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Epigenetic silencing of MicroRNA-503 regulates FANCA expression in non-small cell lung cancer cell

Ning Li [a], Fangfang Zhang [b], Suyun Li [a,*], Suzhen Zhou [a]

[a] Department of Respiratory Medicine, The First Affiliated Hospital of Henan University of Traditional Chinese Medicine, Zhengzhou 450000, China
[b] Department of Internal Medicine-Oncology, The First Affiliated Hospital, Zhengzhou University, China

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ABSTRACT

It is reported that MicroRNA-503 (miR-503) regulates cell apoptosis, and thus modulates the resistance of non-small cell lung cancer cells (NSCLC) to cisplatin. However, the exact role of miR-503 in NSCLC remains unknown. In the present study, the level of miR-503 expression in NSCLC was evaluated using realtime PCR, and the DNA methylation status within miR-503 promoter was analyzed by Combined Bisulfite Restriction Analysis (COBRA) or bisulfite-treated DNA sequencing assays (BSP). We found that the expression of miR-503 was significantly decreased in NSCLC tissues compared to normal tissues. A statistically significant inverse association was found between miR-503 methylation status and expression of the miR-503 in tumor tissues ($P < 0.001$), and expression of miR-503 was restored by the demethylating agent 5-aza-2′-deoxycytidine, suggesting that methylation was associated with the transcriptional silencing. Then, we show that miR-503 targets a homologous DNA region in the 3′-UTR region of the Fanconi anemia complementation group A protein (FANCA) gene and represses its expression at the transcriptional level. Taken together, our results suggest that miR-503 regulates the resistance of non-small cell lung cancer cells to cisplatin at least in part by targeting FANCA.

1. Introduction

Lung cancer is a leading cause of cancer mortality worldwide; it accounts for over a million deaths annually and still has a poor prognosis [1]. Non-small cell lung cancer (NSCLC) is the predominant form of lung cancer and consists of 2 major histological subtypes: squamous cell carcinoma and adenocarcinoma [2].

MicroRNAs (miRNAs) are highly conserved, small noncoding RNAs that can downregulate various gene products by translational repression when partially complementary sequences are present in the 3′ untranslated regions (3′-UTR) of the target mRNAs or by directing mRNA degradation. In general, miRNAs play critical role in variety of biological processes including development, differentiation, apoptosis, cell proliferation, metabolism, and immunity. The downregulation of miRNA subsets implies a tumor-suppressor function, which is often observed in tumour development [3,4]. Studies have extensively shown that hypermethylation of gene promoter is a frequent mechanism of silencing and a finding associated with the prognosis of the diseases and response to therapy in patients with cancer.

It is reported that the miR-503 gene is methylated in liver cancer, and it targets FGF2 and VEGFA and inhibits tumor angiogenesis and growth [5,6]. miR-503 also suppressed the proliferation of liver cancer cells by induction of G1 phase arrest through Rb-E2F signaling pathways [7]. miR-503 also suppressed proliferation and cell-cycle progression of endometrioid endometrial cancer by negatively regulating cyclin D1 [8]. These results suggested miR-503 played a tumor-suppressor role in carcinogenesis. It was reported that miR-503 regulates cell apoptosis, at least in part by targeting Bcl-2, and thus modulates the resistance of non-small cell lung cancer cells to cisplatin [9]. However, it remains unclear if miR-503 methylation is also present in NSCLC.

Fanconi anemia (FA) is a severe genetic disorder characterized by bone-marrow failure, genomic instability, and cellular hypersensitivity to crosslinking agents, including cisplatin [10]. It was reported that disrupted FA pathway results in an improved response towards cisplatin treatment in a subcutaneous (s.c.) xenograft transplantation model in scid mice [11]. Fanconi anemia complementation group A protein (FANCA) is one of the Fanconi anemia disease genes, and approximately 66% of Fanconi anemia patients presents with defective FANCA [12]. FANCA recognizes 5′ flap structures and is involved in DNA repair and maintenance

* Corresponding author. Address: Department of Respiratory Medicine, The First Affiliated Hospital of Henan University of Traditional Chinese Medicine, Renmin Road 19, Zhengzhou 450000, China.
E-mail address: lisuyuntcm@163.com (S. Li).

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of replication forks. It was reported that non-small-cell lung cancer cells could be sensitized approximately two- to three-fold for cisplatin after FANCA was inactivated by a dominant-negative form of FANCA [11]. These results suggested that targeting the FANCA may provide a novel strategy for the sensitization of NSCLC for cisplatin. Using computational and expression analyses, FANCA was identified as a candidate target of miR-503. Here, we analyzed DNA methylation in the miR-503 gene and miRNA expression of miR-503 in patients with NSCLC to assess the effect of miR-503 methylation on the expressions of miR-503 as well as a possible target of miR-503 regulation, FANCA. We also figured out that up-regulation of FANCA was inversely associated with the epigenetic silencing of miR-503 in NSCLC, and exogenous expression of miR-503 down-regulated FANCA expression. We also explored the correlation between miR-503 methylation status and clinical features.

2. Material and methods

2.1. Patient samples and cell lines

Tumor samples were collected from surgical specimens from 65 patients with NSCLC at Department of Respiratory Medicine, the First Affiliated Hospital of Henan University of Traditional Chinese Medicine, China. Non-tumor samples from the macroscopic tumor margin were isolated at the same time and used as the matched adjacent non-neoplastic tissues (>3 cm). Tissue samples were collected, immediately snap frozen in liquid nitrogen, and stored at −80 °C until RNA extraction, all samples were obtained their informed consent and with institutional review board approval of the hospital. All patients obtained a confirmed diagnosis of lung carcinoma after resection. The four different established human NSCLC cell lines used in the study (A549, H466, H1650 and H1299) were maintained in DMEM with 10% FBS and 1% Streptomycin/Penicillin antibiotics. A549/cis cells was also used in our study [13].

2.2. Immunohistochemistry

Immunohistochemical staining was performed on routinely formalin-fixed and paraffin-embedded tissue sections using the streptavidin-biotin complex method. Briefly, tissue sections were deparaffinized in xylene and rehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% H_2O_2 for 30 min. The sections were heated in 10 mmol/L citrate buffer (pH 6.0) in a microwave oven to retrieve the antigen. After nonspecific binding was blocked by a 10-min incubation with 10% rabbit serum, the slides were incubated with primary antibodies at 4 °C overnight. The primary antibodies used were all mouse-antihuman monoclonal antibodies against FANCA (1:100 dilution). Negative controls were treated identically but with the primary antibody omitted. Immunoreactivity was evaluated independently by 3 researchers who were blinded to patient outcome. The percentage of positive tumor cells was determined by each observer, and the average of 3 scores was calculated. We randomly selected 10 high-power fields; and counted 1000 cells in each core. When the mean of percentage of positive cells is close to 0% or 100%, the standard deviation (SD) is close to 0, and when the mean is approximately 50% the SD is approximately 5%. Thus, the SD does not increase with the mean. The following categories were used for scoring: intensity of staining, none (0), mild (1), strong (2); percentage of the positive staining, <5% (0), 5–25% (1), 25–50% (2), >50% (3). Combining intensity and percentage staining resulted in the following score: 0–1, negative (−); 2–6, positive (+).

2.3. Combined bisulfite restriction analysis and bisulfite sequencing

Genomic DNA (2 μg) was modified with sodium bisulfite using EpiTect Bisulfite kit (Qiagen). Methylation status was analyzed by bisulfite genomic sequencing and Combined Bisulfite Restriction Analysis (COBRA). The fragment covering 14 CpG sites from miR-530 promoter region was amplified from bisulfite-modified DNA. The primers for BSP were designed using Methprimer software. The primers used were 5′-TTTTAGGGGAAAAAATTGAGAG-3′ (sense) and 5′-ACAATCCCAAAC TTACCCTAAC-3′ (antisense). Amplified bisulfite-sequencing PCR products were cloned into pMD18-T simple vector (Takara). COBRA was carried out by overnight digestion of the PCR product at 60 °C with the restriction enzyme BstUI (New England BioLabs), which has the recognition sequence 5′-CGCG-3′. The resultant DNA fragments were electrophoresed on agarose gels and stained with ethidium bromide. The proportion of methylated (M) versus unmethylated (U) product (digested versus undigested) was quantitated by using a densitometer to determine the density of methylation. The percent methylation was calculated as follows: $M/(M + U) \times 100$.

2.4. Isolation of miRNA and real time RT-PCR

Total RNA was extracted from cell lines and frozen tissue samples using Trizol reagent (Invitrogen) as described. For detecting mature miR-503, reverse transcription was done following the applied Biosystems Taqman MicroRNA Assay protocol, and the specific primers was designed according to the reference. A 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) was used for testing. All the experiments were performed triplicate. The expression of miR-503 was normalized to U6 and was given by: 2^{-dC_t}. dC_t was calculated as C_t (miR-503)−C_t (U6).

2.5. 5-aza-dC treatment

NSCLC cell lines were incubated for 96 h with 1 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC; Sigma) with medium changed every day. Treated cells were harvested for analysis 2 d after the procedure. Cells were suspended in Trizol for RNA isolation.

2.6. Cell transfection

The pre-miR-503 precursor molecule (miR-503) and negative control RNA-oligonucleotides (NC) were gained from Ambion corporation (Ambion, Austin, USA). The day before transfection, cells were seeded in antibiotic free medium. Transfection of miRNAs was carried out using Lipofectamine 2000 in accordance with the manufacturer's procedure (Invitrogen, California, USA). The level of miR-503 mimics expression in the cells was assayed by real-time PCR.

2.7. Luciferase activity assay

A fragment of the wild-type (WT) FANCA 3′UTR containing the predicted miR-503 binding site was amplified by RT-PCR. Restriction sites are bolded and underlined. Site-directed mutagenesis of the miR-503 target site was carried out using Stratagene QuikChange site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). The construct was sequenced and named FAN-UTR-Mut. The pMIR-report luciferase vector was used for the construction of FAN-UTR or FAN-UTR-Mut plasmids (Ambion, USA). HEK 293T cells were cultured in 24-well plates. In each well, 10 ng of phRL-TK renilla luciferase vector (Promega, USA) was co-transfected to normalize transfection efficiency. 500 ng of FAN-UTR or FAN-UTR-Mut plasmids together with 10 nM pre-miR-503 or negative control was also co-transfected. Transfection was done using

Lipofectamine 2000 and Opti-MEM I reduced serum medium (Life Technologies, California, USA). Firefly luciferase activity was measured using the Dual luciferase assay kit (Promega). Normalized relative luciferase activity (RLA) was calculated as the following formula: RLA = [firefly luciferase]/[renilla luciferase].

2.8. Statistical analysis

Fisher's exact tests were performed to evaluate the significance of the differences between the frequencies of miR-503 promoter hypermethylation status of the various tissue categories, *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Methylation analysis of the miR-503 gene in NSCLC tissues

Methylation of the promoter region of miR-503 in NSCLC tissues was determined by COBRA. COBRA analysis is a well-characterized method for DNA methylation studies and has been done largely before. The area of the CpG-rich region around the transcription initiation site of miR-503 gene which spanned 14 CpG sites and the BstUI recognition sequences were shown in Fig. 1A. The promoter methylation of the miR-503 gene was frequent in the NSCLC tissues, with 52 of 65 (80%) samples positive (Table 1). For the paired adjacent non-tumor tissue samples, 13 of 65 samples were positive (20%). Representative examples of the gel analysis of COBRA are shown in Fig. 1B. In this COBRA study, the proportion of methylated (M) vs. unmethylated (U) products (digested vs. undigested) was semiquantitated by using a densitometer. The percent methylation was calculated as follows: $M/(M + U) \times 100$.

3.2. Association of the miR-503 promoter methylation with transcriptional gene silencing

miR-503 mRNA expression levels in each of the NSCLC tissues were assessed using real-time RT-PCR analysis. To elucidate whether the aberrant methylation of miR-503 is associated with loss of miR-503 expression, the samples were divided into 2 different groups, according to percentage of methylation obtained from COBRA, as <50% and >50%. Correlation of the promoter methylation with miR-503 expression showed a significantly lower expression level in the tumors with >50% methylation compared to the tumors with <50% methylation (Fig. S1). These results suggested that the miR-503 promoter methylation correlated with loss of miR-503 expression.

Table 1
Clinical characteristics of NSCLC patients according to hypermethylation status of miR-503.

		miR-503 methylation		*P* value
Group		+	–	
Cancer tissues		52	13	
Histology	ADC	24	7	
	SCC	28	6	$P = 0.425$
Differentiation	Well	3	4	
	Moderate	27	4	
	Poor	22	5	$P = 0.029$
LN metastasis	Yes	33	4	
	No	19	9	$P = 0.035$
Size (cm)	<3	25	9	
	⩾3	27	4	$P = 0.146$
Smoking status	Yes	32	5	
	No	20	8	$P = 0.107$
FANCA expression	Yes	33	3	
	No	19	10	$P = 0.009$

ADC, adenocarcinoma; SCC, squamous cell carcinoma.

To characterize the correlation between miR-503 methylation and clinical features of lung cancer, several clinicopathological characteristics including age, gender, cell differentiation, tumor size, lymph node (LN) metastases and smoking status were compared between patients with or without miR-503 methylation (Table 1). The prevalence of miR-503 methylation was significantly different between normal and cancerous samples ($P < 0.001$). This result indicated that miR-503 methylation was not associated with histology ($P = 0.425$), tumor size ($P = 0.146$), smoking status ($P = 0.107$), patient's age and gender (data not shown). However, miR-503 methylation was significantly correlated with LN metastasis ($P = 0.035$) and poor differentiation ($P = 0.029$).

3.3. Restoration of miR-503 expression by the demethylation agent

To confirm that this loss of expression was because of the miR-503 promoter methylation, 4 NSCLC cell lines were incubated in the presence or absence of the 5-aza-dC, and methylation status and miR-503 mRNA expression were analyzed by bisulfite DNA sequencing and real time RT-PCR, respectively. As shown in Fig. 2A, NSCLC cells had extensive promoter methylation of the miR-503 gene. miR-503 expression was markedly induced after the treatment with 5-Aza-dC (Fig. 2B), implying that the methylation pattern is associated with transcriptional silencing. Results of COBRA confirmed that treatment with 5-aza-dC led to partial

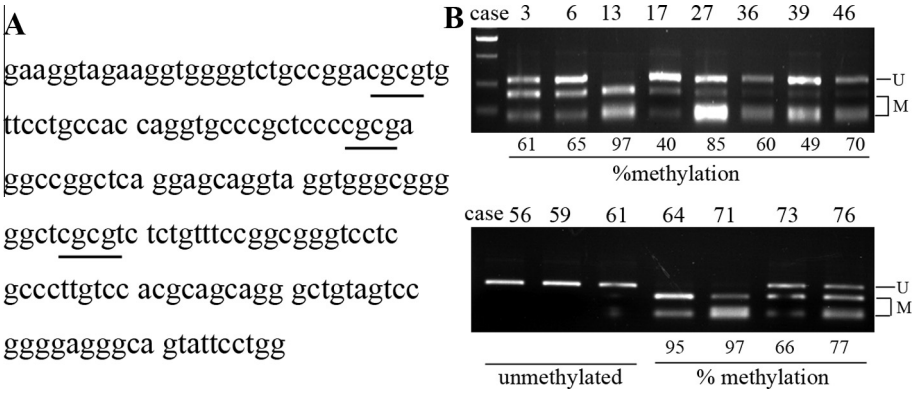

Fig. 1. (A) A map of the CpG islands in relation to the promoter of the miR-503. The cutting sites of BstUI were indicated by underlining. (B) Representative COBRA results of the miR-503 promoter methylation in NSCLC tissues. The PCR products from bisulfite treated DNA were digested with BstU I, which generated digested bands on full or partial digestions. Digested fragments correspond to methylated DNA. Case numbers are shown on top.

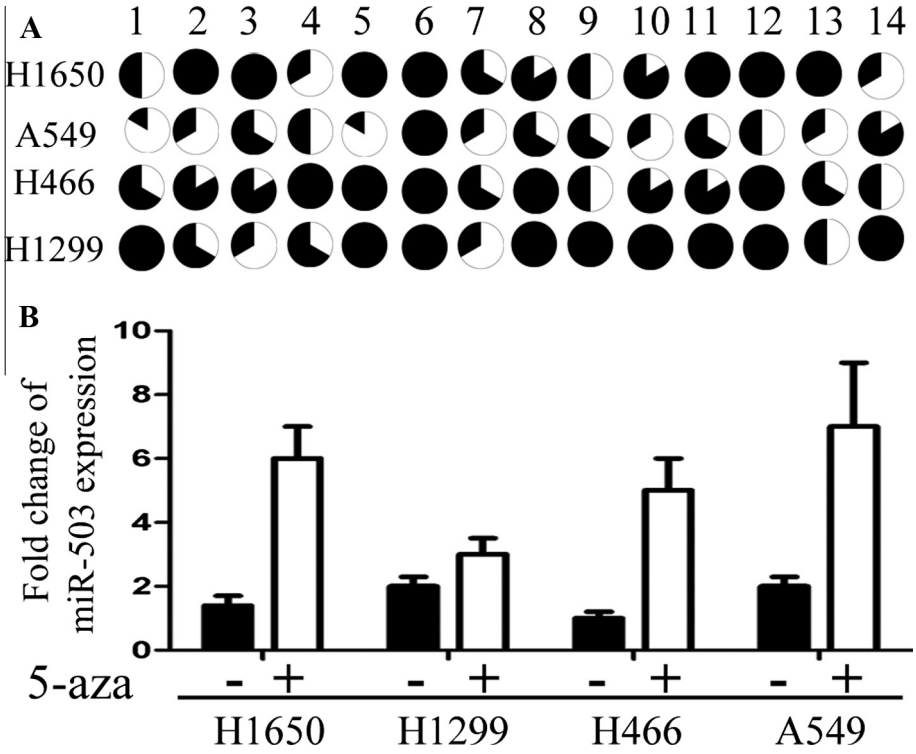

Fig. 2. (A) Methylation patterns of individual bisulfite-sequenced clones of the miR-503 promoter in NSCLC cell lines. Black and white areas represent respectively the percentage of methylated and unmethylated CpG sites out of the colonies sequenced for each case. (B) The expression of miR-503 in NSCLC cell lines treated with or without demethylation agent 5-aza as determined by RT–PCR.

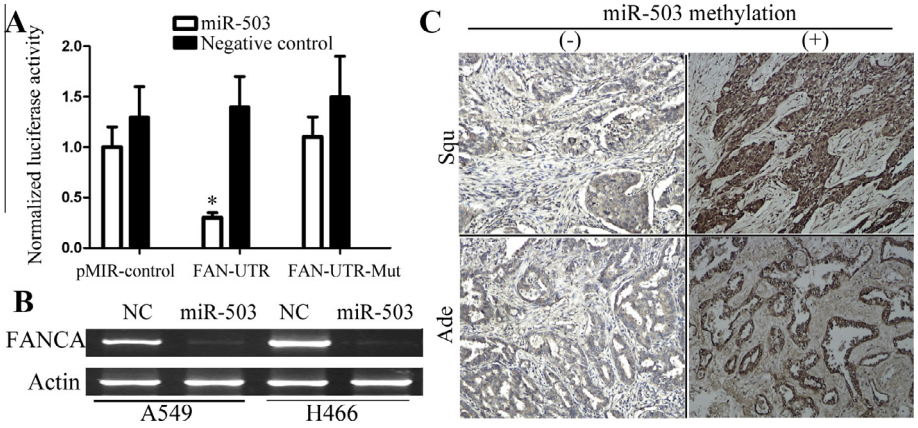

Fig. 3. (A) The firefly luciferase reporter activity is significantly reduced in FAN-UTR vector compared with FAN-UTR-Mut and control ($P < 0.001$). The data were normalized to Renilla luciferase activities. Values are expressed as the mean ± SD of three replicate experiments. (B) Relative levels of FANCA expression in NSCLC cells after transient transfection with the miR-503 precursor molecule or negative control. (C) Inverse relationship between expression of FANCA and the methylation of miR-503 in NSCLC.

demethylation (Fig. S2). These results suggest that the miR-503 gene is methylated and further investigation of the role of miR-503 and it regulation in cancer is warranted.

3.4. FANCA is a candidate target of miR-503

To identify potential gene targets of miR-503 in NSCLC, we applied computational analysis on the down-regulated genes using 3 miRNA target prediction algorithms, miRanda, PicTar, and TargetScan [14,15]. Finally, we chose FANCA as the hypothesized candidate gene. To validate whether miR-503 directly recognizes the 3′UTRs of FANCA mRNA or not, we cloned a sequence with the predicted target sites of miR-503 or a mutated sequence with the predicted target sites to downstream of the pMIR luciferase reporter gene. When the wild-type or mutation-type vector was transfected with miR-503, the luciferase activity of wild-type vector was significantly decreased ($P < 0.001$) compared with mutation-type vector (Fig. 3A). While the wild-type or mutation-type vector was transfected with negative control miRNA, there was no significant difference between the wild-type or mutation-type vector. These data suggest that miR-503 may play a major role in the regulation of FANCA.

To further support FANCA as a miR-503 target, we performed RT-PCR on cells transfected with miR-503 mimic. Our results showed that FANCA protein level was reduced at 24 h post miR-503 transfection (Fig. 3B) as compared with cells transfected with

miRNA negative control. Then, the FANCA in NSCLC tissues were evaluated by analysis immunohistochemistry. As shown in Fig. 3C, up-regulation of FANCA was inversely associated with the hypermethylation of miR-503 in NSCLC tissues ($P = 0.009$) (Table 1).

3.5. Long-term cisplatin exposure promotes methylation of miR-503 gene in human NSCLC Cells

It was reported that the expression of miR-503 was decreased in the cisplatin-resistant non-small cell lung cancer cells, A549/cis, compared with the parental A549 cells. The overexpression of miR-503 sensitized the A549/cis cells to cisplatin, whereas the inhibition of miR-503 in the A549 cells increased resistance to cisplatin, suggesting that miR-503 may be associated with cisplatin resistance [9]. We hypothesized that miR-503 would be methylated in NSCLC cells that are chronically exposed to cisplatin in a methylation-dependent manner. To test this hypothesis, cisplatin resistant cell lines were established, and the changes of miR-503 were detected by RT-PCR. miR-503 was downregulated in cisplatin resistant cell lines compared to the parental cells (Fig. 4A). The percentage of methylation on methylated CpG sites was calculated in A549/cis cells by COBRA. 98% of CpGs were methylated in A549/cis cells, whereas 60% of CpGs were methylated in A549 cells (Fig. 4B). BSP assay was also performed to verify the result of COBRA. The area of the CpG-rich region around the transcription initiation site of miR-503 gene was sequenced. As shown in Fig. 4C, the methylated CpG dinucleotides were detected in A549/cis cells, and they were partially methylated in A549 cells. The expression of DNMT1 was also determined in cisplatin resistant cells and the parental cells. Our result found that the level of DNMT1 was enhanced in cisplatin resistant cells when compared to parental cells, suggesting that increased expression of DNMT1 would be implicated in methylation of miR-503 (Fig. S3).

4. Discussion

Recent demonstration of differential expression of microRNAs (miR) and their target mRNAs in cancer, and the function of some miRs as oncogenes or tumor suppressors has spurred considerable interest in elucidating their role in tumorigenesis [5,6]. It was reported that miR-503 methylation was detected in liver cancer, and the expression of miR-503 was affected by the methylation [7]. It was reported that miR-503 regulates cell apoptosis and thus modulates the resistance of non-small cell lung cancer cells to cisplatin.

In the present, the miR-503 methylation status remains unclear in NSCLC. Here, we analyzed DNA methylation in the miR-503 gene and miRNA expression of miR-503 in 65 patients with NSCLC. We found the promoter methylation of the miR-503 gene was frequent in the NSCLC tissues, with 52 of 65 (80%) tumor samples positive, and 13 of 65 (20%) the paired adjacent non-tumor tissue samples positive, indicating that it is a comment event in NSCLC. The results showed that miR-503 was significantly down-regulated in tumor tissues compared to normal tissues. Correlation of the promoter methylation with miR-503 expression showed a significantly lower expression level in the tumors with hypermethylation compared to the tumors with low methylation. These results suggested that the miR-503 promoter methylation correlated with loss of miR-503 expression. We also found that there was no significant correlation between hypermethylation of miR-503 and vascular invasion, but not some unfavorable variables, such as histological grade and lymph note metastasis.

Analysis of NSCLC cells indicated that miR-503 expression is either undetectable or in low abundance in several lines; the miR-503 promoter methylation correlated with loss of miR-503 expression. miR-503 expression was restored in cells cultured on the DNA demethylating agent 5-aza-2′-deoxycytidine.

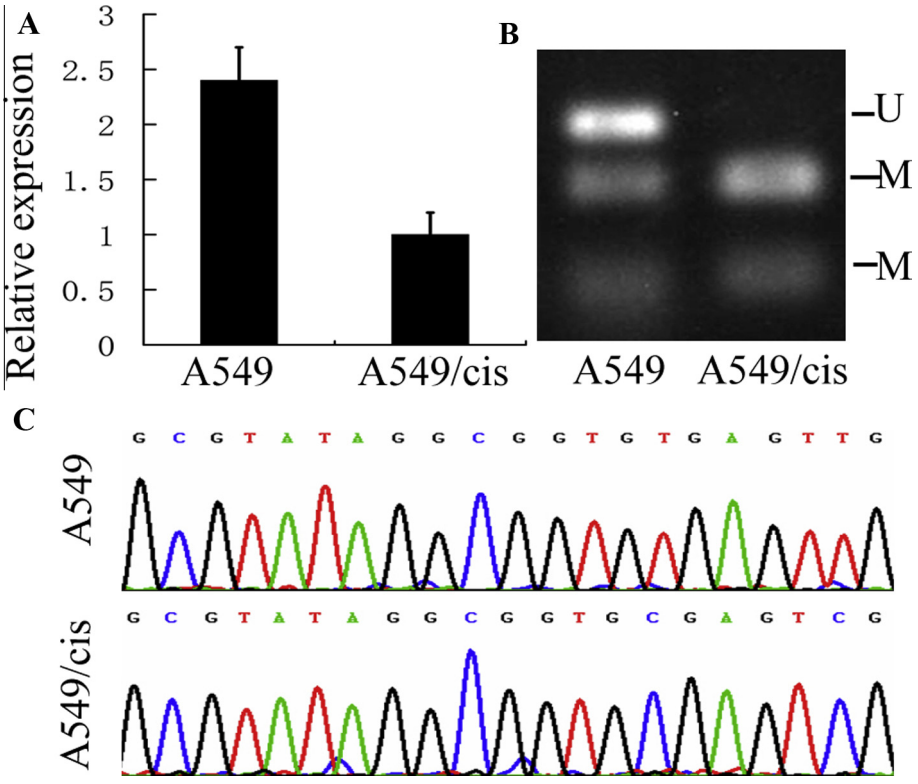

Fig. 4. (A) RT-PCR showing the differential expression of miR-503 gene between the cisplatin resistant cells and parental cells. (B) COBRA data showing differential methylation states in promoter regions of the miR-503 gene between the cisplatin resistant cells and parental cells. (C) An illustrative fragment of the sequencing electropherogram is shown for A549 and A549/cis cells.

Using computational and expression analyses, FANCA was identified as a candidate target of miR-503. It was reported that a panel of cell lines, including non-small-cell lung cancer cells, could be sensitized approximately two- to three-fold for cisplatin after FANCA was inactivated by a dominant-negative form of FANCA [11]. The overexpression of miR-503 sensitized the A549/CDDP cells to cisplatin, whereas the inhibition of miR-503 in the A549 cells increased resistance to cisplatin [9]. We hypothesized that miR-503 regulates the resistance of non-small cell lung cancer cells to cisplatin at least in part by targeting FANCA.

Transfection of miR-503 resulted in down-regulation of FANCA. Reporter assay with 3′untranslated region of FANCA cloned downstream of the luciferase gene showed reduced luciferase activity in the presence of miR-503, providing strong evidence that miR-503 is a direct regulator of FANCA. Expression analysis further revealed that up-regulation of FANCA was inversely associated with the downregulation of miR-503 in NSCLC tissues.

It was reported that the expression of miR-503 was decreased in the cisplatin-resistant non-small cell lung cancer cells, A549/cis, compared with the parental A549 cells. We hypothesized that miR-503 would be methylated in NSCLC cells that are chronically exposed to cisplatin in a methylation-dependent manner. We found that percentage of the methylated CpG dinucleotides was increased in A549/cis cells when compared to A549 cells. DNMT1 plays an important role in DNA methylation and increased DNMT1 expression was also associated with increased resistance to commonly used drugs [16,17]. Our result found that the level of DNMT1 was enhanced in cisplatin resistant cells when compared to parental cells, suggesting that increased expression of DNMT1 would be implicated in methylation of miR-503.

Taken together, epigenetic inactivation of miR-503 is a common event in NSCLC. miR-503 regulates the resistance of non-small cell lung cancer cells to cisplatin at least in part by targeting FANCA, suggesting targeting the FANCA may provide a novel strategy for the sensitization of NSCLC for cisplatin.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.103.

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