MiR-638 inhibits cervical cancer metastasis through Wnt/β-catenin signaling pathway and correlates with prognosis of cervical cancer patients

H. WEI[1], J.-J. ZHANG[2], Q.-L. TANG[3]

[1]Department of Obstetrics and Gynecology, Linyi People's Hospital, Liny, Shandong, China
[2]Department of Obstetrics and Gynecology, Lanling County Hospital of traditional Chinese Medicine, Lanling, Shandong, China.
[3]Department of Obstetrics, Women and Children's Health Care Hospital of Linyi, Linyi, Shandong, China

Abstract. – OBJECTIVE: MiR-638 has been demonstrated to be correlated with several tumor progressions. However, the exact role of miRNA-638 in cervical cancer (CC) has not been investigated. The aim of present study was to explore the prognostic value of miR-638 in patients with CC and analyze molecular mechanisms of miR-638 in CC progression.

PATIENTS AND METHODS: Real-time quantitative RT-PCR was performed to measure miR-638 expression level in 196 paired of CC and matched normal tissues, CC cell lines. The correlation of miR-638 with clinicopathological features and prognosis was analyzed. Furthermore, the effects of miR-638 on tumorigenicity of CC cells were evaluated by functional assays. Finally, Western blot was used to evaluate the activation of Wnt/β-catenin signaling pathway.

RESULTS: We found that miR-638 expression was downregulated in CC tissues and cell lines compared with the adjacent normal tissues and normal cell lines. In addition, low expressions of miR-638 were significantly associated with advanced FIGO stage (p =0.007), lymph node metastasis (p = 0.018) and vascular invasion (p = 0.002). Moreover, the results of Kaplan-Meier method showed that CC patients with lower miR-638 expression had significantly poorer overall survival (p = 0.0023) and progression-free survival (p = 0.0005). In a multivariate Cox model, we found that miR-638 expression was an independent prognostic factor for both overall survival and progression-free survival in patients with CC (both p = 0.001). *In vitro* assay showed that miR-638 overexpression suppressed cell migration and invasion of HeLa cells. The results of Western blot indicated that over-expression of miR-638 inhibited the activation of Wnt/β-catenin signaling pathway.

CONCLUSIONS: Our findings firstly showed that miR-638 might serve as a tumor suppressor. In the future, miR-638 might be regarded as a therapeutic target and a potential prognostic factor in human CC.

Key Words:
    miR-638, Cervical cancer, Wnt/Ð-catenin signaling, Prognosis.

Introduction

Cervical cancer (CC) is the third most common malignancy in women worldwide and responsible for over 20,000 deaths in China annually[1,2]. Although advances in chemotherapy and surgery have been achieved, the prognosis remains poor once cancer has developed[3]. FIGO stage and lymph node metastasis are critical independent prognostic factors, which lead to the failure of surgery, chemotherapy or radiotherapy[4]. Thus, it is extremely urgent to detect CC at an early stage and predict the outcome of CC. However, the molecular mechanism of the pathogenesis of CC remains unclear.

MicroRNAs (miRNA) are a class of non-coding single-stranded RNA molecules, about 22 nucleotides in length, that serve as major important regulators of gene expression at the post-transcriptional level[5]. MiRNAs have been found to be involved in the regulation of various cellular processes, including proliferation, differentiation, cell death, and cell mobility[6,7]. Growing evidence revealed that miRNAs can function as tumor suppressors or oncogenes in development and progression of tumors, including CC[8-10]. Of note, various underlying mechanisms have been revealed.

For instance, Ye et al[11] reported that overexpression of miR-486-3p suppressed cell growth and metastasis by targeting ECM1. Cong et al[12] found that miR-634 inhibits migration and invasion in CC cells by targeting mTOR signal pathway. However, the underlying molecular mechanism of CC has not been fully elucidated.

The biological function of miR-638 has been reported in several tumors, such as hepatocellular carcinoma[13], osteosarcoma[14], and breast cancer[15]. However, the role of miR-638 in the progression of CC has not been investigated. In the present study, we aimed to explore the prognostic value of miR-638 in patients with CC and analyze molecular mechanisms of miR-638 in CC progression. These findings might be helpful in determining potential therapeutic targets for gene therapy in CC.

Patients and Methods

Patients and Specimens

A total of 196 CC tissues and matched adjacent normal tissues were obtained from Linyi People's Hospital and Women and Children's Health Care Hospital of Linyi between July 2008 and January 2012. All specimens were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. The diagnosis was confirmed by two independent pathologists. Only patients without radiotherapy and chemotherapy before operation were collected for this study. A complete follow-up was available for 5 years. The clinicopathological data are summarized in Table I. The study was approved by the Ethical Committee of Linyi People's Hospital and Women and Children's Health Care Hospital of Linyi, and informed consent was obtained from all patients.

Cell Culture and Transfection

CC cell lines (HeLa, SiHa, CasKi and C33A) and HaCaT cells (an immortalized HPV-negative skin keratinocyte line) were brought from Institute of Cell Biology of Chinese Academy of Science (Pudong, Shanghai, China) and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO^2 at 37°C.

The cells were transfected with miR-638 mimic or the miRNA mimics negative control (miR-Ctrl) (Sangon Biotech, Shanghai, Beijing, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

RNA Extraction and Quantitative Real-time PCR

Total RNA from tissues and the cultured cells was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 10 ng of total RNA using TaqManTM. Real-time PCR was

Table I. Correlation between tissue miR-638 expression level and clinicopathological characteristics.

Parameters	Total	High	Low	p-value
MiR-638 expression				
Age				0.449
<45	91	41	50	
≥45	105	53	52	
Tumor size (cm)				0.143
<4.0	63	35	28	
≥4.0	133	59	74	
Histologic grade				0.416
G1 + G2	90	46	44	
G3	106	48	58	
FIGO stage				0.007
Ib-IIa	114	64	50	
IIb-IIIa	82	30	52	
Lymph node metastasis				0.018
Yes	73	27	46	
No	123	67	56	
Vascular invasion				0.002
No	142	78	64	
Yes	54	16	38	

carried out using an ABI 7900HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). For normalization, U6 were used as the endogenous controls. All reactions were performed in triplicate, and the $2^{-\Delta\Delta Ct}$ method was used to quantify the relative expression of miR-638. The primers used for qRT-PCR in this study are as follows: miR-638 forward, 5'-ATCCAGTGCGTG TCGTG-3' and reverse, 5'-TGCTAGGGATCGCGGGCGGGTG-3'. U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Cell Proliferation Assays

Cell proliferation was analyzed using MTT assay. The transfected cells were plated in the transfected cells were plated in 96-well plates (3,000 cells/well). Optical density at 490 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Migration and Invasion Assays

Migration and invasion assays were performed using Transwell chambers. The exact procedure was performed according to the Zeng et al[16].

Western Blotting

All proteins were extracted from tissues or cultured cells using radioimmunoprecipitation assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMSF). Equal amounts of protein were electrophoresed on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene difluoride (PVDF) membrane. Then, the membrane was in-cubated with antibodies against β-catenin, cyclin D1, c-myc and GAPDH overnight at 4°C, followed by horseradish peroxidase (HRP)-conjugated secondary antibody. Detection was performed using a Super Signal Enhanced Chemiluminescence (ECL) kit (NIH, Bethesda, MD, USA). GAPDH was used as a loading control for normalization.

Statistical Analysis

All data were processed with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). All images were made by GraphPad prism 6 (La Jolla, CA, USA). All data were presented as means ± SD. Each experiment was repeated at least three times. Comparisons between two independent groups were analyzed by Student's t-test. Categorical data were evaluated using x^2 test. The survival curves were calculated by the Kaplan-Meier method and the difference by the log-rank test. To determine independent prognostic factor, the Cox multivariate regression analysis was used. $p < 0.05$ was considered as statistically significance.

Results

miR-638 is Downregulated in CC Cell Lines and Tissues

We firstly determined the expression levels of miR-638 in CC tissues, matched adjacent normal tissues and CC cell lines by RT-PCR. As shown in Figure 1A, miR-638 level of CC tissues was lower than in normal tissues ($p < 0.01$). Similarly,
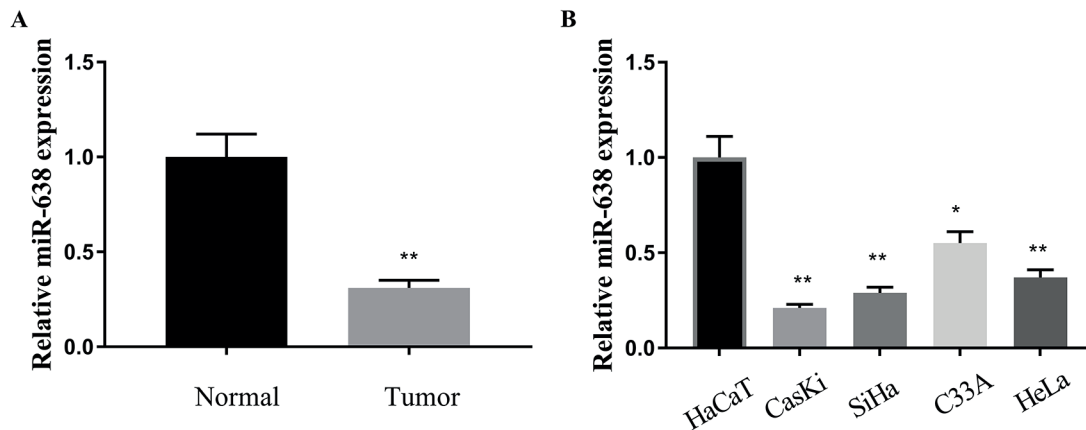

Figure 1. Expression of miR-638 in human CC tissues and cell lines. *(A)* Relative miR-638 expression levels in human CC tissues and the adjacent non-cancerous tissues were detected by PCR analysis. *(B)* qRT-PCR analysis of miR-638 expression level in CC cells (HeLa, SiHa, C-33A, and Caski) and HaCaT cells (an immortalized HPV-negative skin keratinocyte line). *$p < 0.05$, **$p < 0.01$.

we also found that miR-638 expression was significantly decreased in four CC cell lines compared with HaCaT cells (Figure 1B).

Next, we divided CC patients into two groups (High miR-638 expression group and Low miR-638 expression group) according to mean value of miR-638 levels. Statistical assay showed that low expressions of miR-638 were significantly associated with advanced FIGO stage ($p = 0.007$), lymph node metastasis ($p = 0.018$) and vascular invasion ($p = 0.002$). However, there were no significant associations between miR-638 expression and other clinical features (all $p > 0.05$).

Low-Expression Level of miR-638 Predicts Poor Prognosis in CC Patients

To explore the prognostic value of miR-638 in CC patients, we performed Kaplan-Meier analysis. As shown in Figure 2A and 2B, our results showed that patients with low expression of miR-638 had significantly shorter overall survival (OS) and progression-free survival (PFS) than the high expression group ($p = 0.0023$ and $p = 0.0005$). Furthermore, in multivariate Cox model, our results indicated that miR-638 expression was an independent prognostic indicator for OS ($p = 0.001$) and PFS ($p = 0.001$) in patients with CC (Table II).

Up-regulation of miR-638 Suppressed CC Cell Proliferation and Metastasis in vitro

Considering the obvious down-regulation of miR-638 in CC tissues and cells, we wondered whether miR-638 served as a tumor suppressor in CC progression. Then, HeLa cells were transfected with miR-638 mimic or miR-Ctrl with high. The results of RT-PCR showed that miR-638 expression was upregulated in HeLa cells ($p < 0.01$, Figure 3A). MTT showed that knockdown

of miR-638 expression significantly inhibited cell proliferation in HeLa cell lines compared with the control cells (Figure 3B). Then, we further performed transwell assay to explore the effects of miR-638 on migration and invasion of CC cell lines. As shown in Figure 3C and 3D, we found that miR-638 could suppress cancer cell migration and invasion. Taken together, our findings confirmed that miR-638 served as a tumor suppressor in the progression of CC.

Overexpression of miR-638 Decreased Wnt/β-catenin Signaling Pathway in CC Cells

To explore the mechanism by which miR-638 suppressed CC proliferation and metastasis, we focused on the association between miR-638 and Wnt/β-catenin. As shown in Figure 4, our results showed that the β-catenin, c-myc, and cyclin D1 were decreased when miR-638 was overexpressed. Our data indicated that miR-638 served as a tumor suppressor in CC by influencing the activity of Wnt/β-catenin signaling pathway.

Discussion

The human miR-638 is located in the 19p13.2 region. Previous studies have indicated that miR-638 plays an important role in several tumors. For instance, Xia et al[17] reported that miR-638 suppressed invasion and proliferation in non-small cell lung cancer by modulating SOX2 and EMT. Zhang et al[18] showed that ectopic expression of miR-638 inhibited gastric cancer cell growth *in vitro* by targeting phospholipase D1. Wang et al[14] found that high miR-638 was associated with favorable prognosis and

Table II. Multivariate analysis of the associations of prognosis with various clinicopathologic parameters and miR-638 expression in cervical cancer patients.

| Variable	Overall survival			Progression-free survival		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age	1.311	0.723-1.877	0.316	1.116	0.562-1.644	0.278
Tumor size (cm)	1.893	0.823-3.411	0.178	1.466	0.633-2.921	0.113
Histologic grade	2.213	0.663-4.194	0.217	1.894	0.782-3.361	0.167
FIGO stage	3.674	1.643-6.783	0.002	3.127	1.893-7.722	0.001
Lymph node metastasis	3.423	1.452-5.558	0.011	3.893	1.673-4.953	0.008
Vascular invasion	2.783	1.135-5.429	0.006	2.933	1.562-5.982	0.004
MiR-638 expression	2.931	1.673-7.783	0.001	3.342	1.972-8.994	0.001
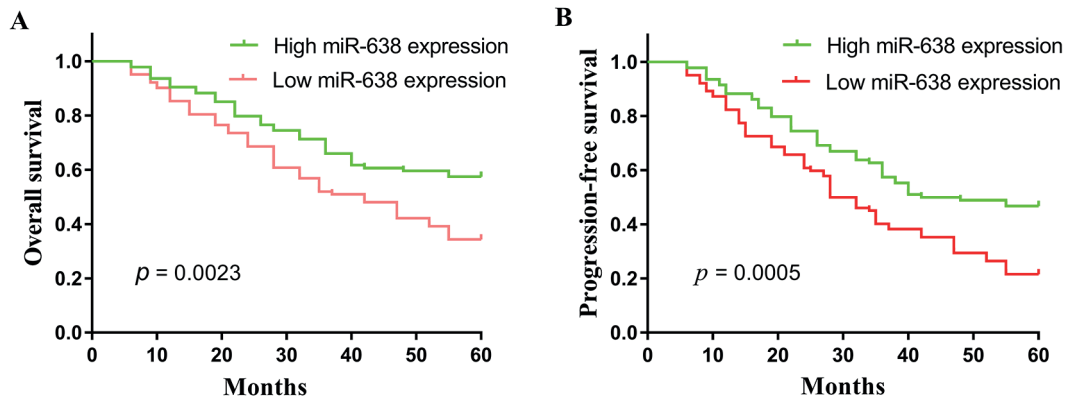
Figure 2. Down-regulation of miR-638 predicts an unfavorable prognosis. *(A)*. Kaplan-Meier survival analysis of OS in 196 patients based on miR-638 expression status. *(B)* Kaplan-Meier survival analysis of PFS in 196 patients based on miR-638 expression status.

suppressed cell proliferation by targeting suppress PIM1 expression in patients with osteosarcoma. Those findings revealed that miR-638 served as a tumor suppressor in above tumors. However, Animesh et al[19] showed that miR-638 expression was significantly high in melanoma tissues. Functional assay indicated that miR-638 functioned as a tumor promoter in the progression of melanoma. Thus, miR-638 may serve as oncogenes or anti-oncogenes according to the kind of tumors. Recently, Xu et al[20] showed that miR-638 expression was down-regulated
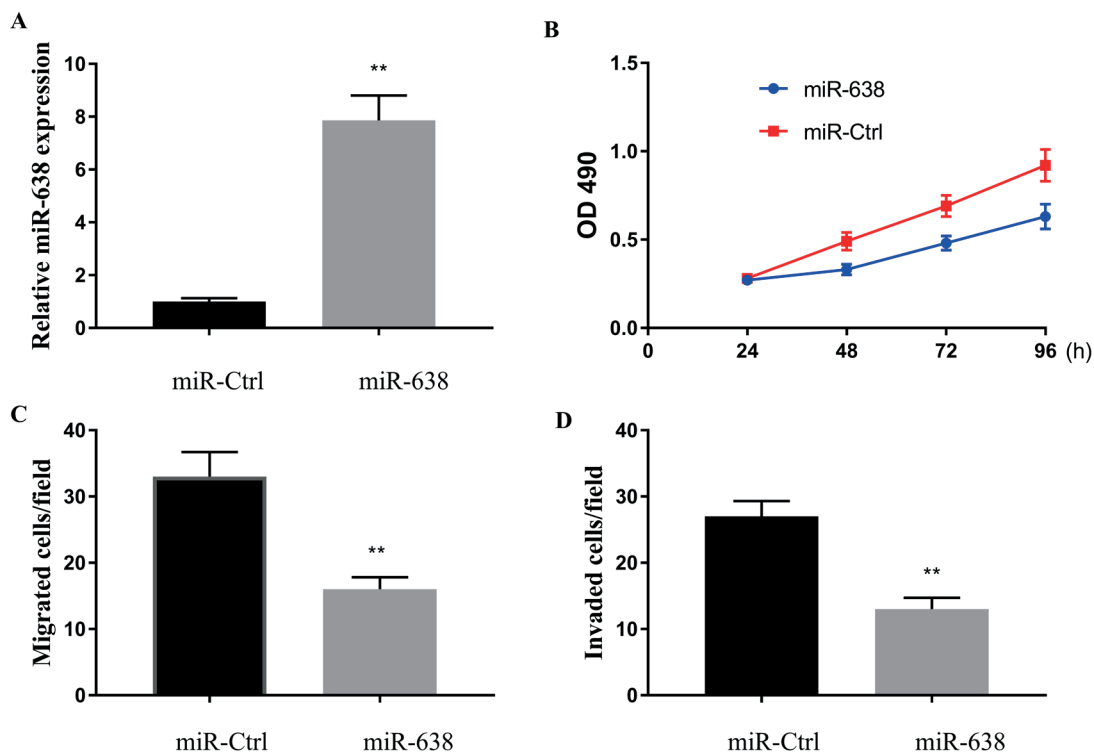

Figure 3. Up-regulation of miR-638 suppressed CC cell growth and metastasis. *(A)* The expression level of miR-638 was significantly upregulated after transfection in HeLa cells, determined by RT-PCR. *(B)* MTT assay was performed to examine HeLa cell proliferation. *(C)* Transwell assay was conducted to analyze the migration of HeLa cells. *(D)* Transwell assay was conducted to analyze the invasion of HeLa cells. *$p < 0.05$, **$p < 0.01$.

miR-Ctrl miR-638
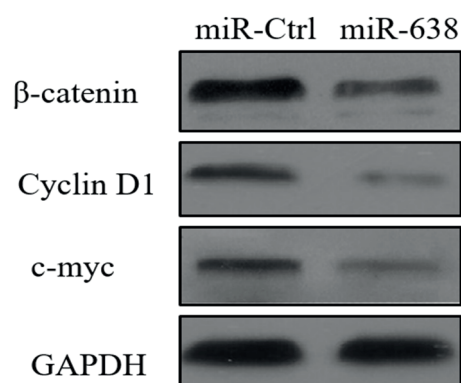
β-catenin

Cyclin D1

c-myc

GAPDH

Figure 4. Over-expression of miR-638 inhibits the activation of Wnt/β-catenin signaling pathway in CC cells.

in CC tissues by microarray analysis. However, the exact function of miR-638 in CC remains unknown.

In the present study, we found that the expression levels of miR-638 were significantly down-regulated in CC tissues compared with normal cervical tissues. The statistical assay showed that low expressions of miR-638 were significantly associated with advanced FIGO stage, lymph node metastasis, and vascular invasion. Furthermore, Kaplan-Meier analysis showed that CC patients with low miR-638 expression tend to have shorter OS and PFS. Further assay by multivariate Cox hazard regression analysis confirmed that miR-638 was an independent prognostic indicator for both OS and PFS. To our best knowledge, this is the first study to explore the prognostic value of miR-638 in CC.

To explore the biological function of miR-638 in CC, we firstly performed *in vitro* experiment and found that over-expression of miR-638 significantly suppressed proliferation and metastasis in CC. To further explore the potential mechanism by which miR-638 inhibited the progression of CC, we focused on Wnt/β-catenin signaling. The Wnt/β-catenin signaling is an important target for the development of novel therapeutics for various tumors[21-23]. Previous works have shown that Wnt/β-catenin pathway activation can promote cell proliferation and suppress cell apoptosis in CC[24,25]. Thus, we wondered whether miR-638 could serve as a tumor suppressor by modulating Wnt/β-catenin signaling. Indeed, the results of Western blot indicated that over-expression of miR-638 inhibited the activation of Wnt/β-catenin signaling pathway.

Conclusions

We demonstrated, for the first time, that miR-638 may act as an indicator of poor survival rate for CC patients. We also exhibited that miR-638 may be a potential inducement to the proliferation of CC cells via modulation of Wnt/β-catenin signaling pathway. Taken together, our study suggested that miR-638 is a critical molecule in CC progression and prognosis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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5593