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Original Article

The FENDRR/miR-214-3P/TET2 axis affects cell malignant activity via RASSF1A methylation in gastric cancer

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Abstract: To explore the effect of fetal-lethal non-coding developmental regulatory RNA (FENDRR) in the initiation and progression of gastric cancer (GC). We detected the levels of FENDRR, microRNA-214-3p (miR-214-3p), and ten-eleven-translocation (TET2) in GC tissues and GC cell lines. In addition, we evaluated the location of FENDRR in GC cell lines by fluorescence in situ hybridization (FISH). Cell proliferation and apoptosis were measured by CCK-8 and Hoechst staining assays. Methylation-specific PCR assay (MSP) was used to evaluate the methylation status of ras-association domain family 1A (RASSF1A). We also observed the direct binding of miR-214-3p on FENDRR by dual-luciferase activity assay in GC cells. FENDRR and TET2 expressions were significantly down-regulated and miR-214-3p was up-regulated in gastric cancer tissues compared to adjacent unaffected tissues. In addition, RASSF1A was hypermethylated in gastric cancer tissues compared to adjacent tissues. The expressions of all the three indicators were influenced by differentiation of tumor, TNM stage of tumors, and lymph node metastasis in patients with GC. A gastric cancer cell line with low FENDRR expression compared to a high FENDRR expressing cell line showed again increased miR-214-3p expression, decreased TET2 and RASSF1A expressions, and RASSF1A hypermethylation, resulting in decreased apoptosis and increased proliferation. Furthermore, we observed a negative correlation between FENDRR and miR-214-3p in GC. The FENDRR/miR-214-3P/TET2 axis plays a critical role in GC progress via methylation of RASSF1A.

Keywords: FENDRR, miR-214-3p, TET2, RASSF1A, gastric cancer, proliferation, methylation

Introduction

Gastric cancer (GC) is a serious disease with an extremely poor prognosis [1, 2]. Recent advances in medicine have decreased GC-associated morbidity in many developed countries [3, 4]. Nonetheless, it is still the fourth most prevalent cancer and the second leading cause of cancer-related death worldwide [5]. The incidence of GC remains high in developing countries, especially in East Asia [6, 7]. During DNA replication, DNA methylation is the most common DNA modification to occur in genome. Aberrant DNA methylation disrupts expression and function of multiple genes involved in regulation of various cancers [8-10]. Recent studies report that DNA methylation of several genes, including N-myc downstream-regulated gene 2 (NDRG2), nasopharyngeal carcinoma-associated gene 6 (NGX6), and Ras-association domain family 1A (RASSF1A), are involved in tumorigenesis of GC [11-13]. Of these genes, RASSF1A (a tumor suppressor gene), is one of the most frequent epigenetically inactivated genes in a wide range of cancers [14, 15]. Another gene, TET2, an important member of the TET (ten-eleven-translocation) family, is involved in promoter demethylation of a diverse range of genes, including RASSF1A [16].

The competing endogenous RNA (ceRNA) network comprises of long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and messenger RNAs (mRNAs) that are involved in the tumorigenesis of GC. Both long non-coding RNAs (lncRNAs) and miRNAs are important regulators of gene expression at transcription, post-transcription, and epigenetic levels [17-

19], and are involved in several biological processes [20]. LncRNAs are a major subgroup of non-coding RNAs (ncRNAs) with more than 200 nucleotides in length [21]. Fetal-lethal non-coding developmental regulatory RNA (FENDRR) is a lncRNA that binds to polycomb repressive complex 2 (PRC2) and TrxG/MLL complexes [22]. Previous studies demonstrated that FENDRR expression is indispensable to development of heart and body wall, and that abnormally expressed FENDRR is found in various cancers, such as GC, osteosarcoma, and lung cancer [23-25]. Moreover, FENDRR epigenetically regulates gene expression by targeting tpromoter of its target gene [22]. The miRNA, miR-214-3p is involved in several biological processes, including cell proliferation, apoptosis, invasion, and metastasis in human GC [26, 27]. Xin R *et al* reported that miR-214-3p promotes peritoneal metastasis by negatively regulating phosphatase and tensin homolog deleted on chromosome ten (PTEN) in GC [26]. In addition, TET2 was shown to be repressed by miR-125b. However, it is unclear whether miR-214-3p is involved in the regulatory process of TET2.

We wanted to address that lncRNAs can act as endogenous miRNA sponges, as a part of the ceRNA network in human GC. Some examples include lncRNA-H19 regulating miR-141, lncRNA-HOTAIR regulating miR-331-3p, and lncRNA-AC130710 regulating miR-129-5p [28-30]. In our study, the major research purpose was to explore if FENDRR interacted with miR-214-3p which regulated tumor formation in GC. We assessed the expression levels of FENDRR, miR-214-3p, and TET2 in GC tissues and GC cells. In addition, we found that FENDRR affects tumor growth by upregulating RASSF1A expression via miR-214-3p.

Materials and methods

Cell lines and tissues

Two human gastric cancer cell lines MGC803 and BGC823, and a human embryonic kidney cell line HEK-293T were obtained from the American Type Culture Collection (ATCC). MGC-803 cells were used for FENDRR overexpression and BGC230 cells were used for FENDRR inhibition. These cells were maintained in RPMI 1640 medium supplemented with 100 U/ml penicillin/streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS) (GIBCO/BRL). Cells were grown at 37°C in an atmosphere of 5% CO_2 and 95% O_2. Gastric cancer (GC) tissues and adjacent normal tissues (n = 100, respectively) were obtained from patients receiving treatment in the Xiangya Hospital of Central South University. Patient clinical information, such as age, sex, differentiation status and TNM stage of tumor, were collected. Half of each specimen was fixed in 4% paraformaldehyde and embedded in paraffin for histological sectioning. The other half of the tissue was immediately transferred into liquid nitrogen, and stored at -80°C. Informed consents were obtained from the participating patients, and the ethics agreement was approved by the Ethics Committee of the Xiangya Hospital.

FENDRR overexpression or knockdown

For FENDRR overexpression, the cDNA of FENDRR was cloned by primers supplemented with 5' BamHI and 3' NotI restriction sites, and PCR products were incubated with two restrictive endonucleases: BamHI and NotI overnight at 4°C. The PCR products were then subcloned into the pcDNA 3.1 vector and were digested with BamHI and NotI. Finally, the established plasmid pcDNA3.1-FENDRR or pcDNA3.1 (negative control) was transfected into MGC803 cells via lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer.

For FENDRR knockdown, small interfering RNA (si-FENDRR) and si-scrambled (negative control) were designed and obtained from GenePharma Co., Ltd (China). BGC803 cells were treated with si-FENDRR or si-scrambled (30 nmol/L) by using lipofectamine 2000 (Invitrogen).

The effects of FENDRR overexpression or knockdown in cell lines were evaluated after the cells were transfected for 48 h.

MiR-214-3p overexpression or knockdown

RNA mimics and inhibitor for miR-214-3p, and the corresponding negative control (NC), were designed and obtained from GenePharma Co., Ltd (China). MGC803 cells were treated with NC, mimics or inhibitor of miR-214-3p by Lipofectamine 2000 (Invitrogen) transfection reagent, as per manufacturer's protocol.

Table 1. The sequence of corresponding primers

Gene	Sequence of Primers (5'-3')
FENDRR F	AGACAAAAACTCACTGCCCA
FENDRR R	TGATGTTCTCCTTCTTGCCTC
miR-214-3p RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACTGCCTG
miR-214-3p F	ACACTCCAGCTGGGACAGCAGGCACAGACAGGC
TET2 F	TAAGTGGGTGGTTCGCAGA
TET2 R	TCCGAGTAGAGTTTGTCAGCC
RASSF1A F	CGCCGCACTTCCTTTTAC
RASSF1A R	GGCTGCTCATCATCCAACA
GAPDH-F	TGTTCGTCATGGGTGTGAAC
GAPDH-R	ATGGCATGGACTGTGGTCAT
U6 F	CGCTTCGGCAGCACATATACTA
U6 R	CGCTTCACGAATTTGCGTGTCA

TET2 knockdown

For the TET2 knockdown assay, siRNA for TET2 (si-TET2) and the corresponding negative control (siRNA-NC) were designed and purchased from GenePharma Co., Ltd (China). MGC803 cells were treated with si-TET2 or siRNA-NC by using lipofectamine 2000 (Invitrogen) according to manufacturer's instruction.

Quantitative real-time PCR (qRT-PCR)

Total RNA obtained from GC tissues or cell lines was extracted using TRIzol reagent (Invitrogen), and was then transcribed by SuperScript III Reverse Transcriptase (Invitrogen), as per the manufacturer's protocol. Quantitative real-time PCR was performed by SYBR® Premix Ex Taq™ II (TaKaRa). U6 was used as an internal control for miRNA, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for both lncRNA and mRNA. The sequences of primers that were used are shown in **Table 1**.

RNA fluorescence in-situ hybridization (FISH)

MGC803 and BGC823 cells grown on a coverslip were fixed by 4% PFA for 30 min at room temperature and washed with PBS for three times. Then cells were immersed into PBS which supplemented with 0.5% Triton X-100 on ice for 15 min, then washed with PBS again for three times. After rinsed in 2 × SSC, hybridization was performed at 37°C in hybridization buffer with 10 nmol/L 5' TYE-665 labelled locked nucleic acid (LNA) detection probe (Exiqon, Woburn, MA) against FENDRR for 18 h. Then, cells were incubated with DAPI (1:10000, ab104139, Abcam) and wash with 2 × SSC for three times.

CCK-8 assay

In vitro cell proliferation was assessed by the Cell Counting Kit-8 (CCK-8) assay, according to the instructions of manufacturer. Briefly, GC cell lines (MGC803 or BGC823) were transfected with either FENDRR/si-FENDRR or miR-214-3p mimics/inhibitor, and were incubated overnight in 96-well plates at a concentration of 1×10^3 cells/well. The CCK-8 solution (10 μl) was added to each well, and incubated for 2 h. The absorbance at 450 nm wavelength was determined at 0, 24, 48, and 72 h after treatment with the corresponding agents.

Hoechst staining

MGC803 or BGC823 cells were seeded in 6-well plates, and Hoechst 33342 (10 μg/mL, Sigma) was added to the culture medium of each well for at least 15 min at 37°C. After two washes with PBS, stained cells were observed using a laser scanning confocal (Leica) microscope at 365 nm. Cells were considered apoptotic if the nuclei were brighter or showed condensed chromatin and nuclear fragmentation.

Transwell

Transwell assay was conducted to measure the invasion and migration abilities of GC cells. For invasion potential assessments, GC cells (2×10^4) were seeded into the upper chamber of a transwell system which was previously coated with 40 μl matrigel at 37°C for 2 h. Then cells were allowed to migrate through membranes for 24 h at 37°C. Afterwards, cells in the upper surfaces of the chambers were completely removed and the lower surfaces of the membranes were stained with 0.5% (w/v) crystal violet. For migration potential assessments, the assay was performed as described above only without matrigel coated.

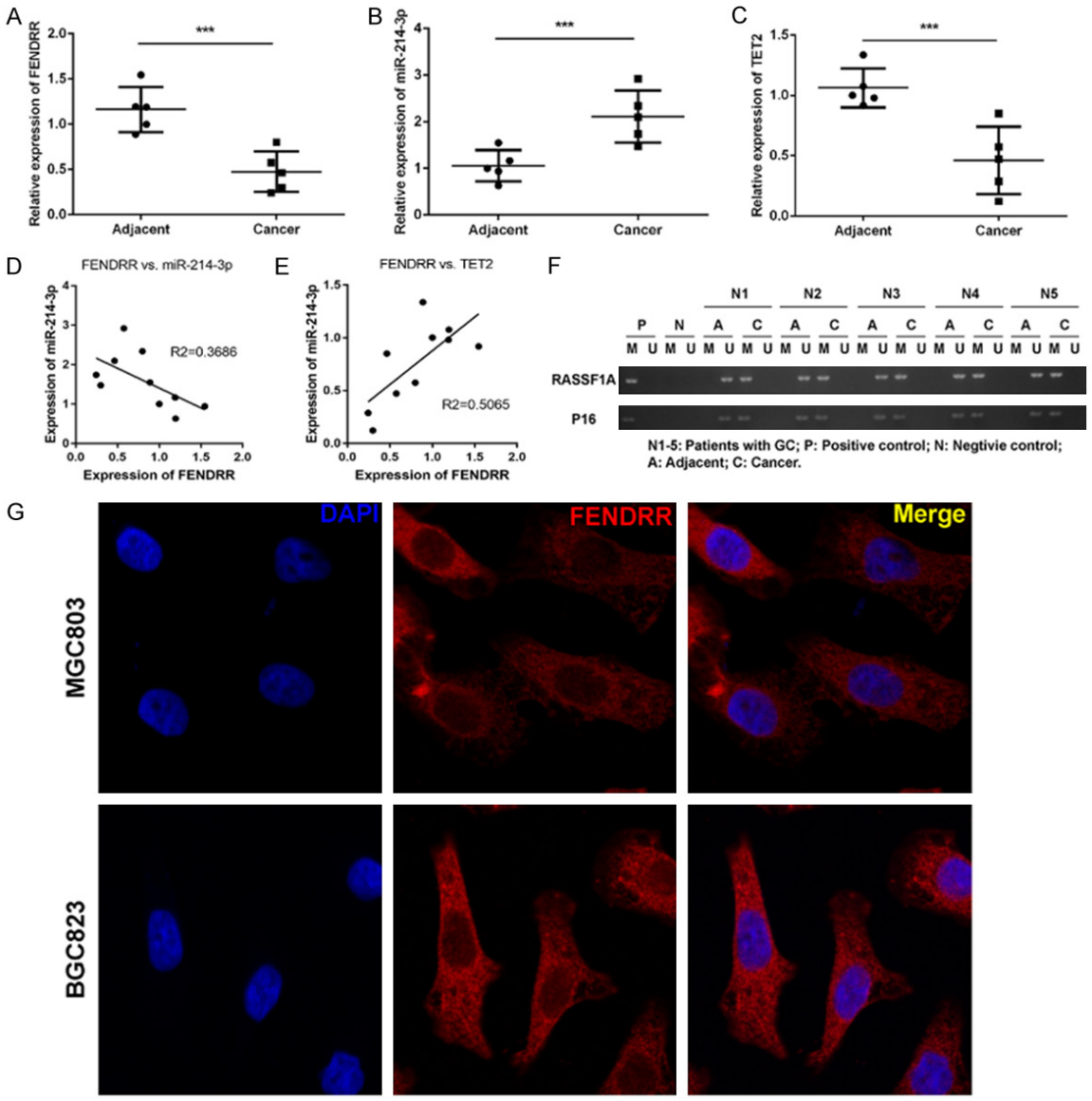

Figure 1. Expression levels of FENDRR, miR-214-3p, and TET2, and methylation levels of RASSF1A in GC and adjacent normal tissues. QRT-PCR assay was used to detect the expression level of (A) FENDRR, (B) miR-214-3p, and (C) TET2 in both GC and adjacent normal tissues. (D) Relationship between expression of FENDRR and miR-214-3p; (E) Relationship between expression of FENDRR and (F) MSP assay was performed to examine the methylation status of P16 and RASSF1A in GC tissues. (G) RNA fluorescence in situ hybridization (FISH) was carried out to determine the intracellular localization of FENDRR in GC cell lines. DAPI was used to stain nuclear. "M" represents methylated and "D" represents unmethylated.

Western blot analysis

Treated cells were lysed with radio immunoprecipitation assay (RIPA) buffer (Beyotime), containing proteinase inhibitors. After centrifugation at 12,000 rpm for 15 min at 4°C, the supernatant was collected and the protein concentrations were determined by a BCA kit (Beyotime). Total protein was separated by 10% SDS-PAGE and then transferred to PVDF membranes (Millipore). After blocking with 5% low-fat dried milk for 2 h at room temperature, membranes were incubated overnight with the appropriate primary antibodies: rabbit polyclonal anti-TET2 (1:500, Santa Cruz), mouse monoclonal anti-human RASSF1A (1:1000, eBioscience), or rabbit polyclonal anti-P16 (1:500, Santa Cruz). The membranes were then washed in PBS for three times, and incubated with the corresponding horseradish peroxi-

Table 2. Correlation between FENDRR, miR-214-3p, TET2 expression level and clinicopathological parameters

Clinicopathological parameters	N	FENDRR expression			*P* value	miR-214-3p expression			*P* value	TET2 expression			*P* value
		Low	High	Positive rate		Low	High	Positive rate		Low	High	Positive rate	
Gender													
Male	59	29	30	50.85%	0.067	23	36	61.02%	0.5384	32	27	45.76%	0.2287
Female	41	28	13	31.71%		19	22	53.66%		17	24	58.54%	
Age (years)													
≥ 50	54	28	26	48.15%	0.154	39	15	27.78%	0.6644	27	27	50.00%	1.000
< 50	46	31	15	32.61%		31	15	32.61%		23	23	50.00%	
TNM stage													
I/II	63	35	40	63.49%	0.045	32	31	49.21%	0.0117	34	29	46.03%	0.035
III/IV	37	28	9	24.32%		9	28	75.68%		28	9	24.32%	
Lymph node metastasis													
YES	65	23	42	64.62%	0.0214	18	47	72.31%	0.0283	47	18	27.69%	0.0103
NO	35	21	14	40.00%		18	17	48.57%		16	19	54.29%	

dase-conjugated secondary antibodies for 2 h at room temperature. GAPDH was used as an internal control, and signals were detected by enhanced chemiluminescent reagents.

Methylation-specific PCR (MSP)

The RASSF1A methylation statuses of GC tissues and cells ewre determined by a methylation-specific PCR (MSP) assay. Genomic DNA of tissues and cells were isolated by the Get pure DNA Kit (Dojindo Molecular Technologies) by following the instruction of manufacturer. Genomic DNA is treated with bisulfate. Bisulfate converts cytosine into a uracil base, but has no effect on methylated cytosine. We thus used the MSP assay to differentiate between methylated and unmethylated DNA in a HotStar TaqE PCR machine (TaKaRa). The primer sequences of methylated RASSF1A were 5'-GGG TTT TGC GAG AGC GCG-3' and 5'-GAT AAC AAA CGC GAA CCG-3', and the primers sequences of unmethylated RASSF1A were 5'-GGG GTT TTG TGA GAG TGTG-3' and 5'-ACT AAC AAA CAC AAA CCA AAC-3' (Gene Bank Accession: AC002481).

Luciferase reporter transfection and dual luciferase activity assay

Bioinformatics predicted a binding site for miR-214-3p in the FENDRR sequence (http://starbase.sysu.edu.cn/index.php), and two binding sites in the TET2 3'UTR (http://www.targetscan.org/vert_71/). A wild-type FENDRR (FENDRR-WT, with a putative miR-214-3p target binding sequence) and a mutant FENDRR (FENDRR-Mut, with mutations in individual bases in the binding site) were synthesized and cloned into the luciferase gene in the pmirGLO luciferase vectors (Promega). We constructed a wild-type TET2 (TET2-WT, position 2181-2188 of 3'UTR) and a mutant (TET2-Mut, with mutations in individual bases in the binding site) with psi-CHECK-2-derived reporter vectors (Promega).

293T cells were seeded into 96-well plates at a density of 2×10^4 cells per well, transfected with 200 ng of FENDRR-WT/FENDRR-Mut or TET2-WT/TET2-Mut in the presence of 50 nM of miR-214-3p mimics or NC by using Lipofectamine 2000 (Invitrogen). Forty-eight hours post-transfection, the cells were harvested. Then, the firefly and renilla luciferase activities were determined by the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activity was normalized to Firefly luciferase activity.

Statistical analysis

All data were presented as mean ± SD. Linear regression analysis of FENDRR vs. miR-214-3p and FENDRR vs. TET2 and statistical analysis was conducted using GraphPad Prism 7. $P < 0.05$ was considered statistically significant.

Results

FENDRR and TET2 expression were downregulated, and miR-214-3p expression was upregulated in GC tissues

LncRNAs have been shown to directly interact with miRNAs to regulate tumorigenesis in

human GC. To investigate the biological functions of FENDRR, miR-214-3p, and TET2 in pathogenesis of GC, we first examined their mRNA expressions by qRT-PCR in cancer tissues. We found that the expression of FENDRR and TET2 were significantly decreased, while the expression of miR-214-3p was remarkably increased in GC tissues compared with adjacent normal tissues (**Figure 1A-C**). As shown in **Figure 1D**, the expression of FENDRR showed negative correlation to miR-214-3p, while presented positive correlation to TET2 (**Figure 1E**). Secondly, we found that the expressions of FENDRR, miR-214-3p, and TET2 were influenced by clinical features of GC (**Table 2**), including differentiation of tumor, TNM stages, and lymph node metastasis, though the correlations were not significant. Moreover, we measured the methylation level of RASSF1A in GC tissues by a methylation-specific PCR (MSP) assay. Results from the MSP assay demonstrated that the methylation levels of RASSF1A were higher in GC tissues than adjacent normal tissues (**Figure 1D**). In addition, to get insight into the functions of FENDRR in GC, we evaluated the intracellular location of FENDRR in two GC cell lines: BGC823 and MGC803 by RNA fluorescence in situ hybridization (FISH). The results showed that FENDRR was predominantly localized in the cytosol of BGC823 and MGC803 (**Figure 1E**).

FENDRR downregulated the expression of miR-214-3p and upregulated the expressions of TET2 and RASSF1A in GC cell lines

To explore the specific role of FENDRR in GC, we established a cell line that overexpressed FENDRR (in MGC803 cells), and a cell line that blocked FENDRR expression (in BGC823 cells). To validate our results, we used qRT-PCR to measure the expressions of FENDRR in MGC803 and BGC823 cells. We found that FENDRR expression was significantly up-regulated in FENDRR-overexpressing MGC803 cells, and FENDRR expression was down-regulated in FENDRR-blocking BGC823 cells compared with their control cells transfected with either pcDNA 3.1 or si-scrambled, respectively (**Figure 2A**). Consistent with these results, miR-214-3p expression level was significant decreased in FENDRR-overexpressing MGC803 cells, and drastically increased in FENDRR-silenced BGC823 cells (**Figure 2B**). Results from qRT-PCR and Western blots showed that the expressions of TET2 and RASSF1A were significantly influenced by FENDRR overexpression or down-regulation in both MGC803 and BGC823 cells (**Figure 2C-E**). We hypothesized that the change in RASSF1A expression was due to DNA methylation. To test this, we used MSP analysis to measure the methylation status of RASSF1A. Our results suggested that RASSF1A had lower methylation levels in MGC803 cells transfected with FENDRR plasmid than those cells transfected with pcDNA 3.1, whereas RASSF1A had higher methylation levels in BGC823 cells treated with si-FENDRR than those cells treated with si-scrambled (**Figure 2F**). These data indicated that FENDRR could inhibit the expression level of miR-214-3p, and promote TET2 expression. In addition, FENDRR increased the expression of RASSF1A by inhibiting the methylation levels of RASSF1A.

FENDRR inhibited cell proliferation, promoted cell apoptosis, and suppressed cell invasion and migration in GC cell lines

We used CCK-8 and the Hoechst staining assays to observe the effect of FENDRR overexpression or knockdown on cell proliferation and apoptosis in MGC803 and BGC823 cells. Cell proliferation in MGC803 cells transfected with FENDRR plasmid, was significantly inhibited, compared with cells transfected with control plasmid (pcDNA 3.1), whereas cell proliferation in BGC823 cells treated with si-FENDRR was significant increased, compared with cells treated with si-scrambled (**Figure 2G**). In addition, overexpression of FENDRR in MGC803 cells promoted cell apoptosis, and knockdown of FENDRR in BGC823 cells inhibited cell apoptosis (**Figure 2H**). Regarding the function of FENDRR on the mobility potential of GC cells, overexpression of FENDRR inhibited the invasion and migration ability of MGC803 cells (**Figure 2I**) while inhibition of FENDRR induced the invasion and migration ability of BGC823 cells (**Figure 2J**).

MiR-214-3p down-regulated the expressions of FENDRR, TET2, and RASSF1A

To investigate the role of miR-214-3p in GC, we treated MGC803 cells with either miR-214-3p mimics or inhibitor. The effect of miR-214-3p overexpression or interference was evaluated by qRT-PCR. We found that the mRNA expression of FENDRR was significantly increased in

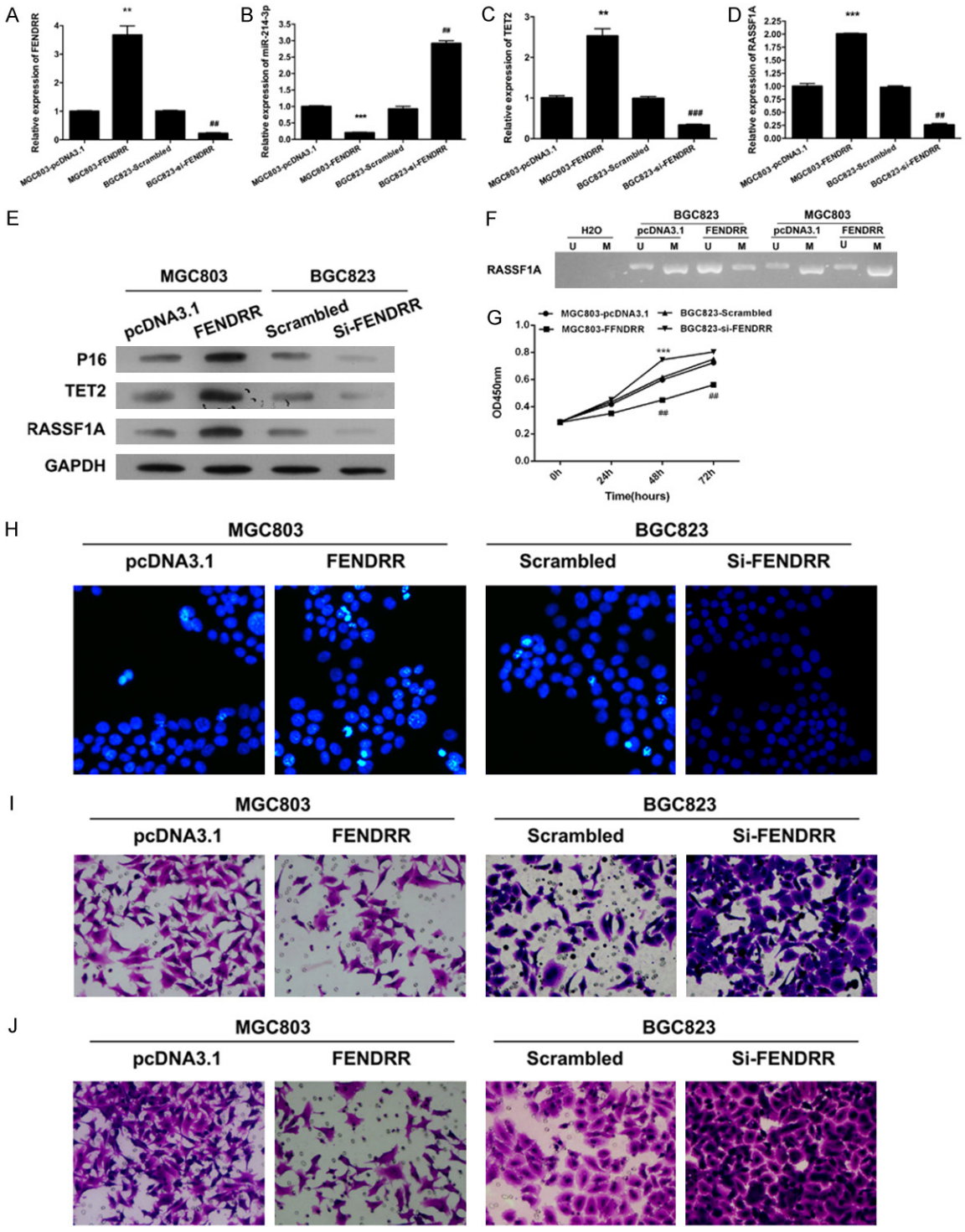

Figure 2. Effects of FENDRR overexpression or knockdown on gene methylation, cell proliferation and apoptosis in GC cell lines. The expression levels of FENDRR (A) miR-214-3p, (B) TET2, and (C) RASSF1A (D) were measured by qRT-PCR in FENDRR-overexpressing MGC803 cells and FENDRR-blocked BGC823 cells. (E) The expression levels of P16, TET2, and RASSF1A were detected by Western blot assay. (F) The methylation status of RASSF1A was detected by MSP. (G) CCK-8 assay was performed to measure the cell proliferation. (H) Hoechst staining was used to detect cell apoptosis in FENDRR-overexpressing MGC803 cells and FENDRR-blocked BGC823 cells. Data represented as means ± SD from three independent experiments. (I) Transwell assay was employed to assess the invasion abilities of FENDRR-overexpressing MGC803 cells and FENDRR-blocked BGC823 cells. (J) Transwell assay was employed to assess the migration abilities of FENDRR-overexpressing MGC803 cells and FENDRR-blocked BGC823 cells. *, $P < 0.05$ vs. MGC803-pcDNA3.1; #, $P < 0.05$ vs. BGC823-scrambled.

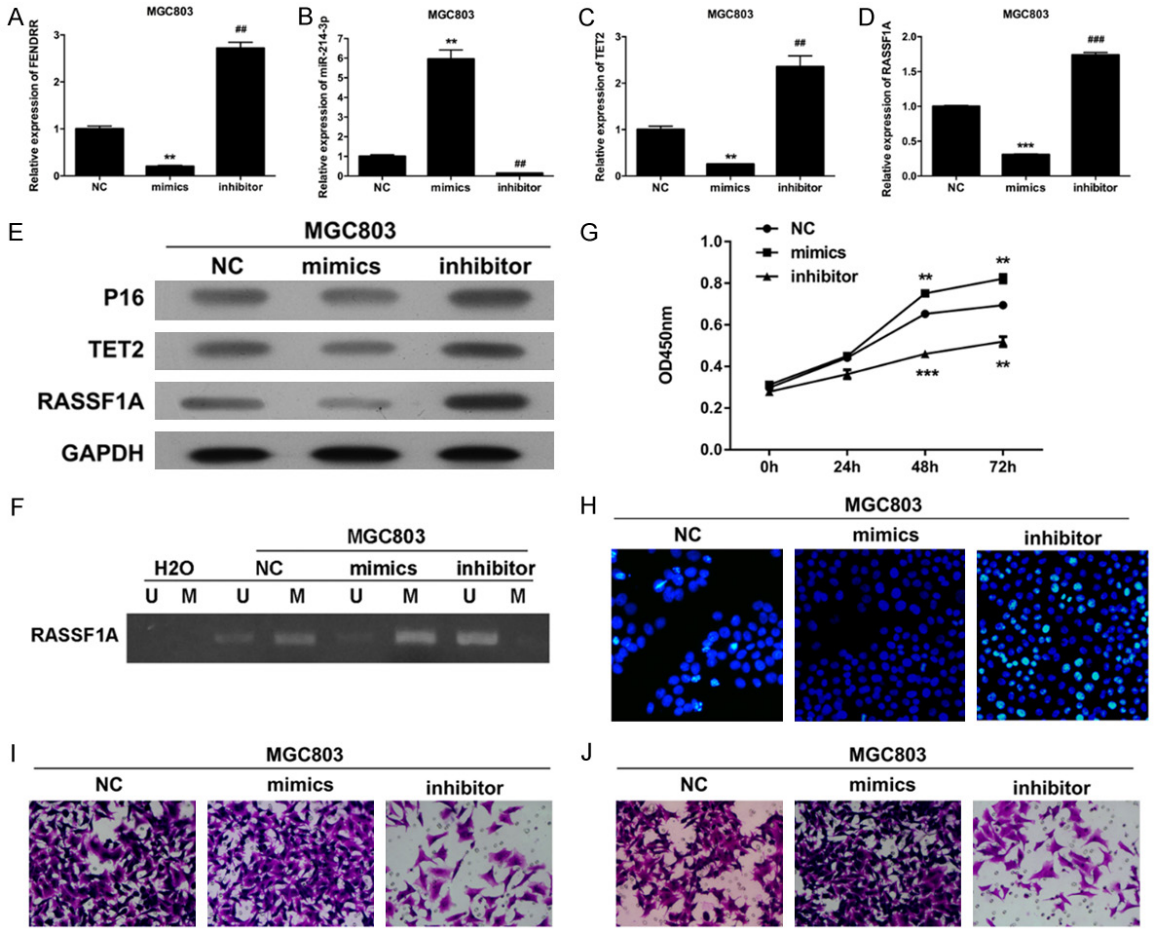

Figure 3. Effects of miR-214-3p overexpression or knockdown on gene methylation, cell proliferation and apoptosis in MGC803 cells. (A) FENDRR, (B) miR-214-3p, (C) TET2, and (D) RASSF1A expression levels were measured by qRT-PCR in MGC803 cells transfected with either miR-214-3P mimics or inhibitor. *, $P < 0.05$ vs. NC; #, $P < 0.05$ vs. NC. (E) Western blot analysis was carried out to detect the protein levels of P16, TET2, and RASSF1A in MGC803 cells after treatment with either miR-214-3p mimics or inhibitor. (F) MSP assay was used to assess the methylation status of RASSF1A after treatment with either miR-214-3p mimics or inhibitor in MGC803 cells. (G) Cell proliferation was assessed by CCK-8 assay in MGC803 cells transfected with either miR-214-3p mimics or inhibitor. *, $P < 0.05$ vs. 0 h. (H) Cell apoptosis was identified via Hoechst staining after treatment with either miR-214-3p mimics or inhibitor in MGC803 cells. (I) Transwell assay was employed to assess the invasion ability of MGC803 cells after treatment with either miR-214-3p mimics or inhibitor. (J) Transwell assay was employed to assess the migration ability of MGC803 cells after treatment with either miR-214-3p mimics or inhibitor. Data represented as means ± SD from three independent experiments.

MGC803 cells treated with the mimics, and markedly decreased in cells treated with the inhibitor, compared to cells treated with negative control (NC) (**Figure 3A**). In addition, the mRNA levels of FENDRR, TET2, and RASSF1A were suppressed by the miR-214-3p mimics, and significantly enhanced by the miR-214-3p inhibitor in MGC803 cells, compared with cells treated with NC (**Figure 3B-D**). Similarly, results from Western blots indicated that the protein expression levels of P16, TET2, and RASSF1A were significantly decreased in MGC803 cells treated with the mimics, whereas the expression levels of P16, TET2, and RASSF1A were markedly increased in cells treated with the inhibitor, compared with NC (**Figure 3E**). We also evaluated the methylation status of RASSF1A by MSP assay. The data suggest that MGC803 cells treated with the mimics increased the methylation levels of RASSF1A, and MGC803 cells treated with the inhibitor significantly decreased the methylation levels of RASSF1A, compared to NC (**Figure 3F**). These results suggested that miR-214-3p inhibited the expressions of TET2 and RASSF1A, and that the expression of RASSF1A was inhibited

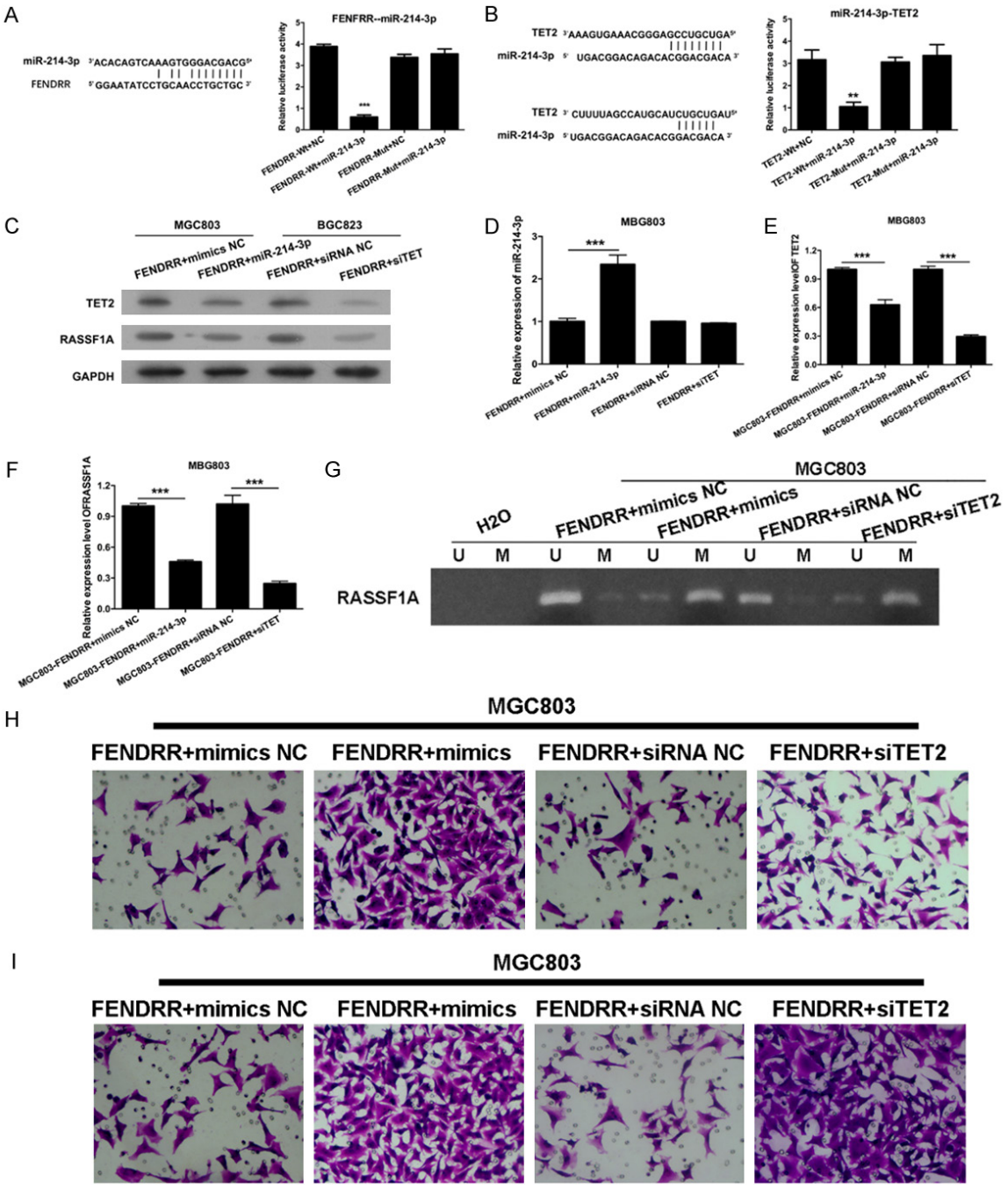

Figure 4. Effects of FENDRR and miR-214-3p on the expression and methylation of TET2 and RASSF1A. (A) FENDRR-WT or FENDRR-Mut plasmids were co-transfected into 293T cells with either miR-214-3p negative control (NC) or miR-214-3p mimics for 24 h and then luciferase activity was assessed. *, $P < 0.05$ vs. FENDRR-WT+NC. (B) TET2-WT or TET2-Mut plasmids were co-transfected into 293T cells with either miR-214-3p NC or miR-214-3p mimics for 24 h and then relative luciferase activity was measured. FENDRR plasmid was co-transfected into MGC803 cells with NC, miR-214-3p mimics, si-scrambled, or si-TET2, and then the expression levels of TET2 and RASSF1A were measured by (C) Western blot and (E and F) qRT-PCR, and (D) the expression of miR-214-3p was detected by qRT-PCR. (G) The methylation of RASSF1A was assessed by MSP in MGC803 cells transfected with FENDRR and NC, miR-214-3p mimics, si-scrambled, or si-TET2. (H) Invasion ability of MGC803 cells transfected with FENDRR and NC, miR-214-3p mimics, si-scrambled, or si-TET2 was assessed by transwell asssy. (I) Migration ability of MGC803 cells transfected with FENDRR and NC, miR-214-3p mimics, si-scrambled, or si-TET2 was assessed by transwell assay. Data represented as means ± SD from three independent experiments.

by increasing the methylation status of RASSF1A.

MiR-214-3p promoted cell proliferation, inhibited cell apoptosis, and induced cell invasion and migration

CCK-8 and Hoechst staining assays were used to detect the biological functions of miR-214-3p in cell proliferation and apoptosis of MGC803 cells treated with either mimics or inhibitor. Cell proliferation was significantly promoted in cells treated with the mimics, whereas cell proliferation was markedly inhibited in cells treated with the inhibitor (**Figure 3G**). Hoechst staining indicated that the cell apoptosis rate was remarkably lower in cells treated with mimics, and was significantly higher in cells treated with inhibitor, than the NC (**Figure 3H**). Moreover, the induced expression of miR-214-3p promoted the invasion and migration potentials of MGC803 cells (**Figure 3I**) while the suppressed expression of miR-214-3p inhibited (**Figure 3J**).

FENDRR regulated the expression of TET2 by sponging miR-214-3p

Bioinformatics predicted a binding site for miR-214-3p in the FENDRR sequence (**Figure 4A**). Based on this information, we posited that FENDRR could directly interact with miR-214-3p and negatively regulate miR-214-3p expression. To test our hypothesis, we constructed dual-luciferase miRNA target sites expression vector for luciferase reporter assay: (1) FENDRR-WT; (2) FENDRR-Mut. The luciferase assay revealed that miR-214-3p mimics significantly suppressed luciferase activity in the FENDRR-WT group (**Figure 4A**). In addition, we found two binding sites for miR-214-3p in the TET2 sequence (**Figure 4B**). We found that miR-214-3p mimics significantly suppressed luciferase activity in the TET2-WT group (**Figure 4B**). These results suggested that FENDRR and miR-214-3p can directly regulate each other and that TET2 is the target gene of miR-214-3p.

Given that FENDRR directly interacts with miR-214-3p, and reduces the expression of TET2, we speculated that FENDRR regulates the expression of TET2 by sponging miR-214-3p. To explore the underlying molecular mechanism of FENDRR, we transferred either miR-214-3p mimics or si-TET2 to the FENDRR overexpressing cells, miR-214-3p NC and si-scrambled (as control), respectively. The qRT-PCR and Western blot results showed that TET2 and RASSF1A expression levels were significantly decreased after miR-214-3p overexpression in FENDRR-overexpressing MGC803 cells (**Figure 4C**, **4E**, **4F**). The FENDRR-induced down-regulation of RASSF1A methylation was reversed by treatment with the mimics of miR-214-3p or si-TET2 in MGC803 cells (**Figure 4G**). As shown in **Figure 2**, overexpressed FENDRR promoted the expression levels of TET2 and RASSF1A, and their expressions were inhibited in the TET2-silenced cells (**Figure 4E**, **4F**). In addition, the expressions of TET2 and RASSF1A were decreased in the miR-214-3p-overexpressing cells (**Figure 4E**, **4F**). These results indicated that the FENDRR-induced upregulation of TET2 and RASSF1A can be inhibited by miR-214-3p. The effect of the above modulations on the invasion and migration abilities of MGC803 cells was also assessed: as shown in **Figure 4H** and **4I**, induced expression of miR-124-3p and inhibited expression of TET2 both increased the cell numbers penetrating the membranes in FENDRR over-expressed cells, confirming the key role of miR-214-3p in the regulation of TET2 by FENDRR.

Discussion

In spite of recent advances in the treatment of GC, it continues to be a leading cause of cancer-associated death worldwide [31]. Long non-coding RNAs (lncRNAs) like H19 and HOTAIR and microRNAs (miRNAs) like miR-508-5p and miR-21 [32-35] have been implicated in the pathogenesis of GC. Furthermore, fetal-lethal non-coding developmental regulatory RNA (FENDRR), identified by Khalil *et al* is associated with poor prognosis of GC [23]. In the present study, we found that FENDRR expression significantly decreased in tissues of patients with GC, consistent with observations reported by Xu TP *et al* [23]. In addition, overexpressed miR-214-3p was shown to regulate metastasis and invasion in gastric cancer and also linked to its poor prognosis [27]. In accordance with these findings, we also observed overexpressed miR-214-3p in GC tissues in our study. The interaction between lncRNAs and miRNAs is also important in multiple human cancers, including colorectal cancer, hepatocellular cancer, and lung cancer [36-38]. Indeed, Zhou X *et al* report

that cell proliferation and migration of GC is regulated by the interaction between miR-141 and lncRNA-H19 [28]. Liu XH *et al* also demonstrat that lncRNA-HOTAIR regulates HER2 expression by directly acting as a "sponge" for miR-331-3p in GC [29]. In the current study, we found that FENDRR was predominantly localized in the cytosol of GC cell lines and overexpression of FENDRR inhibited the expression of miR-214-3p. FENDRR inhibited cell proliferation and promoted cell apoptosis. We also showed that FENDRR directly binds to miR-214-3p.

Inactivation of tumor suppressor genes, caused by hypermethylation of the promoters, also contributes to carcinogenesis in multiple cancers including lung cancer, breast cancer, and gastric cancer. RASSF1A (Ras-association domain family 1A) and P16 (CDKN2A or Ink4a), two critical tumor suppressor genes, are frequently found to be hyper-methylated in many tumors of the liver and prostrate [39-42]. TET2 (Ten-Eleven Translocation 2), a critical enzyme for DNA demethylation, belongs to the TET family, an important regulator of DNA demethylation. Mutations or deletions in TET2 have been identified in various cancers, such as prostate cancer, ovarian cancer, and colorectal cancer [43-45]. However, it was not known if RASSF1A, P16, and TET2 are involved in tumorigenesis of GC. In this study, we found that the expression levels of TET2, RASSF1A, and P16 were significantly decreased, and the methylation levels of RASSF1A and P16 were remarkably higher in tissues of patients with GC. Methylation levels of many tumor-related genes are regulated by the interactions between lncRNAs and miRNAs in various cancers [37, 46]. One study demonstrated that miRNA-29 regulates the expression level of lncRNA gene MEG3 by hypermethylating its promoter in hepatocellular cancer [37]. Another study report that miR-31 and its host gene lncRNA LOC554202 are regulated by promoter hypermethylation in triple-negative breast cancer [47]. Finally, our results demonstrated that the expression and methylation of RASSF1A was regulated by the interaction of FENDRR and miR-214-3p.

Taken together, our data showed that FENDRR inhibited the progress of GC by acting as a miRNA-sponge for miR-214-3p to modulate the expression levels of TET2, P16, and RASSF1A. We have presented a promising new molecular target for the treatment of gastric cancer. However, further work is needed to validate these experiments *in vivo*.

Acknowledgements

Informed consent was obtained from all individual participants included in the study.

Disclosure of conflict of interest

None.

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