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microRNA-214 suppresses the growth of cervical cancer cells by targeting EZH2

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Abstract. A number of studies have revealed the significance of microRNAs (miRs) in tumorigenesis. Cervical cancer (CC) is one of the most malignant cancer types and is associated with a poor overall survival rate. A previous study demonstrated a critical role of miR-214 in the development of multiple cancer types, but its role in CC remains elusive. In the current study, miR-214 was observed to be downregulated in CC tissues compared with the adjacent non-cancerous tissue. Overexpression of miR-214 reduced the proliferation of CC cells, whereas inhibiting its expression resulted in enhanced proliferation. Furthermore, Enhancer of zeste homolog 2 (EZH2) was demonstrated to be a direct target of miR-214 in CC. An MTT assay demonstrated that upregulating miR-214 expression or knocking down the expression of EZH2 impaired the proliferation of a CC cell line. Low expression of miR-214 was positively associated with tumor differentiation (P=0.037) and tumor stage (P=0.012). Notably, low expression of miR-214 predicted poor prognosis of patients with CC. Consequently, the results of the current study demonstrated that miR-214 functions as a tumor suppressor in CC and may be regarded as a potential therapeutic target in CC.

Introduction

Cervical cancer (CC) is the fourth most common type of malignancy in women worldwide, accounting for ~250,000 cancer-associated mortalities annually (1,2). The majority of CC cases are as a result of human papillomavirus (HPV) infections (3), which may explain why ~80% of new CC cases are in developing countries (4,5). An increasing number of studies have revealed that HPV infection alone is not sufficient to initiate the malignant changes that lead to CC, and that other factors contribute to the carcinogenesis and progression of CC (6,7). Therefore, screening the factors involved in tumorigenesis may provide a new way to predict the progression of CC early or to efficiently treat patients with CC.

MicroRNAs (miRNAs or miRs) are a class of small noncoding RNAs that are 18-25 nucleotides in length, and which function as key regulators of gene expression at the post-transcriptional level (8). By targeting the 3'-untranslated regions (3'-UTRs) of target mRNAs, miRNA may lead to either translational repression or degradation of mRNA (9,10). Several studies have demonstrated that miRNAs are involved in regulating various biological processes, including cell proliferation (11), migration (10), invasion (10,12) and drug resistance (13). miR-214, one member of the miR-214 family, has been revealed to be aberrantly expressed in several human cancer types, including breast cancer (14), hepatocellular carcinoma (15), lung cancer (13), esophageal squamous cell cancer (16) and ovarian cancer (17). The dysregulation of miR-214 predicts a poor prognosis in the aforementioned cancers (13-17). Furthermore, the underlying molecular mechanism in these cancers has been explored, and a number of target genes, including PTEN, LHX6, GALNT7 and uncoupling protein 2, have been identified (13,15-17). However, the role of miR-214 in regulating human CC cells remains to be explored.

Enhancer of zeste homolog 2 (EZH2) serves an important role in regulating cell proliferation and the cell cycle via regulating the methylation status of lysine 27 in histone H3 (H3K27) (18,19). A previous study demonstrated that overexpression of EZH2 is associated with worse disease-free survival rates and worse overall survival rates for patients with breast cancer (20). EZH2 was identified as a direct target of miR-214 in breast cancer (21). However, the association between EZH2 expression and miR-214 expression in human CC requires further exploration.

In the present study, the expression and biological function of miR-214 in human CC was evaluated. The expression of miR-214 was identified to be downregulated in CC tissues compared with the adjacent noncancerous tissues and EZH2 was identified as a direct target of miR-214. EZH2 knockdown or miR-214 overexpression could impair the cell proliferation of CC cell lines. Taken together, these results indicate that EZH2 may function as an oncogene and as a mediator of miR-214 in human CC.

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Materials and methods

Clinical tissue samples. A total of 45 patients diagnosed as CC were enrolled in the current study between August 2007 and October 2011, and none of them had received any anti-cancer treatments. Fresh CC tissues and corresponding adjacent noncancerous tissues were obtained from each of the enrolled patients. All tissue samples were stored in liquid nitrogen until further usage. The mean age of these patients was 53.5±7.4 years, ranging between 45 and 69 years. The clinical information of CC cases is presented in Table I. Written informed consent was obtained from all enrolled patients. The current study was performed according to the principles of the Declaration of Helsinki. Ethics approval was granted by the Ethics Committee of the Xuzhou Maternity and Child Health Care Hospital (Xuzhou, China).

Cell culture. Human CC cell line HeLa and normal cervical cell line Ect1/E6E7 were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 95% air and 5% CO_2 at a temperature of 37˚C.

Transient transfection. The miR-214 mimic (5'-UGCCUG UCUACACUUGCUGUGC-3'), miR-214 inhibitor (5'-GCA CAGCAAGUGUAGACAGGCA-3') and negative control miRNA (5'-GUGUCUGUCCUUACGUGCUCCA-3') were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The EZH2-targeting small-interfering RNA (siRNA; 5'-AGUCUCAUGUACGCTGACUCUG-3') and negative control siRNA (5'-GUGUCUUCACGUUACCUAGAGC-3') were also purchased from Guangzhou RiboBio Co., Ltd. All cell transfections were performed using Lipofectamine® 2000 reagent (Invitrogen) according to the manufacturer's protocol and cultured for 48 h prior to the following experiments. The final concentration of miRNAs and siRNAs used for cell transfection was 100 nm.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from cultured cells and fresh-frozen tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. To quantify the expression level of miR-214, a total of 5 μg RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). The temperature protocol of first-strand cDNA synthesis was: 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min. The expression level of miR-214 was normalized to human U6 snRNA. The following PCR primers were used: U6: Forward, 5'-TGCGGGTGCTCG CTTCGGCAGC-3'; reverse, 5'-CCAGTGCAGGGTCCG AGGT-3' and miR-214: Forward, 5'-TGCGGACAGCAGGCA CAGAC-3'; reverse, 5'-CCAGTGCAGGGTCCGAGGT-3'.

The expression level of EZH2 was normalized to GAPDH. The following PCR primers were used: EZH2: Forward, 5'-TTGTTGGCGGAAGCGTGTAAAATC-3'; reverse, 5'-TCCCTAGTCCCGCGCAATGAGC-3' and GAPDH: Forward, 5'-TGAACGGGAAGCTCACTGG-3'; and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. RT-qPCR was performed using SYBR Premix Ex Taq™ kit (Takara Biotechnology Co., Ltd., Dalian, China) on the 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction condition was 95˚C for 3 min, followed by 40 cycles of 95˚C for 30 sec and 60˚C for 30 sec. Relative expression values from three independent experiments were calculated using the $2^{-\Delta\Delta Cq}$ method (22).

Western blot analysis. Total protein was isolated from cultured cells and tissues using RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and was quantified using a BCA protein quantification kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The protein samples (50 μg) were separated using a 12% SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was incubated with fat-free milk for 90 min at room temperature, then incubated with primary antibodies against EZH2 (cat. no. 4905) and GAPDH (cat. no. 2118; both 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 60 min at 4˚C. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074; 1:500; Cell Signaling Technology, Inc.) at room temperature for 60 min. Bands were visualized using the BeyoECL Plus kit (Beyotime Institute of Biotechnology). Densitometric analysis of the protein bands was conducted using ImageJ 1.42 software (National Institutes of Health, Bethesda, MD, USA). Analysis of each sample was repeated three times.

Cell proliferation assay. To determine cell proliferation, an MTT assay was performed. Cells were seeded into 96-well plates at a density of $2x10^3$ cells/well in a volume of 100 μl RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin, and incubated for 0, 24, 48 and 72 h. MTT solution (10 μl) was added to each well at a final concentration of 0.5 mg/ml and the cells were cultured for another 4 h at 37˚C. The medium was removed and the precipitated formazan was dissolved in 100 μl DMSO. The absorbance of each well was measured at 570 nm using the Thermo Multiskan Spectrum spectrophotometer (Thermo Fisher Scientific, Inc.).

Target prediction and luciferase reporter assay. Based on bioinformatics prediction algorithm TargetScan 7.2 (http://www.targetscan.org/vert_72/), EZH2 was selected as a candidate target of miR-214. The 3'-UTR of EZH2 that contains the putative binding sites for miR-214 was amplified from human genomic DNA and cloned into the 3'-UTR of *Renilla* luciferase gene in the psiCHECK-2 receptor vector (Promega Corporation). The putative miR-214 binding sites in EZH2 were then mutated and also cloned into the psiCHECK-2 receptor vector. Following the cloning, the cells were co-transfected with miR-214 mimic or negative control miRNA and the wild-type or mutant 3'-UTR luciferase constructs using Lipofectamine 2000 reagent. Cells were lysed 24 h post-transfection to measure the luciferase activity

Table I. Association between miR-214 expression and clinicopathological features.

Variables	n	miR-214 expression level		P-value
		High	Low	
Age, years				0.252
≥50	25	8	17	
<50	20	6	14	
Differentiation				0.037
Well/Moderate	17	5	12	
Poor	28	9	19	
Tumor stage				0.012
I-II	16	6	10	
III	29	8	21	
Lymph node metastasis				0.075
Negative	18	5	13	
Positive	27	9	18	

miR-214, microRNA-214.

using a Dual-Luciferase Reporter Assay system (Promega Corporation), according to the manufacturer's protocol. Renilla luciferase activity was used to normalize the luciferase activity.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The 75th percentile of the expression level of miR-214 was used to classify the patients into a high (≥75th percentile) or low (<75th percentile) miR-214 expression group. Data are presented as the mean ± standard deviation (three repeats). χ^2 test was used to evaluate associations between the expression of miR-214 and clinicopathological characteristics. Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. Comparisons between two groups were conducted using Student's t-test. Analysis of variance and Tukey's post-hoc test were used when comparing the differences among multiple groups. Cox univariate and multivariate regression analyses were used to identify the independent predictor for the prognosis of patients with CC. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-214 is downregulated in CC tissues and a CC cell line. To explore whether miR-214 expression was altered in CC, miR-214 expression levels in the CC and normal cervical cell lines were examined. As revealed in Fig. 1A, the expression of miR-214 in the CC cell line, HeLa, was significantly lower compared with that in the normal cervical cell line, Ect1/E6E7 (P<0.05). The cell proliferation in HeLa cells was significantly increased compared with the Ect1/E6E7 cells at 72 h (P<0.001; Fig. 1B). The expression of miR-214 was also investigated in the 45 pairs of CC tissues and corresponding adjacent non-cancerous tissues. Compared with the paired normal tissues, miR-214 expression was decreased in 31 of 45 CC tissues (68.89%; Fig. 1C), which was demonstrated to be statistically significant (P<0.01; Fig. 1D). The results demonstrated that the expression of miR-214 was downregulated in CC tissues and HeLa cells, which implies that miR-214 may serve an important role in the progression of CC.

miR-214 expression is associated with clinicopathological features of patients with CC. To investigate the association of miR-214 expression and the clinical outcome of patients with CC, the 45 enrolled patients were classified into two groups based on the expression level of miR-214. The association between miR-214 expression and clinicopathological features was analyzed (Table I). miR-214 expression was significantly associated with tumor differentiation (P=0.037) and tumor stage (P=0.012), while no association was identified between miR-214 expression and age or lymph node metastasis. Patients with high miR-214 expression exhibited significantly higher overall survival probability compared with those with low miR-214 expression (P=0.034; Fig. 2) according to the Kaplan-Meier analysis and log-rank test results. These results indicated that miR-214 may contribute to CC progression. Additionally, a Cox univariate regression analysis revealed that low miR-214 expression (P=0.035), poor tumor differentiation (P=0.044) and high tumor stage (P=0.033) were associated with poorer survival rates of patients with CC (Table II). Similarly, a multivariate Cox regression analysis revealed that low miR-214 expression (P=0.036), poor tumor differentiation (P=0.042) and high tumor stage (P=0.020) could be regarded as independent indicators for poor survival of patients with CC (Table II).

Upregulating miR-214 expression inhibits the proliferation of CC cells. To further understand the biological function of miR-214 expression in CC progression, HeLa cells were transfected with an miR-214 mimic, an miR-214 inhibitor or negative control miRNA to regulate the expression level of miR-214. Successful transfection of the miRNAs was verified by RT-qPCR (Fig. 3A). The expression of miR-214 was significantly downregulated using a miR-214 inhibitor compared with the negative control (P<0.05; Fig. 3A), which significantly promoted HeLa cell proliferation (P<0.01; Fig. 3B) compared with the negative control. Conversely, the expression of miR-214 was significantly upregulated using a miR-214 mimic (P<0.01; Fig. 3A), which significantly inhibited HeLa cell proliferation (P<0.001; Fig. 3B) compared with the negative control. Collectively, the results indicated that miR-214 serves as a proliferation inhibitor, which was consistent with the aforementioned finding that miR-214 expression was reduced in a CC cell line.

EZH2 is a direct target of miR-214. Using the online TargetScan algorithm, the 3'-UTR of EZH2 was identified to contain a putative target sequence for miR-214 (Fig. 4A). To validate EZH2 as a target of miR-214 in CC, the luciferase activities of EZH2 were analyzed using a dual-luciferase reporter assay.

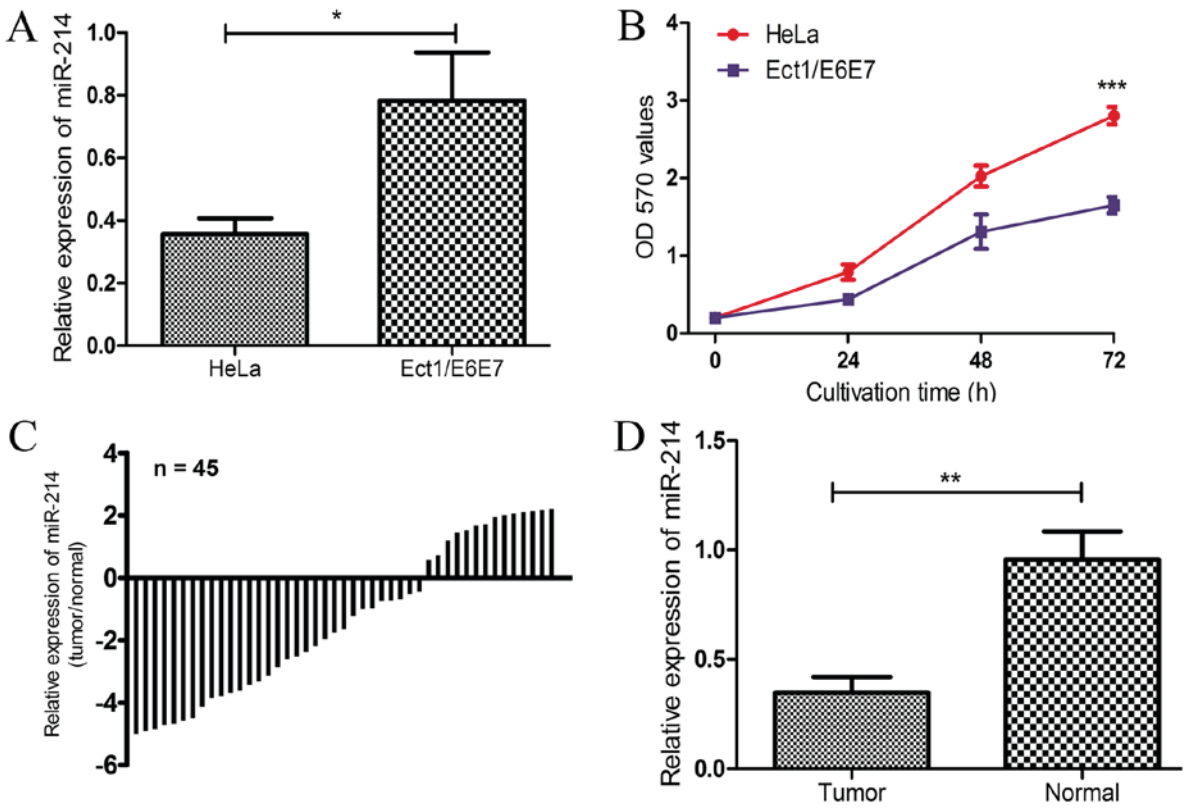

Figure 1. miR-214 is downregulated in human CC. (A) RT-qPCR analysis of miR-214 expression in the CC cell line, HeLa, and the normal cervical cell line, Ect1/E6E7. (B) MTT assay to analyze the cell proliferation rate of the HeLa and Ect1/E6E7 cell lines. RT-qPCR analysis of miR-214 expression was performed in tumor tissues and paired adjacent noncancerous tissues of 45 patients with CC; data are presented as (C) log2-fold change (cancer/normal) and (D) normalized against U6 snRNA. *P<0.05, **P<0.01; ***P<0.01 vs. Ect1/E6E7. miR-214, microRNA-214; CC, cervical cancer; RT-qPCR, Reverse transcription-quantitative polymerase chain reaction; OD, optical density.

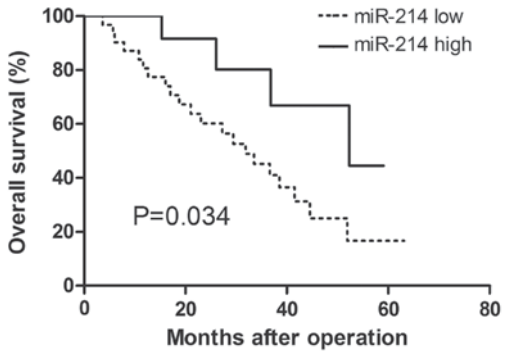

Figure 2. Kaplan-Meier overall survival curve for high and low miR-214 expression. The overall survival rates were measured in the high miR-214 expression group (n=14) and the low miR-214 expression group (n=31). miR-214, microRNA-214.

As expected, the miR-214 mimic significantly reduced the luciferase activity of the wild-type 3'-UTR of EZH2 compared with the negative control miRNA-transfected cells (P<0.001; Fig. 4B). However, no significant differences were identified between cells transfected with negative control miRNAs and the miR-214 mimic when co-transfected with the mutated 3'-UTR of EZH2. The expression of EZH2 in HeLa cells transfected with an miR-214 mimic, an miR-214 inhibitor or negative control miRNA was measured. Upregulating miR-214 expression significantly downregulated the expression of EZH2, whereas downregulating miR-214 expression significantly upregulated the expression of EZH2 in HeLa cells compared with HeLa cells transfected with the negative control miRNA (both P<0.001; Fig. 4C). Additionally, the expression of EZH2 in CC tissues and adjacent noncancerous tissues was examined and, as expected, the expression of EZH2 in CC tissues was significantly higher compared with that of the adjacent non-cancerous tissues (P<0.01; Fig. 4D).

Inhibiting EZH2 expression reduces the proliferation of CC cells. To explore the effect of EZH2 on the proliferation capacity of HeLa cells induced by a miR-214 mimic, an EZH2-specific siRNA was introduced into HeLa cells. As revealed in Fig. 5A and B, EZH2-specific siRNA significantly downregulated the expression of EZH2 at the mRNA and protein levels (both P<0.01). Furthermore, the proliferation of HeLa cells was significantly inhibited by EZH2-specific siRNA at 72 h (P<0.01; Fig. 5C). Taken together, these results demonstrated that miR-214 acts as a cell proliferation inhibitor partly through regulating the expression of EZH2.

Discussion

Dysregulation of miRNAs has been revealed in numerous human cancers and thus increasing research efforts have been made in this field (9-17,23). It has previously been demonstrated that miRNAs may function as a novel class of tumorigenic and tumor-suppressing factors (24). miRNAs are involved

Table II. Univariate and multivariate analyses of overall survival rate.

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
microRNA-214	2.401	1.065-5.413	0.035	2.392	1.060-5.397	0.036
Age, years	2.241	0.821-6.115	0.115	-	-	-
Differentiation	2.342	1.022-5.366	0.044	2.556	1.034-6.314	0.042
Tumor stage	2.422	1.075-5.454	0.033	2.728	1.169-6.366	0.020
Lymph node metastasis	2.438	0.945-6.292	0.066	-	-	-

HR, hazard ratio; CI, confidence interval.

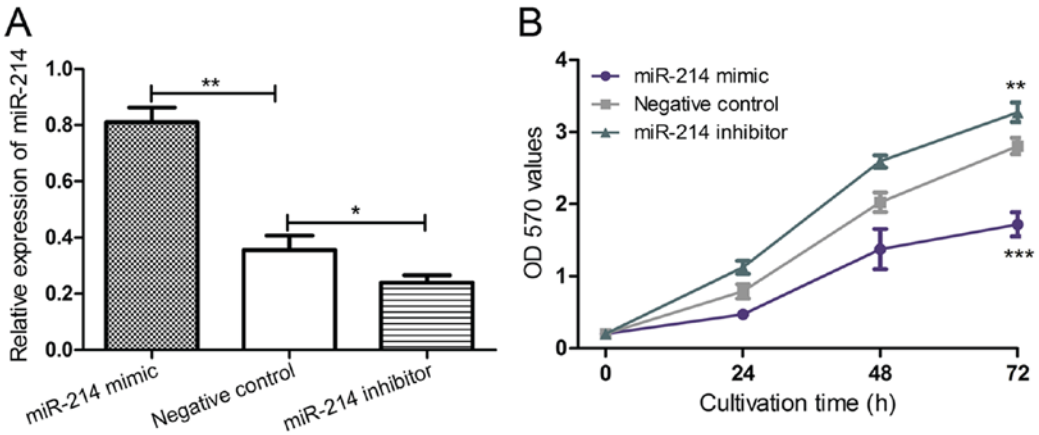

Figure 3. miR-214 overexpression inhibits proliferation of cervical cancer cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-214 expression and (B) cell proliferation analysis using a MTT assay in HeLa cells following miR-214 mimic, miR-214 inhibitor or negative control miRNA transfections. In (A), *P<0.05, **P<0.01. In (B), **P<0.01, ***P<0.001 vs. negative control. miR-214, microRNA-214; OD, optical density.

in the tumor initiation and progression processes with two mechanisms: Certain miRNAs are directly involved in cancer development by controlling cell differentiation and apoptosis, and other miRNAs are involved in cancer by targeting cancer oncogenes and/or tumor suppressors (24). Understanding the molecular mechanisms of miRNAs in cancers may provide new insights into the molecular basis of cancers, and new biomarkers for cancer diagnosis and cancer therapy.

The differential expression of miR-214 in human cancers has been reported previously (13,15-17). In the current study, a decrease in the expression of miR-214 was identified in human CC tissues and the CC cell line, HeLa, which is inconsistent with a previous study (25). In addition, the clinical significance of miR-214 expression in CC was studied. The current study demonstrated that low expression of miR-214 was associated with poor tumor differentiation and high tumor stage, and that patients with low miR-214 expression exhibited a worse 5-year overall survival rate. The multivariate analysis demonstrated that the miR-214 expression, tumor differentiation and tumor stage were independent predictors for the prognosis of patients with CC, which highlighted the importance of miR-214 expression in CC. Zhao *et al* (26) reported that the aberrant expression of miR-214 is associated with the growth of a lung cancer cell line. Therefore, the effect of miR-214 expression on HeLa cell proliferation was also investigated. In the current study, the transfection of an miR-214 mimic into HeLa cells resulted in the upregulation of miR-214, which led to decreased cell proliferation. The transfection of an miR-214 inhibitor into HeLa cells resulted in decreased miR-214 expression, but increased cell proliferation. This finding mirrors the role of miR-214 as a tumor suppressor gene. The deregulation of miR-214 in a CC cell line compared with a normal cervical cell line may account for the aberrant growth behavior of the CC cell line.

Several targets of miR-214, including ARL2, FOXM1 and HMGA1 have been identified in recent years (27-29). To explore the underlying mechanisms of the effects of miR-214 in CC, its potential target genes were explored using bioinformatics analysis and several genes were predicted as target genes of miR-214. The EZH2 gene was selected as a potential target to investigate in the current study as it was widely reported to be ectopically expressed in human cancers and associated with poor prognosis (20,21,30). More importantly, EZH2 may be regulated by miR-214 in skeletal muscle cells (31), erythroid cells (32), cardiac myofibroblasts (33) and in the process of cardiac hypertrophy (34). Recently, Xu *et al* (35) demonstrated that EZH2 may also be regulated by miR-214 in glioma cells. Therefore, a dual-luciferase reporter assay was used in the current study to confirm that EZH2 is a direct target of miR-214 in CC. Notably, EZH2 expression may be regulated

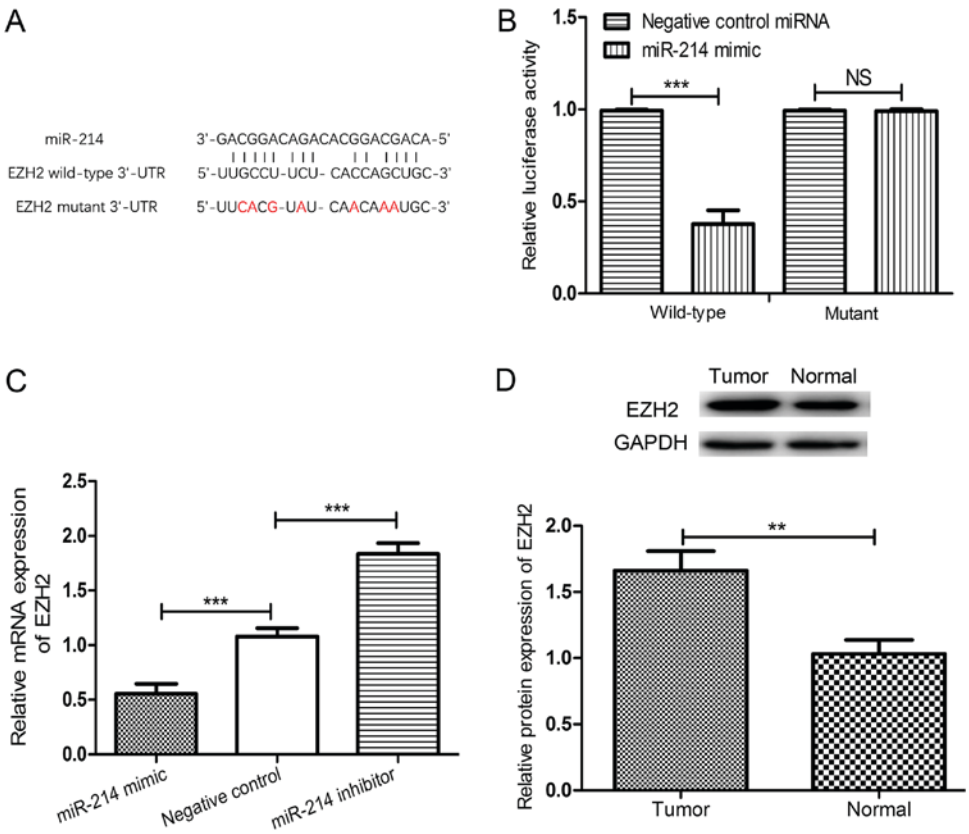

Figure 4. EZH2 is a direct target of miR-214. (A) Putative binding region between miR-214 and EZH2 mRNA. (B) A dual-luciferase reporter assay was performed on HeLa cells to detect the relative luciferase activities of wild type and mutant EZH2 reporters. (C) Reverse transcription-quantitative polymerase chain reaction analysis of EZH2 expression in HeLa cells following miR-214 mimic, miR-214 inhibitor or negative control miRNA transfections. (D) Western blot analysis of EZH2 expression in tumor tissues and paired adjacent non-cancerous tissues. ***P<0.001, **P<0.01. NS, not significant; miR-214, microRNA-214; EZH2, Enhancer of zeste homolog 2; UTR, untranslated region.

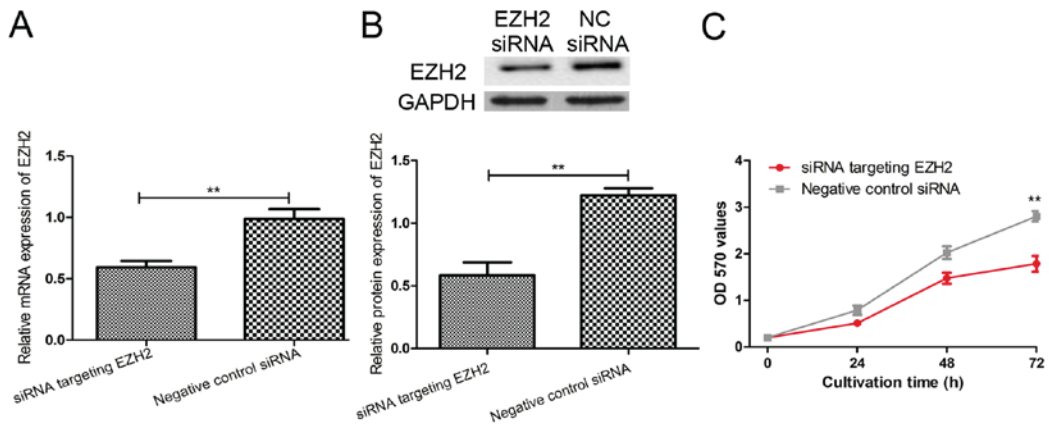

Figure 5. EZH2 downregulation inhibits cell proliferation of cervical cancer cells. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analyses of EZH2 expression in HeLa cells following siRNA targeting EZH2 and negative control siRNA transfections. **P<0.01. (C) An MTT assay was used to analyze the cell proliferation rate of HeLa cells following siRNA targeting EZH2 and negative control siRNA transfections. **P<0.01 vs. negative control. EZH2, Enhancer of zeste homolog 2; siRNA, small-interfering RNA; NC, negative control.

by miR-214, as the upregulation of miR-214 by a miR-214 mimic decreased EZH2 expression and the downregulation of miR-214 by a miR-214 inhibitor increased EZH2 expression in CC. Additionally, the role of EZH2 on HeLa cell proliferation was examined and, and as expected, downregulating the expression of EZH2 decreased cell proliferation, which is in accordance with a previous study (30). Taken together, these results indicate that EZH2 is a target gene of miR-214, which could potentially help to unravel the mechanism of miR-214 in the regulation of CC progression.

In conclusion, miR-214 was expressed at a low level in CC tissues when compared with adjacent non-cancerous tissues, and the overexpression of miR-214 inhibited cell proliferation. A novel target gene of miR-214, EZH2, was revealed to be upregulated in HeLa cells, a CC cell line. These findings indicated that inhibition of miR-214 in CC may contribute to

the malignant phenotype by maintaining a high level of EZH2. Thus, the identification of miR-214 and its target gene, EZH2, in CC may aid in improving the prognosis for CC patients.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YY and YL contributed equally to the study. YY, YL and HS conceived and designed the study. YY, YL, GL, LL, PG and HS performed the experiments. YY, YL and HS wrote the paper. YY, YL and HS reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Ethics approval was granted by the Ethics Committee of the Xuzhou Maternity and Child Health Care Hospital (Xuzhou, China). Written informed consent was obtained from all participants.

Patient consent for publication

Written informed consent was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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