

MicroRNA-206 suppresses proliferation and predicts poor prognosis of HR-HPV-positive cervical cancer cells by targeting G6PD

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Abstract. Cervical cancer (CC) is one of the most frequent gynecological malignancies in females worldwide. Aberrant expression of microRNA (miR)-206 was reported to play an important role in tumor progression. The aim of the present study was to evaluate the potential role of miR-206 and verify its influence on the function of glucose-6-phosphate dehydrogenase (G6PD) in CC. Western blot analysis and RT-PCR were employed to measure miR-206 and G6PD expression. Luciferase assays were performed to validate G6PD as miR-206 targets. CCK-8 assay was performed to examine the regulation of miR-206 and G6PD on CC proliferation. The result showed that miR-206 was downregulated, while G6PD was upregulated in high risk human papillomavirus (HR-HPV)(+) CC. miR-206 directly targeted the 3'-UTR of G6PD. miR-206 overexpressed or G6PD low-expressed suppressed cell proliferation. miR-206 low expressed or G6PD overexpressed predicted poor prognosis. In conclusion, miR-206 reduced cancer growth and suppresses the G6PD expression in CC. This newly identified miR-206 may provide further insight into tumor progression and offers a promising target for the CC therapy.

Introduction

Cervical cancer (CC) remains one of the most frequent gynecological malignancies among females worldwide, which leads to the highest morbidity and mortality in young women,

particularly in developing country (1). Considered as the major etiologic contributor to the pathogenesis of CC, HPVs have been associated with more than 99% of cervical carcinomas (2). It is clear that persistent infection of high-risk human papillomavirus (HR-HPV), especially HPV18 and HPV16 are the most important etiologic agent in cervical carcinogenesis (3,4). The viral oncoproteins HPV18 and HPV16 E7 and E6 can inactivate pRB and p53, thereby influencing their regulation and subsequently contributing to cell cycle checkpoint escape and cervical carcinogenesis (5,6). However, due to the CC complex mechanism, the regulatory mechanism and the biological functions underlying HPV pathogenesis need to be further investigated.

Glucose-6-phosphate dehydrogenase (G6PD) catalyses the first rate-limiting step in the pentose phosphate pathway (PPP) (7). G6PD produces nicotinamide adenine dinucleotide phosphate (NADPH) that affect antioxidant defense and biosynthesis in the cells and is especially important in red blood cells functionally (8). G6PD is taken into account as one oncogene on account of its high expression in a great range of tumors, including breast cancers (9), melanoma (10), colorectal cancer (11) and lung cancer (12). A previous study has demonstrated that the expression of G6PD was high and there is a positive correlation with cervical patients infected with HPV18 and HPV16 of 30 to 40 years female (13). The high expression of G6PD may affect the progression and development of HR-HPV16/18-infected CC. Its underlying molecular mechanisms and the biological functions for its oncogenic roles in HR-HPV16/18 infected CC are still unknown.

MicroRNAs (miRNAs/miRs) are a class of highly conserved, non-coding and endogenous RNAs (ranging in 18-23 nucleotides length) (14,15), which can modulate the physiological process or pathogenesis through partial complementary binding to the 3'-UTR of mRNAs (16). miR-206 has been demonstrated to be involved in different physiological and pathological processes (17). Dysfunctions of miR-206 occurred in a group of tumors, such as hepatocellular carcinoma (18), head and neck squamous cell carcinoma (19) and medulloblastoma (17), which can regulate tumor progression that is involved in cell differentiation, proliferation, metastasis,

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and apoptosis. However, the specific functional molecular mechanisms of miR-206 in CC are still elusive, and the potential of miR-206 as a therapeutic target of CC remains to be evaluated.

We demonstrated that miR-206 was frequently down-regulated while G6PD was upregulated in HR-HPV(+) CC. Overexpression of miR-206 or low expressed G6PD suppressed cell proliferation, and miR-206 low expressed or G6PD overexpressed predicted poor prognosis. Furthermore, we identified G6PD as a direct target of miR-206. We also measured the overall survival (OS) according to the expression of miR-206 and G6PD. The newly identified miR-206/G6PD axis partially elucidates the molecular mechanism of proliferation and is a novel potential therapeutic target for CC treatment.

Materials and methods

Patient selection and human tissues. A total of 56 CC patients (including 42 HPV16/18-positive CCs, 14 HPV-negative CCs) who were treated at the Yantaishan Hospital (Yantai, China) between March, 2014 and August, 2016 participated in this study. Written informed consent was provided by patients. The study was approved by the Ethics Committee of Yantaishan Hospital. All the tissues were independently and histologically diagnosed, and CC was classified based on the International Federation of Gynecology and Obstetrics (FIGO) staging system (20). All the specimens were stored at -80°C. PCR amplification was used to detect cervical HPV infection with the presence of HPV DNA (7).

Cell culture and treatments. HPV16-positive SiHa (HPV16⁺SiHa), and HPV18-positive HeLa (HPV18⁺HeLa) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FCS (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO₂.

miR-206 mimics, G6PD siRNA and the negative controls (NC) were obtained from GenePharma, Co., Ltd. (Shanghai, China). HeLa and SiHa cells were transfected with G6PD siRNA or miR-206 mimics as well as the NC using Lipofectamine 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were used for proliferation after transfection. All transfection was conducted three times.

CCK-8 assay. CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was performed to detect cell proliferation. Cervical cells transfected with miR-206 mimics or G6PD siRNA were seeded into 96-well plates. Then, 10 μ l CCK-8 reagent was added to the wells. The absorbance of each well at 24, 48, 72 and 96 h was detected at 450 nm.

Western blot analysis. Western blot analysis was used to detect the G6PD protein expression in HeLa and SiHa cells transfected with miR-206 mimics. Proteins were isolated from cervical cells with different transfections using RIPA lysis buffer (Thermo Fisher Scientific, Inc.). The protein was then transferred onto a PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and sealed with Tris-buffered saline Tween-20 (TBST). Then, the membranes were blocked by 5%

bovine serum albumin and incubated with specific primary antibody rabbit polyclonal anti-G6PD antibody (1:1,000; cat. no. ab993; Abcam, Cambridge, MA, USA) or GAPDH (1:3,000; cat. no. ab226408). After that, the membrane was incubated in the secondary antibody goat polyclonal anti-rabbit IgG H&L secondary antibody (1:2,000; cat. no. ab150077; Abcam). The ECL detection system was used to detect the protein level (BestBio, Shanghai, China).

RNA isolation and RT-qPCR. Total RNA of cervical cells and tissues were extracted using TRIzol Reagent. RT-qPCR for miR-206/G6PD was performed with SYBR Premix Ex Taq™ (Takara Biotechnology Co., Ltd., Dalian, China). Relative gene expression was determined using the 2^{- $\Delta\Delta$ C_q} method (21). U6 and GAPDH acted as the internal control for the expressions of miR-206 and SPARC. G6PD and GAPDH primers were produced by Invitrogen; Thermo Fisher Scientific, Inc. The transcription primer and PCR primer of miR-206 and U6 were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Primer sequences were as follows: miR-206 forward, 5'-CCAAAGCGGAGTCTCGCAT-3' and reverse, 5'-GCCTAGCATCTTGCTTAGCTC-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCTAT-3'; G6PD forward, 5'-TGCCTTCCATCAGTCGGATACAC-3' and reverse, 5'-TGGTGGGGTAGATCTTCTTCTTGG-3'; and GAPDH forward, 5'-CCCTTCATTGACCTCAACTACATG-3' and reverse, 5'-TGGGATTTCATTGATGACAAGC-3'.

Luciferase assay. The bioinformatics analysis software TargetScan (<http://www.targetscan.org/>) and miRanda (<http://www.microrna.org/microrna/home.do>) were chosen for predicting the targets of miR-206. For luciferase reporter, the wild-type (WT) and mutant type (Mut) 3'-UTR of G6PD were cloned into pcDNA3.1 vector (Ambion; Thermo Fisher Scientific, Inc.) and verified by sequencing. For the luciferase assay, the cells were co-transfected with miR-206 mimics and WT or Mut 3'-UTR of G6PD luciferase reporter plasmid. Then we used Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) for measuring the reporter activities.

Statistical analysis. Statistical analyses were presented as the mean \pm standard deviation using SPSS19.0 software (SPSS, Inc., Chicago, IL, USA). Differences between groups were evaluated by Student's t-test or Tukey's post hoc test after ANOVA in SPSS. Correlation between mRNA and miRNA were estimated using the Spearman's correlation method. In addition, the Kaplan-Meier method with log-rank test was used for analyzing survival. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-206 is downregulated, while G6PD is upregulated in HR-HPV(+) CC. To investigate whether the miR-206 expression was altered in HR-HPV(+) cervical tissues, RT-qPCR was performed in CC and normal cervical tissues, obtained from 46 HPV16/18-positive patients and 10 HPV-negative patients. miR-206 expression was significantly lower in

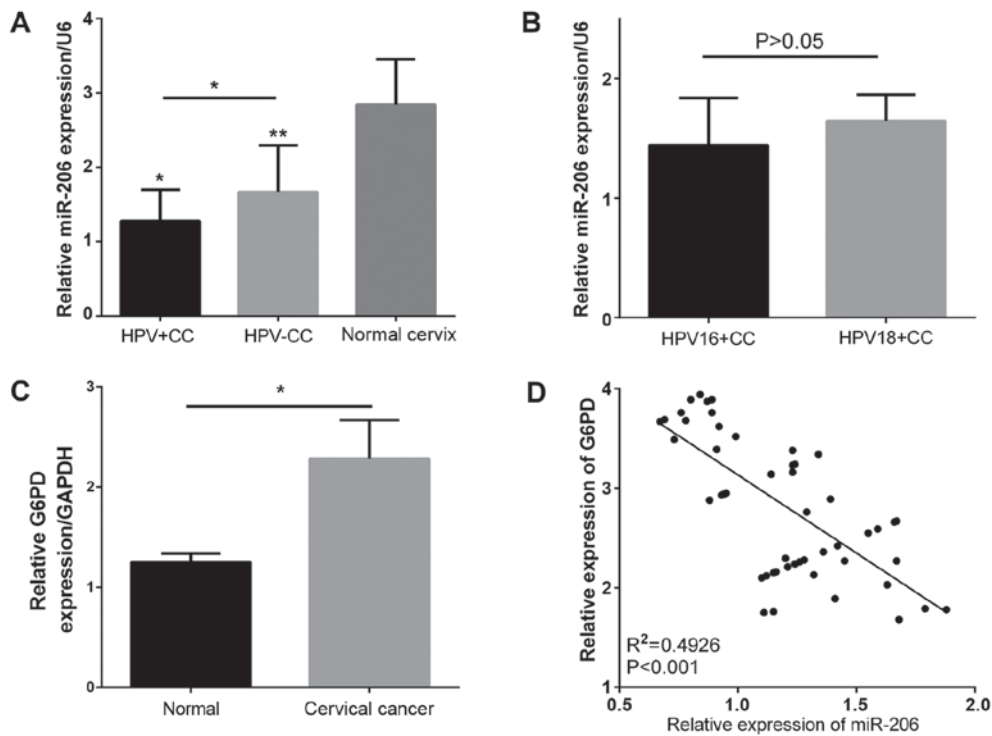


Figure 1. miR-206 was downregulated and inversely connected with G6PD. (A) miR-206 expression in CC tissues compared to normal cervical tissues detected by RT-qPCR. (B) miR-206 levels in HPV16(+) CC and HPV18(+) CC tissues. (C) Relative expression of G6PD in CC tissues. (D) Spearman's correlation analysis of miR-206 and G6PD expression in CC tissues. *P<0.05; **P<0.01. miR, microRNA; G6PD, glucose-6-phosphate dehydrogenase; HPV, human papillomavirus; CC, cervical cancer.

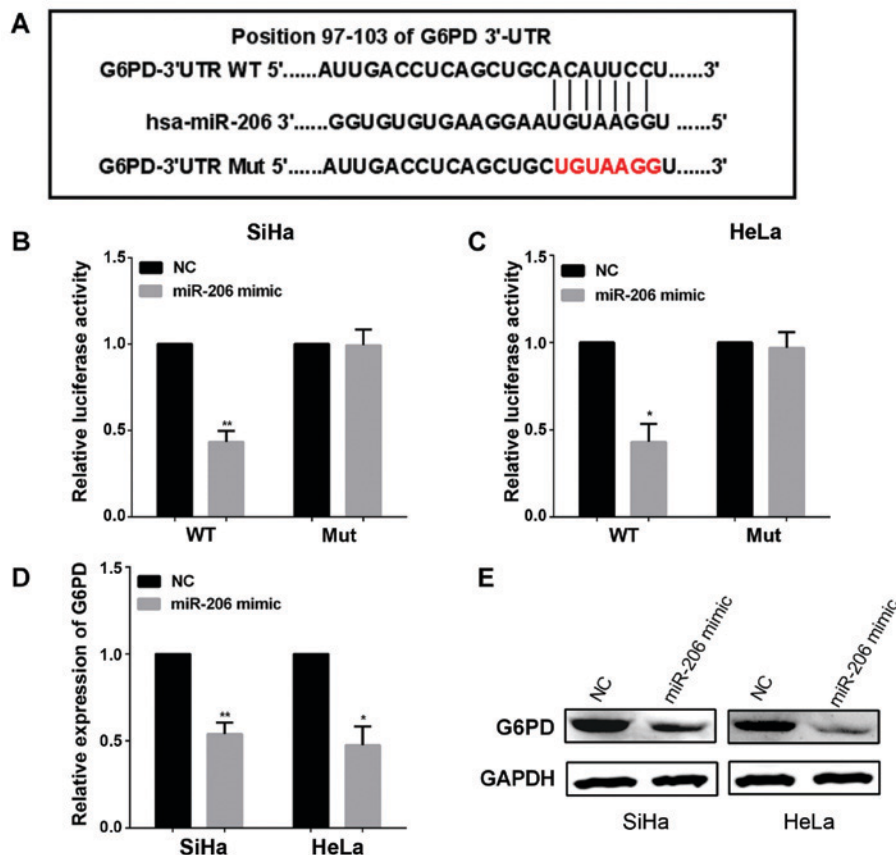


Figure 2. G6PD is the direct target of miR-206. (A) The binding sites of miR-206 on G6PD-3'-UTR. (B and C) Luciferase reporter assay with the pcDNA3.1-G6PD-3'-UTR-WT or pcDNA3.1-G6PD-3'-UTR-Mut were transfected with miR-206 mimics. (D and E) G6PD protein level and mRNA level in HeLa and SiHa cells which transfected with miR-206 mimic. **P<0.01, *P<0.05. G6PD, glucose-6-phosphate dehydrogenase; miR, microRNA; NC, negative controls; Mut, mutant type; WT, wild-type.

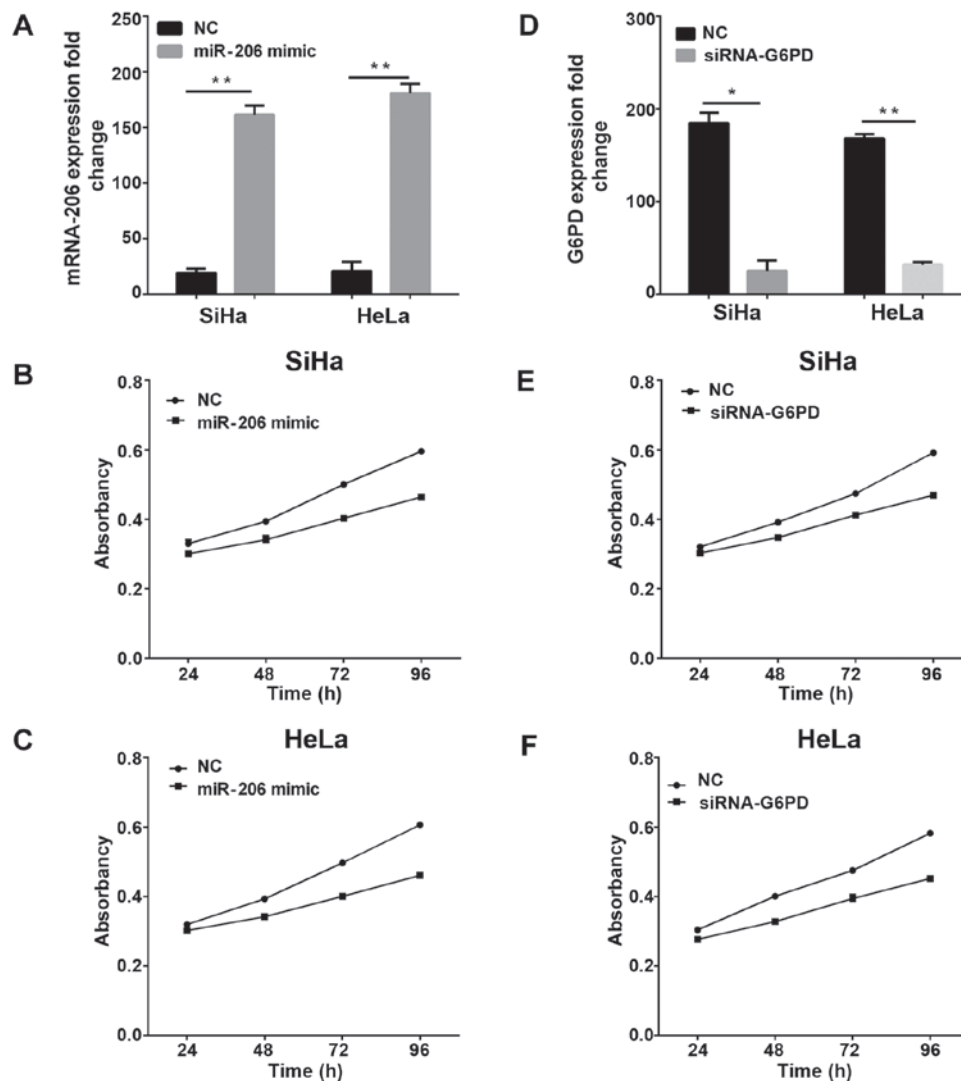


Figure 3. miR-206 overexpressed or G6PD low expressed suppressed cell proliferation. (A) miR-206 was overexpressed in CC cells. (B and C) miR-206 high expression suppressed CC cell proliferation. (D) Interfered G6PD expression by siRNA. (E and F) G6PD low expression suppressed CC cell proliferation. ** $P<0.01$, * $P<0.05$. miR, microRNA; G6PD, glucose-6-phosphate dehydrogenase; CC, cervical cancer; NC, negative controls.

CC tissues (Fig. 1A), and miR-206 expression was lower in HPV16/18-positive CC compared to HPV-negative tissues (Fig. 1A). However, the results showed no significant difference between HPV18(+) CC tissues ($n=20$) and HPV16(+) CC tissues ($n=26$) (Fig. 1B). Generally, G6PD level was significantly higher in CC patients compared to normal control patients (Fig. 1C). An inverse correlation between miR-206 expression and the G6PD level in these clinical specimens ($R^2=0.4926$, $P<0.0001$) (Fig. 1D). Thus, miR-206 and G6PD may have possible roles in modulating the progression of HR-HPV(+) CC.

miR-206 directly targets the 3'-UTR of G6PD. We predicted that G6PD was a downstream target of miR-206 by bioinformatics analysis software TargetScan and miRanda, the binding site of G6PD was at its 3'-UTR located at 97 to 103 as shown in Fig. 2A. To further confirm whether the 3'-UTR of G6PD can be directly targeted by miR-206, we performed luciferase reporter assay. Following the protocol, G6PD 3'-UTR-WT and G6PD 3'-UTR-Mut were cloned into plasmids with either

miR-206 mimic or NC, then following by the measurement of luciferase reporter assays. Luciferase activity decreased when miR-206 mimics were co-transfected with the G6PD 3'-UTR-WT plasmid ($P<0.01$), but there was no change with the G6PD 3'-UTR-Mut plasmid ($P>0.05$) in SiHa and HeLa cells (Fig. 2B and C). These results suggested miR-206 can down-regulate the expression of G6PD by binding to its predicted regions of 3'-UTR. Furthermore, when overexpressed miR-206 by transfected miR-206 mimic, the expression of G6PD was decreased in SiHa and HeLa cell (Fig. 2D). In Fig. 2E, overexpression of miR-206 reduced the protein level of G6PD in the SiHa and HeLa cells. Together, these results demonstrated miR-206 negatively regulated endogenous G6PD expression.

miR-206 overexpressed or G6PD low expressed suppressed cell proliferation. Due to the downregulation of miR-206 and its inverse correlation with G6PD, we hypothesized that miR-206 is a tumor suppressor of CC, and affected CC cell proliferation. For the sake of testing the impact of miR-206 on proliferation, we utilized miR-206 mimic to overexpress

Table I. HPV status and miR-206 levels in women diagnosed as cervical cancer (n=56).

Variable	HPV status (HR-HPV16/18)		miR-206 expression	
	HPV16-/18- negative, n (%)	HPV16+/18+ positive, n (%)	miR-206 low level (≤ median), n (%)	miR-206 high level (> median), n (%)
FIGO stage				
I	2 (3.6)	2 (3.6)	24 (42.8)	2 (3.6)
II	4 (7.1)	8 (14.3)	14 (25)	2 (3.6)
III	2 (3.6)	12 (21.4)	6 (10.7)	2 (3.6)
IV	6 (10.7)	20 (35.7)	2 (3.6)	4 (7.1)
Differentiation				
Well	4 (7.1)	8 (14.3)	30 (53.6)	2 (3.6)
Moderate	2 (3.6)	20 (35.7)	13 (23.2)	2 (3.6)
Poor	8 (14.3)	12 (21.4)	3 (5.4)	6 (10.7)
Tumor diameter (cm)				
≤4	10 (17.8)	19 (33.9)	32 (57.1)	8 (14.3)
>4	4 (7.1)	23 (41.2)	14 (25)	2 (3.6)

miR, microRNA; HR-HPV, high risk human papillomavirus; FIGO, International Federation of Gynecology and Obstetrics.

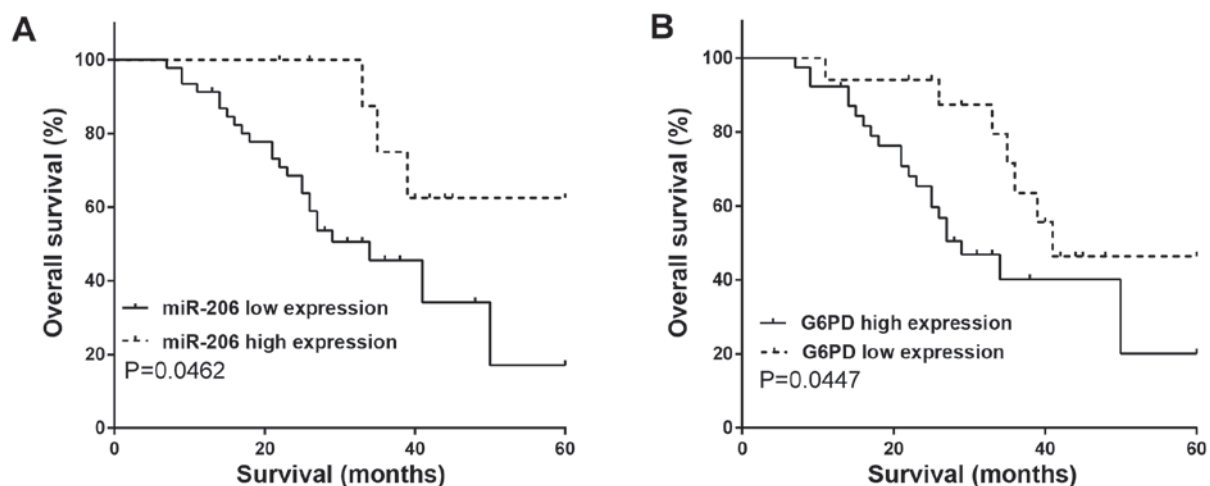


Figure 4. miR-206 low expressed or G6PD overexpressed predicted poor prognosis. (A) OS was calculated between patients according to the expression of miR-206. (B) OS was calculated on the basis of the expression of G6PD. miR, microRNA; G6PD, glucose-6-phosphate dehydrogenase; OS, overall survival.

miR-206 in CC SiHa and HeLa cells and then the expression levels in cells were determined by RT-qPCR (Fig. 3A). Then, we measured cell proliferative ability and found that for overexpressed miR-206 the proliferation ability was decreased both in SiHa and HeLa (Fig. 3B and C). To examine the effect of G6PD on the proliferation of CC, we used siRNA-G6PD to interfere with G6PD expression and the results ($P < 0.01$) were measured by RT-qPCR, as shown in Fig. 3D, and then we calculated the capabilities of cell proliferation. Under these conditions, the results indicated cell proliferative ability was inhibited (Fig. 3E and F).

miR-206 low expressed or G6PD overexpressed predicted poor prognosis. We divided 56 gastric cancer patients into the miR-206 high expression group (n=10) and miR-206

low expression group (n=46) according to miR-206 expression level. In addition, the 56 patients were separated into HPV16-/18- negative group (n=14) and HPV16+/18+ (n=42) according to HPV status. The 56 patients were separated on the basis of FIGO stage, differentiation, tumor diameter, respectively, and the detailed grouping is shown in Table I.

To further evaluate whether miR-206 levels were associated with CC prognosis, we performed Kaplan-Meier analysis to evaluate five year OS in CC. OS was significantly poorer in patients with low tissue miR-206 expression than those with high miR-206 expression (log-rank, $P = 0.0462$; Fig. 4A). In addition, we measured OS according to G6PD expression, and the opposite results were obtained, whereby the OS was lower with G6PD overexpression compared with low expression (log-rank, $P = 0.0447$; Fig. 4B).

Discussion

CC was once considered to be one of the most serious cancers in women worldwide, and almost 90% of CC deaths occurred in developing countries of the world (22). Although cancer treatments have been improved in recent years, the outcomes of patients with CC remain unsatisfactory (23). Thus, identifying new targets for the development of effective therapeutics for CC is urgent. Dysregulation of miRNAs may lead to uncontrolled and progressive cancer growth and has been thoroughly reported in almost all types of human malignancies (24,25), including CC (26). In this study, we found miR-206 was significantly downregulated in CC tissues, and was reduced in HPV16⁺/18⁺ CC. However, there was no significant difference between HPV16(+) CC tissues and HPV18(+) CC tissues. Moreover, G6PD was identified as a direct target of miR-206 and the inverse relationship between them was also observed. We demonstrated that miR-206/G6PD may act as a novel potential therapeutic target and treatment for CC, and a low expression of miR-206 may contribute to tumor progression and cell proliferation in CC patients.

Accumulating evidence has shown that miRNAs can function as a crucial point in gene expressions, and then influence tumor development and progression (27). Mounting evidences have demonstrated that miR-206 is downregulated in breast cancer (28), gastric cancer (29) and various types of human tumors. The low expression of miR-206 may be linked with physiological and pathological processes of tumors, as those researchers proposed. A previous study has reported that miR-206 was downregulated and inhibited cell proliferation, invasion and migration in CC (30), but the underlying molecular mechanisms are still elusive. Our findings were consistent with all the findings, as we have demonstrated that the over-expression of miR-206 could inhibit proliferation by directly targeting G6PD in SiHa and HeLa. In addition, we identified that miR-206 downregulation and/or G6PD upregulation predicted poor prognosis.

This study revealed the relative expression of G6PD was higher in HPV16⁺/18⁺ CC tissues. G6PD can be found widely expressed in tumors and could function as an important member in regulating cell invasiveness, survival and oxidative stress (10,31). The bioinformatics analysis software was used for predicting the targets of miR-206. And then G6PD was looked for as the potential gene effectors which may participate in the function of miR-206. We confirmed that G6PD was a direct target of miR-206, and it was confirmed that a higher G6PD expression played a significant role in tumor proliferation and predicted poor prognosis. G6PD may therefore be independent prognostic factors for OS of patients suffering from CC.

In conclusion, we have indicated that miR-206 acts as a tumor suppressor in CC by inhibiting cancer proliferation. Furthermore, we demonstrated that miR-206 has an inverse correlation with G6PD and directly targets it. This newly identified miR-206 may provide new insight into the progression of CC and offer a promising therapeutic target for the treatment of CC. Nevertheless, further investigation to examine the function miR-206/G6PD axis in tumorigenesis and progression of CC is needed.

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Availability of data and materials

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

Authors' contributions

JC and YP contributed to the study design, data acquisition and analysis and drafted the manuscript; JW and YL contributed to the conception of the study. HW and HL contributed significantly to the data analysis and study preparation. All authors have read and approved the final study.

Ethics approval and consent to participate

Written informed consent was obtained from all the patients. The study was approved by the Ethics Committee of Yantai Shan Hospital (Yantai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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