


# MiRNA-873-5p Acts as a Potential Novel Biomarker and Promotes Cervical Cancer Progression by Regulating ZEB1 via Notch Signaling Pathway

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Chen-Xia Wen<sup>1</sup>, Hai-Li Tian<sup>1</sup>, E Chen<sup>2</sup>, Jin-Fang Liu<sup>3</sup>, and Xiao-Xing Liu<sup>1</sup> 

## Abstract

**Objective:** Our group aimed to investigate the expression pattern of miRNA-873-5p in cervical cancer (CC) patients and its association with CC progression.

**Methods:** Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was applied for the examination of the expressions of miRNA-873-5p in both CC specimens and cell lines. The clinical significance of miRNA-873-5p was statistically analyzed. MTT, colony formation, Transwell and flow cytometry assays were used to detect cell proliferation, metastasis, and apoptosis changes of Hela and Siha cell line. Luciferase reporter assays and Western blots were utilized to identify the target genes of miRNA-873-5p. Western blot and RT-PCR were used to judge the dysregulation of Notch signaling.

**Results:** Our results indicated that miRNA-873-5p expression was distinctly reduced in CC patients. Low miRNA-873-5p expressions were distinctly correlated with positively distant metastasis, The International Federation of Gynecology and Obstetrics (FIGO) stage and poor prognosis of CC patients. A functional assay using miRNA-873-5p mimics indicated that overexpression of miRNA-873-5p distinctly suppressed CC cells proliferation and metastasis, and promoted apoptosis. Bioinformatic assays revealed that miRNA-873-5p may target the 3'-UTR of ZEB1 and lead to the suppression of its translation, which was verified by the use of luciferase assays. Finally, overexpression of miRNA-873-5p suppressed the expressions of Jag1, Maml2 and Hey1.

**Conclusion:** Taken together, we firstly provided evidence that miRNA-873-5p expression was a poor favorable factor for CC patients, and the use of miRNA-873-5p may represent a and potential biomarker and promising therapeutic approach for CC.

## Keywords

miRNA-873-5p, cervical cancer, ZEB1, biomarker, notch signaling, metastasis

## Introduction

Cervical cancer (CC) is a common gynecological malignancy that brings about approximately 233,000 deaths per year worldwide.<sup>1</sup> It is a heterogeneous multifocal disease and the incidence rate is increasing.<sup>2</sup> Persistent infection with high-risk human papillomaviruses (HPV), such as HPV16, has been confirmed to be an elevated risk element for CC.<sup>3</sup> Although widespread implementation of screening programs has decreased the incidence and mortality of this disease, clinical outcomes in CC patients vary distinctly and are very challenging to predict accurately.<sup>4,5</sup> The long-term survival of CC is closely related to the clinical stage of neoplasm at diagnosis.<sup>6</sup> Therefore, more sensitive biomarkers for early diagnosis and more efficient and safer treatments are urgently needed.

<sup>1</sup> Department of Obstetrics and Gynecology, Dongying People's Hospital, Dongying, Shandong, China

<sup>2</sup> Department of Oncology, Dongying People's Hospital, Dongying, Shandong, China

<sup>3</sup> Department of Rongjun, Dongying Rongjun Hospital, Dongying, Shandong, China

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### Corresponding Author:

Xiao-Xing Liu, Department of Obstetrics and Gynecology, Dongying People's Hospital, No. 317 Nanyi Road, Dongying 257091, Shandong, China.  
Email: liuxiaoxing0546@163.com



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MicroRNAs (miRNAs) are defined as a group of noncoding RNAs, ranging between 18 and 28 nucleotides in length.<sup>7</sup> It has been confirmed that miRNAs control genes expressions via binding to the targeting mRNAs at their 3'-untranslated region, resulting in mRNA degradation or translation suppression.<sup>8</sup> Growing studies show that miRNAs play a critical role in various physiological processes, such as cellular growth, immune response, differentiations, metastasis and apoptosis.<sup>9,10</sup> More importantly, previous studies have demonstrated that miRNAs acting as tumor promoters or anti-oncogenes are involved in the regulation of various tumors.<sup>11,12</sup> Recently, growing researches highlighted the importance of miRNAs as a potential diagnostic and prognostic biomarker for cancers, including CC. Several functional miRNAs, such as, miRNA-196a, miRNA-214 and miRNA-92a, have been identified.<sup>13-16</sup> However, the expression pattern and biological function of a large number of miRNAs remain to be elucidate.

MiRNA-873-5p, located in 9p21.1, has been frequently demonstrated to be dysregulated and play an important role in several disease.<sup>17,18</sup> Previous studies reported that miRNA-873-5p was significantly overexpressed and served as a potential anti-oncogenic miRNA in several tumors, such as gastric cancer,<sup>19</sup> breast cancer<sup>20</sup> and glioblastoma.<sup>21</sup> However, the expression and biological functions of miRNA-873-5p in CC remained largely unclear. Here, we firstly analyzed the expression, clinical significance and tumor-related function of miRNA-873-5p in CC. MiRNA-873-5p might be a novel target for CC diagnosis and treatment.

## Patients and Methods

### Patients and Tissue Collection

A total of 138 patients who were diagnosed as CC and underwent surgery in Dongying People's Hospital from January 2011 to February 2014 were enrolled in this study. Informed consent was received from all the subjects. None of the patient received chemotherapy or radiotherapy prior to biopsy. All the specimens were immediately snap frozen for RT-PCR assays. This study was approved by the Medical Ethics Committee of Dongying People's Hospital.

### Cell Culture

Ect1/E6E7 cells (as controls) were purchased from Kang Lang Biotechnology Co., Ltd. (Zhejiang, Hangzhou, China). The human CC cells: HeLa, Caski, ME-180, C33A, SiHa and SW756, were purchased from the CAS Cell Bank (Xuhui, Shanghai, China). The cells were cultured in T25 flasks using DMEM media (DMEM; BasalMedia Technologies, Zhuque, Nanjing, China), supplemented with 10% FBS (Excell Bio, Chengdu, Sichuan, China). a humidified atmosphere chamber (Thermo, Pudong, Shanghai, China) containing 5% CO<sub>2</sub> at 37°C was used for maintenance of all cells.

### Bioinformatics Analysis

The binding sites between miRNA-873-5p and ZEB1 were predicted by "miRDB" (<http://www.mirdb.org/>).

### Microarray and Computational Analysis

Our group collected the microarray information by measuring 5 pairs of CC specimens and matched normal specimens. The genechip Affymetrix Human Genome U133 Plus 2.0 Array GPL570 was used to generate the collected data. The exclusion criterion was the *P*-value regulated to be more than 0.05. A method of hierarchical clustering was used to create the heat map of the abnormally expressed miRNAs. Limma package (version: 3.40.2) of R software was used to study the differential expression of miRNAs.

### Cell Transfection

For cell transfection, HeLa or SiHa cells ( $2 \times 10^5$  cells per well) were cultured in 6-well plates overnight and then transfected with plasmids or microRNA mimics (miR-NC or miRNA-873-5p mimic), which were designed by Genepharma Co., Ltd. In short, 16  $\mu$ l Lipofectamine 2000 reagents in 400  $\mu$ l of Opti-MEM (Invitrogen Co., Carlsbad, CA, USA) were mixed with 12  $\mu$ l microRNA mimics (20  $\mu$ M) in 400  $\mu$ l Opti-MEM for 15-20 min at room temperature. Thereafter, the complexes were added into the plates and the HeLa or SiHa cells were maintained for 4-5 h in a humidified atmosphere chamber containing 5% CO<sub>2</sub> at 37°C. Then, the medium was replaced by fresh medium containing 10% FBS. The cells were used for experiments after transfection of 24 h.

### Real-Time-PCR

Total RNAs were extracted from cultured cells or clinical samples using Trizol reagent (SHRBio, Jiangning, Nanjing, China). For mRNA detection, approximately 2  $\mu$ g total RNA was reverse transcribed to cDNA using FastKing-RT Super Mix kits (TIANGEN, Beijing, China), and quantitative real-time PCR (qRT-PCR) assay was conducted using SYBR Green qPCR Master Mix kits (Excell Bio, Taicang, Jiangsu, China). For miRNA detection, the miRNA was isolated using miRNA Purification Kit (CWBIO, Kunming, Beijing, China). Then, the miRNA cDNA Synthesis Kit (CWBIO, Kunming, Yunnan, China) and TransScript Green miRNA Two-Step qRT-PCR SuperMix kits (TRANSGEN Biotech, Chengdu, Sichuan, China) were applied for the detection of the expressions of mature miRNA-873-5p. GAPDH and U6 were used as internal references for mRNA and miRNA, respectively. These data were analyzed using the comparative Ct method. The related primers were listed in Table 1.

### Western Blot

Cells were lysed with ice-cold radio immunoprecipitation assay (RIPA) buffer (TRANSGEN Biotech, Haidian, Beijing,

**Table 1.** The Primer Sequences Included in This Study.

Name	Primer sequences (5'–3')
miR-873-5p: forward	GCAGCAGGAACUUGUGAG
miR-873-5p: reverse	TGCCGCCTGAACTTCACTCC
ZEB1: forward	CAGCTTGATACCTGTGAATGGG
ZEB1: reverse	TATCTGTGGTCGTGTGGGACT
GAPDH: forward	GGGTGTGAACCATGAGAAGT
GAPDH: reverse	TGAGTCCTTCCACGATACCAA
U6: forward	CTCGCTTCGGCAGCACA
U6: reverse	AACGCTTCACGAATTTGCGT

China) containing protease inhibitor cocktail (TRANSGEN Biotech, Haidian, Beijing, China). Proteins which was concentrated by a BCA Kit (Pierce, Hangzhou, Zhejiang, China) in the lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The 5% (wt/vol) nonfat milk was used for membranes block. Subsequently, the membranes were incubated with primary antibodies specific for ZEB1 (1:600; Abcam, Cambridge, MA, USA), GAPDH (1:2000; Yunshan Technology, Pudong, Shanghai, China), Jag1 (1:700; CST, Danvers, MA, USA), Maml2 (1:600; Abcam, Cambridge, MA, USA) or Hey1 (1:700; CST, Danvers, MA, USA). PBS containing 0.05% Tween was used to wash the membranes which further used for the incubation of the appropriate secondary antibodies. The levels of proteins were visualized using ECL reagents (Beyotime, Pudong, Shanghai, China) and imaged by ECL System (GE Healthcare, Milan, Italy). ImageJ software was applied to analyze the optical density of the protein bands.

### Cell Viability Assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (GeFan Bio, Baoding, Hebei, China). Briefly, CC cells were transfected with miR-NC or miRNA-873-5p mimics, respectively. Twenty-four hours after transfection, cells were transferred into 96-well plates (Excell Bio, Taicang, Jiangsu, China) with density of 2000 cells per well. After culturing for 24 h, 20  $\mu$ l MTT solution (0.5 mg/ml) were added into each well at the indicated time-points and continued to culture for 4 h. Subsequently, 100  $\mu$ l dimethyl sulfoxide (DMSO; Sangon, Pudong, Shanghai, China) was added into the well following removal of the supernatant. Finally, optical density (OD) values of each well were determined and recorded with a microplate reader.

### Colony Formation Assay

HeLa or SiHa cells were seeded at the density of 500 cells per well using 6-well plates (Excell Bio, Taicang, Jiangsu, China) and maintained for two weeks in a humidified atmosphere chamber (Thermo, Pudong, Shanghai, China) containing 5%

CO<sub>2</sub> at 37°C until visible colonies formed. Afterwards, the colonies were fixed in 4% paraformaldehyde (Aladdin, Shanghai, China) and stained with 0.3% crystal violet solution (YESEN, Pudong, Shanghai, China) before observation. The number of colonies was counted under an inverted microscope (IX71, Olympus, Tokyo, Japan).

### Apoptosis Assay

Cell apoptosis were analyzed by flow cytometry analysis using Annexin V/propidium iodide (PI) Apoptosis Assay Kit (Geni Technology, Xicheng, Biejing, China). Briefly, the HeLa or SiHa cells transfected with miR-NC or miRNA-873-5p mimics were collected and resuspended in appropriate binding buffer. Thereafter, Annexin V-FITC (5  $\mu$ l) and PI reagents (5  $\mu$ l) were incubated with the cells in the dark for 15-20 min. Results were analyzed by an FACS Calibur system (BD Biosciences, San Jose, CA, USA).

### Transwell Assay

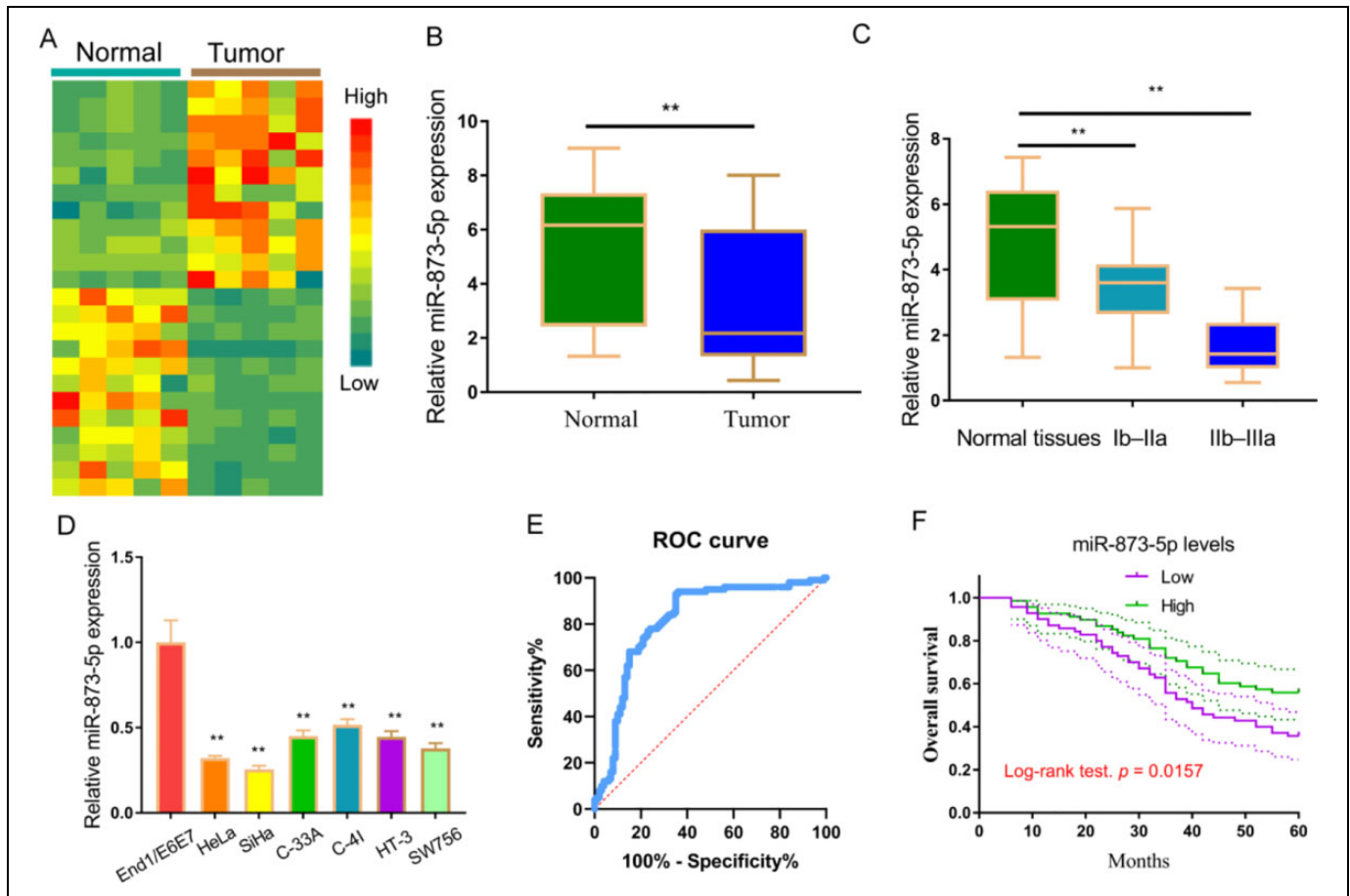
For the determination of the ability of cellular invasion, Transwell assay was performed according to a previously described method.<sup>22</sup>

### Luciferase Reporter Assay

Dual luciferase reporter assays were carried out to assess the binding efficiency between miRNA-873-5p and the 3'UTR of ZEB1. Sequences corresponding to the 3'-UTR of ZEB1 mRNA containing the wild-type (ZEB1 WT) or mutated (ZEB1 MUT) miRNA-873-5p binding sequence were synthesized by Sangon Biotech Co., Ltd. and related reporter plasmids (pMIR-ZEB1 WT, pMIR-ZEB1 MUT) were constructed by GeneCh Technology (Kunming, Yunan, China) through standard molecular cloning methods. Subsequently, HeLa or SiHa cells were plated in 24 well plates at the density of  $1 \times 10^5$  cells per well. Then, pMIR-ZEB1 WT (400 ng) or pMIR-ZEB1 MUT (400 ng) plasmids were transfected into HeLa or SiHa cells with 40 ng pRL-TK vectors (Promega, Madison, WI, USA), as well as miRNA-873-5p mimic or miR-NC, using Lipofectamine 2000 reagent (Invitrogen Co., Carlsbad, CA, USA). Twenty-four hours after transfection, the activities of luciferases in cell lysates were technically measured.

### Statistical Analysis

Statistical analyses were performed with the SPSS 17.0 statistics software (SPSS, Inc., Chicago, IL, USA). The statistical significance of the studies was analyzed using Student's t test or one-way ANOVA test. Receiver operating characteristic (ROC) curves were applied the determine the diagnostic value of miRNA-873-5p in CC patients. Differences in patient survival were determined by the Kaplan-Meier method and log-rank test. Survival data were evaluated using univariate and multivariate Cox proportional hazards models. A value of  $P < 0.05$  was considered as statistically significant.



**Figure 1.** Analysis of miRNA-873-5p expression in CC tissues and clinical parameters. (A) Heatmap showed the mostly differently expressed miRNAs by analyzing 10 pairs of CC and adjacent normal cervical tissues. High expression: red color; low expression: green color. (B and C) miRNA-873-5p was determined in 138 pairs of CC tissues using real-time PCR (normalized to GAPDH). (D) miRNA-873-5p expression in five CC cell lines and End/E6E7 by RT-PCR. (E) The diagnostic value of miRNA-873-5p expression in CC patients were determined by ROC assays. (F) Kaplan-Meier survival analyses. \* $P < 0.05$ , \*\* $P < 0.01$ .

## Results

### Decreased Expressions of miRNA-873-5p in Human CC Patients

For identifying the miRNAs dysregulated in CC, we performed Chip analysis using five pairs of CC and their adjacent non-cancerous samples. The top 24 dysregulated lncRNAs were shown using Heat Map (Figure 1A). Among these miRNAs, miRNA-873-5p was certified to be significantly downregulated in CC tumor specimens. Then, we detected miRNA-873-5p expressions in CC patients and discovered that miRNA-873-5p levels were significantly lower in CC samples than in adjacent paired normal specimens in 138 CC patients (Figure 1B,  $6.15 \pm 0.83$  vs  $2.31 \pm 0.42$ ,  $P < 0.01$ ). Furthermore, miRNA-873-5p levels in tumors with high FIGO stage were significantly lower than those in those with high FIGO stage (Figure 1C). Then, we also detected miRNA-873-5p levels in CC cells and the data proved that its levels were dramatically decreased in various CC cells, including HeLa, SiHa, C-33A, C-4I, HT-3 and SW756, when compared with End/E6E7

(Figure 1D). Because HeLa and SiHa cells exhibited a relatively higher level of miRNA-873-5p compared with other four CC cells, they were used for further functional assays. Moreover, we also examined the diagnostic value of miRNA-873-5p in CC patients. As presented in Figure 1E, the results of ROC assays revealed that low miRNA-873-5p expression had an AUC value of 0.8168 (95% CI 0.7546 to 0.8790) for CC tissues. The sensitivity and specificity of miRNA-873-5p for distinguishing CC specimens from non-tumor tissues was 70.44%/82.23%. In conclusion, our data proved that miRNA-873-5p might be related to CC progression.

### Prognostic Values of miRNA-873-5p Expression in CC Patients

To delve into whether dysregulation of miRNA-873-5p displayed possible association with clinicopathological features in CC, 138 CC patients were divided into two groups in accordance with the median relative miRNA-873-5p expressing value that was used as the cut-off: high miRNA-873-5p group

**Table 2.** Correlation Between miR-873-5p Expression and Different Clinical Features in CC Patients.

Parameters	Number of cases	miR-873-5p expression		P-value
		Low	High	
Age (y)				0.398
< 65	66	31	35	
≥ 65	72	39	33	
HPV				0.387
(+)	64	35	29	
(-)	74	35	39	
Histology				0.176
Squamous	63	28	35	
Adenocarcinoma	75	42	33	
Tumor size				0.163
< 4 cm	79	36	43	
≥ 4 cm	59	34	25	
Histological grade				0.100
Well/moderate	88	40	48	
Poor	50	30	20	
Lymphatic invasion				0.020
Yes	52	33	19	
No	86	37	49	
Distant metastasis				0.006
Yes	46	31	15	
No	92	39	53	
FIGO stage				0.004
Ib-IIa	51	34	17	
IIb-IIIa	87	36	51	

(n = 68) and low miRNA-873-5p group (n = 70). As presented in Table 2, we discovered that low miRNA-873-5p levels were dramatically associated with lymphatic invasion ( $P = 0.020$ ), distant metastasis ( $P = 0.006$ ) and FIGO stage ( $P = 0.004$ ). However, no significant differences were discovered between miRNA-873-5p expressions and other clinical factors such as patients' age, HPV, histology and histological grade (All  $P > 0.05$ ). Then, the data from Kaplan-Meier assays revealed that downregulation of miRNA-873-5p in tissues was relevant with worse overall survival ( $P = 0.0157$ , Figure. 1F). Moreover, univariate analyses suggested that miRNA-873-5p expression, lymphatic invasion, distant metastasis and FIGO stage may influence the survival rate in CC patients (Table 3). More importantly, multivariate analyses confirmed that miRNA-873-5p levels may be an independent prognostic indicator for overall survival of CC patients ( $P = 0.005$ , Table 3). In summary, the above findings revealed that miRNA-873-5p had potentials to be a prognostic biomarker for CC patients.

### MiRNA-873-5p Regulated the Proliferation and Apoptosis of CC Cells

Based on the fact that miRNA-873-5p was downregulated in human cervical carcinoma, we wondered whether it played critical roles in modulating the tumor behaviors. Firstly, we

tested the efficiency of miRNA-873-5p mimic in CC cells with qPCR, which confirmed that the relative miRNA-873-5p levels were dramatically increased in both HeLa and SiHa cells when miRNA-873-5p mimics was used (Figure 2A). Subsequently, MTT was performed to evaluate the cell proliferation of CC cells which were altered by miRNA-873-5p. As the data presented in Figure 2B and C, transfection of miRNA-873-5p mimics notably suppressed the cell growth of CC cells (Inhibition ratio: 61.4% in HeLa cells and 53.4% in SiHa cells). Besides, in colony forming assays, Colony formation in CC cells was dramatically inhibited after miRNA-873-5p was overexpressed (Surviving fraction: 34.5%, Figure 2D and E). In addition, our group also performed cell apoptosis assays, finding that overexpression of miRNA-873-5p caused a dramatically increased apoptotic rates of CC cells (Figure 2F). Overall, these data demonstrated that overexpression of miRNA-873-5p inhibited the CC cells proliferation and induced cell apoptosis.

### MiRNA-873-5p Suppresses Metastasis of CC Cells In Vivo

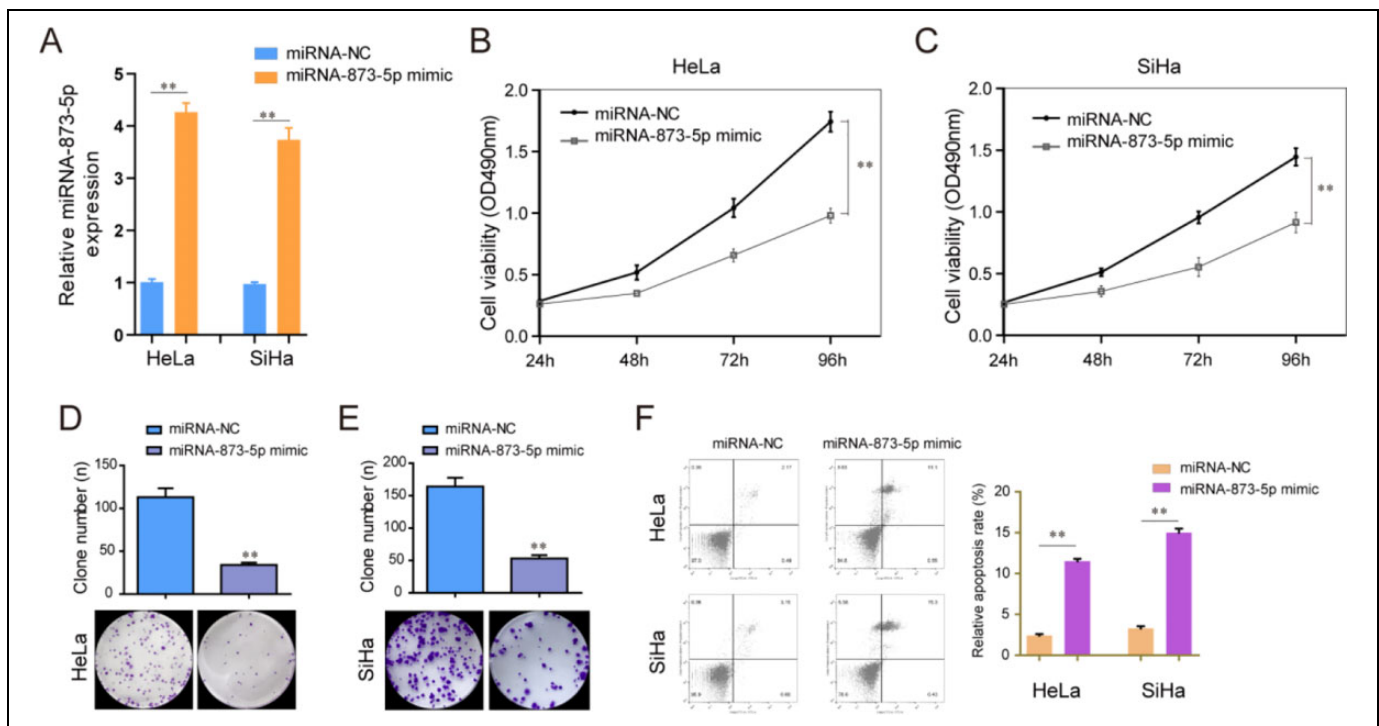
We next attempted to explore whether miRNA-873-5p was able to act as a modulator in the mobility of CC. To achieve that, we respectively conducted transwell and wound-healing assays using CC cells. Transwell assays indicated that overexpression of miRNA-873-5p resulted in a distinct reduction of cell invasive abilities in CC cells (Figure 3A and B). Moreover, wound-healing assays demonstrated that enhancing expressions of miRNA-873-5p contributed to a remarkable inhibition of cell migratory capabilities (Figure 3C). Overall, the above findings validated that miRNA-873-5p served as a crucial modulator in the progress of CC metastasis.

### ZEB1 Was a Direct Target of miRNA-873-5p in CC

Then, the potential target genes of miRNA-873-5p were predicted by miRDB (<http://www.mirdb.org/>) for the exploration of the molecular mechanisms by which miRNA-873-5p impaired proliferation and metastasis of CC cells. Among these genes, we found that miRNA-873-5p potentially targeted ZEB1, which had confirmed to serve as a tumor promoter in multiple types of cancers.<sup>23,24</sup> The data suggested that the 3'UTR of gene ZEB1 harbored a potential miRNA-873-5p binding site (Figure 4A). To further ascertain this prediction, luciferase assays were performed, which suggested that ectopic expression of miRNA-873-5p remarkably decreased the luciferase activity of the reporter plasmids containing wild-type ZEB1 3'-UTR, while there were no significant depression effects on that of the mutant ZEB1 3'-UTR in CC cells (Figure 4B and C). Meanwhile, transfection of miRNA-873-5p mimics in CC cells resulted in a reduction of ZEB1 mRNA expressions (Figure 4D). Moreover, western blot assays revealed that the protein levels of ZEB1 in CC cells were significantly decreased by miRNA-873-5p mimic transfection, which was consistent with the mRNA detection data (Figure 4E and F). On the other hand,

**Table 3.** Univariate and Multivariate Analysis of Clinical Factors for Overall Survival in 138 Patients With CC.

Risk factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Age (y)	1.214	0.534-1.978	0.135	-	-	-
HPV	1.556	0.783-2.215	0.334	-	-	-
Histology	1.329	0.967-1.884	0.126	-	-	-
Tumor size	1.667	0.634-2.441	0.119	-	-	-
Histological grade	1.379	0.775-2.214	0.188	-	-	-
Lymphatic invasion	2.854	1.433-3.774	0.014	2.548	1.216-3.328	0.033
Distant metastasis	3.556	1.569-5.428	0.003	3.167	1.238-4.564	0.009
FIGO stage	3.784	1.477-4.879	0.008	3.237	1.138-3.896	0.011
miR-873-5p expression	3.564	1.633-5.894	0.001	3.038	1.215-4.776	0.005



**Figure 2.** Ectopic expression of miRNA-873-5p inhibited cervical cell proliferation and promoted apoptosis. (A) Relative expression levels of miRNA-873-5p in HeLa or SiHa cells when transfected with negative control miRNA mimic (miR-NC) or miRNA-873-5p mimic. (B and C) The proliferation rates of HeLa or SiHa cells determined by MTT assays. (D and E) Representative images of the colony formation assays and statistical analysis of HeLa or SiHa cell colony number. (F) Flow cytometry analysis was applied to detect the apoptotic rates of HeLa or SiHa cells transfected with miR-NC or miRNA-873-5p mimic.

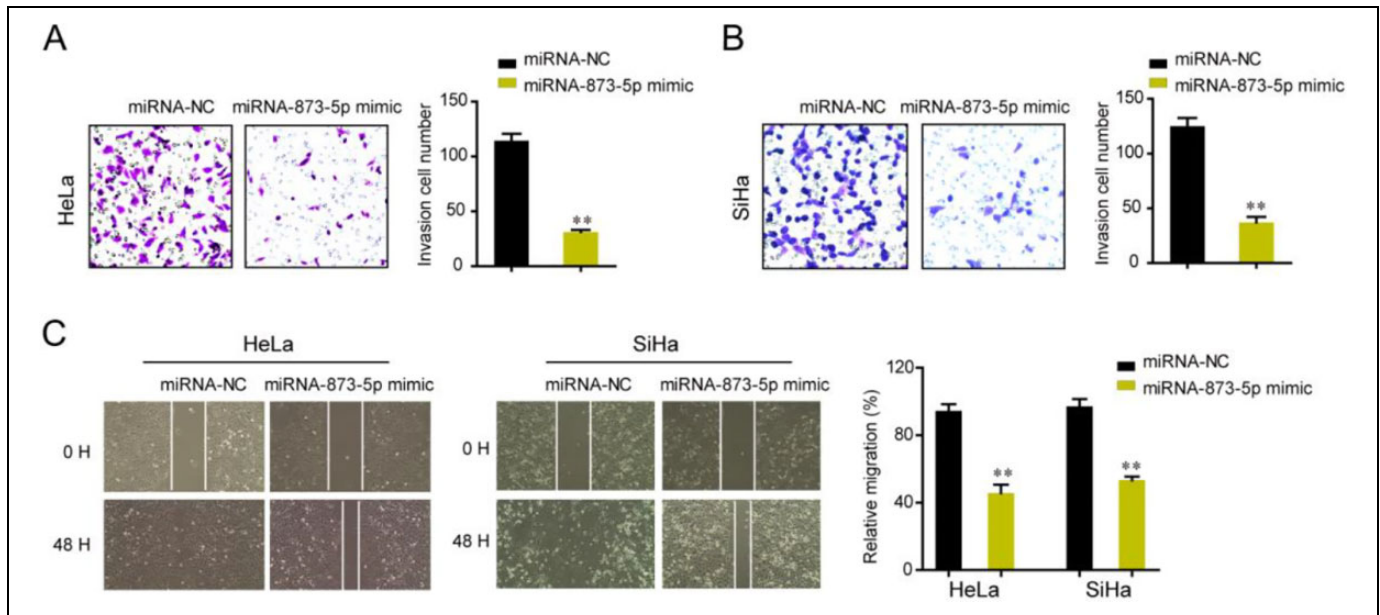
Pearson's correlation assays demonstrated that ZEB1 mRNA expression was negative correlated with the expression of miRNA-873-5p in CC tissue samples (Figure 4G). Overall, our findings demonstrated that ZEB1 was a direct target of miRNA-873-5p in CC cells.

### ZEB1 Reversed miRNA-873-5p Induced Inhibition of Growth and Invasion in CC Cells

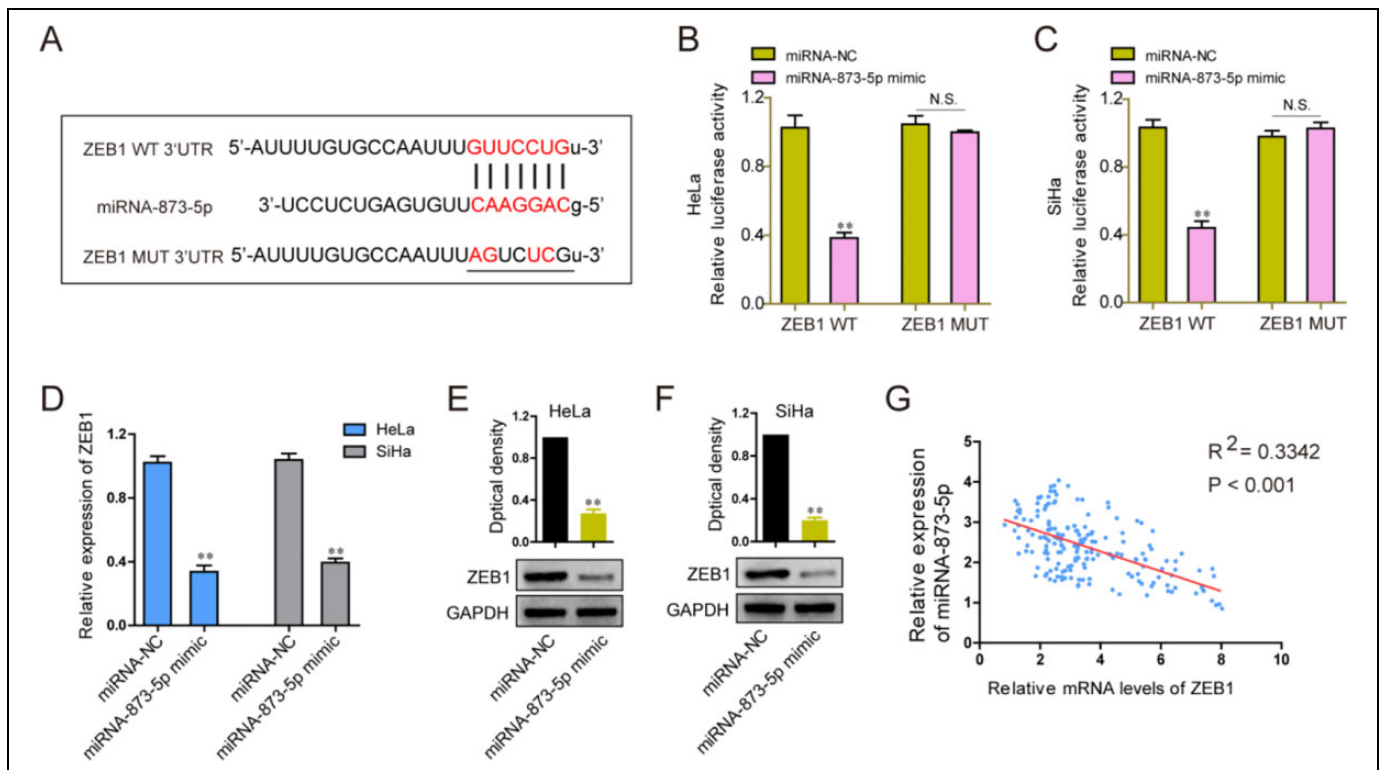
To confirm whether miRNA-873-5p exhibited its tumor-promotive roles via modulating ZEB1, gain-of-function

experiments were conducted by restoration of ZEB1 expression. Specifically, a plasmid vector expressing ZEB1 was constructed, which resulted in constitutive expression of ZEB1. As a result, the levels of ZEB1 were reduced by transfecting miRNA-873-5p mimic, while overexpression of ZEB1 could significantly reverse the expression of ZEB1 in CC cells compared with cells transfected with miRNA-873-5p mimic (Figure 5A). Additionally, the analysis of MTT assays revealed that reintroduction of ZEB1 remarkably reversed the suppressor influences of miRNA-873-5p mimic on the proliferation of HeLa and SiHa cells (Figure 5B and C). Similarly,

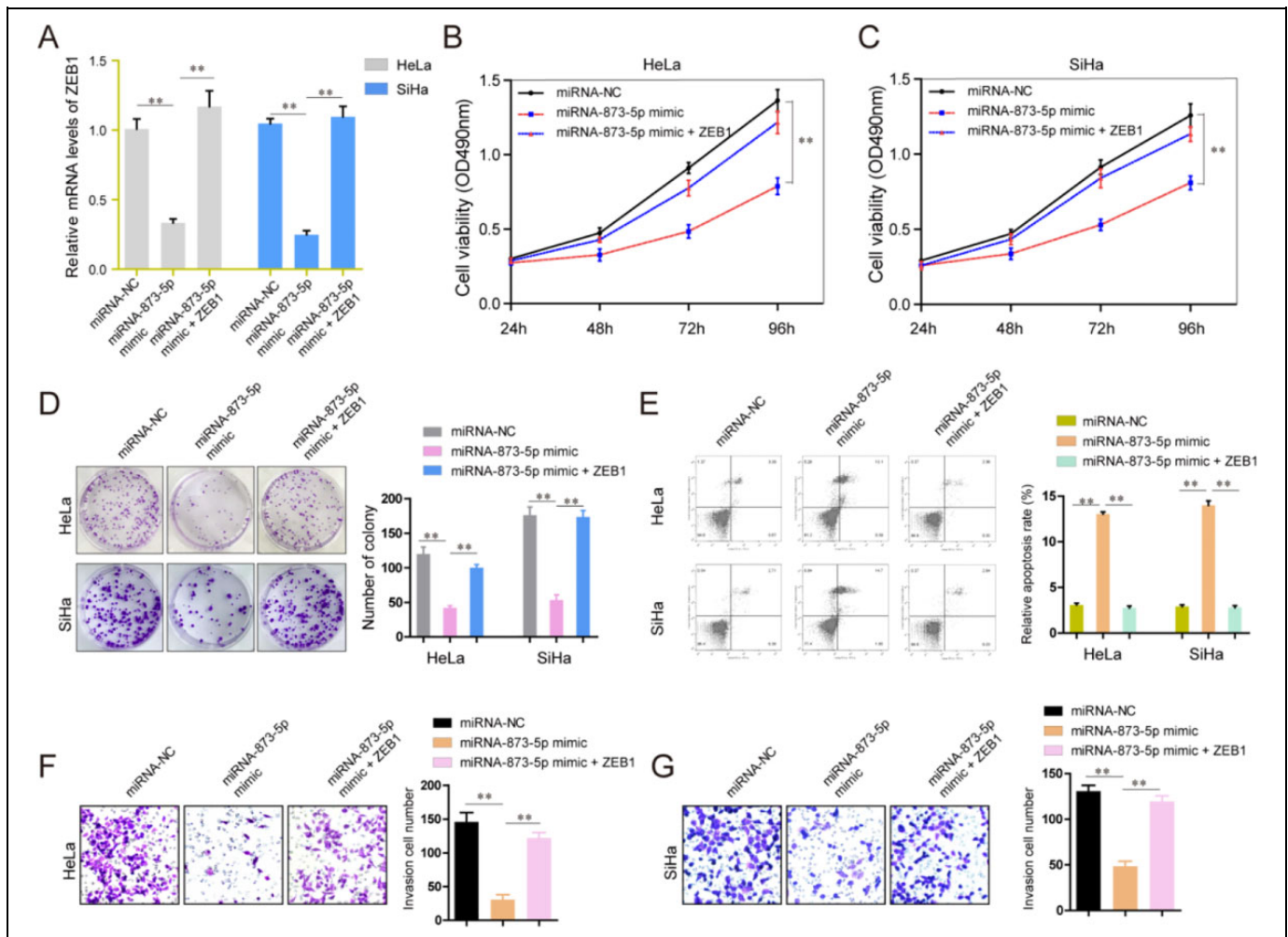




**Figure 3.** MiRNA-873-5p overexpression suppressed cervical cell mobility. (A and B) Representative images of transwell assays and statistical analysis of the invasion cell number in HeLa or SiHa cells. (C) Wound-healing assays detected the cell migration. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 4.** MiRNA-873-5p directly targeted ZEB1. (A) ZEB1 mRNA 3'-UTR harbored the miRNA-873-5p binding site, which was predicted by bioinformatics tool "miRBD". (B and C) Dual-luciferase reporter assays determined the luciferase activity of HeLa or SiHa cells under various experiments condition. (D-F) qPCR and western blot assays were conducted to evaluate the effects of miRNA-873-5p on ZEB1 mRNA and protein levels. (G) The relationship between miRNA-873-5p and ZEB1 expression using Pearson's correlation analysis. \* $P < 0.05$ , \*\* $P < 0.01$ .



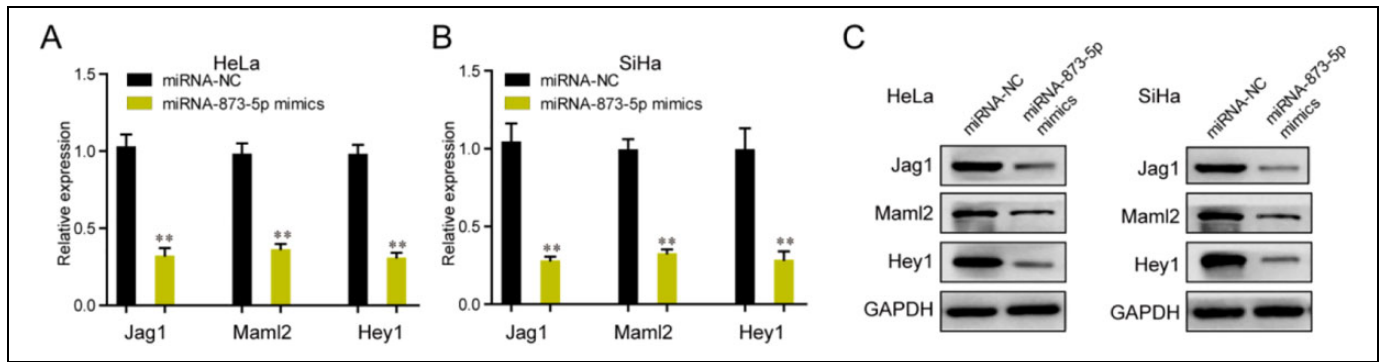
**Figure 5.** Overexpression of ZEB1 abrogated the tumor suppressive roles of miRNA-873-5p. (A) Relative mRNA expression levels of ZEB1 in HeLa and SiHa cells transfected with miR-NC, miRNA-873-5p mimic or co-transfected with miRNA-873-5p mimic and ZEB1 overexpression plasmids. (B and C) MTT assays determined the proliferation rates of HeLa and SiHa cells under various experiments condition. (D) Representative images of the colony formation assays and statistical analysis of HeLa and SiHa cell colony number in different groups. (E) Flow cytometry analysis detected the apoptotic rates of HeLa or SiHa cells transfected with miR-NC, miRNA-873-5p mimic or co-transfected with miRNA-873-5p mimic and ZEB1 overexpression plasmids. (F and G) Representative images of the transwell assays and statistical analysis of HeLa and SiHa cell invasive number in different groups. \* $P < 0.05$ , \*\* $P < 0.01$ .

it was also found that inhibition in cell colony formation by overexpression of miRNA-873-5p was dramatically impaired by re-introduction of ZEB1 (Figure 5D). Moreover, flow cytometry analysis confirmed that upregulation of miRNA-873-5p resulted in a remarkable increase in cell apoptotic rates, while enforcing ZEB1 expression notably rescued the apoptosis of CC cells (Figure 5E). Besides, transwell assays indicated that the restoration of ZEB1 expression mostly blocked the inhibitory influences of miRNA-873-5p on the invasion of CC cells (Figure 5F and G). In summary, these results validated that ZEB1 was a direct target of miRNA-873-5p, and the growth as well as invasion suppressive effects of miRNA-873-5p were mainly via abrogating the expressions of ZEB1.

### MiRNA-873-5p Modulated Notch Signaling in CC Cells

To further uncover the detail molecular mechanisms by which miRNA-873-5p repressed the malignant behaviors of CC, we sought to investigate whether miRNA-873-5p was capable to regulate Notch signaling in CC cells, because previous studies had indicated that the downstream target gene, ZEB1, could activate Notch signaling in cancer.<sup>25</sup> Therefore, we first transfected miRNA-873-5p mimics into CC cells, and subsequently performed qPCR to detect the levels of key components of Notch signaling: Jag1, Maml2 and Hey1. The results validated that forced expression of miRNA-873-5p caused markedly decreased expression of Jag1, Maml2 and Hey1 (Figure 6A and B). Afterwards, we conducted western blot which





**Figure 6.** MiRNA-873-5p influenced Notch signaling in cervical cancer cells. (A and B) qPCR examined the relative expression of key components involved in Notch signaling: Jag1, Maml2, and Hey1. (C) Western blot measured the protein levels of Jag1, Maml2, and Hey1. \* $P < 0.05$ , \*\* $P < 0.01$ .

exhibited similar results that enhancing miRNA-873-5p expression led to a remarkable suppression of protein levels of Jag1, Maml2 and Hey1 (Figure 6C). Therefore, these data provided preliminary evidence that miRNA-873-5p could suppress the activation of Notch signaling in CC.

## Discussion

Human CC is one of the deadliest gynecological neoplasm in China<sup>26</sup> Such high mortality rates are ascribed to CC recurrence despite aggressive treatment including surgical resection for CC with advanced stages.<sup>27</sup> Prediction of CC patients prognosis is very important for design of individual treatment.<sup>28</sup> Although distant metastasis and International Federation of Gynecology and Obstetrics (FIGO) staging may serve as biomarkers for predicting clinical outcome of CC patients, they may not be sufficient to estimate patient prognosis.<sup>29</sup> Recently, increasing studies focused on the possibility of miRNAs as novel biomarkers and several miRNAs had been reported to display associations with prognosis of CC patients.<sup>30,31</sup> In this study, we performed microarray analysis to screen differentially expressed miRNA, finding that miRNA-873-5p expressions were substantially decreased in CC specimens, which were further demonstrated using RT-PCR in CC cells and specimens. ROC assays revealed miRNA-873-5p as a potential diagnostic biomarker for CC patients. In addition, the levels of miRNA-873-5p in tumors with high FIGO stage were significantly lower than those in those with low FIGO stage. Clinical assay indicated that CC patients with low miRNA-873-5p levels exhibited a shorter five-year overall survival. Moreover, multivariate analysis via analyzing several prognosis factors revealed that miRNA-873-5p expression acted as a strong independent predictor of poor survival in CC. Our present study firstly explored and confirmed the prognostic value of miRNA-873-5p in a large number of CC patients.

With enormous successes of next-generation sequencing and bioinformatics technology, growing miRNAs were identified to be important modulators in various tumors.<sup>32,33</sup> Recently, several studies have reported the expression and

function of miRNA-873-5p in several types of cancers. For instance, Gao et al<sup>34</sup> revealed that miRNA-873 was highly expressed in lung adenocarcinoma and its forced upregulation resulted in the promotion of the abilities in the metastasis of lung adenocarcinoma cells via controlling SRCIN1. Liang et al<sup>35</sup> suggested that the expressions of miR-873 were distinctly down-regulated in esophageal cancer and its forced expression distinctly suppressed cells growth and metastasis. Cao et al<sup>19</sup> indicated that overexpression of miRNA-873-5p decreased gastric cancer cell viability VIA targeting hedgehog-GLI. The above observation suggested that miRNA-873-5p played different role in different cell situation. In this study, we further explored whether miRNA-873-5p could influence CC behavior. We reported that overexpression of miRNA-873-5p had an inhibitory effect on CC cell growth by suppressing cell proliferation and inducing apoptosis. Further in vitro assay also confirmed that overexpression of miRNA-873-5p could suppress the abilities of invasion of CC cells. Overall, our findings revealed that miRNA-873-5p served as a tumor suppressor in CC, suggesting it as a potential therapeutic target for CC patients.

ZEB1, belonging to the ZEB1 family of transcription factors, is a transcription factor that have been demonstrated to be involved in the clinical modulation of metastasis by inducing EMT in cancer cells.<sup>23</sup> Dysregulation of ZEB1 has been demonstrated in various human cancers, where it is generally believed to contribute to the progression of migration and invasion.<sup>36,37</sup> Recently, accumulating evidence has indicated that miRNAs suppressed cancer cells proliferation and metastasis by targeting ZEB.<sup>38,39</sup> For further study of the potential mechanism by which miRNA-873-5p exhibited its function in CC, we identified ZEB1 as a potential target gene of miRNA-873-5p via a bioinformatics method. The results of luciferase report assays proved that miRNA-873-5p directly modulated ZEB1 by binding its 3'-UTR. Moreover, we also found that transfecting miRNA-873-5p mimics into CC cells resulted in decreased expression of ZEB1 and ZEB1 expression was negative correlative with the expression of miRNA-873-5p in CC tissue samples. Moreover, in functional assay, we confirmed that forced ZEB1 expression reversed the suppressor

roles of miRNA-873-5p on CC proliferation and invasion. Taken together, we supposed that miRNA-873-5p inhibited CC carcinogenesis via targeting ZEB1. In the future, other targets of miRNA-873-5p, besides ZEB1, need to be identified in CC.

Notch signaling is an evolutionarily conserved mechanism that acts as an important regulator in cervical homeostasis, injuries and repairs, but in contrast to other signal pathways such as Hedgehog, TGF- $\beta$  and Wnt, Notch signaling was observed to occur through cell-cell communication, whether transmembrane receptors on one cell were activated by transmembrane ligands a juxtaposed cell.<sup>40,41</sup> In recent years, growing evidence had demonstrated that aberrant activation of Notch signaling pathway was involved in tumor progression via influencing cellular growth, metastasis and EMT progress.<sup>42,43</sup> In addition, it was previously reported that the EMT activator ZEB1 may promote the activation of Notch signaling.<sup>23</sup> Thus, we wondered whether miRNA-873-5p displayed its anti-cancer effects by targeting ZEB1 via the Notch signaling pathway. To demonstrate our hypothesis, we performed RT-PCR and Western blot for the examination of the expressions of three key factors of Notch signaling (Jag1, Maml2 and Hey1) after miRNA-873-5p levels were upregulated. As expected, overexpression of miRNA-873-5p distinctly suppressed the protein and mRNA levels of Jag1, Maml2 and Hey1, indicating that miRNA-873-5p served as an anti-oncogene in CC progression by targeting ZEB1 via the Notch signaling.

## Conclusion

In summary, we firstly reported that miRNA-873-5p was downregulated in CC and associated with unfavorable prognosis in CC patients. Elevated expression of miRNA-873-5p suppressed the CC cells progression and metastasis through targeting ZEB1 via the Notch signaling. MiRNA-873-5p could be a potential therapeutic target and novel molecular biomarker for CC.

## Author Contributions

Chen-Xia Wen and Hai-Li Tian contributed equally to this work.


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## ORCID iD

Xiao-Xing Liu  <https://orcid.org/0000-0002-6081-4650>

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