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Methylation-associated silencing of miR-495 inhibit the migration and invasion of human gastric cancer cells by directly targeting PRL-3

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ABSTRACT

Phosphatase of regenerating liver-3 (PRL-3) is believed to be associated with cell motility, invasion, and metastasis. Our previous work found that PRL-3 is highly overexpressed in gastric cancer (GC) tissue with peritoneal metastasis and directly involved in the pathogenesis of GC peritoneal metastasis. Moreover, we further found that the down-regulation of endogenous miR-495 expression plays a causative role in over expression of PRL-3 in GC peritoneal metastasis. However, the molecular regulation mechanisms by which endogenous miR-495 expression is down-regulated and PRL-3 promotes GC peritoneal metastasis remain to be clearly elucidated. Some studies have shown that the promoter methylation is closely related to the miRNA gene expression. Therefore, in present study, based on our previous findings, we will analysis whether DNA methylation is a major cause of the down-expression of endogenous miR-495, which results in PRL-3 overexpression in GC peritoneal metastasis. Methylation specific PCR (MSP) and sodium bisulfite sequencing method (BSP) detected miR-495 gene promoter methylation status. We treated GC cell lines with 5-Aza-2′-deoxycytidine (5-Aza-dC) to make the gene promoter methylation inactivation. By treating with 5-Aza-dC the migration and invasion of GC cells were significantly inhibited. And the miR-495 was overexpressing, corresponds to the mRNA and protein levels of PRL-3 were reduced, the ability of invasion and metastasis was inhibited. This study suggest that miR-495 have tumor suppressor properties and are partially silenced by DNA hypermethylation in GC, will provide new strategies for prevention and treatment of GC peritoneal metastasis.

1. Introduction

Over the past 70 years the incidence of GC has fallen notably in most parts of the world [1]. The long-term survival rate also has improved in past decades [2]. Despite of this, GC remains an important component of the global cancer burden [3]. And it is the second most common cause of cancer-related cancer due to its poor prognosis [4]. The 5-year survival rates among patients are disappointing, one of the predominant cause is metastasis [5,6]. But there has no effective method to predict metastasis of GC on the current, elucidate the molecular mechanism of invasion and metastasis is crucial for the prognosis of GC [7]. Thereby, numbers of studies on GC metastasis prediction have been carried out and some biomarkers have been used for screening GC [8–11].

Our previous work showed that in primary GC with peritoneal metastasis the PRL-3 expression was significantly higher than in corresponding primary GC [12]. In addition, some studies also indicated that the expression levels of PRL-3 were associated with GC invasion and metastasis [13–15]. MicroRNAs (miRNAs) are a category of small noncoding RNAs, and they are highly conserved and endogenously expressed. MiRNAs can participate in the cell proliferation, differentiation and apoptosis by repressing their target genes [16]. Furthermore, Evidence has shown that miRNAs play pivotal roles in cancer initiation and progression because of theirs function as oncogenes or tumor suppressors [17,18]. Some studies have demonstrated that miRNAs are correlated with the metastasis and prognosis of gastric cancer [19–21]. Therefore, we carry out a further research between miRNAs and the expression of PRL-3 gene, and the result show that miR-495 can inhibit GC cell migration and invasion, and act as tumor suppressors by targeting the PRL-3 oncogene [22]. But the molecular regulation mechanisms by which endogenous miR-495 expression is down-regulated and

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PRL-3 promotes GC peritoneal metastasis remain to be clearly elucidated.

The mechanism responsible for miRNA dysregulation in cancer has not been fully investigated. DNA methylation is important in regulating the expression of genes, and plays an essential role in cancer development. DNA methylation in the promoter CpG island suppresses the downstream gene transcription [23]. Recently, some studies have shown that the promoter methylation is closely related to the miRNA gene expression [24,25]. And numerous studies have demonstrated that a high frequency of aberrant DNA methylation occurs in GC [26]. Additionally, emerging evidence shows that the DNA methylation is one of the main mechanisms miRNA expression silence in GC [27,28]. MiRNAs expression is regulated by the DNA methylation; meanwhile miRNAs can also regulate the epigenetic mechanisms. Furthermore, recently studies have shown that 5-Aza-dC could inhibit the methyltransferase activity of DNA methyltransferase enzyme 1 (DNMT1). And treated with 5-Aza-dC the migration and invasion of GC cells were significantly inhibited [29].

In addition, we used the CpG island online prediction software (http://cpgislands.usc.edu/) and found that the miR-495 gene promoter region exist the CpG island. Thus, in the current study, we hypothesized that DNA methylation was a major cause of the down-expression of endogenous miR-495, which results in PRL-3 overexpression in GC peritoneal metastasis. Considering this, we examined the miR-495 gene promoter methylation status and the miR-495 expression. And we also analyzed the protein and mRNA expression levels of the PRL-3 gene both in normal and malignant gastric cell and tissue specimens. Then we treated the GC cell lines with 5-Aza-dC to make the gene promoter methylation inactivation. The results showed that the miR-495 was overexpressing, corresponds to the mRNA and protein levels of PRL-3 were reduced, and the ability of invasion and metastasis was inhibited in GC cells. Our study reveals that miR-495 which targeted PRL-3 have tumor suppressor properties and are partially silenced by DNA hypermethylation in GC.

2. Materials and methods

2.1. Gastric cells and culture and gastric tissues

The human GC cell lines SGC7901, MKN45, MKN28, and BGC823 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The human gastric mucosa cell lines GES-1 and HFE-145 were obtained from our central laboratory. The GC cells were cultured in RPMI-1640 medium (Gibco, USA), the gastric mucosa cells were maintained in Dulbecco's Modified Eagle's medium (DMEM, Gibco, USA), cells were supplemented with 10% fetal bovine serum (FBS), in a humidified atmosphere of 5% CO_2 at 37 °C. The human GC tissues and the normal gastric tissues were obtained from the first affiliated hospital of Nanchang University (Nanchang, China). None of the patients received radiotherapy or chemotherapy before the operation. The samples were fresh-frozen and stored in liquid nitrogen after surgical. All samples were obtained with informed consent and approved by the Ethics Committee of the hospital.

2.2. 5-Aza-dC treatment

The GC cells were seeded in 6-well plates (5×10^5 cells/well), and cultured in RPMI-1640 medium supplemented with 10% FBS, in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. Then, GC cell lines were treating with 5 μmol/l 5-Aza-dC (Sigma, USA) for 72 h, and replaced 5-Aza-dC containing medium every 24 h. Exponentially growing cells were used for Western Blot, RT-PCR, MSP and BSP, and detected the ability of invasion and metastasis.

2.3. Western blot analysis

The protein concentration was detected by the BCA kit. Proteins were separated by a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred onto polyvinyl fluoride (PVDF) membranes (Millipore Corporation, USA) by using Bio-rad Mini-PROTEAN Tetra Electrophoresis System

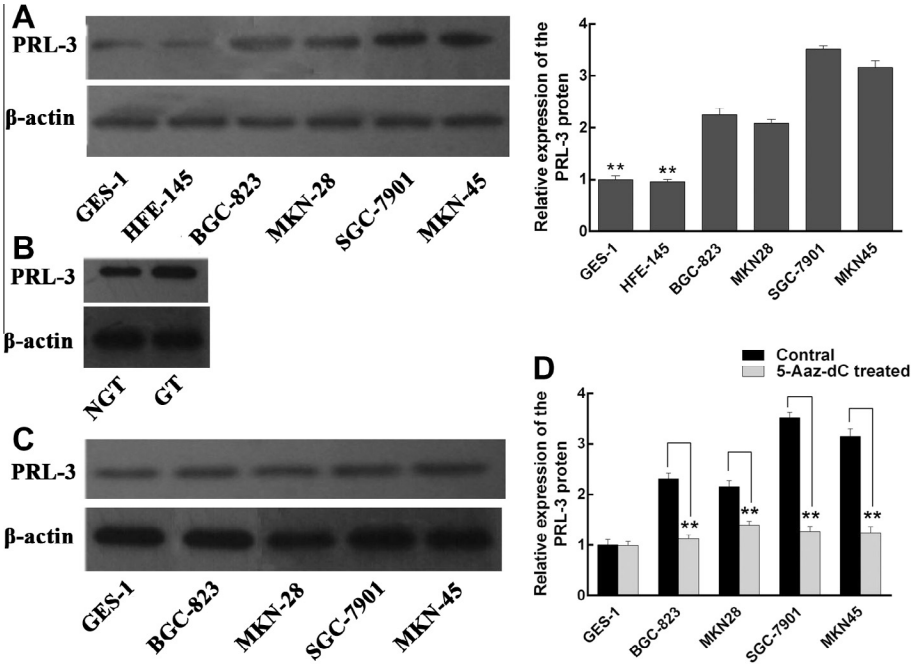

Fig. 1. Expression of the PRL-3 protein in GC samples and normal gastric samples. (A) PRL-3 protein was up-regulated in GC cell lines. β-Actin was used as an internal loading control. (B) PRL-3 protein was overexpressed in gastric malignant tissues (GT) and normal gastric mucosa tissues (NGT). (C) Expression of the PRL-3 protein in the four GC cell lines treated with 5-Aza-dC. (D) The PRL-3 protein was dramatically decreased compared with untreated cells ($^{**}P < 0.01$). Each bar represents mean values (±SD) from three independent experiments.

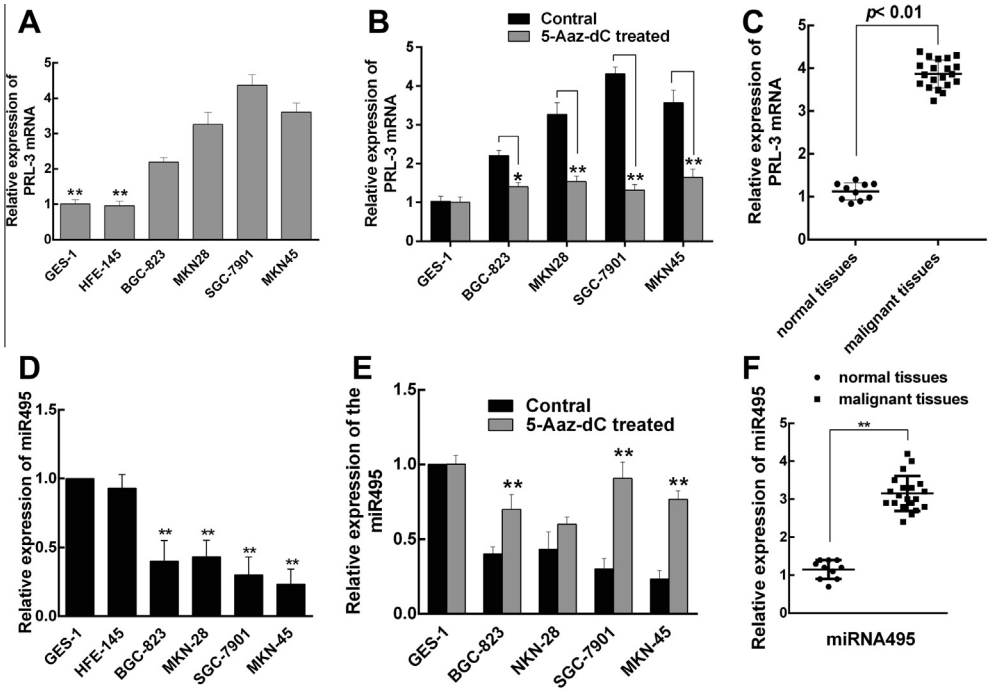

Fig. 2. Expression of the PRL-3 mRNA and miR-495 in GC samples and normal gastric samples and re-expressed by 5-Aza-dC treatment. (A) PRL-3 mRNA was up-regulated in GC cell lines ($^{**}P < 0.01$). (B) Treatment with 5-Aza-dC the PRL-3 mRNA was significantly decreased compared with untreated cells ($^{*}P < 0.05$, $^{**}P < 0.01$). (C) PRL-3 mRNA was overexpressed in GC tissues compared with the normal gastric samples ($^{**}P < 0.01$). (D) MiR-495 was significantly downregulated in the GC cell lines ($^{**}P < 0.01$). (E) MiR-495 was significantly increased in BGC-823, SGC-7901 and MKN-45 by treated with 5-Aza-dC compared with untreated cells ($^{**}P < 0.01$). (F) MiR-495 was significantly downregulated in normal gastric tissues ($P < 0.01$). Results are presented as fold differences based on $2^{-\Delta\Delta Ct}$ calculations, obtained from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, error bars represent the standard deviation.

(Bio-Rad, USA). After that the membranes were blocked with 5% skim milk in Tris-buffered saline for 1 h and incubated with anti-PRL-3 antibody (Sigma, USA) at 4 °C overnight. Then washed the membranes with TBST (TBS with 0.1% Tween20), the membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit at room temperature. After washing, we used the ECL detection kit (Thermol Biotech Inc, USA) to detect the immunoreactive protein bands.

2.4. Real-time quantitative PCR (RT-PCR) analysis of mRNA and miRNA levels

Trizol reagent (Invitrogen, USA) was used to extract the total RNA, and converted into cDNA by using an Expand Reverse Transcriptase Kit (Takara, China). The PCR primers was just the same as previously described [22]. Real-time quantitative RT-PCR was performed using ABI Prism 7700 Sequence Detector (Applied Biosystems, USA) with the SYBR Green PCR Master Mix (Applied Biosystems, USA) to detect mRNAs, β-actin mRNA was used as an endogenous control. Expression of the mature miRNAs was analyzed using 7900HT Fast Real-Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems, USA). The relative expressions of miRNAs were normalized by U6 SnRNA. The data was analyzed using comparative delta Ct method.

2.5. Methylation specific PCR (MSP) and sodium bisulfite sequencing method (BSP)

Normal human peripheral lymphocyte DNA treated with Sss I methyltransferase (New England Biolabs, USA) was used as methylated positive control. The DNA was extracted using AxyPrep™ Multisource Genomic DNA Miniprp Kit (Axygen, China). Genomic DNA was treated with EZ DNA Methylation-Gold™ Kit (Zymo research, USA), and analyzed by MSP, collected the BSP product for sequencing. PCR amplification was performed with HotStar Taq Polymerase (Qiagen, Germany) and consisted of initial incubation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 60 s, 60 °C for 30 s and 72 °C for 60 s, followed by one cycle of 72 °C for 10 min. PCR products were electrophoresed in 3% agarose gels (containing ethidium bromide), and then visualized by ultraviolet (UV) illumination. The primer sequences were designed by the MethPrimer (http://www.urogene.org/methprimer/), and were listed in Supplementary Table 1.

2.6. Cell migration and invasion assays

The cells treated with 5-Aza-dC were seeded on upper chamber of an 8 μm Matrigel-coated membrane matrix (Corning, USA), and 600 μl of 20% FBS–DMEM was added to the lower chamber. The cells were cultured for 24 h and non-migrating or non-invading cells were removed. Invasive cells located on the lower surface of the chamber were stained with 0.1% crystal violet at 37 °C for 30 min and then washed redundant crystal violet with PBS. Cells were then soaked in 33% ice-cold acetic acid and oscillated for 10 min, the ice-cold acetic acid was assessed by measuring absorbance at 570 nm using a microplate reader (Tecan, Shanghai, China) [23].

2.7. Statistical analysis

Each experiment was repeated at least three times. The results were expressed as means ± SDs. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., USA). The Student's *t*-test, one-way ANOVA, two-way ANOVA and χ^2 test was used to generate *P* values. Statistical significance was accepted at $P < 0.05$.

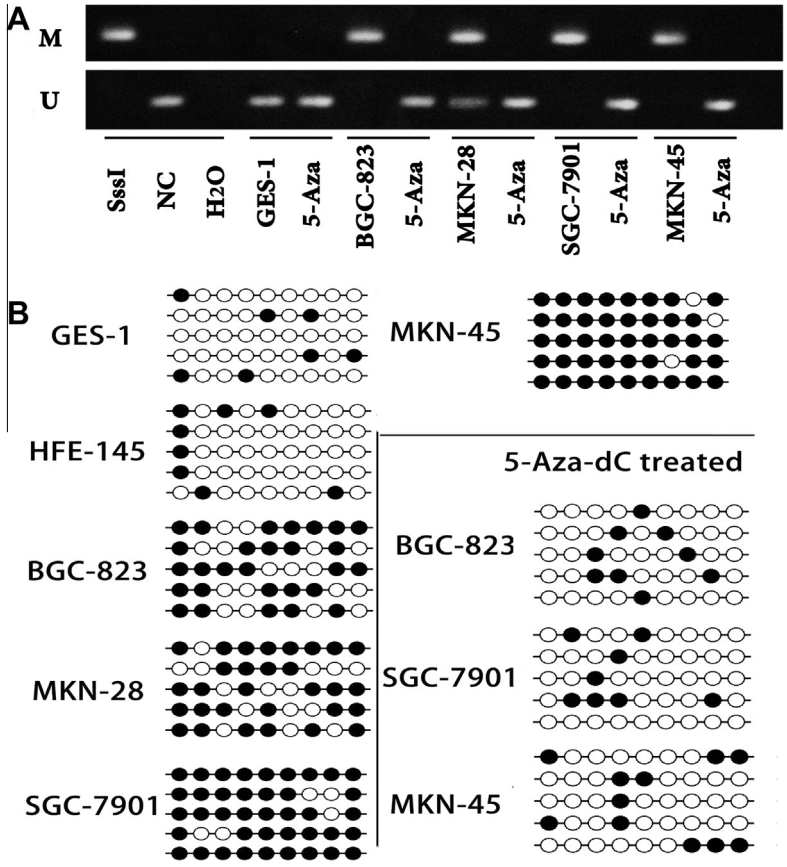

Fig. 3. Analysis of the methylation status of the miR495 gene promoter in GC cell lines and it's correlation with miR-495 expression. (A) The hypermethylation of the miR-495 gene promoter in GC, and can be significantly reversed by treated with 5-Aza-dC. (B) BSP analysis of miR-495 in GC cell lines, with or without 5-Aza-dC treatment. Open and filled circles represent unmethylated and methylated CpG sites, respectively. The black line between circles represent the base pairs between each CpG sites, each horizontal row represents a single clone. There are 9 CpG sites.

3. Results

3.1. The significantly high expression of the PRL-3 and down-regulated of miR-495 in GC

To detect the expression of PRL-3 and miR-495 in GC, we analyzed ten normal gastric mucosa tissues and twenty malignant tissues, two normal gastric cell lines, four different differentiation degrees of GC cells by qRT-PCR and Western blot. The result shown that compared with the normal gastric tissues, PRL-3 protein was overexpressed in GC cell lines (Fig. 1A). The similar result appeared in the gastric tissues (Fig. 1B). In addition, the PRL-3 mRNA was significantly up-regulated in BGC823 cells, MKN28 cells, SGC-7901 cells MKN45 cells compared to GES-1 cells (2.17-fold, 3.23-fold, 4.33-fold and 3.57-fold, respectively, $P < 0.01$, Fig. 2A). And in GC tissues the expression of the PRL-3 mRNA was 3.4-fold compared to the normal gastric mucosa tissues ($P < 0.01$, Fig. 2C). Meanwhile, the PRL-3 expression between GES-1 and HFE-145 cell lines has no statistical significance.

In addition, the expression of miR-495 was decreased to 2.50-fold in BGC823 cells, 2.31-fold in MKN28 cells, 3.33-fold in SGC7901 cells and 4.29-fold in MKN45 cells compared to GES-1 cells, respectively ($P < 0.01$, Fig. 2D). And the result shown that the miR-495 in malignant tissues were significantly down-regulated of 2.74-fold in comparison with normal gastric mucosa tissues ($P < 0.01$, Fig. 2F). The results are consistent with our previous study.

3.2. Hypermethylation of the miR-495 gene promoter suppressed miR-495 expression and resulted in the high expression of PRL-3

To verify the interaction between miR-495 and DNA methylation, we assessed the methylation level of the miR-495 gene promoter by MSP. The methylated DNA molecules were measured by BSP. And the result showed that the methylation proportion of miR-495 was significantly higher in GC tissues than those in normal gastric tissues (15/20 *vs* 2/10, $P < 0.05$). And the methylation levels of miR-495 were much higher in GC cell lines than human gastric mucosa cell lines (Fig. 3). The high methylation status was negatively correlated with miR-495 gene expression. Therefore, just as the result shown, the hypermethylation of the miR-495 gene promoter may be responsible for the high expression of PRL-3.

3.3. Re-expression of the miR-495 by 5-Aza-dC treatment suppressed the expression of PRL-3

To further assess whether miR-495 gene was functionally methylated in GC cells, we treated the GC cell lines with a demethylating agent, 5-Aza-dC. The expression of miR-495 in GC cells was significantly restored by 5-Aza-dC, and the expression of the miR-495 was markedly up-regulated except for MKN-28 (Fig. 2E, $P < 0.01$). And then detect the methylation levels, as shown in the result, after treated with 5-Aza-dC, the methylation level of the miR-495 gene promoter was significantly down-regulated compared with untreated (Fig. 3). Furthermore, we detected the

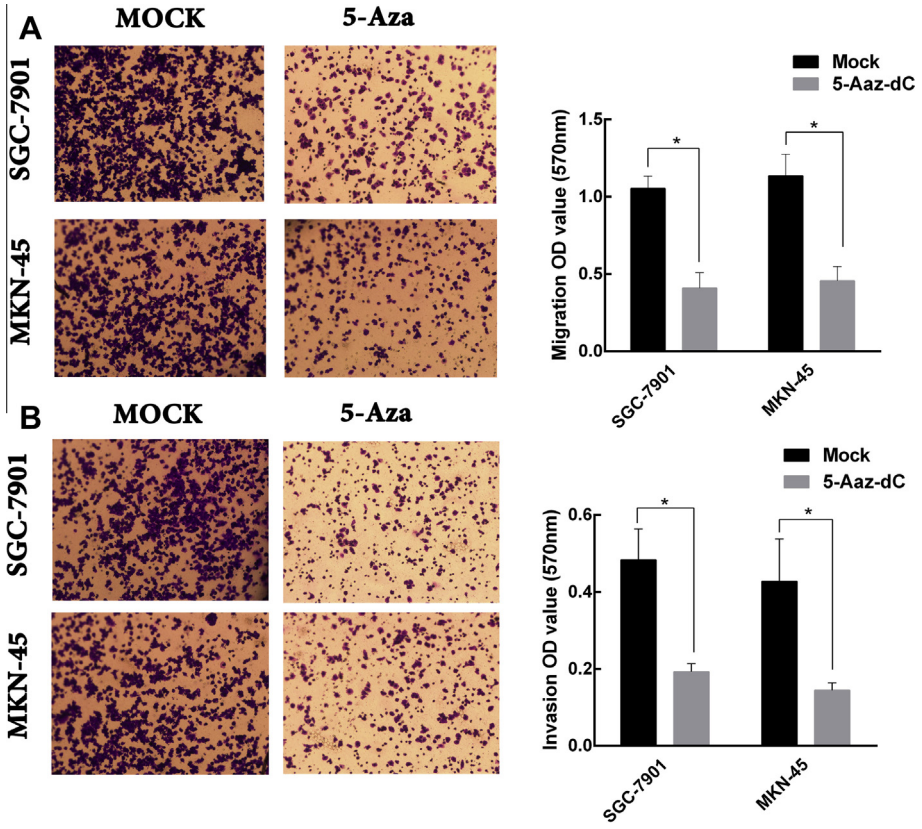

Fig. 4. Treatment with 5-Aza-dC resulted the up-regulation of the miR-495, inhibited the mobility of the GC cells. After treated with 5-Aza-dC, the GC cells were subjected to transwell assays. Representative photographs of migrating and invading cells. Average OD values for migrating and invading cells (±SD) from three independent experiments are shown above.

expression levels of PRL-3 in order to reveal the relationship between DNA methylation with the expression of PRL-3. Fig. 1D and Fig. 2B exhibited that PRL-3 protein and mRNA was dramatically decreased compared with untreated cells ($P < 0.05$). All of these results demonstrated that the DNA hypermethylation suppressed miR-495 expression and resulted in the high expression of PRL-3, and could be significantly reversed by 5-Aza-dC.

3.4. Treatment with 5-Aza-dC suppressed gastric cancer invasion and metastasis

Since our previous studies indicate the important roles of PRL-3 in the invasion and metastasis of GC [21]. We further found that miR-495 inhibits the migration and invasion of human GC cells by directly interacting with PRL-3 [22]. We hypothesized that the change of the miRNA495 promoter methylation level lead to the change of GC cells migration and invasion. In transwell migration assay, GC cells treated with 5-Aza-dC presented a significantly decrease migration capacity than those untreated cells (Fig. 4A). And the invasiveness of the GC cells was obviously reduced in matrigel invasion assay (Fig. 4B). These results indicated that the DNA hypermethylation of the miR-495 negative correlation with the migration and invasion of GC cells.

4. Discussion

The present study showed that methylation level of the miR-495 gene was significantly higher in GC tissues and cells. Corresponds to these, the expression level of miR-495 was significantly down-regulated in GC. Moreover, just as we confirmed before, the down-regulation of miR-495 result in the low-expression of PRL-3.By treating with 5-Aza-dC in GC cells, the methylation levels of the miR-495 gene was decreased significantly, and the expression of miR-495 was increased, which resulted in significant down-regulation of the PRL-3 mRNA and protein levels, and the decrease in cell migration and invasion. The result of this study demonstrated that the DNA hypermethylation of the miR-495 gene was a major cause of the down-expression of endogenous miR-495, which results in PRL-3 overexpression in GC peritoneal metastasis.

To date, a number of studies have shown that miRNAs can function as oncogenes or tumor suppressors, and play critical roles in tumor development and progression [30]. In GC, some miRNAs have been proved associated with invasion and metastasis [19,20,31]. MiRNAs directly degrade target mRNAs or inhibit gene translation to regulate the expression of protein-coding genes [32]. Nonetheless, the mechanism of which miRNAs promote or suppress tumor growth has not fully revealed. Meanwhile, some studies have demonstrated that DNA methylation in promoter regions of these miRNAs genes is a possible mechanism responsible for it [33]. Furthermore, the promoter region of DNA methylation represses transcription of the downstream genes [34]. Additionally, in our previous study, we found that miR-495 can inhibit GC cell migration and invasion, and act as tumor suppressors by targeting the PRL-3 oncogene. However, the molecular regulation mechanisms by which endogenous miR-495 expression is down-regulated and PRL-3 promotes GC peritoneal metastasis remain to be clearly elucidated.

To further explore the correlation between PRL-3 expression and methylation status of the miR-495 gene promoter, MSP and BSP amplification were performed to detect the promoter methylation status of the miR-495 gene. 5-Aza-dC, a methylation inhibitor, belongs to a class of cytosine analogues. 5-Aza-dC

sequesters DNMT enzyme activity by irreversibly binding DNMT [35]. And it has therapeutic value for the treatment of cancer. Recently, many studies have confirmed that the miRNA expression can be restored by 5-Aza-dC treatment [36,37], and treatment with 5-Aza-dC decreases cell proliferation and induces apoptosis in cancers [38]. In present study, we treated the cells with 5-Aza-dC to change the miR-495 promoter methylation status. The results of the MSP and BSP showed that high methylation status was negatively correlated with miR-495 gene expression. The hypermethylation of the CpG island of the miR-495 gene promoter region suppress the expression of miR-495. And by treating with 5-Aza-dC, the hypermethylation of the miR-495 gene promoter were significantly reversed. And we further investigate the methylation status of the miR-495 gene promoter impact on PRL-3 expression. The results indicated that the hypermethylation of the miR-495 gene promoter lead to the high expression of PRL-3. Additionally, the ability of invasion and metastasis of the GC cells was detected by transwell chamber assay, meanwhile, the inhibitory effect of 5-Aza-dC on the invasion and metastasis of GC cells was detected in the same way. The invasiveness of cells treated with 5-Aza-dC was dramatically decreased compared with the untreated cells.

In summary, our study shows the highly overexpress of the PRL-3 in primary GC with peritoneal metastasis was due to the hypermethylation of the miR-495 gene promoter. And treatment with a DNMT1 inhibitor can significantly reverse the methylation status of the miR-495 gene promoter. As a result, the decrease of methylation status of miR-495 will rescue the expression of PRL-3 and inhibit the invasion and metastasis of GC cells. This study extends our knowledge about the mechanism of the GC metastasis, and will provide new strategies for prevention and treatment of GC peritoneal metastasis. Further study is needed to fully elucidate the mechanism of the GC peritoneal metastasis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.083.

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