DNA methylation downregulated mir-10b acts as a tumor suppressor in gastric cancer

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Abstract

Background MicroRNAs act as tumor suppressors or oncogenes. The pathological roles of miRNAs in gastric tumorigenesis are largely unknown. Although miR-10b was identified as an miRNA deregulator expressed in gastric cancer (GC), there also exists some debate on whether miR-10b is acting as tumor suppressor or oncogene in GC.

Methods Quantitative RT-PCR was employed to investigate the level of miR-10b in GC tissues and matched adjacent normal tissues ($n = 100$). In vitro cell proliferation, apoptosis assays, cell migration, and invasion assays

were performed to elucidate the biological effects of miR-10b. Because silencing of miRNA by promoter CpG island methylation may be an important mechanism in tumorigenesis, GC cells were treated with 5-aza-2'-deoxycytidine and trichostatin A, and expression changes of miR-10b were subsequently examined by quantitative RT-PCR. Furthermore, the methylation status of the CpG island upstream of miR-10b was analyzed by methylation-specific PCR in GC tissues ($n = 29$).

Results We showed here that miR-10b was significantly downregulated in GC cell lines and tissues as demonstrated by quantitative real-time PCR. Overexpression of miR-10b in MGC-803 and HGC-27 dramatically suppressed cell proliferation, migration, invasion, and induced apoptosis. Moreover, we demonstrated that T-cell lymphoma invasion and metastasis (Tiam1) was a target of miR-10b. Furthermore, 5-aza-2'-deoxycytidine and trichostatin A increased miR-10b expression, and the methylation level was high in the CpG islands upstream of miR-10b gene.

Conclusions Taken together, these findings suggest that miR-10b may function as a novel tumor suppressor and is partially silenced by DNA hypermethylation in GC.

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Introduction

Gastric cancer is the fourth most common cancer and the second most frequent cause of cancer death worldwide [1]. Advances in diagnostic and therapeutic approaches have led to excellent expectations for long-term survival for early gastric cancer, whereas the outlook for individuals with advanced gastric cancer is still disappointing. The

poor prognosis is frequently explained by lack of early diagnostic biomarkers and effective therapeutic treatments. Because the prognosis of gastric cancer is closely related to the stage of disease at diagnosis, novel diagnostic modalities for early stages and new therapeutics are urgently needed [2].

MicroRNAs (miRNAs) are a large family of endogenous non-protein-coding small RNAs that are 20–25 nucleotides in length [3]. Through downregulating the expression of target genes, miRNAs can direct a wide repertoire of normal biological mechanisms such as embryonic development, cell growth, apoptosis, and differentiation [4]. Emerging evidence supports that miRNAs are novel players in carcinogenesis [5]. In this regard, miRNAs are dysregulated in most, if not all, types of human cancers examined so far. A large number of miRNAs is overexpressed in human tumors compared to normal tissues, and gene silencing by miRNAs enhances tumor cell proliferation, survival, motility, and invasiveness, angiogenesis, and drug resistance [6, 7]. These findings suggest that this class of regulators includes enhancers of tumor progression.

Accumulating evidence suggests that miR-10b may behave as novel oncogenes in human cancers [3]. miR-10b has been identified as one of the most upregulated miRNAs in human cancers. In 2007, Ma et al. [3] showed that miR-10b was highly expressed in metastatic breast cancer cells, compared with normal mammary tissue, and positively regulated cell migration and invasion. Overexpression of miR-10b in otherwise nonmetastatic breast tumors initiated robust invasion and metastasis [8]. miR-10b is a unique miRNA expressed specifically in glioma tumors but not in normal brain cells: neither neural progenitor cells nor mature glia or neurons [9, 10]. It has been reported that miR-10b was upregulated in all glioma samples compared with nonneoplastic brain tissues; the expression level of miR-10b was associated with higher-grade glioma and the tumor invasive factors uPAR and RhoC [9]. Moreover, the overexpression level of miR-10b was observed in many other cancers, including neurofibromatosis type 1, esophageal cancer, pancreatic cancer, nasopharyngeal carcinoma, hepatocellular carcinoma, and colorectal cancer [11–16]. These findings suggested that miR-10b was strongly expressed in highly metastatic cancer cells and played a central role in cancer metastasis.

There also exists some debate on whether miR-10b acts as tumor suppressor or oncogene in GC. Recently, Kim et al. [17] found that miR-10b may act as a tumor suppressive gene in gastric carcinogenesis. miR-10b was silenced in gastric cancer cells by promoter methylation, gastric cancer cells that were transfected with precursor miR-10b showed a significant decrease in colony formation and growth rates, and miR-10b promoter methylation

increased with patient age and occurred significantly more frequently in intestinal type than in diffuse type. Also, the regulation of miR-10b in human gastric cancer and its potential molecular mechanism have not been investigated. However, the study of Liu et al. [18, 19] reported that miR-10b was highly expressed in 15 human gastric tumor species with a tendency to metastasis, which was further confirmed by Wang et al. [18, 19] by real-time reverse transcription-polymerase chain reaction (RT-PCR). In this study, we focused on the expression and roles of miR-10b in human gastric cancer. The results showed that miR-10b was downregulated in GC tissues. Overexpression of miR-10b inhibited proliferation, migration, and invasion of GC cells and induced cell apoptosis. These data suggested that miR-10b was a candidate tumor suppressor in GC. Further methylation analysis of miR-10b promoter indicated that its expression was regulated by methylation of correlated CpG islands to some extent. Finally, we found that Tiam1 was the target of miR-10b in GC. This finding indicated that miR-10b may act as a tumor suppressor by targeting Tiam1.

Materials and methods

Ethics statement

All patients agreed to participate in the study and gave written informed consent. This study was approved by the ethical board of the institute of Basic Medical Sciences, Chinese Academy of Medical Science, and complied with Declaration of Helsinki.

Samples and cases

Gastric tumors and their morphologically normal tissues (located >3 cm from the tumor) were obtained between 2009 and 2011 from 100 GC patients undergoing surgery at the Cancer Hospital of the Chinese Academy of Medical Sciences ($n = 32$), the Chinese PLA General Hospital ($n = 34$), and The First Affiliated Hospital of Shanxi Medical University ($n = 34$). Tissue samples were cut into two parts: one was fixed with 10 % formalin for histopathological diagnosis, and the other was immediately snap-frozen in liquid nitrogen and stored in liquid nitrogen until RNA extraction. None of the patients received radiotherapy or chemotherapy before surgery. Because of individual differences between patients, we lacked information about some clinicopathological data. Use of the tissue samples for all experiments was approved by all the patients and by the Ethics Committee of the institution. The characteristics of the patients are described in Table 1.

Table 1 Clinicopathological characteristics of patients with gastric cancer (GC)

Parameter	Total samples	Percentage (%)	95 % CI of mean log2 fold change $\pm$ SEM	<i>P</i>
Age (years)	100			0.428
≥ 60		50	−0.955 (−1.863 to 0.047)	
<60		50	−0.478 (−1.365 to 0.206)	
Gender	100			0.933
Male		82	−0.728 (−1.346 to 0.110)	
Female		18	−0.662 (−2.569 to 1.245)	
Location	97			0.766
Proximal		48.5	−0.73 (−1.617 to 0.156)	
Body		51.5	−0.913 (−1.772 to 0.054)	
Classification	92			
Ulcer		75	−0.756 (−1.464 to 0.049)	
Protrude		6.5	−3.433 (−5.999 to 0.867)	
Erosion		18.5	−0.698 (−2.264 to 0.866)	
Grade	57			0.734
Well and moderately differentiated		28.1	−0.99 (−1.950 to 0.031)	
Poorly differentiated		71.9	−1.296 (−2.907 to 0.315)	
Tumor size	80			0.710
T1–T2		25	−0.339 (−1.82 to 1.14)	
T3		36.3	−0.986 (−1.758 to 0.214)	
T4		38.7	−0.916 (−2.128 to 0.294)	
Lymphatic invasion	98			0.376
N0–N1		66.3	−0.608 (−1.303 to 0.086)	
N2–N3		33.7	−1.173 (−2.337 to 0.009)	
Nodal involvement	98			0.029
Negative		90.8	−0.591 (−1.18 to 0.002)	
Positive		9.2	−2.849 (−5.887 to 0.189)	
Stage	97			0.400
I–II		37.1	−0.435 (−1.378 to 0.507)	
III–IV		62.9	−0.963 (−1.751 to 0.176)	
Perineural invasion	99			0.712
Negative		59.6	−0.6779 (−1.438 to 0.082)	
Positive		40.4	−0.9038 (−1.886 to 0.078)	

Cell lines and cell culture

The following human GC cell lines were used in this study: MGC-803 (mucinous gastric cancer, poorly differentiated), HGC-27 (metastatic lymph node, undifferentiated carcinoma), MKN-45 (signet-ring carcinoma, poorly differentiated), and SGC-7901 (adenocarcinoma, moderately differentiated). The MGC-803 cell line was purchased from the Cell Resource Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China), and was propagated in Dulbecco's modified Eagle's medium (Gibco; Invitrogen; Life Technologies, Germany), supplemented with 10 % fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), streptomycin (100 μ g/ml), and

penicillin (100 U/ml). The HGC-27, MKN-45, and SGC-7901 cell lines were provided by American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in RPMI 1640 medium (PAA) supplemented with 10 % FBS (Gibco).

Cell transfection

The miR-10b mimics, miR-10b inhibitor, and the scramble mimics, which are nonhomologous to the human genome, were synthesized by GenePharma (Shanghai, China; Table 2) and transfected into the cells to a final oligonucleotide concentration of 10 nmol/l. All cell transfections were introduced by DharmaFECT1 reagent (Dharmacon, Austin, TX, USA) according to the manufacturer's

Table 2 Primer/mimics/probe sequences

Name	Sequence (5'-3')
miRNA reverse transcription prime	
miRNA-10b	GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGAC CACAAA
U6 snRNA	AAAATATGGAACGCTTCACGAATTTG
Real-time PCR primer sequence	
miRNA-10b	GATTTAGGTATTTTATTTTGGGTGG CTCCATATCGCACTTTAATCTCTAACT
U6 snRNA	CTCGCTTCGGCAGCACATATACT ACGCTTCACGAATTTGCGTGTC
miRNA mimics sequence	
miRNA-10b	UACCCUGUAGAACCGAAUUUGUG CAAAUUCGGUUCUACAGGGUAUU
Negative control	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT
MSP primer sequence	
Methylated	GAATCGAATTTGTGTGGTATTC CTAACCAACGCCGACTACT
Unmethylated	AGAATTGAATTTGTGTGGTATTT ACTAACCAACACCAACTACT
Taqman probe sequence	
miR-10b	CTGGATACGACCACAAA
U6 snRNA	CCATGCTAATCTTCTCTGTA

instructions for use. For each cell transfection two or three replication experiments were performed.

TaqMan RT-PCR for miRNA expression

Total RNA was extracted from the cells and tissues with Trizol reagent (Invitrogen, Calsbad, CA, USA). MicroRNAs were quantitated by real-time PCR using TaqMan MicroRNA assay (Invitrogen). First-strand complementary DNA (cDNA) synthesis was carried out from 1 µg total RNA in 12 µl final volume containing 2 M stem-loop primer, 10 mM dNTP Mix (Invitrogen). The mix was plated at 65 °C for 5 min, and then mixed with 5× RT buffer, 0.1 M DTT, 200 U/µl MultiScribe reverse transcriptase, and 40 U/µl RNase inhibitor (Invitrogen). The mix was plated at 37 °C for 55 min, at 70 °C for 15 min, and then held at −20 °C. Real-time PCR was performed using a standard TaqMan PCR protocol. The 20-µl PCR reactions included 1 µl RT product, 1 µl Universal TaqMan Master Mix, and 1× TaqMan probe/primer mix (Invitrogen) (Supplementary Table S1). All RT reactions including no-template controls were run in triplicate. All mRNA quantification data were normalized to U6. The relative amount of transcript was calculated using the comparative Ct method.

5-Aza-CdR and trichostatin A treatment of cell lines

GC cell lines MGC-803 were treated with 5-aza-2'-deoxycytidine (5-Aza-CdR; Sigma-Aldrich) at 0.7, 1.5, and 3 µmol/l; HGC-27 were treated with 5-Aza-CdR at 0.5, 1, or 1.5 µmol/l for 3 days or 300 nmol/l trichostatin A (TSA; Sigma-Aldrich) for 24 h. For the combination treatment, cells were first treated with 5-Aza-CdR for 48 h; then TSA (300 nmol/l) was added, and the cells were treated for an additional 24 h. Culture medium containing the drug was replaced every 24 h. RNA of cell lines was purified with TRIzol reagent following instructions from the manufacturer. cDNA synthesis was carried out as described earlier, and 1 ml of the diluted cDNA for each sample was amplified by RT-PCR using a protocol previously described.

DNA isolation and bisulfite modification

Genomic DNA was obtained from primary tumors at −196 °C in liquid nitrogen and matched adjacent normal tissues ($n = 22$; included 11 patients in whom expression of miR-10b was downregulated and 11 others in whom expression was upregulated), using a Biomed DNA Kit (Biomed, Beijing, China) according to the manufacturer's instructions. Bisulfite treatment and recovery of samples were carried out with the Epitect Bisulfite kit (Qiagen, Hilden, Germany). Genomic DNA (2 µg) in 20 µl water was used for each reaction, mixed with 85 µl bisulfite mix and 35 µl DNA protect buffer. Bisulfite conversion was performed on a thermocycler as follows: 99 °C for 5 min, 60 °C for 25 min, 99 °C for 5 min, 60 °C for 85 min, 99 °C for 5 min, 60 °C for 175 min, and 20 °C indefinitely. The bisulfite-treated DNA was recovered by an Epitect spin column and subsequently sequenced to confirm the efficiency of bisulfite conversion.

Methylation analysis

MSP was used to analyze methylation of miR-10b promoter in cell lines and tissues. Methprimer (<http://www.urogene.org/methprimer/index1.html>) was used to design the MSP primer (Supplementary Table S1). MSP reactions on new primers were optimized using methylated positive control (M-DNA), which used normal human peripheral lymphocyte DNA treated in vitro with Sss I methyltransferase (New England Biolabs, Beverly, MA, USA). The DNA of two normal human peripheral lymphocytes was used as the normal control. Touchdown PCR consisted of two phases: phase 1 included an initial denaturation of 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at variable temperatures for 30 s, and extension at 72 °C for 40 s. In the first cycle, the

annealing temperature was set to 58 °C and, at each of the 10 subsequent cycles, the annealing temperature was decreased by 0.6 °C. Phase 2 consisted of 35 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 40 s. MSP products were analyzed on 3 % polyacrylamide gels.

Cell proliferation and apoptosis assay

Cells were incubated in 10 % CCK-8 (Dojindo, Kumamoto, Japan) diluted in normal cultured medium at 37 °C until visual color conversion occurred. Proliferation rates were determined at 0, 12, 24, 48, 72, and 96 h after transfection. All experiments were performed in quadruplicate. First, 1×10^6 cells were plated into 6-well plates, and, after transfection with each oligonucleotide for 48 h, the apoptosis assay was conducted using the Annexin V-FITC double-stained detection kit (BD Biosciences, San Jose, CA, USA). Annexin V⁻ and PI⁻ cells were used as controls. Annexin V⁺ and PI⁻ cells were considered as apoptosis, and Annexin V⁺ and PI⁺ cells were designated as necrotic.

Cell migration and invasion assays

A wound-healing assay was done to assess cell migration. An artificial wound was created 24 h after transfection using a 200- μ l pipette tip on the confluent cell monolayer, and mitomycin C was added to the culture wells. To visualize migrated cells and wound healing, images were taken at 0, 12, 24, 48, and 72 h.

Invasion was assayed by the ability of cells to pass through a Matrigel-coated membrane matrix (BD Biosciences). Cells were seeded onto a Matrigel-coated membrane matrix present in the insert of a 24-well culture plate 24 h after transfection. Fetal bovine serum was added and the noninvasive cells were removed. Invasive cells located on the lower surface of the chamber were stained with 0.1 % crystal violet (Sigma) and counted.

Dual luciferase assays

These cells were cotransfected with 0.4 μ g reporter construct, 0.2 μ g pGL-3 control vector, and miR-10b or negative controls. Cells were harvested 24 h post transfection and assayed with the Dual Luciferase Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Firefly luciferase values were normalized to Renilla and the ratio of Firefly/Renilla values was reported. All transfection assays were carried out in triplicate.

Western blotting analysis

Western blot analysis was carried out using standard methods. Proteins were separated on 10 % sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinyl fluoride (PVDF) membranes (Amersham, Buckinghamshire, UK). Membranes were blocked overnight with 5 % nonfat dried milk and incubated for 2 h with anti-Tiam1 antibody (Abcam, England) at 1:1,000 dilution and anti-GAPDH antibody (Proteintech, Chicago, IL, USA) at 1:50,000 dilution. After washing with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1 % Tween20), the membranes were incubated for 2 h with goat anti-rabbit antibody (zsgb-bio, Beijing, China) at 1:5,000 dilution and 1:50,000 dilution.

Histology

Histological diagnosis was according to the triple-site gastric biopsy method. Tissues were fixed overnight in buffered formalin, embedded in paraffin, cut to 3- μ m thickness, and stained with hematoxylin and eosin (H&E).

Statistical analysis

Each experiment was repeated at least three times. Statistical analysis was performed using SPSS 15.0. Data are presented as the mean $\pm$ standard deviation. Statistical analyses were done by analysis of variance (ANOVA) or Student's *t* test; statistical significance level was set at $\alpha = 0.05$ (two-sided).

Results

miR-10b is downregulated in gastric cancer cells

The expression of mature miR-10b was examined in four human GC cell lines (HGC-27, MGC-803, SGC-7901, and MKN-45): four GC tissues and four adjacent nonneoplastic tissues (Fig. 1a). These GC cell lines exhibited extraordinarily low expression of miR-10b compared to the four pairs of primary cancer tissues or adjacent tissues. Furthermore, the expression of miR-10b in GC tissues decreased noticeably compared with the adjacent tissues, except in one patient (Fig. 1b).

Expression of miR-10b in clinical GC patients and correlation analysis with clinicopathological characteristics

To study the relationship of miR-10b with GC occurrence, the expression of miR-10b was detected in 100 clinical patients using Taqman real-time PCR. Of 100 GC samples, miR-10b was downregulated in 60 cases (60/100; 60 %) compared with adjacent tissues when the cutoff was set at 2.0 (Fig. 1c), and miR-10b was upregulated in 40 cases (40/

Fig. 1 Expression of miR-10b in human gastric cancer cell lines and tissues. **a** Four patients who were diagnosed as gastric cancer in hematoxylin and eosin (H&E) staining. $\times 100$. **b** Expression of miR-10b in four human gastric cancer cell lines (HGC-7, MGC-803, SGC-7901, MKN-45) and four primary tissues (C) and adjacent nonneoplastic tissues (N) using real-time PCR with Taqman probes. **c** miR-

10b was detected in 100 GC patients by real-time PCR. Data are presented as log 2 of fold change of GC tissues relative to non-tumor adjacent tissues. **d** Expression of miR-10b in GC tissues was lower than that in non-tumor adjacent tissues. $P < 0.05$. Experiments were performed three times. All data used *t* test and are shown as mean $\pm$ SD

100; 40 %). In general, the expression of miR-10b in GC tissues was significant lower than in adjacent tissues (Fig. 1d, $p < 0.05$). To evaluate the correlation between miR-10b expression and clinicopathological characteristics, patients were divided into groups with high and low expression. As shown in Table 1 and Fig. 1, a statistically significant association was observed between the low miR-10b group and GC clinical lymphatic invasion. A lower level of miR-10b expression in the patients seemed to be associated with more lymphatic invasion ($p = 0.029$, independent-samples *t* test). These data suggested that alterations of miR-10b could be involved in GC progression.

miR-10b inhibits GC cell proliferation and survival

To study the role of miR-10b in gastric carcinogenesis, MGC-803 and HGC-27 were transfected with miR-10b mimics, both of which showed great transfection efficiency (Fig. 2a). CCK-8 proliferation assay showed that cell growth rate was reduced in miR-10b mimics-transfected MGC-803 and HGC-27 cells compared with scramble-transfected cells

or untreated cells; conversely, miR-10b inhibitor significantly accelerated the cell proliferation of MGC-803 and HGC-27 (Fig. 2b). To address whether upregulation of miR-10b would induce GC cells apoptosis and death, the number of early apoptotic MGC-803 and HGC-27 cells following treatment with miR-10b mimics was determined (Fig. 2c). As expected, few early apoptotic cells (22.0 % in MGC-803 or 4.5 % in HGC-27) were detected in the scramble-treated cells, whereas miR-10b mimics treatment increased the percentage of early apoptotic cells (28.3 % in MGC-803 or 6.9 % in HGC-27) as judged by annexin V staining.

miR-10b inhibits cell migration and invasion in vitro

To analyze the role of miR-10b on cell migration and invasion, which were the key determinants of malignant progression and metastasis, wound healing and trans-well assays were performed in MGC-803 and HGC-27 cells. Both of two cell lines treated with miR-10b mimics were distinctively less migratory than scramble control or untreated cells at 12, 24, and 36 h after scratching. In

Fig. 2 Overexpression of miR-10b inhibits gastric cell growth and affects cell apoptosis. **a** Expression levels of miR-10b were examined by real-time PCR after transfection of 50 nmol/l miR-10b mimics, or scramble or no transfection. **b** Growth of MGC-803 and HGC-27 cells shown after transfection with 50 nmol/l miR-10b mimics, miR-10b

inhibitor, or scramble or no transfection. Growth index as assessed at 1, 2, 3, 4, and 5 days. **c** MGC-803 and HGC-27 cells were stained with PE Annexin V and 7-AAD 72 h after treatment with miR-10b mimics or scramble or no transfection. Early apoptotic cells are shown in right quadrant. * $p < 0.05$; ** $p < 0.01$

contrast, miR-10b inhibitor significantly accelerated wound closure (Fig. 3a). Furthermore, we conducted a cell invasion assay with Matrigel and stained the invaded cells to measure the directional invasion ability of the cells after ectopically expressing miR-10b in MGC-803 and HGC-27 cells. The invasiveness of cells transfected with miR-10b mimics was dramatically decreased compared with the scramble control and untreated cells, whereas miR-10b inhibitor increased both cell migration and invasion (Fig. 3b, c).

miR-10b expression is epigenetically regulated

To analyze the regulation of miR-10b by DNA methylation, we searched the human genome database for the

presence of CpG islands around miR-10b and identified a large CpG island (Fig. 4a). To elucidate whether the lower expression of miR-10b in GC was from epigenetic alteration, we treated MGC-803 and HGC-27 cells with the DNA methylation inhibitor 5-aza-CdR (AZA) and a histone deacetylase inhibitor trichostatin A (TSA). The expression of miR-10b was upregulated in both MGC-803 and HGC-27 cells with AZA or AZA combined with TSA treatment but not in TSA treatment alone (Fig. 4b). To further detect whether miR-10b expression is associated with methylation of GC, the methylation status of the miR-10b promoter was examined using methylation-specific PCR (MSP; Fig. 4c). We chose 29 pairs of tissues (primary tumors and their matched adjacent normal tissues),

Fig. 3 Overexpression of miR-10b inhibits gastric cancer cell migration and invasion. **a** MGC-803 and HGC-27 cells were not transfected or transfected with 50 nmol/l miR-10b mimics, miR-10b inhibitor, or scramble for 24 h, and wounds were made. Relative ratio of wound closure per field is shown. **b, c** MGC-803 and HGC-27 cells

were not transfected or were transfected with 50 nmol/l miR-10b mimics, miR-10b inhibitor, or scramble for 24 h, and trans-well invasion assay was performed. Relative ratio of invasive cells per field is shown. Magnification: migration $\times 400$; invasion $\times 40$. All data are mean $\pm$ SD

Fig. 4 Downregulation of miR-10b in gastric cancer cells is associated with methylation of miR-10b upstream region. **a** Schematic illustration of deletion of a segment (130–180 M) of chromosome 2q34.11 where miR-10b genes were located. **b** Effect of 5-Aza-CdR and TSA on miR-10b expression in MGC-803 and HGC-27 gastric

cancer cell lines. 5-Aza-CdR combination with TSA significantly increased miR-10b levels. **c** Representative MSP results of miR-10b methylation in primary gastric cancer tumors and normal adjacent tumor tissues. Case numbers shown on top. M methylated primers, U unmethylated primers. Cases with hypermethylation are marked

including 15 patients who possessed a lower miR-10b level (low miR-10b group) and 14 patients who demonstrated a higher miR-10b level (high miR-10b group). Concordant with our proposition that miR-10b expression in gastric cancer was suppressed by DNA methylation, the hypermethylation percentage of promoter of the miR-10b gene was 73 % (11/15) in the “low miR-10b group” versus 29 % (4/14) in the “high miR-10b group”).

miR-10b targets Tiam1 in GC

MiRNAs perform biological functions through negatively regulating their target genes. It has been reported that the oncogene Tiam1 is a direct target of miR-10b in breast carcinoma cells. As predicted by PicTar, there was complementarity between has-miR-10b and Tiam1 3'-UTR (Fig. 5a). The effect of miR-10b on the translation of Tiam1 mRNA into protein was assessed by luciferase reporter assay in NP cells (Fig. 5b). To assess the regulation of miR-10b in Tiam1 expression, the protein level of Tiam1 was analyzed in six miR-10b downregulated GC tissues. Tiam1 was upregulated in four GC tissues (Fig. 5d). Furthermore, the protein level of Tiam1 in MGC-803 and HGC-27 cells was examined after miR-10b

overexpression. Tiam1 was obviously decreased in the presence of miR-10b mimics compared with the scramble control in both MGC-803 and HGC-7 cells (Fig. 5c).

Discussion

Several studies have shown that miRNAs play critical roles in tumor development and progression [20]. Although oncogenic and tumor suppressive roles of several miRNAs have been characterized in several different types of tumors, possible roles of miRNAs in mediating GC tumorigenesis and tumor progression remain largely unexplored. In this study we demonstrate that miR-10b was frequently downregulated in human GC cancer, and that miR-10b was significantly associated with lymph node metastasis. Further studies showed that overexpression of miR-10b suppressed GC cell proliferation, migration, and invasion in GC cells MGC-803 and HGC-27. As for the mechanism, Tiam1 was identified as a target of miR-10b in gastric carcinogenesis. These findings suggest that miR-10b has an important role in inhibiting the development and progression of GC.

miR-10b was first found to be downregulated in breast cancer and further confirmed by others, but Ma et al. [3, 21,

Fig. 5 Tiam1 is a direct target of miR-10b. **a** Predicted duplex formation between human Tiam1 3'-UTR and miR-10b: Tiam1 3'-UTR is highly conserved in different species. *Upper panel*: sequence alignment of miR-10b with binding site on the Tiam1 3'-UTR. *Lower panel*: sequence of the miR-10b-binding site within the Tiam1 3'-UTR of four species. **b** Luciferase activity of wild-type (WT-UTR) or

mutant (MUT-UTR). **c** Tiam1 protein expression in MGC-803 and HGC-27 transfected with 50 nmol/l miR-10b mimics, scramble, or not transfected. **c** Western blot analysis of Tiam1 protein expression in six patients whose miR-10b expression was downregulated in GC tissues compared to **(d)** corresponding adjacent nonneoplastic tissues (N). *nt* nucleotide

22] found that miR-10b was upregulated in metastatic breast cancer. In this connection, miR-10b has been shown to mediate its oncogenic effect through downregulating HOXD10. However, miR-10b did not show remarkable regulation on HOXD10 in GC cells (data not shown), indicating the oncogenic or tumor suppressive effects of miR-10b might be tissue specific. Some debate exists on

whether miR-10b is upregulated or downregulated in GC. Li et al. [23] first reported that seven miRNAs were significantly associated with patient survival using Cox proportional hazard regression in a training data set. Hazard ratios from the univariate Cox regression analysis showed that the levels of expression of seven miRNAs correlated with death from any cause: four (miR-10b, miR-21,

miR-223, and miR-338) were risk miRNAs. The study of Liu et al. [18, 19] also reported that miR-10b was highly expressed in 15 human gastric tumor species with a tendency to metastasis, which was further confirmed by Wang et al. [18, 19] by real-time RT-PCR. However, Kim et al. [17] showed that miR-10b may act as a tumor suppressor in GC. Thus, we measured the level of miR-10b in four GC cell lines and 100 GC samples. Our results showed that miR-10b was downregulated in 60 (60/100; 60 %) GC tissues compared with the adjacent tissues and that the expression of miR-10b in GC tissues was significantly lower than in adjacent tissues. The contrasting results may be because of the different quantity of clinical samples and the indistinctive change. We also found that the lower expression of miR-10b in GC specimens was correlated with lymph node metastasis. All this evidence indicated that miR-10b might contribute to a gastric cell's malignancy.

To further verify the role of miR-10b in the development of GC, cell transfection was performed. Upregulation of miR-10b significantly inhibited cell proliferation, migration, and invasion, enhanced cell apoptosis, and reduced cell viability in GC cell lines, indicating that repression of miR-10b might promote tumor progression in gastric carcinogenesis. Further studies are needed to elucidate this mechanism.

Tumor invasion and metastasis is a complex and multistep process: miR-10b may play different roles via different targets [18, 22, 24, 25]. Previous studies indicated that miR-10b restrained Tiam1 by translational inhibition, in which the 3' UTR miR-10b-binding site is crucial [26]. We found overexpression of miR-10b likely decreased Tiam1 protein level in the both GC cell lines, suggesting that Tiam1 was a target of miR-10b in gastric cancer.

Tiam1, a member of the DbI gene family of guanine nucleotide exchange factors (GEFs), was first identified in 1994 by proviral tagging in combination with in vitro selection for invasiveness from murine leukemia cells [27]. Tiam1 has been implicated as a ubiquitous Rac activator that participates in several cellular processes [28, 29]. More and more investigations have revealed that Tiam1-Rac pathway activation can promote cell adhesion, migration, invasion, and metastasis in a variety of cancers, such as prostate cancer, breast cancer, hepatocellular carcinoma, colon carcinoma, and renal carcinoma, suggesting that Tiam1 plays an important role in tumorigenesis and carcinoma progression [30]. Moreover, overexpression of Tiam1 is considered to be a new potential or even an independent predictor of poor prognosis for clinical patients in multiple types of cancer. Previous study has shown that Tiam1 mRNA expression is upregulated in GC compared with adjacent pair-matched non-tumor tissues, and postoperative survival analysis indicated that patients with strong Tiam1 expression had lower disease-specific

survival rates than those with negative Tiam1 expression [31, 32]. However, the underlying mechanisms are unclear. Our data showed that the ability of miR-10b to target Tiam1 may provide one such mechanism of posttranscriptional control of Tiam1.

Various molecular mechanisms lead to miRNA dysregulation, such as genetic mutation, epigenetic aberration, and deregulated transcriptional activity [33, 34]. Among them, epigenetic mechanisms play critical roles in the transcriptional silencing of tumor suppressor genes or suppressor miRNAs by specific DNA methylation and histone modification in cancer cells [35]. Our data showed that miR-10b was markedly upregulated when GC cells (MGC-803 and HGC-27) were treated with both 5-Aza-CdR and TSA. In addition, computational analysis reveals that miR-10b is located in a CpG island on chromosome 2q34.11. Therefore, it seems possible that DNA methylation may be associated with miR-10b regulation. By MSP samples, methylation frequencies detected in the promoter of miR-10b were higher in the miR-10b downregulated group than in the upregulated group. This specificity furnished the hypothesis of a relationship between miR-10b expression and DNA methylation. Overall, these results suggested that methylation was an important mechanism of miR-10b downregulation in GC.

In conclusion, our studies suggested that miR-10b acts as a tumor suppressor in GC and is regulated by DNA methylation. Furthermore, the lower expression of miR-10b in GC specimens was correlated with lymph node metastasis. In addition, miR-10b overexpression suppressed cell proliferation, migration, and invasion and induced apoptosis, indicating that an miRNA-based therapeutic pattern might serve as a basis for the development of novel potential therapies in gastric cancer.

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