MicroRNA-148a Can Regulate Runt-Related Transcription Factor 3 Gene Expression via Modulation of DNA Methyltransferase 1 in Gastric Cancer

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Underexpression of the gene runt-related transcription factor 3 (RUNX3), an important tumor suppressor, is known to contribute to gastric cancer progression. However, the mechanism underlying aberrant *RUNX3* expression has not been fully elucidated. We investigated the role of miR-148a (miR-148a) and DNA methyltransferases (DNMTs) in *RUNX3* promoter methylation and gene expression. *RUNX3* mRNA, RUNX3 protein, and methylation levels were assayed in human gastric cancer tissues and matched normal tissues, and AGS and BGC-823 cells by real-time reverse transcription PCR, Western blot, and methylation-specific PCR, respectively. A correlation between *RUNX3* mRNA levels and that of miR-148a was also investigated in gastric cancer tissues. We found that *RUNX3* mRNA levels were significantly downregulated in gastric cancer tissues compared with their matched normal tissues, and were closely associated with miR-148a expression. After treatment of human gastric cancer AGS and BGC-823 cells with the DNA methylation inhibitor 5-aza-2'-deoxycytidine, a significant increase in *RUNX3* mRNA, RUNX3 protein, and the non-methylated form of the *RUNX3* promoter were observed relative to untreated cells. Enforced expression of miR-148a, which can modulate DNMT1 and DNMT3B, also increased the expression of RUNX3 in gastric cancer cells. Knockdown of DNMT1 was associated with increased levels of *RUNX3* mRNA and RUNX3 protein, while knockdown of DNMT3B did not have any effect on these in BGC-823 cells. Our results show that miR-148a may regulate *RUNX3* expression through modulation of DNMT1-dependent DNA methylation in gastric cancer and highlight a miRNA-epigenetics regulation mechanism of gene expression.

INTRODUCTION

Gastric cancer is the second most frequent cause of cancer death worldwide, with an estimated one million new cases per year (Hartgrink et al., 2009). The genesis and progression of human gastric cancer is thought to be crucially influenced by genetic and epigenetic alterations, including the activation of oncogenes and the inactivation of tumor suppressor genes (Wu et al., 2012). Runt-related transcription factor 3 (RUNX3), a member of the family of transcription factors that contain the runt domain, is located at human chromosome 1p36 and has been identified as a tumor suppressor in gastric cancer (Li et al., 2002) as well as breast (Huang et al., 2012), bladder (Kim et al., 2005), and lung (Araki et al., 2005) cancers. A loss or substantial decrease in RUNX3 expression may be causally related to gastric cancer, as it correlates with differentiation, lymph node metastasis, and poor prognosis of this disease (Hsu et al., 2009).

Methylation of CpG islands in gene promoters is an important regulatory mechanism for gene expression, and aberrant patterns of DNA methylation are important in carcinogenesis (Jones, 2012). Several cancer-associated genes, such as *adenomatous polyposis coli* (APC), *E-cadherin* (CDH1), cyclin-dependent kinase 4 inhibitor B (p15/INK4B), cyclin-dependent kinase inhibitor 2A (p16/INK4A), and RUNX3, may be inactivated in gastric carcinogenesis due to hypermethylation (Sato and Meltzer, 2006), and hypermethylation of the *RUNX3* promoter region in particular is frequently found in gastric cancer and other human cancers (Kim et al., 2004; Li et al., 2002). However, it is not clear what causes aberrant methylation of *RUNX3*.

The methylation of DNA is established and maintained by DNA (cytosine-5-)-methyltransferases (DNMTs). There are three enzymatically active mammalian DNMTs, namely DNMT 1 (DNMT1), DNMT 3 alpha (DNMT3A) and DNMT 3 beta (DNMT3B),

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Received December 3, 2012; revised February 5, 2013; accepted February 12, 2013; published online March 29, 2013

Keywords: DNA methyltransferase 1, gastric cancer, miR-148a, RUNX3

and one related regulatory protein, DNMT 3-like (DNMT3L). The latter lacks catalytic activity (Denis et al., 2011). The most abundant of these is DNMT1, which is primarily responsible for maintenance and regulation of DNA methylation patterns, while DNMT3A and DNMT3B establish *de novo* methylation patterns which are then maintained by DNMT1 (Jones and Baylin, 2002; Okano et al., 1999). Previous studies have shown that significant increased expression of DNMT1 protein positively correlates with the hypermethylation status of CpG islands of several genes in gastric cancers, including *p16*, *human MutL homologue 1 (hMLH1)*, *CDH1*, and *thrombospondin-1 (THBS1)* (Etoh et al., 2004).

MicroRNAs (miRNAs) are endogenous small noncoding RNA fragments that regulate gene expression and major cellular processes such as proliferation, differentiation, and apoptosis, with an important role in tumorigenesis (Lujambio and Lowe, 2012). There are recent reports that specific miRNAs participate in human cancers by regulating DNA methylation via DNMTs, resulting in the silence or promotion of methylation-sensitive tumor suppressor genes. For example, Fabbri et al. (2007) demonstrated that an increase in miR-29 expression in non-small-cell lung carcinoma cell lines inversely correlated with DNMT3A and DNMT3B levels. In addition, DNA methylation and methylation-silenced tumor suppressor gene expression (fragile histidine triad [*FHIT*] and WW domain-containing oxidoreductase [*WWOX*]) were restored to normal, and tumorigenicity was inhibited *in vitro* and *in vivo*. In our previous studies, we confirmed that miR-148a was significantly downregulated in gastric cancer (Zhu et al., 2012). Furthermore, it has been verified that DNMT1 is a target of miR-148a (Braconi et al., 2010; Pan et al., 2010). Therefore, we hypothesized that miR-148a could regulate *RUNX3* gene expression through modulation of DNMT1-dependent DNA methylation in gastric cancer.

Our overall objective in the present study was to search for the mechanism by which *RUNX3* expression is affected by miRNA-induced regulation of gene promoter methylation. First we investigated the associations of *RUNX3* expression and *RUNX3* CpG-methylation with the expression of miR-148a in gastric cancer tissues, relative to corresponding normal mucosae. Then, by transfecting *in vitro* a miR-148a overexpression plasmid or DNMT small interfering RNA (siRNA) plasmids into gastric cancer cells, we demonstrated the effect of miR-148a and DNMTs, respectively, on *RUNX3* expression.

MATERIALS AND METHODS

Cell lines

The human gastric cancer cell lines BGC-823 and AGS and the human gastric epithelium immortalized cell line GES-1 were obtained from the Shanghai Institute of Digestive Surgery (China). Cells were cultured separately in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen) in 75-cm³ flasks at 37°C in a 5% CO₂ incubator.

Tissue samples

Thirty-eight human gastric cancer samples and corresponding noncancerous tissues were obtained from gastric cancer patients who underwent gastrectomy between January 2009 and December 2009 at Affiliated Wuxi Second Hospital, Nanjing Medical University, Wuxi, China. None of these patients received radiotherapy or chemotherapy before surgery. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA, DNA, or protein could be extracted.

Each patient provided written informed consent prior to surgery. The Ethics Committee of Nanjing Medical University, China approved the study.

RNA isolation and real time reverse transcription PCR (qRT-PCR)

Total RNA from the gastric tissue specimens and cultured cells were isolated using Trizol Reagent (Invitrogen) in accordance with the manufacturer's protocol. To detect the gene expression of *RUNX3*, 100 ng of total RNA was reverse transcribed using PrimeScript RT Master Mix kit (Takara, Japan) and then analyzed by qRT-PCR on an ABI 7900HT Fast real-time PCR system (Applied Biosystems) using SYBR Premix Ex Taq II (Takara) at 95°C for 2 min, and then 40 cycles of 95°C for 15 s and 60°C for 35 s. The primers used for *RUNX3* have been described previously (Lin et al., 2012). The sense strand was 5'-TCTGTAAGGCCCAAAGTGGGTA-3' and the antisense strand was 5'-ACCTCAGCATGACAATATGTCACAA-3'. Results were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and changes in expression were calculated using the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001).

Sodium bisulfite modification and MS-PCR

Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Qiagen, USA). The bisulfite conversion was performed using an EpiTect Bisulfite (Qiagen) in accordance with the product manual. The methylation status of *RUNX3* was analyzed by methylation-specific PCR (MSP) as described previously (Kim et al., 2004). MSP assays were repeated at least twice to verify the reproducibility of the assay.

Protein extraction and Western blot

Total protein was extracted using a Qproteome Mammalian Protein Prep Kit (Qiagen) in accordance with the manufacturer's instruction. Typically, 20 μg of the protein were loaded per lane. Protein samples were resolved using SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk for 1 h at room temperature and incubated at 4°C overnight with human specific anti-RUNX3 antibody (sc-376591, Santa Cruz), anti-DNMT1 antibody (sc-271729, Santa Cruz), and anti-DNMT3B antibody (Abcam, USA). Sequentially, the secondary antibodies were conjugated to horseradish peroxidase and the proteins were visualized via chemiluminescence (Beyotime, China). β-actin (ACTB; sc-47778, Santa Cruz) was used to normalize the quantity of the protein.

Plasmid construction and translation

The miR-148a overexpression plasmid was constructed as previously described (Zhu et al., 2012). *DNMT1* siRNA (sc-35204) and *DNMT3B* siRNA (sc-37759) were bought from Santa Cruz Biotechnology (USA). AGS and BGC-823 cells were seeded in 6-well plates at 1 × 10⁵ cells/well and transfected with pRNAi-CMV4.3/Neo-pre-miR-148a (CMV4.3-miR-148a), the blank plasmid pRNAi-CMV4.3/Neo-Neg (CMV4.3-Neg), *DNMT1* siRNA, *DNMT3B* siRNA, or a blank control at concentrations of 100 nM using Lipofectamine 2000 Transfection Reagent (Invitrogen) in accordance with the manufacturer's instructions. Cells were harvested 48 h after transfection for RNA extraction, or after 72 h for protein extraction.

Treatment with 5-aza-dC

For treatment with DNA methylation inhibitor 5-aza-2'-deoxycy-

Fig. 1. *RUNX3* mRNA and *RUNX3* protein expression in 38 gastric cancer tissues and cell lines. (A) qRT-PCR analysis results of *RUNX3* mRNA in gastric cancer tissues and adjacent normal tissues. Data are presented as log₂ fold change in cancer tissues relative to matched normal tissues. The positions of 2-fold underexpression are shown as a dashed line. (B) *RUNX3* protein expression in clinical gastric tissues and gastric cancer cell lines was assessed by Western blot. The adjacent normal gastric tissues and normal gastric epithelial cell line GES-1 were included as controls, respectively. β-actin was the loading control. N = normal gastric tissue, T = gastric tumor. (C) Positive correlation between miR-148a levels and *RUNX3* mRNA levels.

Fig. 2. Methylation-dependent regulation of *RUNX3* expression. (A) Methylation status of the *RUNX3* promoter region was analyzed by MSP in three typical gastric cancer patients. For each sample primers were used to recognize the methylated (M) and non-methylated (U) sequence portions of *RUNX3*. N = normal gastric tissue, T = gastric tumor. (B) In cancer tissues, *RUNX3* mRNA and miR-148a levels were significantly downregulated in those in which the *RUNX3* promoter region was methylated compared with those tissues with *RUNX3* promoter regions that were not methylated. Points represent mean, boxes represent standard deviation and error bars indicate 25-75 percentile. (C) AGS and BGC-823 cells were treated (or not) with the DNA methylation inhibitor 5-aza-dC and evaluated for *RUNX3* mRNA and *RUNX3* protein using qRT-PCR and Western blot, respectively. The data represent the mean and standard errors of treated cells in three independent experiments, relative to the non-treated control. * $P < 0.05$. (D) *RUNX3* methylation status was analyzed by MSP after treatment with 5-aza-dC.

tidine (5-aza-dC) (Sigma, USA), the cells were seeded into a 24-well plate at 2×10^4 /ml and cultured with 5-aza-dC at a final concentration of 10 μ M for 72 h. Fresh medium containing 5-aza-dC was added every 24 h.

Statistical analyses

All experiments were independently repeated at least three times. Continuous data are presented as mean $\pm$ standard deviation, and comparisons between two groups were con-

Fig. 3. Overexpression of miR-148a suppresses *RUNX3* gene expression in gastric cancer cells. (A) The expression of miR-148a significantly increased after transfection with CMV4.3-miR-148a relative to the control in both AGS and BGC-823 cells. (B) AGS and BGC-823 cells were transfected with CMV4.3-miR-148a or CMV4.3-Neg. *RUNX3* mRNA and *RUNX3* protein expression was assessed by qRT-PCR and Western blot, respectively. The mRNA data represent the mean and standard errors of three independent experiments, relative to the control. * $P < 0.05$.

ducted using Student's unpaired *t*-test. Spearman's coefficient of correlation (*r*) was used to calculate the correlation between the expressions of miR-148a and *RUNX3* mRNA in gastric cancer tissues. All statistical analyses were carried out using SPSS version 17.0 (SPSS, USA). The results were considered to be statistically significant at $P < 0.05$.

RESULTS

Decrease of *RUNX3* expression was closely associated with miR-148a expression in gastric cancer

We analyzed the expressions of *RUNX3* mRNA in 38 patients, that is, 38 pairs of gastric cancer tissues and their matched adjacent non-tumor tissues, using qRT-PCR. In 27 (71.1%) cases, mRNA levels of *RUNX3* were as much as 50% less in gastric cancer tissues than in matched normal tissues (Fig. 1A).

Using Western blot analyses we evaluated the expressions of *RUNX3* in human gastric tumor and matched normal tissue specimens, and in cells of the human gastric cancer cell lines AGS and BGC-823 and one human gastric epithelial immortalized cell line, GES-1. Consistent with previous studies, *RUNX3* was expressed in normal gastric tissues and cells, but was decreased or absent in gastric cancer tissues and cells (Fig. 1B).

In previous studies we used qRT-PCR to assay the same 38 pairs of gastric cancer tissues and matched adjacent non-tumor tissues to detect the expression of miR-148a. In those studies we found that miR-148a was significantly downregulated in gastric cancer tissues (Zhu et al., 2012). Interestingly, in the present study an obvious correlation was observed between the expression of miR-148a and *RUNX3* mRNA in the 38 pairs of tissue samples ($r = 0.581$, $P < 0.001$, Fig. 1C).

Hypermethylation of *RUNX3* induced the loss of *RUNX3* gene expression

We examined promoter methylation of *RUNX3* in 38 pairs of gastric cancer tissues and adjacent normal tissues by MSP. Twenty-one of 38 (55.3%) gastric cancer tissues showed *RUNX3* DNA hypermethylation, while only 2 normal mucosal tissues were hypermethylated (Fig. 2A).

Furthermore, in cancer tissues in which the *RUNX3* promoter region was methylated, the *RUNX3* mRNA and miR-148a levels were significantly less than in those in which this region lacked methylation (Fig. 2B). To further examine the role of aberrant methylation in the silence of *RUNX3* gene expression in gastric cancer, the gastric cancer cell lines AGS and BGC-823 were treated with the methylation inhibitor 5-aza-dC, and

Fig. 4. The expression of RUNX3 after knockdown of DNMT1 or DNMT3B. (A) Protein levels of DNMT1 and RUNX3 were examined by Western blot after transfection with DNMT1 siRNA plasmid or blank plasmid for 72 h. (B) Protein levels of DNMT3B and RUNX3 were examined by Western blot after transfection with DNMT3B siRNA plasmid. β-actin was used for the loading control. (C) The expression of *RUNX3* mRNA was examined by qRT-PCR after transfection with DNMT1 siRNA or DNMT3B siRNA for 48 h. The data represent the mean and standard errors of three independent experiments, relative to the control. * $P < 0.05$.

then analyzed for the expressions of *RUNX3* mRNA and RUNX3 protein using qRT-PCR and Western blot, respectively. A significant increase in the levels of both mRNA and protein were observed after treatment with 5-aza-dC (Fig. 2C). Then we further detected the methylation level of CpG islands of the promoter region in 5-aza-dC-treated cells, and found that treatment with 5-aza-dC increased expression of the non-methylated form of *RUNX3* (Fig. 2D). These results confirm that hypermethylation of *RUNX3* results in the downregulation of *RUNX3* expression.

Overexpression of miR-148a downregulated *RUNX3* gene expression

To examine the effects of miR-148a on *RUNX3* gene expression, we transfected gastric cancer cells with the miR-148a overexpression plasmid CMV4.3-miR-148a or blank plasmid CMV4.3-Neg before qRT-PCR and Western blot analysis. In both AGS and BGC-823 cell lines, those cells transfected with the miR-148a overexpression plasmid had significantly increased levels of miR-148a compared with the control group ($P < 0.05$, Fig. 3A). The expressions of *RUNX3* mRNA and RUNX3 protein were also increased in gastric cancer cells (Fig. 3B).

Complementary binding regions between miR-148a and *RUNX3* have not been predicted by any of the databases, including TargetScan, PicTar, miRanda, and miRbase Target. This suggests that miR-148a may not directly target *RUNX3*. Previous studies have already confirmed that miR-148a could modulate DNMT1 and DNMT3B protein expression (Braconi et al., 2010; Duursma et al., 2008; Pan et al., 2010; Zhu et al., 2012). Therefore, our results show that overexpression of miR-148a in gastric cancer cells might upregulate RUNX3 expression through the modulation of DNMTs.

DNMT1, not DNMT3B, contributed to the aberrant methylation of *RUNX3*

To further study a DNMT-dependent mechanism of *RUNX3* alteration, we assayed the expression of RUNX3 after knockdown of DNMT1 or DNMT3B in BGC-823 cells. The downregulation of DNMT1 or DNMT3B was verified by Western blot analysis after treating BGC-823 cells with *DNMT1* siRNA or *DNMT3B* siRNA for 72 h (Figs. 4A and 4B). An increase in *RUNX3* mRNA and RUNX3 protein expression was observed in *DNMT1* siRNA-treated cell lines (Figs. 4A and 4C). In contrast, *DNMT3B* siRNA-treated cells did not show any deregulation of *RUNX3* (Figs. 4B and 4C). Therefore, DNMT1, but not DNMT3B, contributes to the aberrant methylation of *RUNX3* and furthermore causes downregulation of *RUNX3* expression. Altogether, our studies confirm that miR-148a can indirectly regulate *RUNX3* gene expression via modulation of DNMT1-dependent DNA methylation.

DISCUSSION

RUNX3 is regarded as a tumor suppressor gene, and involved both in the oncogenic Wnt and anti-oncogenic transforming growth factor beta (TGF-β) signaling pathways which induce cell cycle arrest and apoptosis (Ito, 2011). *RUNX3* also can suppress gastric cancer metastasis by inactivation of matrix metalloproteinase 9 (MMP9) through the upregulation of tissue inhibitor of metalloproteinases 1 (TIMP1) (Chen et al., 2011). Previous studies showed that *RUNX3* expression was reduced in 40% of early-stage and nearly 90% of advanced-stage gastric cancers (Li et al., 2002). Therefore, *RUNX3* can be considered a strong tumor suppressor candidate for human gastric cancer.

Identification of the molecular mechanism responsible for the inactivation of *RUNX3* gene expression may contribute to the field of gastric cancer therapy. The silencing of *RUNX3* has been attributed to several mechanisms, including mutation, promoter hypermethylation, and aberrant localization of the protein to the cytoplasm (Ito et al., 2005; Li et al., 2002). About 45% to 60% of gastric cancers do not express *RUNX3*, mainly due to hypermethylation of the *RUNX3* promoter (Li et al., 2002). Furthermore, *RUNX3* methylation was associated with the histological type and differentiation grade in gastric cancer

(Fan et al., 2011). Consequently, hypermethylation may have a significant role in the downregulation of RUNX3 expression. However, the regulatory mechanism of aberrant RUNX3 promoter hypermethylation has not been fully elucidated.

Mature miRNAs can directly regulate the expressions of various genes either by mRNA cleavage (i.e., slicing) or by translational repression, depending on the degree of complementarity between the seed sequence in the 5' end of the miRNA and the seed-match sequence (generally) in the 3' untranslated region (UTR) of the target mRNA (Lujambio and Lowe, 2012). Kitago et al. (2009) found that miR-532-5p, which had a sequence complementary to the 3' UTR of RUNX3, was highly expressed in melanoma and could suppress RUNX3 expression. In addition, overexpression of miR-130b could downregulate RUNX3 protein expression, and miR-130b expression was inversely associated with RUNX3 hypermethylation in gastric cancer. This suggests that RUNX3 can be regulated by miRNAs, possibly as an alternative mechanism to hypermethylation (Lai et al., 2010).

In our previous studies, we confirmed that miR-148a was significantly downregulated in gastric cancer and an increase in miR-148a levels was associated with lower DNMT1 levels in gastric cancer cells. In this study, we further demonstrated that downregulation of miR-148a expression was not only closely associated with decrease of RUNX3 expression but also inversely related to hypermethylation of RUNX3 in gastric cancer. Also, RUNX3 was upregulated by treatment with the methylation inhibitor 5-aza-dC in gastric cancer cell lines. It has been accepted that the deoxycytidine analog 5-aza-dC is a DNA methylation inhibitor, due to the covalent binding with DNMTs during the methylation reaction (Jüttermann et al., 1994). Consequently, silence of RUNX3 might be brought about through promoter methylation catalyzed by DNMTs.

In the present study we transfected gastric cancer cells with a miR-148a overexpression plasmid, and RUNX3 mRNA and RUNX3 protein levels were significantly upregulated. Thus miR-148a could downregulate DNMT expression, which decreased the methylation of RUNX3, and RUNX3 protein levels increased. Our findings are consistent with several studies that provide evidence for miRNA-induced regulation of promoter methylation through DNMTs (Braconi et al., 2010; Garzon et al., 2009; Li et al., 2012; Pan et al., 2010; Wang et al., 2012; Zhao et al., 2011).

In this study we also assayed the expression of RUNX3 after knockdown of DNMT1 or DNMT3B in gastric cancer cells. Interestingly we observed that DNMT1, but not DNMT3B, contributes to the aberrant methylation of RUNX3 and caused the downregulation of RUNX3 expression. These data are similar to those in a previous study (Jung et al., 2007). Consequently, we suggest that miR-148a indirectly regulates RUNX3 gene expression via modulation of DNMT1-dependent DNA methylation. We note that DNMT1 and DNMT3B could cooperate to maintain DNA methylation and silence genes in human cancer cells (El-Osta, 2003; Rhee et al., 2002). Therefore, we speculate that DNMT3B, which is downregulated by miR-148a, could cooperate with DNMT1 to further regulate RUNX3 expression in gastric cancer. However, Jung et al. (2007) found that dual inhibition of DNMT1 and DNMT3B did not have a synergistic effect on gene reexpression and demethylation in cancer cells. Therefore, the cooperative effect of DNMT1 and DNMT3B in gene expression needs to be further investigated.

Our previous results showed that the silence of miR-148a and overexpression of DNMT1 may constitute an epigenetic-miRNA feedback mechanism in gastric cancer, as overexpres-

sion of DNMT1 leads to hypermethylation in the promoter region of miR-148a thus causing the silence of miR-148a, and the silence of miR-148a reduces its suppression of DNMT1 resulting in higher levels of DNMT1 and hypermethylation of miR-148a (Zhu et al., 2012). We believe that in gastric cancer this miRNA-epigenetic feedback may further lead to the downregulation of methylation-sensitive genes, such as P16, RUNX3, and E-cadherin, through the hypermethylation of promoters. We must note that miR-152 (Wang et al., 2012), miR-126 (Zhao et al., 2011), and miR-29 (Garzon et al., 2009; Li et al., 2012) have also been observed to target DNMT1. Consequently, our further studies will focus on these miRNAs, which may cooperatively target DNMT1 and further regulate the expression of methylation-sensitive genes.

ACKNOWLEDGMENTS

This work was supported by the Key Program for Technology Development Fund of Nanjing Medical University (NJMU04).

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