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ORIGINAL ARTICLE

miR-218 suppresses the growth of hepatocellular carcinoma by inhibiting the expression of proto-oncogene Bmi-1

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Summary

Purpose: *To identify microRNAs (miRNAs) directly regulating the proto-oncogene Bmi-1 expression in the development of hepatocellular carcinoma (HCC) and to explore the underlying molecular mechanisms.*

Methods: *Four HCC cell lines, including HepG2, Bel7404, Huh7, and PLC5, the normal hepatocellular cell line MIHA, and 30 HCC biopsies were included in this study. Potential miRNAs, which interact with Bmi-1 and are involved in the development of HCC were identified through bioinformatic analyses. The expression of miRNA and Bmi-1 in HCC cell lines and HCC tissues was analyzed using fluorescence protein analysis, real-time quantitative PCR (RT-qPCR), and Western blotting.*

Results: *Bioinformatic analysis suggested that miR-218 is a potential miRNA regulating Bmi-1 expression. Fluorescence protein analysis, RT-qPCR, and Western blotting confirmed the direct interaction between miR-218 and Bmi-1. In addition, increased expression of Bmi-1 was detected in HCC cell lines and HCC tissues. In most HCC tissues, the expression of miR-218 was decreased and was associated with increased expression of Bmi-1.*

Conclusion: *miR-218 downregulates the expression of the proto-oncogene Bmi-1 in HCC, and it may be an effective target for the treatment of this disease.*

Key words: *Bmi-1, hepatocellular carcinoma, miR-218*

Introduction

HCC is the fifth most common malignancy and accounts for the third cause of cancer deaths, with >95% 5-year mortality [1,2]. In recent years, an increasing incidence of HCC has been observed globally. In China, HCC has a relatively high incidence compared to other cancers. Approximately 82% of HCC cases are registered in developing countries, with 55% of them occurring in China [3]. In addition, half of the new cases of HCC in the world were identified in China according to the World Health Organization (WHO) reports [4]. Hepatitis B, which is associated with liver cancers, poses a great public challenge in China, in which approximately 94 million hepatitis B virus carriers have been reported [5]. Furthermore, the incidence of hepatitis C patients is gradually increasing in China. The large and increasing number of hepatitis B and C patients in China is a big economic and social burden [6]. While surgery is an effective approach for the treatment of HCC, numerous HCC patients are diagnosed at advanced stages, and most of them suffer a relatively low 5-year overall survival rate. Therefore, alternative therapeutic approaches for the treatment of HCC are urgently needed.

The B-cell-specific moloney murine leukemia virus insertion site 1 (Bmi-1) protein, which is en-

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coded by a proto-oncogene, is a member of the polycomb family proteins. Bim-1 is directly involved in regulating cell growth and proliferation, and is essential for self-renewal of adult stem cells and leukemia stem cells. Abnormal expression of Bmi-1 has been shown in the development of various human tumors, such as leukemia [7], breast cancer [8], gastric cancer [9], lung cancer [10], bladder cancer [11], colorectal cancer [12], and esophageal cancer [13]. Overexpression of Bmi-1 has been identified in both HCC tissues and cell lines [14,15]. Inhibition of the expression of Bmi-1 arrested the cell cycle and significantly suppressed cell growth, suggesting that Bmi-1 plays an important role in carcinogenesis and is a potential target for effective treatment of human cancers [16,17].

miRNAs are small non-coding RNAs (~22 nucleotides in length) found in plants, animals and some viruses [18]. It has been confirmed that miRNAs play important roles in post-transcriptional regulation of gene expression [19]. Recently, several studies have reported that some miRNAs inhibited the growth of tumor and tumor cells through specifically suppressing the expression of Bmi-1. For example, miRNAs miR-15a and miR-16 inhibit the proliferation of ovarian cancer cells by downregulating Bmi-1 expression [20]. miR-128 inhibits the proliferation and self-renewal of glioma cells by directly suppressing Bmi-1 expression [21]. Downregulation of miR-128 results in increased resistance to chemotherapy [22]. It was reported in 2011 that miR-708 directly targeted Bmi-1 and ZEB2 to induce apoptosis of nephroblastoma and tumor formation [23]. In addition, it has been reported that miR-194, miR-200b, and miR-15b inhibited epithelial to mesenchymal transition (EMT) through suppressing Bmi-1 expression [24,25]. However, miRNAs associated with Bmi-1 in the development of HCC have been identified while the underlying mechanisms are not understood.

In the present study, we identified several miRNAs and investigated their interaction with Bmi-1 and roles in HCC. We believe that our study is useful for identifying novel targets for the treatment of HCC, especially advanced HCC.

Methods

Ethics statement

This study was conducted according to the Declaration of Helsinki. Informed consent was obtained from all the study participants, and ethical clearance to conduct the study was obtained from the Shenzhen Hospital, Peking University.

Reagents, cell lines and biopsies

Fetal bovine serum (FBS), trypsin-EDTA, Trizol, and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma (St.Louis, USA). NCode™ miRNA First-Strand cDNA Synthesis Kit was purchased from Invitrogen (Carlsbad, CA, USA). SYBR Green PCR master mix used in RT-qPCR assay was purchased from Roche (Rotkreuz, Switzerland). Thirty pairs of HCC and para-cancer tissues were surgically dissected from HCC patients in the Department of Hepatobiliary Surgery, Shenzhen Hospital, Peking University for follow-up experiments.

Prediction of miRNAs targeting Bmi-1 based on bioinformatic analyses

Targetscan (www.targetscan.org) and miRanda (www.microrna.org) were firstly used to identify potential miRNAs, which directly target Bmi-1. Then, DINAN-microT software and Findtar were used to further analyze selected miRNAs.

Evaluation of Bmi-1 expression in HCC cell lines using RT-qPCR

Total RNA was isolated from the HCC cell lines, including HepG2, Bel7404, Huh7, and PLC5 as well as normal hepatocellular cells MIHA using Trizol. Reverse transcription (RT) was conducted using the Promega RT kit and RT-qPCR was performed using the Fast SYBR® Green Master Mix kit from ABI on a ABI 7500 Real Time PCR equipment. The primers for qPCR assay of Bmi-1 were the forward primer 5' AATGTGTGTCCTGTGTGGAGGGT 3' and the reverse primer 5' GCTGACGGGTGAGCTGCATAAA 3'. GAPDH was used as internal control in RT-qPCR assay using the forward primer 5' TATTCAGGAAGGTGTTACTTAA 3' and the reverse primer 5' TAAGGCACCCTTCTGAGTAGA 3'.

Evaluation of miR-218 expression in HCC cell lines using RT-qPCR

Total RNA was isolated from the HCC cell lines, including HepG2 and Bel7404. Reverse transcription was conducted to synthesize cDNA using the NCodeTM miRNA First-Strand complementary DNA Synthesis kit from Invitrogen (Burlington, Canada). RT-qPCR was performed using the Fast SYBR® Green Master Mix kit from ABI on an ABI 7500 RT-PCR equipment. The primers for qPCR assay of miR-218 were the forward primer 5' TTGTGCTTCATCTAACCATGT 3' and the reverse primer included in the NCodeTM kit. U6 was used as internal control in RT-qPCR assay using the forward primer 5' TTCACGAATTTGCGTGTCAT 3' and the reverse primer 5' CGCTCGGCAGCACATATAC 3'.

Construction of fluorescence reporting vector and mutant

The Bmi-1 3'UTR fragment containing a potential miRNA218-binding site was amplified using PCR and inserted into the pmiR-Glo plasmid to obtain a fluorescent reporter vector of wild-type (WT). A fluorescent reporter vector mutant (Mu) was also constructed using the Promega Site Mutation Kit to introduce mutation on the miR-218 binding site.

Western blotting

HepG2 cells were transfected with miR-218 or miR-218 inhibitor (anti-miR-218). The cells were harvested and treated with RIPA lysis buffer to obtain proteins. The concentration of proteins from HepG2 cells were measured with bicinchoninic acid (BCA) assay. Proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocked with fat-free milk, the membrane was incubated with anti-Bmi-1 antibody overnight. The membrane was washed with PBS and incubated with the secondary antibody for 2-4 hrs. Then, the membrane was washed with PBS twice and exposed with enhanced chemiluminescence (ECL) to detect and measure Bmi-1.

Statistics

Statistical analyses were conducted using the SPSS13.0 package. All experiments were repeated for over three times, and the data were presented as mean ± standard deviation. Comparison of mean values between different groups was conducted using the *t*-test. A p value less than 0.05 was considered to show significant difference. Correlation analysis between the expression of miR-218 and Bmi-1 mRNA was evaluated with the Spearman's correlation co-efficient.

Results

Increased Bmi-1 expression in HCC cells and tissues

Previously, increased Bmi-1 expression has been reported in several tumors. In the present study, we observed significantly increased expression of Bmi-1 in the four types of HCC cells (HepG2, Bel7404, Huh7, and PLC5) (Figure 1A,1C), which is consistent with the proto-oncogene feature of Bmi-1. In addition, increased expression of Bmi-1 was also observed in HCC tissues compared with that in the corresponding para-cancer tissues (Figure 1B).

miR-218 was selected as the miRNA directly regulating Bmi-1

Bmi-1 was used as the target to *in silico* identify miRNAs, which potentially target Bmi-1. A total of 45 miRNAs were identified to target Bmi-1 based on the miRNA screening software targetscan and miRanda, and statistical analyses. Then, DINAN-microT and Findtar selected nine miRNAs, which had the highest possibility of regulating Bmi-1 expression (Figure 2A). Then, the expression of six miRNAs, including miR-218, miR-128, miR-27a,

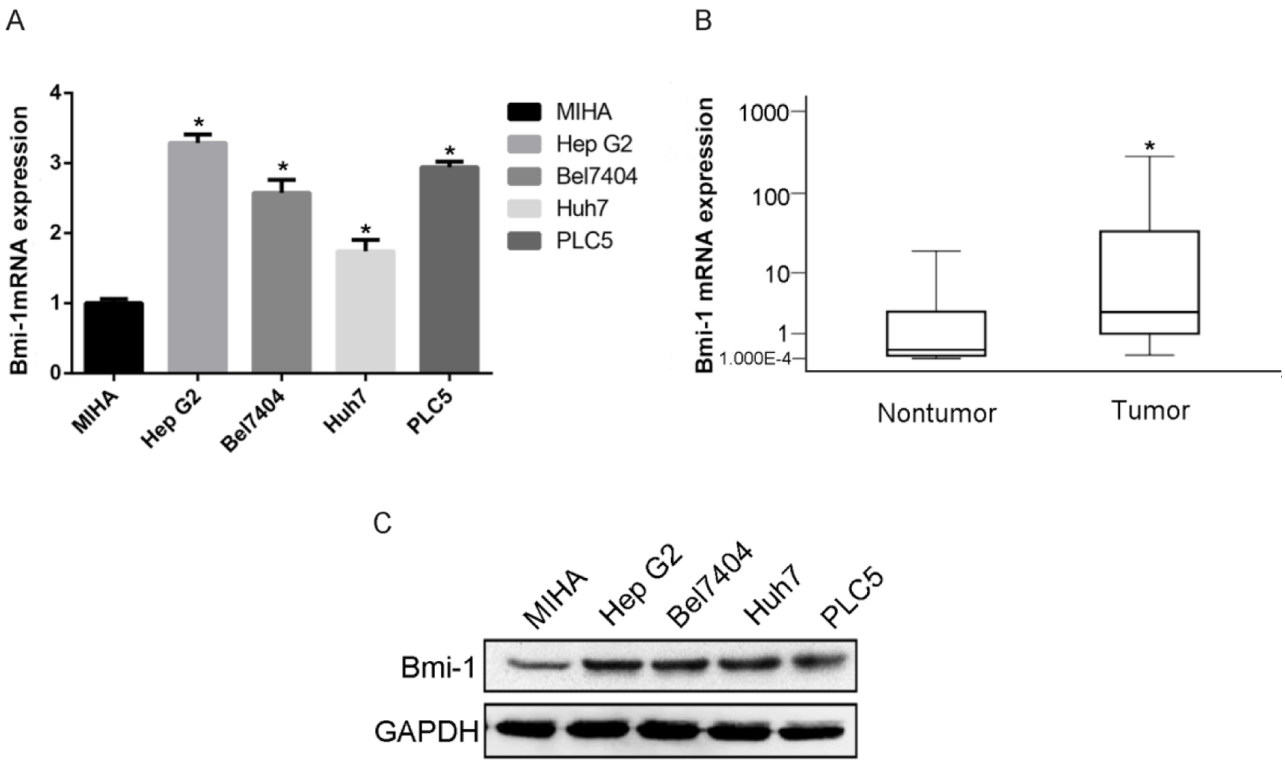

Figure 1. Increased Bmi-1 expression in hepatocellular carcinoma cells and tissues. **A:** The relative levels of Bmi-1 mRNA in HCC cell lines HepG2, Bel7404, Huh7, and PLC5 and normal hepatocellular MIHA cells based on RT-qPCR. *p<0.05 Hep G2, Bel7404, Huh 7, PLC5 cells vs MIHA cells. **B:** The relative levels of Bmi-1 mRNA in HCC and para-cancer tissues based on RT-qPCR. *p<0.05 tumor vs nontumor. **C:** The relative levels of Bmi-1 protein in HCC cell lines HepG2, Bel7404, Huh7, and PLC5 and normal hepatocellular MIHA cells, based on Western blotting. GAPDH was used as internal control.

A

miRNA No	*Position (common)*	*Conservation (DINAN)*	*Precision (DINAN)*	*Loop Score (Findtar)*	*Energy (Findtar)*	*Score (Target scan)*
miR-27a	469-489	10	0.93	25	-22.1	87
miR-27b	471-489	10	0.93	20	-22.5	87
miR-128	469-488	10	0.91	25	-21.8	96
miR-218	1459-1477	11	0.81	20	-13.9	97
miR-221/222	454-476	9/8	0.79/0.78	25	-19.4	76/67
miR-200b	704-726	-	-	25	-17.4	70
miR-200c	700-726	-	-	25	-15.2	70
miR-340	736-748	10	0.75	-	-	38

B

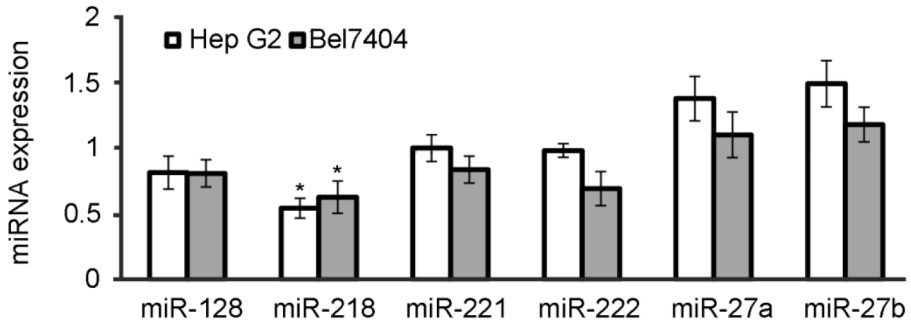

Figure 2. Identification and selection of miRNA targeting Bmi-1 in hepatocellular carcinoma. A: Nine miRNAs were identified to potentially regulate Bmi-1 in HCC based on the miRNA screening software targetscan, miRanda, DINAN-microT, and Findtar. B: The expression of six identified miRNAs in HCC cell lines HepG2 and Bel7404.* p<0.05 miR-218 vs miR-128, miR-221,miR-222, miR-27A and miR-27B.

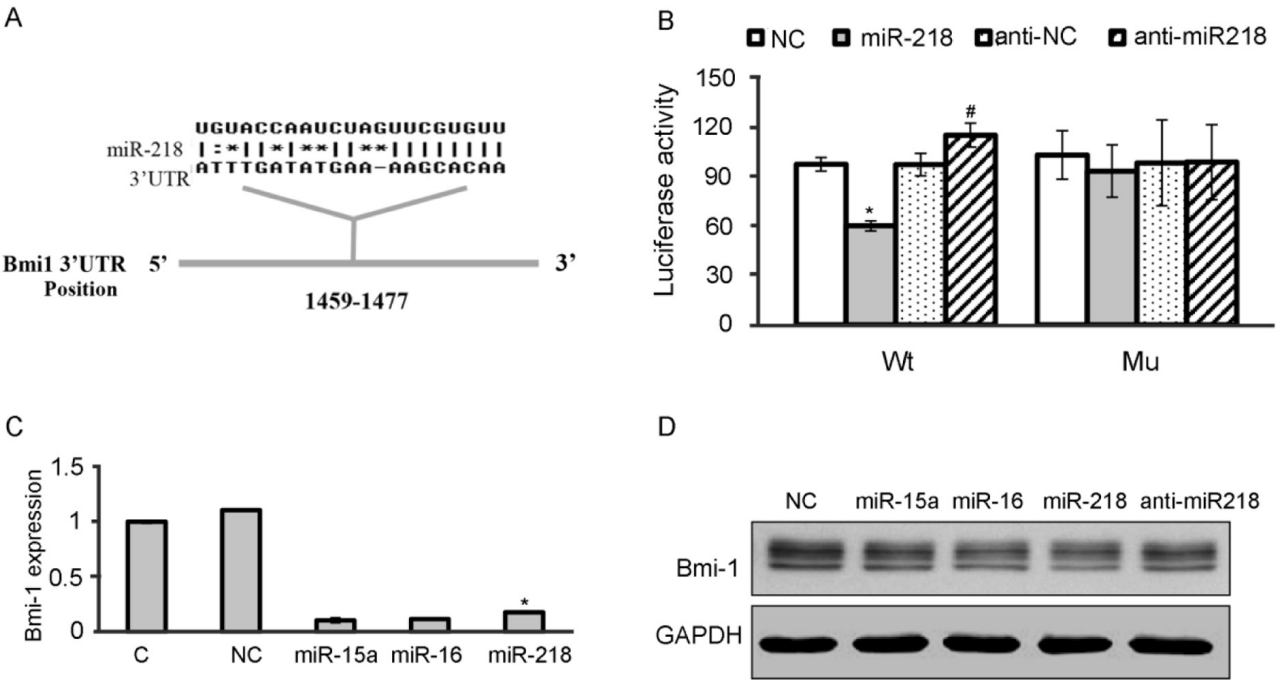

Figure 3. Identification of Bmi-1 as target of miR-218 in HepG2 cells. **A:** *In silico* analyses identified a binding site (1459-1477 nt) on the 3'-UTR of Bmi-1. **B:** Luciferase activity of the wild-type or mutant 3'-UTR of Bmi-1 reporter gene in HepG2 cells transfected with control or miR-218 mimics. *p<0.05 miR-218 vs NC; #p<0.05 anti-miR218 vs anti-NC; **C:** The relative level of Bmi-1 based on RT-q-PCR. miR-15 and miR-16 were used as controls. *p<0.05 miR-218 vs C, NC, miR-15A, miR-16. **D:** The relative level of Bmi-1 based on Western blotting assay. GAPDH was used as internal control.

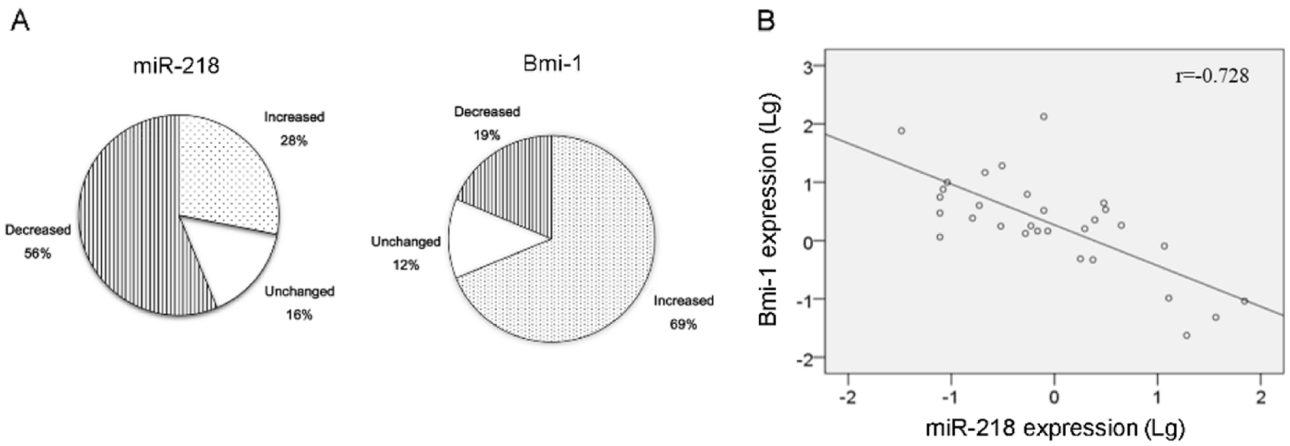

Figure 4. Association between miR-218 and Bmi-1 in hepatocellular carcinoma biopsies. **A:** The expression of miR-218 and Bmi-1 in 30 HCC biopsies. **B:** Negative correlation between miR-218 and Bmi-1 mRNA levels in the 30 HCC tissue samples shown by Spearman's correlation analysis.

miR-27b, miR-221, and miR-222 in HCC cell lines HepG2 and Bel7404 was evaluated using RT-qPCR, the results of which showed decreased expression of miR-218 and miR-128 in both cell lines but miR-218 mRNA level was lower than that of miR-128. However, it has been reported that miR-128 inhibits the proliferation of glioma cells by targeting Bmi-1, and the changes of miR-128 are more significant in hepatoma cell models. Therefore, miR-218 was finally selected as the miRNA targeting Bmi-1 in HCC in the follow-up experiments.

miR-218 directly targeted Bmi-1 in HCC cells

A miR-218-binding site on the 3'-UTR of Bmi-1 (1459-1477) was identified based on *in silico* analysis (Figure 3A). Then, a fluorescence reporting vector containing the Bmi-1 3'UTR including the miRNA218-binding site was constructed to test the interaction between miR-218 and Bmi-1. The fluorescence reporting vector and miRNA were co-transfected into HepG2 cells and the luciferase activity was measured after 28-30 hrs of transfection. The luciferase activity of the plasmid was significantly inhibited by miR-218, but anti-miR-218 increased the luciferase (Figure 3B), suggesting that miR-218 bound with Bmi-1 3'-UTR. In addition, RT-qPCR and Western blotting demonstrated that miR-218 mimics decreased Bmi-1 expression (Figure 3C,3D). In the experiment, miR-15 and miR-16 were used as positive controls according to a previous study [12]. These results suggested that Bmi-1 is a direct target of miR-218.

Negative correlation between miR-218 and Bmi-1

The expression of miR-218 and Bmi-1 was examined in 30 HCC biopsies. The results showed that miR-218 was downregulated in 56.3% of the biopsies and Bmi-1 was upregulated in 68.8% of the biopsies (Figure 4A). In addition, a negative correlation was identified between miR-218 and Bmi-1 (Figure 4B).

Discussion

miRNAs are a group of endogenous non-coding RNAs, which is widely found in animals, plants, and viruses. miRNAs regulate the expression of target genes at post-transcriptional levels. The role of miRNAs in gene expression regulation has been extensively studied. Numerous studies provide evidence showing that miRNAs are widely involved in most physiological processes such as development, organogenesis, and metabolic processes, and are closely linked to tumorigenesis and viral replication [26,27]. Recent studies demonstrated that miR-218 plays critical roles in the development of a number of tumors. Reduced miR-218 has been observed in gastric cancer [28], cervical cancer [29] and nasopharyngeal carcinoma [30]. In addition, it has been reported that miR-218 inhibited the growth and induced apoptosis of tumor cells. miR-218, which is a highly conserved miRNA, is located on human chromosome 5 and has been shown that inhibited the development and metastasis of many tumors [31]. As a tumor suppressor factor, miR-218 has been reported to inhibit the proliferation of HCC cells [32]. Furthermore, reduced expression of miR-218 was found in 56.3% of HCC tissues, which is consistent with previous studies. Taken together, these studies suggest that miR-218 plays an important role in the development of HCC.

As mentioned above, increased expression of Bmi-1 in various tumors, which is associated

with the development and invasion, metastasis and prognosis, has been observed in many tumors [8,12]. A study by Wu et al. confirmed that Bmi-1 knockdown in human BCa T24 cells inhibited cell proliferation and invasion [33]. In the present study, we also found increased Bmi-1 expression in HCC cell lines and tissues, which is consistent with previous studies. These results suggest that Bmi-1 and abnormal Bmi-1 expression play important roles in the development of HCC. In order to investigate the molecular mechanisms underlying the downregulation of Bmi-1 in HCC and whether miRNAs are involved in Bmi-1 expression, we first identified a number of potential miRNAs, which may regulate Bmi-1 expression, based on bioinformatic analyses. Our results suggest that miR-218 directly regulated Bmi-1 expression. Fluorescence enzyme activity analysis, RT-qPCR, and Western blotting assays demonstrated that miR-218 directly regulated Bmi-1 expression. To further study the interaction between miR-218 and Bmi-1 in HCC, we determined the expression of miR-218 and Bmi-1 in 30 HCC tissues and the results demonstrated that the level of miR-218 was negatively associated with Bmi-1 expression in these tissues, which is consistent with previous studies in other tumors. It has been reported that miR-218 may serve as a prognostic biomarker and induce apoptosis and growth arrest by downregulating BMI-1 in HCC [34]. As a member of the polycomb group (PcG) family, Bmi-1 was shown to regulate the AKT phosphorylation in breast cancer [35]. Furthermore, Cheng et al. found that miR-218 inhibited the proliferation, migration and invasion of bladder cancer through the miR-218/BMI-1/PTEN/AKT axis [36]. In addition, miR-218, which is negatively regulated by the long-noncoding RNA CCAT1, affects the lung cancer cell cycle via Bmi-1 [37]. However, the relationship between miR-218 and BMI-1 is still not fully understood in HCC.

In summary, our results showed reduced expression of miR-218 and increased expression of Bmi-1 in HCC and identified a negative correlation between miR-218 and Bmi-1. Therefore, the miR-218/BMI-1 axis may be a potential therapeutic target for effective treatment of HCC.

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Conflict of interests

The authors declare no conflict of interests.

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