miR-186 Regulates the Initiation and Development of Breast Cancer via SHP-1 Methylation

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Abstract

**Background** Change in the methylation status of genomic DNA, especially in CpG islands in the promoter region, is considered to be an early event in tumor initiation, leading to silencing of gene expression, subsequent abnormalities in gene structure and function, and malignant transformation of the cell. Due to the abnormal expression of miR-186 and SHP-1 in breast cancer tissues and cells, we propose that miR-186 is closely related to the methylation of SHP-1

**Method** Using 5-azacytidine as a de-methylation agent and Validating with Setylation-specific polymerase chain reaction (MSP) after treatment. Measurement of the viability of breast cancer cells using the CCK-8 method Measurement of the apoptotic rate of breast cancer cells using annexin V-FITC/PI double labeling. Cell metastasis were measured by wound healing assay. Luciferase reporter assays was used to confirm the target of MiR-186. SHP-1 and miR-186 expression was measured by RT-PCR and western blot.

**Results** In the present study, we found that SHP-1 expression was reduced to various degrees in all 5 cell lines (UACC-812, MDA-MB-213, MDA-MB-468, SK-RB-3 and MCF-7). 5-azacytidine can remove the methylation from the SHP-1 promoter region. Apoptosis was observed in MCF-7 cells after demethylation of the SHP-1 gene promoter region by 5-azacytidine, and the effect was time- and concentration-dependent. Luciferase reporter assays showed that miR-186 promotes methylation through binding with the 3’ UTR of the SHP-1 promoter region. Western blot showed miR-186 regulates the initiation and development of tumor cells through the SHP-1-JAK-STAT axis. In animal models, low expression of miR-186 can cause significantly limited tumor growth.

**Conclusion** The low SHP-1 expression may be an important factor in the initiation of breast cancer, and that miR-186 could serve as an excellent molecular diagnostic marker and a possible therapeutic target.

**Background**

Breast cancer is a substantial threat to women's health. Clear genotyping is a major characteristic of the pathological classification of breast cancer that distinguishes it from other tumors, and there are marked differences in prognosis based on genotype. Notably, more discoveries are being made through breast cancer genomics research. Changes in gene activity, especially abnormalities in the DNA methylation of tumor suppressor genes, play important roles in the initiation and development of breast cancer. Changes in methylation status\(^1\), including a global reduction in methylation in the genome and an abnormal localized increase in the methylation of CpG islands, are crucial factors in carcinogenesis and lead to genome instability, such as the activation of mobile genetic elements, the instability of chromosomes, and the expression of proto-oncogenes\(^2\).

SHP-1 is a recently discovered tumor suppressor gene encoding a protein tyrosine kinase that is mainly expressed in hematopoietic cells and, therefore, is considered a hematopoietic cell phosphatase (HCP). It is located on chromosome 12p13 and is a key regulator of cellular phosphorylation levels. The SHP-1
phosphatase participates in the adjustment of phosphorylation levels in cells and functions as a negative regulator in cell signal transduction. The SHP-1 protein dephosphorylates proteins, regulates the tyrosine phosphorylation level of cell signaling proteins, and inhibits the proliferation, differentiation and viability of cells.

MicroRNAs (miRNAs) are short (20–24 nt) noncoding RNAs that participate in the posttranscriptional regulation of gene expression in multicellular organisms through their effects on the stability and translation of mRNAs and play important regulatory roles in the growth, development and apoptosis of cells. The gene encoding microRNA 186 (miR-186) is located at region 3, band 1 on the short arm of chromosome 1 and encodes a noncoding RNA molecule that is approximately 22 nucleotides in length. We detected elevated miR-186 expression in breast cancer tissues, and elevated miR-186 expression was also reported in esophageal squamous cell carcinoma and pancreatic cancer. This type of abnormal miRNA expression is frequently concurrent with the progression and malignant transformation of tumors. There is abundant evidence indicating that cancer-related miRNAs can serve as reliable diagnostic markers as well as potential therapeutic targets. It has been noted in the literature that miRNAs can inhibit the degradation or translation of target mRNA by recognizing and binding to complimentary sites on the 3'-untranslated region (3'-UTR). Therefore, we speculate that miR-186 may regulate the initiation and development of breast cancer cells through its effects on SHP-1 methylation.

5-Azacytidine is a cell cycle-specific agent with anti-metabolic and anti-tumor effects. Due to its ability to associate with DNA and RNA, it interferes with ribonucleic acid metabolism and subsequently causes a decrease in protein synthesis. 5-Azacytidine is also a DNA methyltransferase inhibitor and an epigenetic modifier that activates multiple tumor suppressor genes by inhibiting DNA methylation of the promoters of these genes and induces tumor cell apoptosis. We utilized this function of 5-azacytidine to explore the relationship between miR-186 and SHP-1.

Although the inhibitory effects of SHP-1 on tumors have been confirmed, the effects of SHP-1 on breast cancer cells remain unclear, and the regulatory mechanisms of its expression have not been completely elucidated. This study used the breast cancer cell line MCF-7 to assess the methylation status of the SHP-1 gene in breast cancer; performed demethylating treatment on MCF-7 cells and observed the effects of the SHP-1 gene in tumor cells on the gene and protein expression of SHP-1 in MCF-7 cells, as well as on cell proliferation and apoptosis; discussed the causal factors of SHP-1 gene methylation and the association of SHP-1 with miR-186 and the roles of these 2 factors in the initiation and development of breast cancer; and provided epigenetic information for breast cancer diagnosis.

**Materials And Methods**

1. **Culturing of human breast cancer cells**

The UACC-812, MDA-MB-231, MDA-MB-468, SK-RB-3, and MCF-7 breast cancer cell lines were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Medical Sciences (Shanghai,
China). The MCF-7 human breast cancer cell line was a donation from the Central Laboratory of the First Affiliated Hospital of Dalian Medical University.

2. Reagents

The EZ DNA Methylation-Direct™ kit was obtained from ZYMO Research (USA). CpG methyltransferase (M. Sssl) was obtained from Biolabs (USA). TaKaRa Taq™ HotStart Version, DNA marker DL500, RT-PCR kits, DNA marker DL2000, and primers for target genes and endogenous reference gene were obtained from TaKaRa Bio (Dalian, China). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Japan). 5-Azacytidine was obtained from Sigma-Aldrich (USA). Trizol reagent was obtained from GIBCO (USA).

3. PCR analysis specific for methylated and unmethylated DNA

3.1 Methylation modification and DNA isolation

(1) Cells ($2 \times 10^3$) were collected and treated as follows: [10 µl of M-digestion buffer + 9 µl of sample (2×10³ cells) + 1 µl of proteinase K + X µl of H₂O = 20 µl total volume]. (2) The samples were incubated at 50°C for 20 min. (3) Twenty microliters of each sample was added to 130 µl of CT conversion reagent solution in a PCR tube; the samples were mixed and centrifuged to ensure that no liquid was on the top or side of the tube. (4) The PCR tubes were placed in a thermal cycler and incubated under the following conditions: j 98°C for 8 min; k 64°C for 3.5 h; and l 4°C (no more than 20 h). (5) M-Binding Buffer (600 µl) was added to each Zymo-Spin™ IC column in a collection tube. (6) Each sample (step 2) was transferred into a Zymo-Spin™ IC column containing M-Binding Buffer and mixed by inverting the column several times with the lid closed. (7) M-Wash Buffer (100 µl) was added to each column, and the sample was centrifuged at maximum speed for 30 s. (8) M-Desulphonation Buffer (200 µl) was added to each column, and the column was allowed to sit at room temperature (20-30°C) for 15-20 min. After incubation, the column was centrifuged at maximum speed for 30 s. (9) M-Wash Buffer (200 µl) was added to each column, and the column was centrifuged at maximum speed for 30 s. M-Wash Buffer (200 µl) was added to each column again, and the column was centrifuged at maximum speed for 30 s. (10) Each column was placed in a 1.5 ml centrifuge tube, and M-Elution Buffer (10 µl) was added directly to the column matrix. The column was centrifuged at maximum speed for 30 s to elute DNA. (11) One microliter of sample was removed, and the optical density 260 (OD₂₆₀) and OD₂₈₀ values were determined using a spectrometer. The purity of the DNA was assessed based on the OD₂₆₀/OD₂₈₀ ratio, and samples with ratios of 1.8-2.0 were considered to be of acceptable quality and were used immediately or stored at -20°C for future use.

4. PCR amplification

The following were the SHP-1 gene primers specific for methylated DNA: forward, 5′–GAA CGT TAT TAT AGT ATA GCG TTC–3′ (nt: 6857-6880); reverse, 5′–TCA CGC ATA CGA ACC CAA ACG–3′ (nt: 7015-6995); the length of the amplification product was 159 bp. The following were the SHP-1 gene primers specific
for unmethylated DNA: forward, 5’–GTG AAT GTT ATT ATA GTA TAG TGT TTG G–3’ (nt: 6855-6882); reverse, 5’–TTC ACA CAT ACA AAC CCA AAC AAT–3’ (nt: 7016-6993); the length of the amplification product was 159 bp. The reaction conditions were as follows: initial denaturation, 95°C for 5 min; denaturation (40 cycles), 94°C for 30 s, 55°C for 40 s, and 72°C for 1 min; and annealing, 72°C for 5 min. The products were analyzed via 20 g/L agarose gel electrophoresis, and the results were visualized with an ultraviolet light gel imaging system.

5. Preparation and storage of 5-azacytidine

The instructions for preparation were as follows: 1 mg of 5-azacytidine was dissolved in 1 ml of 50% acetic acid solution (acetic acid:water, 1:1) for a stock solution of 4100 µmol/L; the stock solution was diluted to the desired concentration for immediate use or stored at -20°C for future use.

6. Demethylation treatment

MCF-7 cells were cultured in high-glucose DMEM containing 100 mL/L fetal bovine serum. The cells were cultured at an initial density of 5×10^4/mL in tissue culture flasks, and 4-6 h later, the demethylating agent 5-azacytidine was added to the medium, with a final concentration of 5 µmol/L. Fresh medium and demethylating agent were replaced every 24 h. Cells cultured in the absence of the demethylating agent were used as a control.

7. Methylation-specific polymerase chain reaction (MSP) after treatment

Cells were cultured in medium containing 5 µmol/L 5-azacytidine (demethylating agent) as described above, and fresh medium and demethylating agent was provided every 24 h. After 3 d of culture, methylation modification and DNA isolation were performed as described above, and PCR was carried out as previously described.

8. Analysis of the mRNA expression of SHP-1 in MCF-7 breast cancer cells via RT-PCR

Total RNA from MCF-7 cells was isolated using Trizol, and cDNA synthesis was performed using random primers and MMLV reverse transcriptase according to the product manual. The primers for SHP-1 were as follows: forward, GACTGTGACATTGACATCCAG; reverse, CTTCCTCTTGAGGGAACCCTT; the product length was 350 bp. The primers for β-actin were as follows: forward, 5’-CACTGTGTGGCGTGACAGAGT-3’; reverse, 5’-TCATCACCATTGGACAGAG-3’; the product length was 154 bp. The amplification conditions were as follows: initial denaturation, 95°C for 5 min; 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min; and extension, 72°C for 10 min. β-actin was used as the endogenous reference gene. The products were analyzed via 20 g/L agarose gel electrophoresis, and the results were visualized using an ultraviolet light gel imaging system.

9. Measurement of the viability of breast cancer cells using the CCK-8 method
The stock solution of 5-azacytidine was diluted to 0.1, 1, 5, 10 and 20 μmol/L. Plates (96-well) were labeled as blank (equal volume of medium without cells), control and experimental, and each group contained 6 replicates. MCF-7 breast cancer cells in the log growth phase were collected, digested and seeded in 96-well plates at 1 × 10⁴ cells per well in 200 μl of medium, with 5 plates total. MCF-7 cell suspension (50 μl) was seeded into each well of the plates for the control and experimental groups; the plates were shaken gently, placed in a 37°C incubator with 5% CO₂ and incubated for 4h. Then, the medium was removed, and 200 μl of medium containing 0.1, 1, 5, 10, or 20 μmol/L 5-azacytidine was added to each experimental well; 200 μl of medium was added to each blank and control well. After shaking the plates gently, they were placed in a 37°C incubator with 5% CO₂, and fresh medium with the same concentration of 5-azacytidine was provided every 24 h. One plate was removed every 24 h, and 10 μl of CCK-8 solution was added into each well. After an additional incubation of 2.5 h in the CO₂ incubator, the plate was placed in an enzyme-linked immunosorbent assay (ELISA) analyzer, and the A values (OD) at 450 nm were measured. Cell viability was calculated as follows: viability (%) = (experimental A value – blank A value)/(negative control A value – blank A value) × 100%. Each experiment was repeated 3 times.

10. Measurement of the apoptotic rate of breast cancer cells using annexin V-FITC/PI double labeling

Annexin V-FITC is a calcium-dependent phospholipid-binding protein conjugated with fluorescein. It has a very strong affinity for phosphatidylinerine, to which it specifically binds while the cell maintains cell membrane integrity. Therefore, it was used as a more sensitive tool to detect early apoptotic cells and calculate the percentage of apoptotic cells. MCF-7 breast cancer cells in the log growth phase were digested with 2.5 g/L trypsin for passage, and the medium was replaced with high-glucose DMEM containing 5-azacytidine at 1, 5, or 10 μmol/L after 24 h. Cells were provided with fresh medium containing 5-azacytidine (at the same concentrations) every 24 h, and after 3 d of treatment, the cells were cultured in fresh medium without 5-azacytidine for 24 h before measurement. Cells incubated in the same volume of complete media without 5-azacytidine were used as controls. MCF-7 cells treated with 5 μmol/L 5-azacytidine for 1, 3 or 5 d were analyzed in the same manner, with cells incubated in the same volume of complete medium without 5-azacytidine as controls. Each experiment was repeated 3 times.

11. Statistical analysis

The data were analyzed using SPSS 11.0 software, and all measurement data are presented as the mean±standard deviation (±s). One-way ANOVA was performed, and the least significant difference (LSD) test was used for pairwise comparisons. P<0.05 was considered to be statistically significant.

Results

1. The expression of SHP-1 in breast cancer cells is suppressed

To select the cell line with the most pronounced change in gene expression, we assessed SHP-1 gene expression in 5 human breast cancer cell lines (UACC-812, MDA-MB-213, MDA-MB-468, SK-RB-3 and MCF-7; Figure 1 A) and found that SHP-1 expression was reduced to various degrees in all 5 cell lines. MCF-7
cells exhibited the most pronounced reduction, which was significantly different from SHP-1 expression in UACC-812 cells (Figure 1 B, p < 0.0001). To further explore the mechanism of action of SHP-1 in the initiation and development of breast cancer, we selected MCF-7 cells for subsequent experiments.

To investigate whether SHP-1 expression is only suppressed in tumor tissues, we selected 4 pairs of tumor tissues and the corresponding adjacent tissues from triple-negative breast cancer patients and analyzed SHP-1 protein expression using Western blot after protein isolation from tissues. SHP-1 expression was significantly reduced in tumor tissues compared with normal tissues in all 4 patients, indicating that the expression of this gene was reduced on the protein level in breast cancer tissues (Figure 1 C). Quantification of the Western blot results confirmed that the expression of SHP-1 was suppressed in tumor tissues compared with adjacent tissues; the difference was statistically significant (p < 0.0, Figure 1 D).

2. Removal of SHP-1 methylation could inhibit tumor growth

The abnormal expression of SHP-1 in breast cancer tissues is possibly related to the hypermethylation of its promoter region. We performed methylation-specific PCR (MSP) on the SHP-1 gene using primers specific for methylated and unmethylated DNA. The MSP amplification products were electrophoresed on a 2% agarose gel, and a distinct positive band was visible in the M lane but not the U lane. Moreover, the band was consistent in size with the band of the product from the methylation positive control reaction (normal human peripheral blood DNA modified by methyltransferase SssI, lane M3), indicating that the experimental techniques, primers and reagents were correct and that the results were reliable. The MSP assay verified the methylation of the SHP-1 promoter region in MCF-7 human breast cancer cells (Figure 1 A-1).

To restore SHP-1 gene expression, we used 5-azacytidine to remove the methylation from the SHP-1 promoter region and examined the apoptosis status of the cancer cells. First, the demethylating effect of 5-azacytidine was verified on the RNA level. Using MCF-7 cells treated with 5 μmol/L 5-azacytidine for 48 h, MSP confirmed that the promoter region of the SHP-1 gene was methylated in MCF-7 cells that did not undergo demethylation treatment, showing a positive band in the MSP lane but not in the UN-MSP lane; analysis of lane U after treatment revealed demethylation, with no band in the MSP lane and a positive band in the UN-MSP lane (Figure 1 A-2). These results confirmed the demethylating function of 5-azacytidine. Subsequently, the mRNA expression of SHP-1 in 5-azacytidine-treated MCF-7 cells was measured. The mRNA expression of SHP-1 significantly increased after treatment with 5 μmol/L and 10 μmol/L 5-azacytidine, and the expression level increased with increasing doses of 5-azacytidine (Figure 2 B-1). Quantification of the results revealed statistically significant differences (p < 0.05, Figure 2 B-2).

After the demethylating effects of 5-azacytidine on the promoter region of the SHP-1 gene was confirmed, the dose- and time-dependent inhibitory effects of 5-azacytidine on the proliferation of MCF-7 breast cancer cells was further explored. Cells in the experimental group were cultured in media containing various concentrations of 5-azacytidine, and the absorbance at 450 nm was measured after 1, 3 and 5 d of treatment. The results indicated that cell growth was inhibited at all the concentrations of 5-
azacytidine tested and that the inhibitory effect was time- and concentration-dependent. At similar concentrations of 5-azacytidine, the inhibitory effect on MCF-7 cells increased with the increasing treatment duration (p < 0.05). With similar treatment durations, there were significant differences in the rate of inhibition among cells treated with different concentrations of the drug (p < 0.05), indicating that the inhibitory effect of 5-azacytidine on cell proliferation increased with increasing concentrations of the drug (Figure 2 C). Measurements of absorbance at 450 nm after 1, 3 and 5 d of 5 μmol/L 5-azacytidine treatment showed that the absorbance value was negatively correlated with MCF-7 cell growth. This experiment was repeated 3 times, and the results indicated that the proliferation of MCF-7 cells treated with 5-azacytidine was significantly reduced compared with that of MCF-7 cells not treated with 5-azacytidine (Figure 2 C), and pairwise comparisons revealed statistically significant differences in the effect of 5-azacytidine among cells treated for 1, 3 and 5 d (p < 0.05; Table 1).

Apoptosis was observed in MCF-7 cells after demethylation of the SHP-1 gene promoter region by 5-azacytidine, and the effect was time- and concentration-dependent. The apoptotic rates of cells treated with 1, 5 and 10 μmol/L 5-azacytidine for 24 h were 10.1%, 16.63% and 23.97%, respectively, showing significant differences (p<0.05). The apoptotic rates of MCF-7 cells treated with 5 μmol/L 5-azacytidine for 1, 3 and 5 d were 11.77%, 17.73% and 25.95%, respectively (Figure 2 D, Table 2).

3. miR-186 promotes methylation through binding with the 3’ UTR of the SHP-1 promoter region

The hypermethylation of the SHP-1 promoter region in breast cancer cells appears to be a key causal factor of decreased gene expression and the initiation and development of tumors. What is the cause of the decreased expression? There are indications in the literature that miR-186 is closely associated with SHP-1. miR-186 is a microRNA that can target SHP-1 and destabilize the target mRNA or inhibit its translation. First, we used RT-PCR to assess the difference in miR-186 expression in breast cancer tissues and adjacent normal tissues (Figure 3 A, p < 0.05), and the results suggested that the elevated expression of miR-186 is closely associated with tumor initiation. Subsequently, to confirm the interaction between miR-186 and the SHP-1 gene, we designed small interfering RNA (simiR-186) to knock down miR-186 and 2 breast cancer cell lines harboring SHP-1 mutations (Figure 3 C). First, 2 types of control miR-ctrl and simiR-186 were transfected into MCF-7 breast cancer cells after serum starvation, and Western blot analysis was conducted to confirm the function of simiR-186 in the 3 groups of cells (Figure 3 B). Then, the interaction between miRNA-186 and SHP-1 was explored through a luciferase reporter assay (Figure 3 D). The 3 cell lines, wildtype, SHP-1 mut-1 and SHP-1 mut-2, were each divided into 2 groups that were transfected with miR-ctrl and miR-186, respectively, and the changes in SHP-1 gene activity was assessed by measuring the correlating luciferase activity. We found that the binding of miR-186 to the SHP-1 promoter region is key to SHP-1 gene methylation (p < 0.05).

4. miR-186 regulates the initiation and development of tumor cells through the SHP-1-JAK-STAT axis

As a tumor suppressor gene, the anti-tumor effects of SHP-1 are weakened by the methylation of its promoter, and this process is closely related with miR-186. Therefore, to further investigate the effects of miR-186 on breast cancer cells, we knocked down miR-186 using simiR-186 and assessed the effects on
the viability of tumor cells through a phenotypic analysis. Figure 4 A-1, 2 shows the results of a wound healing assay. After 24 h, the wound areas in the simiR-186 group were significantly larger (p < 0.05). To further investigate whether a similar reduction in migration ability would also occur across a membrane, we employed a transwell cell assay (Figure 4 B-1, 2). The number of cells in the same-sized measurement area was lower in the simiR-186 group (p < 0.05), indicating that the migration ability of breast cancer cells decreased after miR-186 was knocked down.

Which cellular pathways or molecular targets are involved in this phenomenon? The JAK/STAT signal transduction pathway participates in the regulation of biological processes such as cell proliferation, differentiation, embryonic development, and immunity. Preliminary results (Figure 4 C) from this study showed that that SHP-1 gene is closely related to the JAK/STAT cellular signaling pathway and that the elevated expression of this gene inhibited the expression of downstream proteins of this pathway, including CREB-binding protein (CBP), CIS, interferon alpha (IFN-α), proviral integration site for Moloney murine leukemia virus-1 (Pim-1), phosphorylated Janus kinase (p-JAK) and phospho-signal transducer and activator of transcription 3 (p-STAT3), leading to a reduction in the ability of tumor cells to grow, migrate and invade. In addition to breast cancer, the methylation of the SHP-1 gene promoter occurs at a high frequency in B-cell lymphoma, and further study is necessary to elucidate whether this phenomenon is related to the deregulation of the JAK/STAT signal transduction pathway due to the downregulation of SHP-1 gene expression.

5. Explorative studies in animal models

To verify these results in animal models, we used normal MCF-7 breast cancer cells (control group) and MCF-7 breast cancer cells in which miR-186 was knocked down (treatment group) as tumor sources and injected the cells subcutaneously into the inguinal regions of nude mice (4-6-week-old nude mice, 1*10^7 cells/200 µl/mouse). The results in Figure 5 B reveal that the treatment group exhibited smaller tumor sizes, slower tumor growth, and longer latency before tumor formation. Subsequent Western blot and immunohistochemistry analyses of the expression of SHP-1 in tumor tissues (Figure A, C) confirmed that the change in tumor size stemmed from the relatively elevated expression of SHP-1.

Discussion

The most basic genetic model of tumor formation is that the overamplification of proto-oncogenes and the silencing of tumor suppressor genes result in the disruption of the balance of cell proliferation, inducing cell malignancy. Studies have found that inactivation or deletion of tumor suppressor genes will place tumor suppressor genes at a disadvantage in the battle against oncogenes, usually resulting in the overproliferation of cells and subsequent occurrence of malignant tumors. Epigenetic mechanisms are closely related to the initiation and development of tumors. Changes in the methylation status of genomic DNA, especially CpG islands in the promoter region, can lead to gene expression silencing, which results in abnormalities in gene structure and function and subsequent carcinogenic transformation of
cells. CpG islands are found in the 5' promoter regions of 60% of human genes. The methylation of these CpG islands regulate the configuration, structure and stability of DNA and alter normal gene expression.

At the end of the 20th century, several research groups, including the group at Washington University, isolated and purified the human SHP-1 gene in succession. The SHP-1 protein can specifically bind to phospho-tyrosine (Tyr-P) and catalyze its dephosphorylation. The SHP-1 gene has recently been recognized as a candidate tumor suppressor gene, and the SHP-1 protein exerts negative regulatory functions on cell proliferation in signal transduction networks. Studies by Koyama et al. on lymphoma and leukemia cell lines and patient samples revealed that the protein and mRNA levels of SHP-1 were decreased to various degrees and that the transfection of SHP-1 gene into leukemia cells led to the inhibition of cell proliferation activity. Yip et al. found variation in SHP-1 mRNA expression in their study of 18 breast cancer cell lines, and abnormal SHP-1 expression was later observed in other tumor cells. SHP-1 exhibited tumor-suppressing activity in the initiation of liver cancer, and this suppressive effect was also observed in medullary thyroid carcinoma. This experiment used MSP to verify the existence of abnormal SHP-1 gene promoter methylation in MCF-7 breast cancer cells; abnormal methylation may be one of the reasons why SHP-1 mRNA expression in breast cancer is abnormal.

Due to current advances in DNA methylation profiling studies and breakthroughs in the utilization of demethylating agents in leukemia treatment, increasingly more people have realized the necessity of DNA methylation research. As studies have progressed, the occurrence of hypermethylated regions and gene silencing were frequently observed during the initiation and development of multiple types of tumors. However, methylation inhibitors can change this phenomenon and restore gene expression.

Since we had previously uncovered SHP-1 gene hypermethylation in breast cancer through extensive experimental studies, we have further explored the endogenous cause of this phenomenon — the upstream factor miR-186 — and further confirmed the association between miRNA-186 and SHP-1 through a luciferase reporter assay. miR-186 binds to the 3'UTR of the SHP-1 promoter region, leading to the methylation of SHP-1 and the inhibition of the function of the tumor suppressor gene SHP-1, as well as subsequent tumor initiation and development. Although the elevated expression of miR-186 in breast cancer suggested its association with SHP-1, the expression of miR-186 was suppressed in other tumor types. Recent studies on miR-186 found that it regulates the growth, invasion, cell cycle, and apoptosis of tumor cells; it can also serve as a noninvasive marker for malignant tumors, and it is closely related to patient prognosis. miR-186 provides a new direction to explore tumor treatment.

In summary, miR-186 induces the abnormal methylation status of the SHP-1 gene, which is closely associated with tumor initiation and development, and provides new diagnosis and treatment methods for breast cancer. Currently, investigations into various new targets through increasingly more detailed studies on the mechanisms of action of gene methylation in tumor cells is of great importance.
Conclusion

In summary, in this study, we found that SHP-1 gene has reduced expression in breast cancer cells and breast cancer tissue, and that methylation of the gene is an important cause of low expression. In addition, we have found that miR-186 is highly expressed in breast cancer tissue and is the key to the methylation of SHP-1 gene. Once the methylation of the SHP-1 gene is removed, or the expression of miR-186 is inhibited, the growth and invasion of breast cancer cells is significantly inhibited, and animal models are validated. This process is associated with JAK/STAT pathway.

Declarations

Authors’ contributions

Xia Zhang and Weiguo Zhang conceived of the study. Yongjian Zheng and Lifei Tong contributed the materials used in this study. Kun Wang, Chunxia Zhou, Di Wang and Bin Zhang performed research and analyzed data. Di Wang wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Due to technical limitations, table 1,2 is only available as a download in the Supplemental Files section.
**Figures**

**Figure 1**

A. Western blot analysis of the protein expression status of SHP-1 in 5 human breast cancer cell lines. B. Quantification of the results in A. C. The Western blot results for the expression of SHP-1 and β-actin in 4 pairs of tumor tissues and adjacent tissues from patients. D. Histogram showing the expression level of SHP-1, p < 0.05.
Figure 2

A-1. Negative control. M: methylated product; U: unmethylated product; DNA marker DL500 (top-bottom: 500 bp, 400 bp, 300 bp, 200 bp, 150 bp, 100 bp, 50 bp). Data were derived from 3 independent experiments. *p < 0.05. MSP analysis of the methylation status of the SHP-1 gene promoter. 1, 2: the methylation status of MCF-7 cells; 3, 4: positive control; 5, 6: negative control. A-2. Changes in the methylation status of the SHP-1 gene in MCF-7 breast cancer cells after 5-azacytidine treatment: 1, 2: the
mecylation status of MCF-7 cells after treatment; 3, 4: the methylation status of MCF-7 cells without demethylation treatment; 5, 6: negative control; M: methylated product; U: unmethylated product. DNA marker DL500 (top–bottom: 500 bp, 400 bp, 300 bp, 200 bp, 150 bp, 100 bp, 50 bp). B-1. RT-PCR analysis of the mRNA expression of SHP-1 in MCF-7 cells. Unit: µmol/L. B-2. Histogram of the quantification of SHP-1 mRNA, p < 0.05. C. Curves demonstrating the relationship between the viability of MCF-7 breast cancer cells and the concentration of 5-azacytidine, as well as the treatment duration. D. Flow cytometry results depicting the apoptotic rate of MCF-7 breast cancer cells after treatment with various concentrations of 5-azacytidine for various durations. a-d: representative flow cytometry results for cells treated with 0, 1, 5 and 10 µmol/L 5-azacytidine, respectively, for 24 h. e-h: representative flow cytometry results for cells treated with 5 µmol/L 5-azacytidine for 0, 1, 3 and 5 d, respectively.

Figure 3

A. RT-PCR analysis indicates that the miRNA-186 level in breast cancer tissues is higher than that in adjacent tissues; the difference is statistically significant (p < 0.01). B. Western blot results indicate that the expression level of SHP-1 increases in cells transfected with simiR-186. C. The nucleotide sequences
of the SHP-1 gene, Has-miR-186 and the mutated SHP-1 gene in 2 mutant cell lines. D. After miR-ctrl and miR-186 transfection into wildtype, SHP-1 mut-1, and SHP-1 mut-2 cells, wildtype cells transfected with miR-186 show significantly lower viability (p < 0.05), while SHP-1 mut-1 and SHP-1 mut-2 cells do not exhibit any significant difference.

Figure 4

A-1. Wound healing assay demonstrating that the wound widths in the simiR-186 group are wider than those in the control group after 48 h. A-2. Histogram of the wound areas for the 2 groups, p < 0.05. B-1. Comparison of transwell assay results. B-2. Histogram of the number of cells in the measured area, p < 0.05. C. Both the upregulation of SHP-1 and downregulation of miR-186 result in the reduced expression of downstream proteins in the JAK/STAT pathway, including CBP, CIS, IFN-α, Pim-1, p-JAK and p-STAT3. D. Mechanism diagram of cellular signaling pathways.
Figure 5

A The protein expression of SHP-1 in two tumor tissues. B. The tumors are smaller in the treatment group after tumor formation. C. Immunohistochemistry results indicating that the expression of SHP-1 is higher in the treatment group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- table1.jpg
- table2.jpg