

## Down-Regulated Mir-22 as Predictive Biomarkers for Prognosis of Cervical Cancer

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### Abstract

**Background:** microRNA-22 (miR-22) was frequently altered in numerous cancers and was involved in various cellular processes related to carcinogenesis. However, the role of miR-22 in cervical cancer haven't been investigated.

**Methods:** 62 pairs of fresh cervical cancer tissue were collected between May 2012 and March 2015. Real-time quantitative RT-PCR assay was performed to evaluate the expression levels of miR-22. The survival curves were determined using the Kaplan-Meier method and Cox regression for statistics. The roles of miR-22 in cell proliferation, migration and invasion were identified using miR-22 mimic-transfected cells. In addition, the regulatory effect of miR-22 on suppressor of cancer cell invasion (SCAI) was evaluated using qRT-PCR a dual luciferase reporter assay.

**Results:** miR-22 expression in cervical cancer tissues was significantly lower than that in normal tissues (mean  $\pm$  SD:  $1.944 \pm 1.026$  vs.  $4.981 \pm 1.507$ ,  $P < 0.0001$ ). Low miR-22 expression level was correlated with FIGO stage ( $P = 0.00004$ ), tumor differentiation ( $P = 0.0002$ ), and lymph-node-metastasis stage ( $P = 0.0099$ ). Kaplan-Meier analysis with the log-rank test indicated that low miR-22 expression had a significant impact on overall survival ( $P = 0.0016$ ) and progression-free survival ( $P = 0.0005$ ). Moreover, ectopic miR-22 expression potently inhibited cervical cancer cell growth ( $P < 0.01$ ), migration ( $P < 0.01$ ) and invasion ( $P < 0.01$ ) *in vitro*. miR-22 overexpression in cervical cancer cell lines decreased MMP1 and MAPK1 expression at the translational level and reduced MMP1/MAPK1-driven luciferase-reporter activity ( $P < 0.01$ ).

**Conclusions:** Our data demonstrated that the expression of miR-22 was downregulated in cervical cancer, and associated with overall survival as well as progression-free survival, suggesting that miR-22 could serve as an efficient prognostic factor for cervical cancer patients.

**Keywords:** miR-22; Prognosis; Cervical cancer

### Background

Cervical cancer is a highly prevalent cancer in women all over the world, which causes about 300,000 deaths each year, continues to be the leading cause of death among gynecological malignancies [1]. Although the cervical cytology screening benefits a lot in the early diagnosis and treatments, cervical cancer outcomes vary significantly [2]. The exactly molecular mechanisms of cervical cancer initiation and progression have not yet been fully elucidated. More importantly, it tends to present at more and more young cases in recent years [3]. So there is an urgent need for understanding more sensitive molecular prognostic biomarkers to better predict the outcome of cervical cancer.

MicroRNAs (miRNAs) are small, conserved, non-coding short RNAs of 18–25 nucleotides in length that bind to target mRNAs mainly at their 3'-UTR [4]. These small molecules have been found to regulate genes involved in diverse biological processes such as cell proliferation, development, differentiation, apoptosis and others [3]. Numerous studies have shown that alterations in miRNAs expression in human cancers are often associated with tumor development, progression and metastasis [5]. Recently, microRNA-22 (miR-22) has been shown to be related with several kinds of cancers, such as prostate cancer, breast cancer, and etc [6-9]. In this study we focus on the expression and clinical significance of miR-22 in cervical cancer. Understanding the molecular mechanisms of miR-22 in the development and progression of human cervical cancer could provide the promising point for developing a therapeutic strategy.

### Methods patients and tissue samples

This study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Xi'an Jiao Tong University. Written informed consent was obtained from all patients. All specimens were handled and made anonymous according to the ethical and legal standards. 62 cervical cancer tissues and 20 normal cervix tissues were collected and immediately placed in liquid nitrogen and then stored at -80°C until the isolation of RNA. Clinical-pathological characteristics including age, pathological stage, histology, lymph node metastases and tumor grade were collected and shown in Table 1. None of the patients recruited in this study had undergone preoperative chemotherapy or radiotherapy.

The duration of follow-up was calculated from the date of surgery to death or last follow-up, and patients were excluded if they

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Characteristic	n=62	miR-22 expression		P value
		High	Low	
Age(yr)				0.28
<55	36	20	16	
≥55	26	10	16	
FIGO stage				0.0002
I - II	40	12	28	
III-IV	22	18	4	
Pathology				0.21
endometrioid	54	24	30	
serous	8	6	2	
Differentiation				0.00004
High	38	9	29	
Middle+Low	24	21	3	
Lymph node metastasis				0.0099
Negative	40	14	26	
Positive	22	16	6	

**Table 1:** Clinical-pathological characteristics were collected and shown.

had incomplete medical records or inadequate follow-up. Patient's conditions were staged according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO).

### Cell lines and transfections

Cervical cancer cell lines, Hela and SiHa, were obtained from American Type Culture Collection (ATCC) and cultured in T-medium with 5% Fetal Bovine Serum at 37°C, 5% CO<sub>2</sub>. Hela and SiHa cells were transfected with Lipofectamine 2000 (Invitrogen, CA, USA), according to the manufacturer's protocol. miR-22 mimics and its negative control were purchased from Genepharma Company (Shanghai, China). A scrambled sequence without significant homology to any rat, mouse or human gene was used as a negative control (vector group) (ABI PRISM, Carlsbad, CA). The double-stranded oligonucleotide encoding miR-22 mimic and negative control were annealed and inserted into the linearized eukaryotic pFU-GW-009 vector (GeneChem). All these vectors were confirmed by sequencing. SiHa and HeLa cells were transfected with the miR-22-expressing plasmids using Lipofectamine 2000 (Invitrogen, CA, USA), and G418 (400 µg/ml, Sigma) was used to select the clones stably overexpressing miR-22.

### Clonogenic assays

Cell viability was examined using the CellTiter 96 Non-Radioactive

Cell Proliferation Assay (MTS assay, Promega), according to the manufacturer's protocol. For clonogenic assays, transfected cells were calculated and seeded 48 hrs post-transfection at low density in 6-well plates. Cells were incubated for 10-14 days, then fixed and stained with 0.2% methylene blue in 50% methanol. The surviving fraction was calculated by comparison with control cells.

### Cell wound healing assay

The migration ability of the cervical cancer cells was determined using a wound healing assay. At 24 h post-transfection, a monolayer of cells was scratched with the angularity of a small X-ray film. Wound closures were photographed every 4 h.

### Luciferase assay

The miR-22 binding sites from 3'-UTR MMP1/MAPK1 or mutant 3'-UTR were cloned into the pGL3 reporter luciferase vector (GeneChem). For reporter assay, 100 nM miR-22 mimic or control miRNA was co-transfected with 0.1 µg of the pGL3-3'UTR wildtype or mutant plasmid DNAs into Hela cells in 96-well plates using Lipofectamine 2000. Luciferase activity was measured 48 hours posttransfection as described previously.

### Statistical analysis

All data were processed using PASW Statistics 16. Data were presented as mean ± SEM, using t tests for 2-group comparisons.

While the results did not display normal distribution, we chose to analyze the data with non-parametric methods. (Mann-Whitney U test between two groups and Kruskal-Wallis H test for three or more groups). A P value less than 0.05 is considered statistically significant. The relationship between miR-22 and MMP1/MAPK1 expression was explored by Spearman's correlation analysis. Significant associations between miR-22 changes and clinicopathologic parameters were assessed using a  $\chi^2$  test. Two-sided P-values < 0.05 were considered statistically significant.

### Results

#### Relative expression of microRNA-22 is associated with advanced clinicopathologic characteristics of patients with cervical cancer

Relative miR-22 expression was detected by qRT-PCR between paired tumor tissues and normal tissues from 62 patients with cervical cancer. Of the 62 patients with cervical cancer, 32 were placed in the low miR-22 expression group and 30 were placed in the high miR-22 expression group. The associations between clinicopathologic features and miR-22 expression were summarized in Table 1. Our results demonstrated that the relative expression of miR-22 were markedly downregulated in cervical cancer samples compared with the normal tissues ( $2.162 \pm 0.2044$  vs  $5.819 \pm 0.3157$ ), ( $P < 0.0001$ ; Figure 1A). To

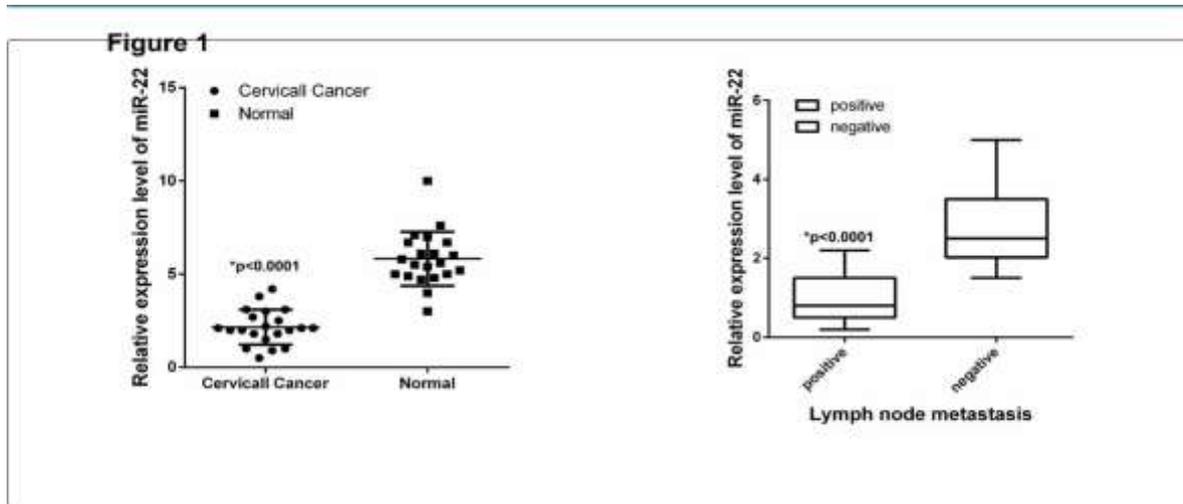
evaluate the clinical value of miR-22 in cervical cancer patients, we divided the patients into different groups and the association between relative miR-22 expression and clinicopathologic information was then analyzed (Table 1). A significant low miR-22 expression level was correlated with FIGO stage ( $P = 0.00004$ ), tumor differentiation ( $P = 0.0002$ ), and lymph-node-metastasis stage ( $P = 0.0099$ ) (Figure 1B, Table 1). No significant association was found between miR-22 Expression and other clinical characteristics, such as age and pathology (Table 1). Hence, downregulated miR-22 expression was closely related to cervical cancer metastasis.

## Invasion assays

Briefly, cells ( $0.5 \times 10^6$  cells/well) were seeded with medium containing low serum (1%) in the upper chamber. The lower chamber was filled with medium containing high serum (20%) as a chemoattractant. Cells were incubated for 24 hrs and then membranes were stained using Diff-Quick (Siemens). A light microscope was used to count the number of invading cells. Invasion assays were performed using the BD BioCoat Matrigel Invasion Chambers and Control Inserts (BD Biosciences), respectively.

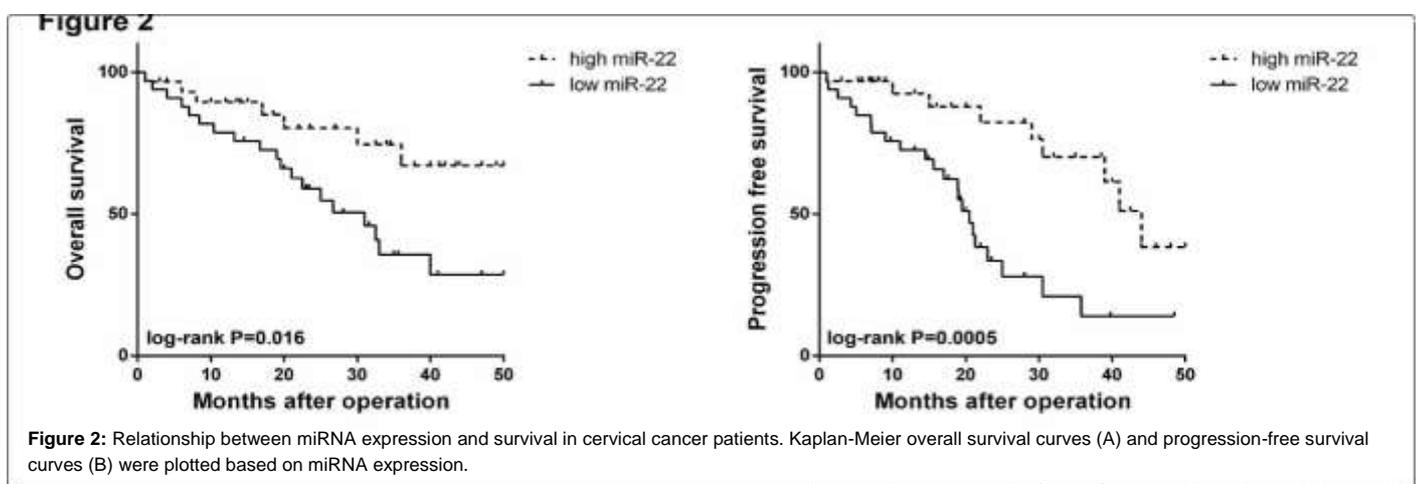
## The expression of miR-22 is associated with survival of cervical cancer patients

To further analyze the significance of miR-22 in terms of clinical prognosis, Kaplan Meier analysis with the log-rank test was performed, using patient survival (Figure 2). Our results indicated that low miR-22 expression had a significant impact on overall survival ( $P = 0.0016$ ;



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**Figure 1:** miR-22 expression is downregulated in clinical specimens. **A:** qRT-PCR analysis showed that miR-22 expression was downregulated in cervical cancer tissues compared with the normal tissues; **B:** miR-22 expression was significantly lower in cervical cancer patients with lymph node metastasis compared with patients without lymph metastasis. Small nuclear RNA U6 was used as a normalization control.



**Figure 2:** Relationship between miRNA expression and survival in cervical cancer patients. Kaplan-Meier overall survival curves (A) and progression-free survival curves (B) were plotted based on miRNA expression.

shown in Figure 2A) and progression-free survival ( $P = 0.0005$ ; shown in Figure 2B). Multivariate analysis revealed that miR-22 expression level

was an independent prognostic factor for overall survival ( $HR = 2.754$ , 95% CI: 1.201-5.591), as well as progression-free survival ( $HR =$

3.460, 95% CI: 1.805-7.536) of cervical cancer patients. This supports the notion that low levels of miR-22 in tumor tissues could lead to worse survival rates in cervical cancer patients.

### miR-22 promotes colony formation, migration and invasion *in vitro*

To determine whether miR-22 had any effects on cervical cancer cell lines (SiHa and HeLa cells), which are both cervical squamous cell carcinoma lines, we analyzed colony formation, migration and invasion. Both cell lines were transfected with premiR negative control (miR-NC) or pre-miR-22 (miR-22). miR-22 significantly inhibited colony formation compared with the NC control in both cell lines (both  $P < 0.05$ ; Figure 3A).

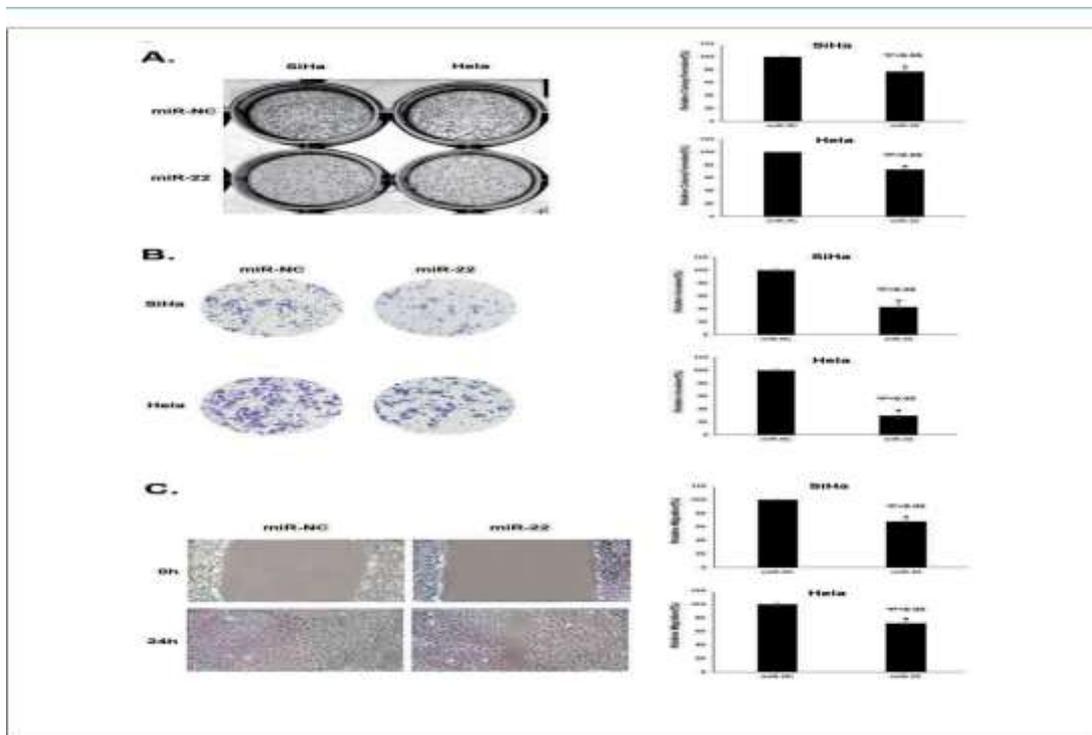
Because miR-22 down-regulation was observed to be associated with lymph node metastasis and recurrence in our patients, we performed migration and invasion assays. Consistent with the clinical data, miR-22 overexpression reduced migration and invasion capacities of both HeLa and SiHa cells ( $P < 0.05$ ; Figures 3B and 3C).

Taken together, these results suggest an oncogenetic role for miR-22 in regulating cervical cancer cell survival, invasion and motility in both HeLa and SiHa cells.

### miR-22 Directly Targets and Inhibits MMP1 and CXCR4

To understand how miR-22 facilitates cervical cancer growth and metastasis, we used four algorithms (Targetscan, miRanda, miRDB and miRWalk) to help identify miR-22 targets in human cervical cancers. Of these target genes that were predicted by all algorithms (Figure 4A), matrix metalloproteinase 1 (MMP1) and mitogen-activated protein kinase 1 (MAPK1) attracted our attention immediately as they have been implicated in tumorigenesis and metastasis. We cloned the MMP1 and MAPK1 3'-UTR into a luciferase reporter vector. Luciferase assay revealed that miR-22 directly bound to MMP1 and MAPK1 3'-UTR, and by which it remarkably reduced luciferase activities (Figure 4B). However, mutation of the putative miR-22 sites in the 3'-UTR of MMP1 and MAPK1 abrogated luciferase responsiveness to miR-22. To directly assess the effect of miR-22 on MMP1 and MAPK1 expression, we performed western blot analysis. As seen in (Figure 4C), overexpression of miR-22, through infection of miR-22 mimic, in HeLa cells increased are direct downstream targets for miR-22 in HeLa cells. The above results prompted us to examine whether miR-22 inhibits

**Figure 3:** Cell growth characteristics, colony formation, cell invasion and migration experiments were performed. **A:** Colony formation of the cervical cancer cell lines after miR-22 mimic or miR-NC transfected were analyzed. **B:** Transwell cell invasion assay in the cervical cancer cell lines after miR-22 mimic or miR-NC transfected. Scale bar, 50  $\mu$ m. **C:** The cell-wound-healing assay were used to assess the migrating ability in the cervical cancer cell lines after miR-22 mimic or miR-NC transfected. Each well was examined in duplicate with at least three experiments, and the results are means  $\pm$  S.E.M. \* $P < 0.05$  and \*\* $P < 0.01$  using a Student's t test.

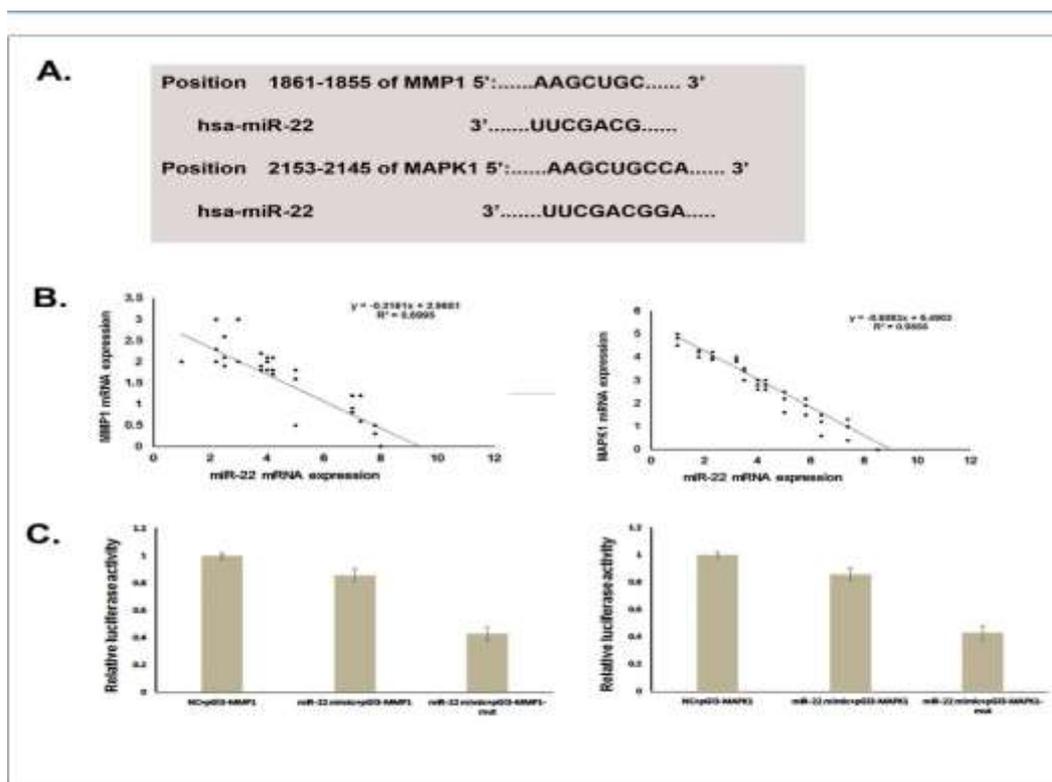


## Discussion

### Discussed

Evidence has shown miRNAs may play an important role in cancer initiation, development and metastasis in many types of cancers, including cervical cancer [10-12]. Thus, studies have focused on cancer-specific miRNAs and its target genes to elucidate biological mechanism [13,14]. Previously, researchers have found that dysregulation of several miRNAs might be associated with the prognosis in patients with cancer.

In this study, we analyzed the expression of miR-22 in cervical cancer tissues, explored the effects of miR-22 on cervical cancer cell lines, and determined the target genes regulated by miR-22. We have discovered that expression levels of miR-22 were downregulated with an increase in the grade of FIGO stage. First, we found that patients with cervical cancer have a much lower level of miR-22 compared with normal cervical tissue (Figure 1). Although considerable evidence has revealed the importance of miR-22 in cancer, its role as an ontogenetic factor is still controversial: it was downregulated in breast cancer, inhibits cell proliferation, cell metastasis, impairs migration in ovarian cancer cells [15,16]. In contrast, miR-22 expression was suggested to be upregulated in prostate cancer [6].



**Figure 4:** Suppressor of cancer cell invasion is a direct target of miR-22. **A:** Putative miR-22 binding sequence in the 3'-UTR of the mRNA of MMP1 and MAPK1 were shown; **B:** Inverse correlation between miR-22 and mRNA levels of MMP1 and MAPK1 in tissue samples was illustrated; **C:** Dual-luciferase reporter assay showed a significant decrease in the relative luciferase activity of wt- MMP1/MAPK1 (wild-type MMP1/MAPK1 3'-UTR) co-transfected with miR-22 mimic compared with that of mut- MMP1/MAPK1 (mutant-type MMP1/MAPK1 3'-UTR).

However, the clinical significance and prognostic value of miR-22 in cervical cancer haven't been investigated. Hence, our present study focused on the expression and clinical significance of miR-22 in cervical cancer.

Furthermore, survival analyses showed that post-operative patients with lower levels of miR-22 had a poorer prognosis in both overall survival and progression free survival compared with those with higher miR-22 level (Figure 2). Multivariate Cox analysis also revealed that the post-operative level of miR-22 is an independent prognostic factor for cervical cancer. Taken together, our results support a notion that miR-22 may have potential as a promising biomarker for the prognosis of cervical cancer. However, it still needs to be explored further, as our sample size and the duration of follow-up are limited.

Then the expression of the miR-22 with various clinical features of cervical cancer were analyzed (Table 1). We found that low miR-22

expression was correlated with FIGO stage and lymph node metastasis, suggesting that miR-22 might be involved in the carcinogenesis and metastasis of cervical cancer. In our present study, we confirmed the role of miR-22 in cervical cancer via its ability to inhibit cell colony formation, migration and invasion *in vitro*. We identified two tumor suppressor genes targeted by miR-22: matrix metalloproteinase 1(MMP1) and mitogen-activated protein kinase 1 (MAPK1). Out of these genes, MMP1 could have a specific suppressive impact as it suppresses cell proliferation and metastasis, correlates with cell apoptosis and proliferation, and is associated with lymph node metastasis in cervical cancer [17-20]. Thus we conclude that abnormally low expression of miR-22 in cervical cancer that specifically suppresses the function of MMP1 could be a common carcinogenetic mechanism in various cervical cancer. This may also help to explain why miR-22 is depressed in most cervical cancer tissue samples. However, the mechanisms of miR-22 regulation in cervical cancer are complex.

Comprehensive studies on the carcinogenic effect of miR-22 are needed to be established as a biomarker for use in both clinical diagnosis and prognosis of patients with cervical cancer.

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