsa-miR-195	0.446	0.003
hsa-miR-376c	0.225	0.003	hsa-miR-15a	0.447	0.009
hsa-miR-218	0.227	0.002	hsa-miR-27a	0.467	0.003
hsa-miR-143	0.257	0.002	hsa-miR-342-3p	0.479	0
hsa-miR-210	0.306	0.001	hsa-miR-99a	0.481	0
hsa-miR-98	0.313	0.005	hsa-miR-151-3p	0.487	0.003
hsa-miR-150	0.322	0.007	hsa-miR-150*	2.047	0.004
hsa-miR-140-5p	0.333	0.005	hsa-miR-494	2.138	0.006
hsa-miR-365	0.34	0.008	hsa-miR-572	2.385	0.003
hsa-miR-20b	0.343	0.007	hsa-miR-1207-5p	2.683	0.009
hsa-miR-1274a	0.357	0.005	hsa-miR-638	2.71	0.003
hsa-miR-19b	0.39	0	hsa-miR-1268	2.845	0.001
hsa-miR-455-3p	0.391	0.01	hsa-miR-939	3.509	0
hsa-miR-224	0.399	0.009	hsa-miR-671-5p	3.576	0
hsa-miR-127-3p	0.402	0.002	hsa-miR-188-5p	4.404	0.002
hsa-miR-106b	0.405	0	hsa-miR-765	8.028	0.009
hsa-let-7f	0.409	0.008	hsa-miR-483-5p	8.237	0.001
hsa-miR-199b-5p	0.41	0.004	hsa-miR-1300_v13.0	8.245	0.004
hsa-miR-497	0.414	0.001	hsa-miR-663	9.402	0

[a] Mean fold changes of miRNA expressions in HSIL tissues compared to those in normal cervical tissues

[b] The lowest false discovery rate of differential expression with statistically significance

483-5p were the most upregulated miRNAs, and miR-218, miR-376c, miR-374b, miR-149, miR-152 and miR-142-3 were the most downregulated miRNAs (Fig. 1b; Table 3).

However, when we compared miRNA expression in LSIL tissues to normal cervical tissues, we could not identify significant differential expression (Fig. 1c).

Fig. 2 The relative expression of 9 miRNAs in cervical cancer, HSIL and LSIL compared with normal cervical tissue by real-time RT-PCR. Each miRNA expression level was normalized to that of U6 and normal cervix RNA. *$P < 0.05$, **$P < 0.01$
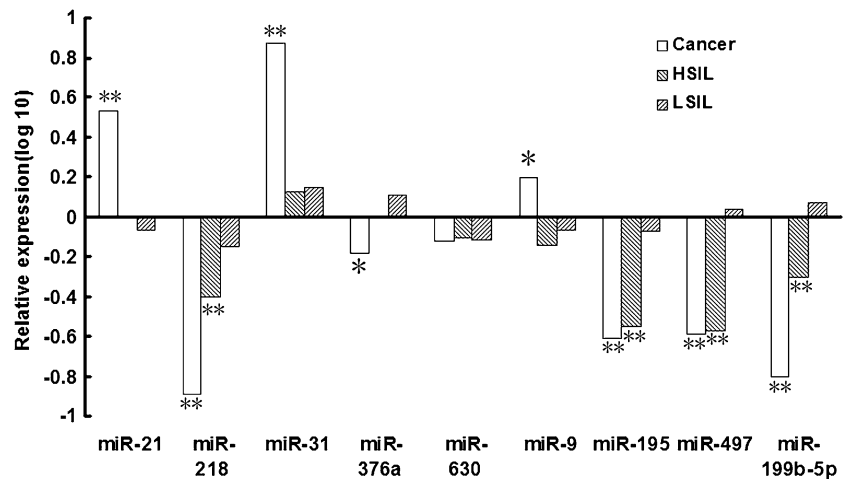
Validation of miRNAs with real-time RT-PCR

To validate the microarray data, 9 miRNAs (miR-21, miR-218, miR-376a, miR-31, miR-630, miR-9, miR-195, miR-497, miR-199b-5p) that were differentially expressed in SCC or HSIL were selected and analyzed in 40 cervical cancers, 35 HSIL, 15 LSIL and 13 normal tissues using qRT-PCR. The real-time RT-PCR results demonstrated that the expression of miR-21, miR-31 and miR-9 were significantly upregulated in cervical cancer. The expression of miR-218, miR-195, miR-497, miR-199b-5p were significantly downregulated in cervical cancer and HSIL, and miR-376a was significantly downregulated in cervical cancer. No significant difference in miR-630 expression was observed in cervical cancer, HSIL, LSIL compared with normal cervical tissues. The validation results were consistent with the miRNA assay (Fig. 2).

Target gene prediction of miR-218 and miR-21 and GO analysis

MicroRNAs can regulate a large number of target genes. The identification of miRNA gene targets is critical to understanding the direct regulatory roles of miRNAs in cervical cancer. Several databases based on various algorithms are available for predicting the targets of selected miRNAs. TargetScan 4.2, PicTar and miRanda were used to predict the gene targets of miR-218 and miR-21. There are 2890 and 2155 predicted targets for miR-218 and miR-21, respectively. Four of the predicted gene targets were selected for GO analysis, which were LAMB3 and ROBO1 for miR-218, PTEN and PDCD4 for miR-21 (Table 4). These candidate targets were selected based on their target prediction scores as well as their known involvement in human cancers. GO analysis revealed that these gene targets are involved in cell adhesion, cell proliferation, angiogenesis, apoptosis, and cell migration, which are all

functions related to cancer initiation and progression. Therefore, miR-218 and miR-21 may be involved in the cervical carcinogenesis mechanism.

Effects of miR-218 and miR-21 overexpression on migration and invasion in Hela cells

After demonstrating a significant downregulation of miR-218 and an upregulation of miR-21 in cervical cancer, we further investigated the roles of miR-218 and miR-21 in cervical cancer progression. Synthetic miR-218 and miR-21 mimics were transiently transfected into HeLa cells, and transwell migration and invasion assays were performed. The results demonstrated that the invasion and migration capabilities were inhibited in HeLa cells with miR-218 overexpression (Fig. 3a). In contrast, these abilities were increased in cells with miR-21 overexpression (Fig. 3b). These data suggest that both miR-218 and miR-21 are likely to be involved in cervical cancer progression.

Discussion

In the present study, we performed a miRNA microarray analysis and profiled miRNA expression in cervical SCC, HSIL, LSIL and normal cervical tissues. Our study demonstrated that miRNAs are aberrantly expressed in human cervical cancer and cervical preneoplastic lesions. We identified 16 upregulated and 10 downregulated miRNAs between SCC and normal cervices. miR-21, miR-21*, miR-15b and miR-16 were the most overexpressed miRNAs, and miR-218 and miR-376 were the most underexpressed miRNAs. We also validated these data by performing qRT-PCR analyses of nine miRNA with altered expression in SCC or HSIL. These results indicated aberrant miRNA expression profiles in cervical cancer and HSIL. Several recent studies have shown that miRNAs

Table 4 Gene ontology annotation of target genes of miR-218 and miR-21

| MiRNAs	Target genes	Description	Gene ontology
miR-218	LAMB3	Laminin, beta 3	GO:0032403 protein complex binding
			GO:0007155 cell adhesion
			GO:0044420 extracellular matrix part
			GO:0005198 structural molecule activity
	ROBO1	Roundabout, axon guidance receptor, homolog 1	GO:0007155 cell adhesion
			GO:0002042 cell migration involved in sprouting angiogenesis
			GO:0005515 protein binding
			GO:0008046 axon guidance receptor activity
miR-21	PTEN	Phosphatase and tensin homolog	GO:0005515 protein binding
			GO:0004721 phosphoprotein phosphatase activity
			GO:0008283 cell proliferation
			GO:0001525 angiogenesis
			GO:0006915 apoptotic process
			GO:0016477 cell migration
	PDCD4	Programmed cell death 4	GO:0006915 apoptotic process
			GO:0005515 protein binding
			GO:0003723 RNA binding

have altered expression profiles in cervical cancer [13–15]. Studies have also shown that several downregulated miRNAs are associated with advanced-stage disease, lymph node metastasis and poor prognosis in cervical cancer [16]. However, the identified miRNAs from different studies exploring cervical cancer were inconsistent, which may be due to differences in the high-throughput platforms and methods used in different laboratories [17]. Furthermore, sample collection procedures, data normalization and filtering methods may affect the final results. In our studies, the prevalence of HPV positive is relatively low, which may be because only six common HPV types were detected and the number of sample is relative small. These may also affect the results. It will be necessary to increase the number of sample and improve the method of HPV detection in the future.

Some studies have demonstrated aberrant miRNA expression in precancerous lesions [15, 17]. In this study, we also identified 13 upregulated and 31 downregulated miRNAs in HSIL when compared with normal cervical tissues. Several miRNAs, namely miR-218, miR-195, miR-199b-5p, and miR-497, were downregulated in both SCC and HSIL. We also observed that some miRNAs were overexpressed in SCC but not in HSIL. We hypothesize that the miRNAs that are downregulated in both SCC and HSIL may be involved in the abnormal transformation from precancerous lesions to cancer. However, we did not observe differential miRNA expression between LSIL and normal cervical tissue, which may account for the reversibility between LSIL and normal cervical tissue. Thus,

further investigation is required with a larger number of patient samples.

In our study, miR-218 was the most downregulated miRNA and miR-21 was the most upregulated miRNA in cervical cancer tissues. miR-218 is known to be underexpressed in a variety of cancerous tissues and is involved in cancer invasion, metastasis and proliferation [18–20]. miR-218 was also observed to be downregulated in cervical cancer, CIN tissues and cervical cancer cell lines. Furthermore, infection with high-risk HPV-16 also lowers the expression of miR-218 [21–23]. miR-21 expression has been shown to be significantly upregulated in head and neck cancer [24], endometrioid endometrial cancer [25], and lung cancer [26]. Studies have also reported that miR-21 expression increases in cervical cancer tissues and cells [27–29].

The role of miRNA in the process of tumorigenesis is not yet clear. The functional analysis of target genes will help us to understand the molecular mechanisms of miRNAs involved in cervical carcinogenesis. Among the predicted target genes, LAMB3 and ROBO1 have been validated as targets of miR-218 [30–32], and PDCD4 and PTEN have been identified as targets of miR-21 in several cancers [33–35]. A gene ontology analysis demonstrated that these target genes may be involved in cell growth and proliferation, apoptosis, adhesion and migration. Our in vitro studies indicated that the overexpression of miR-218 can significantly reduce cervical cancer cell motility and invasion. This result confirmed recent studies [36, 37]. Our studies also showed that the overexpression of miR-21
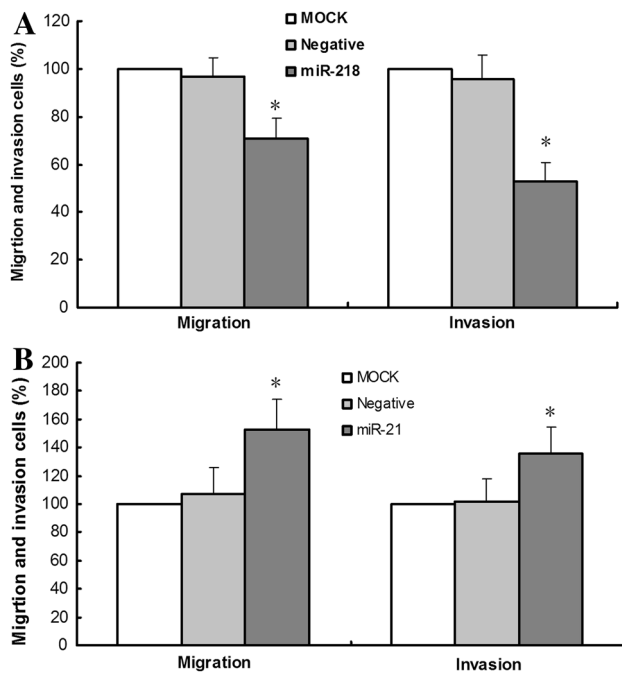
Fig. 3 The effects of miR-218 and miR-21 overexpression on cervical cancer cell migration and invasion. miRNA mimics were transfected into cervical cancer HeLa cells, and the effects on cell migration and invasion were evaluated by Transwell migration invasion assays. **a** Overexpression of miR-218 led to a significant reduction of cervical cancer cell migration and invasive capacity. Compared with the mock cells, the rate of cell migration and invasion in the transfected cells was 71.01 ± 8.5 and 52.6 ± 6.0 %, respectively. **b** Overexpression of miR-21 led to a significant increase in cervical cancer cell migration and invasive capacity. Compared with mock-transfected cells, the rate of cell migration and invasion in the transfected cells was 152.81 ± 16.8 and 154.9 ± 8.1 %, respectively. Data represent mean ± SEM; *P < 0.05. *SEM* standard error of the mean

can significantly increase cervical cancer cell motility and invasion. These studies suggest that miR-218 may function as a tumor suppressor in cervical cancer by targeting multiple cancer-related genes and miR-21 likely acts as an oncogene in cervical cancer.

Our study demonstrated that there is aberrant miRNA expression in cervical cancer and precancerous lesions and that the deregulation of miR-218 and miR-21 is involved in cervical cancer cell invasion and migration. The identification of aberrant miRNA expression patterns in cervical cancer and CIN contributes to the understanding of the pathogenesis and progression of cervical neoplasm at the molecular level. The altered miRNAs may play an important role in the malignant transformation of cervical cancer as a tumor suppressor gene or oncogene. We hope that further studies on these microRNAs and their target regulation will lay a theoretical foundation for therapeutic interventions of cervical cancer using microRNAs.

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