

Decreased miR-30b-5p expression by DNMT1 methylation regulation involved in gastric cancer metastasis

Fengchang Qiao · Kun Zhang · Pihai Gong ·
Ling Wang · Jiaojiao Hu · Sen Lu ·
Hong Fan

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Abstract miRNAs have emerged as crucial regulators in the regulation of development as well as human diseases, especially tumorigenesis. The aims of this study are to evaluate miR-30b-5p expression pattern and mechanism in gastric carcinogenesis due to which remains to be determined. Expression of miR-30b-5p was analyzed in 51 gastric cancer cases and 4 cell lines by qRT-PCR. The effect of DNA methylation on miR-30b-5p expression was assessed by MSP and BGS. In order to know whether DNMT1 increased miR-30b-5p promoter methylation, DNMT1 was depleted in cell lines AGS and BGC-823. The role of miR-30b-5p on cell migration was evaluated by wound healing assays. Decreased expression of miR-30b-5p was found in gastric cancer samples. In tumor, the expression level of miR-30b-5p was profound correlated with lymph node metastasis ($P = 0.019$). The level of

miR-30b-5p may be restored by DNA demethylation and DNMT1 induced miR-30b-5p promoter methylation. In vitro functional assays implied that enforced miR-30b-5p expression affected cell migration, consistent with tissues analysis. Our findings uncovered that miR-30b-5p is significantly diminished in gastric cancer tissues, providing the first insight into the epigenetic mechanism of miR-30b-5p down-regulation, induced by DNMT1, and the role of miR-30b-5p in gastric cancer carcinogenesis. Overexpression of miR-30b-5p inhibited cell migration. Thus, miR-30b-5p may represent a potential therapeutic target for gastric cancer therapy.

Keywords miR-30b-5p · DNMT1 · Migration · Gastric cancer

Abbreviations

qRT-PCR Quantitative reverse-transcription polymerase chain reaction
DNMT1 DNA methyltransferase 1

Fengchang Qiao and Kun Zhang have equal contribution to this manuscript.

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F. Qiao · P. Gong · L. Wang · J. Hu · H. Fan (✉)
Key Laboratory of Developmental Genes and Human Diseases,
Ministry of Education, Department of Genetics and
Developmental Biology, The Medical School of Southeast
University and the Institute of Life Science, Southeast
University, Nanjing 210009, China
e-mail: fanh@seu.edu.cn

F. Qiao
e-mail: fengchang-qiao@163.com

P. Gong
e-mail: gongpeihai666@163.com

L. Wang
e-mail: wangling1615@126.com

J. Hu
e-mail: joycehu1120@foxmail.com

K. Zhang
The 3rd Affiliated Hospital of Harbin Medical University
(Harbin Medical University Cancer Hospital), Harbin 150081,
China
e-mail: L068925@126.com

S. Lu
The First Affiliated Hospital with Nanjing Medical University,
Nanjing 210029, China
e-mail: lusen68@yahoo.com.cn

Background

Gastric cancer is the fourth most common cancer and one of the leading causes of cancer related deaths in the world, though its incidence has decreased in the last decade [1]. The poor prognostic methods used to detect gastric cancer and the major clinical limitation in detecting cancer at an early stage lead to less than 5 percent people survive for more than five years [2]. In recent times, gastric cancer is generally considered as the results of deregulation in complex biological processes including many genes which regulate activities such as cell growth, death or apoptosis, DNA repair, etc. The alterations in gene regulation activities result from various underlying genetic instabilities and epigenetic changes [3, 4]. However, current tumor markers are not ideal in relatively low specificity and sensitivity. Thus, there is an urgent need to identify more specific and sensitive novel markers for clinical management of gastric cancer.

MicroRNAs (miRNAs) are non-coding small RNAs of 19–25 nucleotides that have a crucial function in post-transcriptional regulation by binding to the 3'UTR of mRNA, inhibiting translation or mRNA degradation. It has been reported that as many as 30–60 % of protein-encoding genes may be regulated by miRNAs [3, 4]. Depending on their mRNA target, miRNAs can function as a tumor suppressor or promoter of oncogenesis. Many studies have focused on aberrantly expressed miRNAs and their effects on tumorigenesis. Human microRNA-30b (miR-30b), a member of miR-30 family, has been reported to be associated with various human malignancies. In TRAIL-resistant glioma cells, miR-30b expression is particularly increased and impairs TRAIL-dependent apoptosis by targeting caspase-3 [5]; High miR-30b expression in combination with other miRNAs significantly predicts a shorter recurrence-free survival in hepatocellular carcinoma [6]; Up-regulation of miR-30b plays a role in blocking of terminal B cell differentiation in primary central nervous system lymphoma (PCNSL) [7]; However, Van Laere et al. found that miR-30b was significantly down-regulated in breast cancer patients in comparison with non-inflammatory breast cancer [8]; Also in Ehrlich ascites tumor cells, miR-30b expression was low, contributing to the cells with drug-resistant phenotype [9]; miR-30b was significantly dysregulated between parathyroid carcinoma and parathyroid adenoma [10]. Thus the expression pattern of miR-30b is different due to various tumors. However, most of previous study reported miR-30b expression and function in different tumors; they didn't distinguish miR-30b-5p from miR-30b-3p, two mature miRNAs spliced from 5' or 3' end of pre-miR-30b. miR-30b-5p may be a novel biomarker for pancreatic ductal adenocarcinoma [11]. Nevertheless, the expression profile and clinical significance of miR-30b-5p in gastric cancer are not clear, not to mention the mechanism that miR-30b-5p deregulation.

In the present study, we set out to evaluate the expression of miR-30b-5p in gastric cancer tissues and analyze its correlation with clinicopathological factors of gastric cancer patients, and the epigenetic mechanism of miR-30b-5p down-regulation in gastric cancer cell lines, which will provide a potential molecular therapeutic target for human gastric cancer.

Materials and methods

Cell culture and tissue specimens

Human gastric adenocarcinoma cell lines AGS, MGC-803, SGC-7901, BGC-823 were purchased from the Cell Bank of Chinese Academy of Sciences. Cells were routinely cultured with RPMI-1640 medium supplemented with 10 % fetal bovine serum at 37 °C in a humidified atmosphere with 5 % CO₂. Gastric cancer and adjacent non-cancerous tissue specimens were obtained from the 3rd Affiliated Hospital of Harbin Medical University. The samples and our study were approved by the Committees for Ethical Review of Research at the 3rd Affiliated Hospital of Harbin Medical University in China and the patients signed informed consent forms. The clinicopathological features are shown in Supplementary Table 1.

Real-time reverse transcriptase quantitative PCR (qRT-PCR)

Total RNA was extracted from cells and tissues samples with Trizol reagent (Invitrogen, USA). The quality and quantity of the RNA samples were assessed by standard electrophoresis and spectrophotometric methods. The first-strand cDNA was synthesized using reverse transcription kit (TAKARA, Japan) according to the manufacturer's instruction. Hsa-miR-30b-5p gene expression was examined using a SYBR-Green PCR kit (TAKARA, Japan). The primers were purchased from RIBO Corporation (Guangzhou, China) and U6 small nuclear RNA was used as a normalizer. PCR was performed at 95 °C for 30 s, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The relative expression ratio of hsa-miR-30b-5p in gastric cancer tissues and cells was quantified by the $2^{-\Delta\Delta CT}$ method.

5'-Aza treatment, Methylation-Specific PCR (MSP), Bisulfite Genome Sequencing (BGS)

A total of 1.5×10^5 AGS or BGC-823 cells were plated in 6-well plates. The cells were cultured in medium containing 0, 10 or 50 μ M of the DNA methyltransferase inhibitor 5'-Aza (Sigma, USA) for 72 h. Then RNA was extracted and first-strand cDNA was synthesized for qRT-PCR.

Genomic DNA was extracted from the cells using the phenol–chloroform method followed by bisulfite modification. Methylation-specific PCR (MSP) and bisulfite

genome sequencing (BGS) were performed as previously described [12]. Bisulfite-treated genomic DNA from AGS-cMT1 and AGS-SiMT1 cells was amplified using methylated miR-30b-5p primers: MF: 5'-TCGGGTATCGGATATGTTTGTAGTAAC-3'; MR: 5'-TTAAAAAATAATTTAAACCTCCGCC-3' and unmethylated primers: UF: 5'-TGGGTATTGGATATGTTTGTAGTAATGT-3'; UR: 5'-TTAAAAAATAATTTAAACCTCCACC-3'. Primers for BGS were: 5'-GATTGAGTTGGTTTGGTTGAGTATT-3' (forward) and 5'-TCCCATTTTAAAACTCTCCTACTC-3' (reverse). PCR products for the bisulfite sequencing were gel-purified, sub-cloned into a pMD19-T vector system (TAKARA, Japan). At least ten colonies were sequenced to assess the degree of methylation in each CpG site.

A vector-based DNMT1 siRNA construct transfection to gastric cancer cell lines AGS and BGC-823

SiRNAs targeting DNMT1 were designed and prepared as previously described [13]. The siRNA sequences against DNMT1 were designed as sense and anti-sense oligonucleotides corresponding to nucleotide position 2,620–2,638 of human DNMT1 (GenBank Accession No. NM001379.1). There was no homology with other human genes by scanning the GenBank of NCBI using this siRNA. The human gastric cancer cell lines were seeded one day before transfection in order that they were 70–80 % confluent the next day. 4 μ g of DNMT1 siRNA construct was transfected by transfection reagent from Roche. Control cells were treated with pSUPER-GFP plasmid. Cells were grown and selectively cultured in 0.4 mg/ml Geneticin (Life Technologies, USA) for 2 months after the initial transfection. AGS and BGC-823 cells transfected with DNMT1 siRNA construct were labeled as AGS-SiMT1, BGC-SiMT1; those transfected with DNMT1 scramble sequence were labeled as AGS-cMT1 or BGC-cMT1.

Western blot analysis

Western blot analysis was performed to detect DNMT1 protein expression in gastric cancer cell lines. The protein concentration of each extract was standardized using the BCA assay (Pierce, USA). The DNMT1 primary antibody (1:1000, Santa, USA) and the mouse monoclonal anti- β -actin antibody (1:8000, Sigma, USA) were used to detect DNMT1 protein levels. The intensities of specific protein bands were quantified with Gel Pro 3.2 (UVP, CLL, USA), corrected for the intensity of the respective β -actin band.

Wound healing detection

Cell migration was assessed by measuring the movement of cells into a scraped area created by a 200- μ l pipette tip, and the spread of the wound closure was observed after the cells

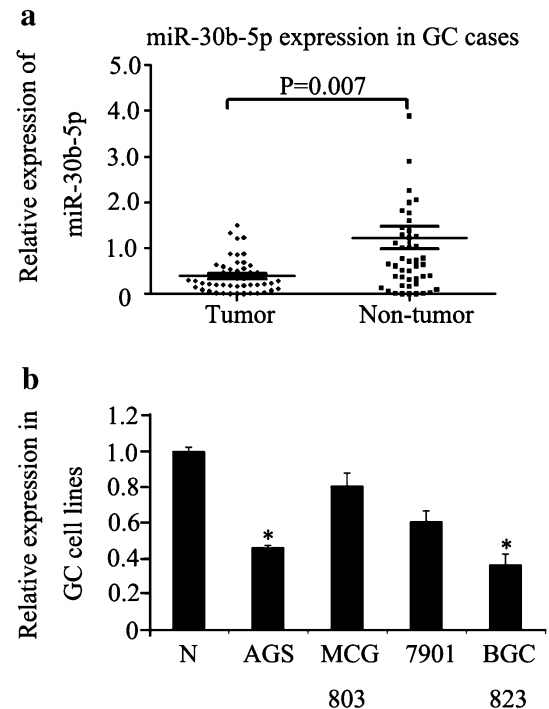


Fig. 1 Relative miR-30b-5p expression is determined in gastric cancer tissues and cell lines by qRT-PCR. Actin was used as an internal control. **a** The top and bottom horizontal lines of the box indicate the 25th and 75th percentiles, respectively. The bold lines within the box indicate the median values. Every dot indicates one sample. N : non-tumor tissues. **b** Detection of miR-30b-5p expression in gastric cancer cell lines and 5 pooled normal tissues (N). * $P < 0.05$

transfected with miR-30b-5p mimics (100 nM) for 24 h. miR-30b-5p mimics (UGUAAACAUCUACACUCAGCU) and negative control were designed and synthesized by GenePharma (Shanghai, China). The cells were photographed under a microscope.

Statistical analysis

Correlations between the miRNA expression levels and pathological features were analyzed with the χ^2 test using SPSS 13.0 software for Windows. Differences were analyzed by Fisher's exact test. The independent Student's t-test was used to compare the results, which were expressed as the mean \pm SD between any two preselected groups.

Results

Expression of miR-30b-5p is significantly down-regulated in gastric cancer and associated with cancer metastasis

The miR-30b-5p levels were detected by qRT-PCR in gastric tumor, matched non-tumor tissue specimen and 4

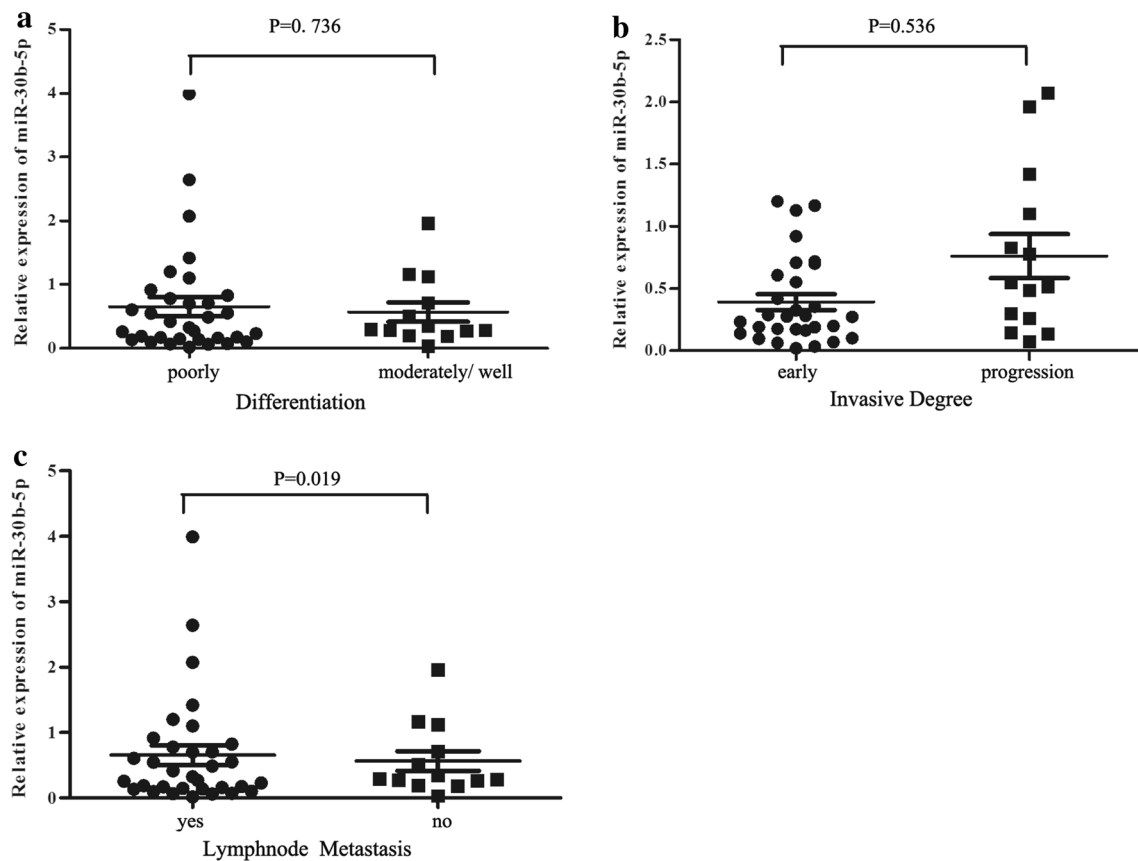


Fig. 2 The correlation between miR-30b-5p expression levels and clinical characters. There was statistical significance between the level of miR-30b-5p and lymphnode metastasis ($P = 0.019$), no difference in differentiation ($P = 0.736$) and invasive degree ($P = 0.536$)

gastric cancer cell lines. We confirmed that reduced expression of miR-30b-5p was a frequent event in gastric cancer tissue and the frequency of miR-30b-5p down-regulation was 62.75 % (32/51). The expression levels of miR-30b-5p in gastric cancer were lower than that in non-tumor tissues ($P = 0.007$) (Fig. 1a). The decreased expression of miR-30b-5p was found in AGS and BGC-823 cells, compared to normal tissues (five pooled normal tissues) ($P = 0.023$; $P = 0.017$) (Fig. 1b).

In order to correlate the expression pattern of hsa-miR-30b-5p with clinicopathological features of patients in gastric cancer, patients were categorized into two groups according to the expression level difference (≥ 1.5 -fold) of miR-30b-5p. However, there was no statistically significant association between miR-30b-5p and clinicopathological features, including age, gender, differentiation, invasion degree and lymph node metastasis (Supplementary Table 1).

While the 51 pairs specimens were categorized based on clinical feature, differentiation and invasive degree had no relationship with miR-30b-5p expression ($P = 0.736$;

$P = 0.536$) (Fig. 2a, b), but we found the expression level of miR-30b-5p was profound correlated with lymph node metastasis ($P = 0.019$) (Fig. 2c). These data indicated that decreased miR-30b-5p expression may play an important role in the metastasis process of gastric cancer.

Silenced miR-30b-5p was restored by methylation inhibitor 5'-Aza

Corcoran et al. [14] predicted the promoter of miR-30b contains CpG islands and has a statistically significant Pol II signal. To examine whether miR-30b-5p down-regulation due to epigenetic inactivation, gastric cancer cell lines AGS and BGC-823 were treated with DNA methylation inhibitor 5-aza-2'-deoxycytidine (5'-Aza). miR-30b-5p expression was restored with different dose of 5'-Aza treatment in AGS (Fig. 3a, $P = 0.011$, $P = 0.005$) and BGC-823 cell lines (Fig. 3b, $P = 0.045$, $P = 0.011$) depending on dosage. These findings imply the expression of miR-30b-5p is affected by methylation in gastric cancer cells.

Fig. 3 miR-30b-5p expression was detected in gastric cancer cell lines AGS (a) and BGC-823 (b) after 5'-Aza treatment (0, 10 and 50 μ M) for 72 h by qRT-PCR

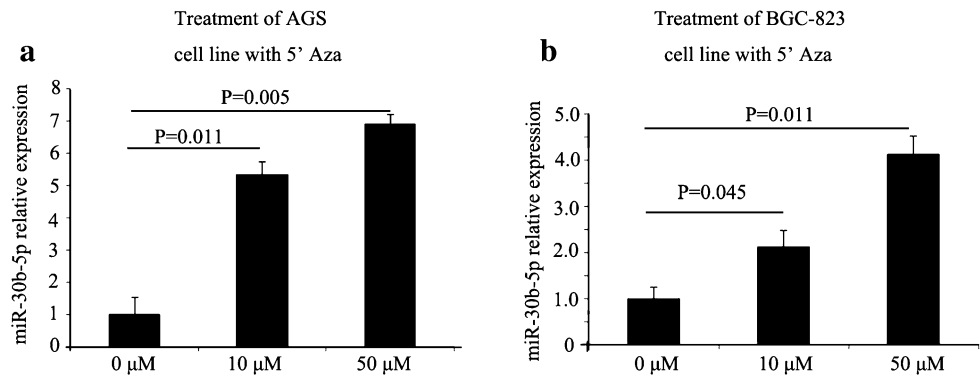
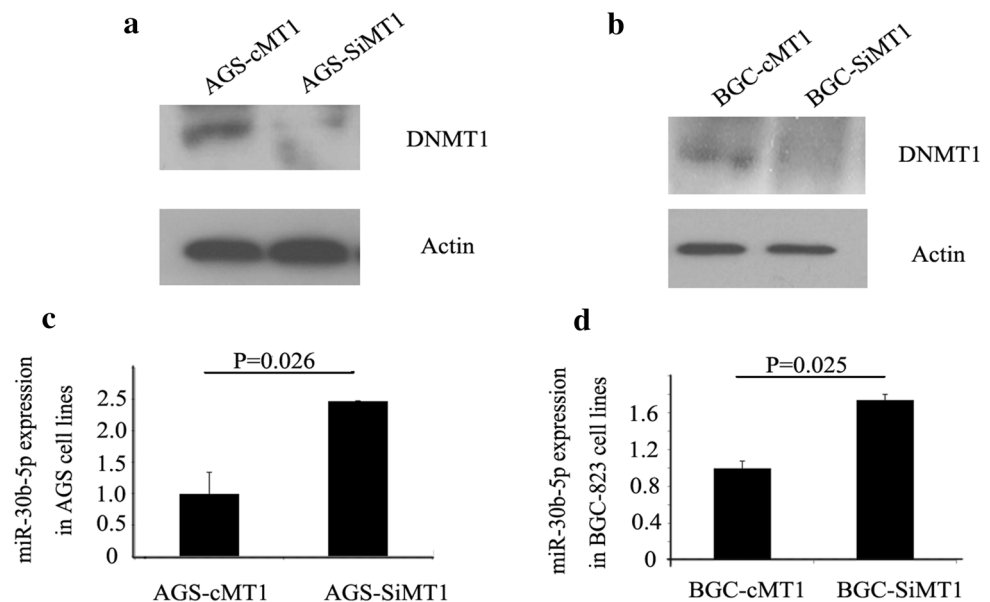


Fig. 4 miR-30b-5p expression increased in DNMT1 knocked-down gastric cancer cell lines. **a** and **c**, DNMT1 expression of protein level was inhibited in AGS and BGC cell lines by western blot detection. **b** and **d**, miR-30b-5p expression increased in AGS-SiMT1 and BGC-SiMT1 compared to scramble SiRNA transfected cell lines AGS-cMT1 and BGC-cMT1



miR-30b-5p was induced after DNMT1 knockdown in gastric cancer cell lines

5'-Aza, a potent inhibitor of DNMT1, is known to induce demethylation and reactivation of silenced genes. 5'-Aza incorporates into DNA forming covalent adducts with cellular DNMT1, thereby depleting the cells from enzyme activity and causing de-methylation of genomic DNA as a secondary consequence [15, 16]. Thus we knocked down DNMT1 protein expression in AGS and BGC-823 cell lines (Fig. 4a, c) to further explore whether miR-30b-5p expression was affected by DNMT1. The results showed that miR-30b-5p expression increased in AGS-SiMT1 and BGC-SiMT1 compared to scramble SiRNA transfected cell lines AGS-cMT1 and BGC-cMT1 (Fig. 4b, $P = 0.026$ and 4d, $P = 0.025$).

Depletion of DNMT1 induced miR-30b-5p expression via demethylation of the promoter

In order to evaluate whether promoter methylation status contribute to induced miR-30b-5p by DNMT1 RNAi, MSP

and BGS were employed to detect methylation status in miR-30b-5p promoter region (Fig. 5). MSP showed that the restoration of miR-30b-5p was associated with its promoter demethylation in AGS-SiMT1 and BGC-SiMT1 cell lines (Fig. 5b, $P < 0.05$ and Fig. 4). Subsequently, a 218 bp (−3164/−2947) fragment that contained part of the miR-30b-5p promoter was chosen for further methylation analysis by bisulfite sequencing (Fig. 5c). Ten individual colonies were sequenced to identify methylated cytosine residues. We found that all 4 CpG sites were methylated in AGS-cMT1. In contrast, different CpG sites (S1, S2 or S4) were unmethylated in the AGS-SiMT1 cell line. The results indicated that DNMT1 knockdown changed the methylation status of certain CpG sites within the promoter of miR-30b-5p gene. Of note, methylation levels of CpG sites at the predicted binding sites for the STATx transcription factors was altered in AGS-cMT1 and AGS-SiMT1 cells. For the STATx transcription factors binding sites, the methylation ratio of the CpG sites was 100 and 60 % in AGS-cMT1 and AGS-SiMT1 cells, respectively. Collectively, these data suggested that miR-30b-5p

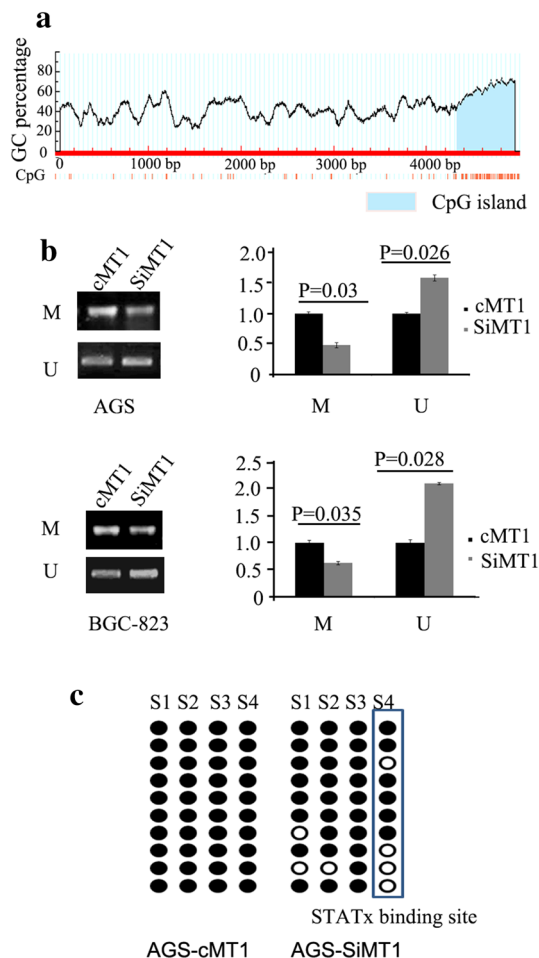


Fig. 5 DNMT1 suppresses miR-30b-5p expression via DNA methylation. **a** CpG island in the miR-30b-5p gene promoter (5,000 bp upstream of TSS). **b** The methylation status of the miR-30b-5p promoter was detected in AGS-SiMT1 and AGS-cMT1 cells by methylation-specific PCR. M, methylation-specific primer amplification; U, unmethylation-specific primer amplification. **c** Mapping of the methylation status of individual CpG sites in the miR-30b-5p promoter by bisulfite genomic sequencing in AGS-SiMT1 and AGS-cMT1 cells. The regions spanning the CpG island with 4 CpG sites were analyzed. Black represents the methylated CpG site; white represents the unmethylated CpG site; each circle represents bisulfite sequencing of 1 CpG site for 10 random clones from AGS-SiMT1 and AGS-cMT1 cells. The boxed regions show the schematic of transcription factor binding sites

expression is regulated in a methylation-dependent manner and that miR-30b-5p may be preferentially methylated by DNMT1.

miR-30b-5p inhibits migration of gastric cancer cell lines

To investigate the role of miR-30b-5p in gastric cancer metastasis, we transfected miR-30b-5p mimic into AGS and BGC-823 cells and performed in vitro cell migration assay. The in vitro migration assay showed that AGS cells

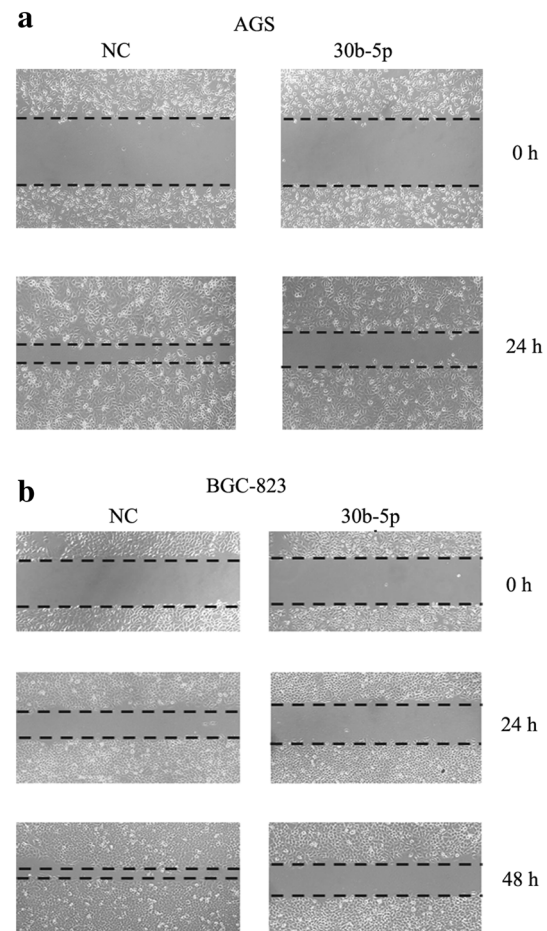


Fig. 6 Migration detection in gastric cancer cell lines. **a** miR-30b-5p overexpression inhibited cell migration in wound healing assays of AGS cell lines at 24 h. **b** miR-30b-5p overexpression inhibited cell migration in wound healing assays of BGC-823 cell lines at 24 h

migrated significantly slower after transfection of miR-30b-5p mimic at 24 h, compared to control cells (Fig. 6a); BGC-823 cells with miR-30b-5p overexpression also markedly inhibited cell migration ability at 24 h after transfection, in contrast to BGC-823 with control (Fig. 6b). Collectively, these results imply that miR-30b-5p inhibits the migration of gastric cancer cell lines.

Discussion

Studies have shown that miR-30b participated in tumorigenesis of several tumor types, including glioma [5], hepatocellular carcinoma [6], primary central nervous system lymphoma (PCNSL) [7], breast cancer patients [8] and Ehrlich ascites tumor [9], however, the expression profile is different varying the tumors. In present study, we detected miR-30b-5p expression in gastric cancer cases. The result demonstrated a markedly attenuation of miR-30b-5p expression in 62.75 % of gastric cancer tissues.

While Fukushima R [17] et al. reported that miR-30b expression was up-regulated in pooled RNA samples of primary gastric cancer from 5 patients. Based on our findings, no consensus was reached may due to the differences in ethnic groups, geographical areas or sample size between individual studies.

Given that more than 90 % of cancer patients mortality is attributed to metastasis [18], miR-30b has been shown to play a role in metastasis and epithelial-to-mesenchymal transition (EMT) [19, 20]. Interestingly, we found down-regulation of miR-30b-5p expression significantly correlated with lymph node metastasis in gastric cancer patients. When we examined the metastatic ability of gastric cancer cells after miR-30b-5p mimic and negative control (NC) treatments, we found enhanced expression of miR-30b-5p can inhibit cancer metastasis. With the help of bioinformatics prediction (Target scan, miRanda, miRWalk and miRDB), Snail1 [19], MMP21 [21, 22], RECK [23], VIM, Bcl-9 [24], Bcl-10 [25] were identified as direct targets of miR-30b-5p. Thus, the function of miR-30b-5p in metastasis and EMT depends on its target regulation. However, future studies are required to validate the association between miR-30b-5p and targets. These results indicate that miR-30b-5p may serve as a potential predictor for prognosis of gastric cancer patients. Further studies with more clinical samples from different regions need to be warranted.

Expression of miR-30b-5p was down-regulated in gastric cancer, but the mechanisms underlying remain unknown. To further explore the reason of miR-30b-5p down-regulation in gastric cancer, we considered an alternative mechanism of miRNA down-regulation by epigenetic transcriptional silencing regulation [26]. We found that the level of miR-30b-5p could be induced with dose-dependent after treated with 5'-Aza. Thus, we suggest that decreased expression of miR-30b-5p in human gastric cancer cell lines are, at least in part, due to methylation of miR-30b-5p promoter.

The epigenetic regulation is essential for mammalian development and its abnormalization leads to a series of diseases including cancer [27–29]. Mammalian DNA is predominantly methylated at the C-5-position of CpG islands by concerted action of three DNA methyltransferases, namely, DNMT1, DNMT3A, and DNMT3B [30]. Several studies have suggested that 5'-Aza restores the expression of silenced genes by selective degradation or the partial influence of DNMT1 [15, 16], moreover, miRNAs were modulated by DNMT1 [31, 32]. We found DNMT1 knock-down induced miR-30b-5p expression. DNMT1 altered methylation status of specific transcription factor binding sites such as STATx. Methylation of STATx binding sites via DNMT1 maybe associated with impaired binding of STATx at the miR-30b-5p promoter and the

inactivation transcription of miR-30b-5p. Up to date, there are no studies on the mechanisms of miR-30b-5p down-regulation in gastric cancer; the present study is the first report showing depletion of DNMT1 restored miR-30b-5p expression by demethylating the promoter in gastric cancer cell lines.

Conclusions

In conclusion, our study demonstrates for the first time that epigenetic mechanism of decreased expression of miR-30b-5p in gastric cancer. DNMT1 could suppress miR-30b-5p expression partly through methylation of its promoter. Diminished miR-30b-5p might be a poor prognostic factor for gastric cancer patients. Overexpression of miR-30b-5p inhibits cell metastasis.

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Conflict of interest We declare that we have no conflict of interest.

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