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Original Article

Methylation-associated silencing of MicroRNA-335 contributes tumor cell invasion and migration by interacting with RASA1 in gastric cancer

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Abstract: MicroRNAs (miRNAs) are small non-coding RNAs that function as endogenous silencers of target genes, previous studies have shown that miR-335 play an important role in suppressing metastasis and migration in human cancer including gastric cancer (GC). However, the mechanisms which result in aberrant expression of miR-335 in GC are still unknown. Recent studies have shown that the silencing of some miRNAs is associated with DNA hypermethylation. In this study, we find the promoter of miR-335 we embedded in CpG island by accessing to bioinformatics data and the low expression of miR-335 in 5 gastric cell lines can be restored by 5-aza-2'-deoxycytidine (5-Aza-dC) treatment. So we postulated that the miR-335 genes undergo epigenetic inactivation in GC. Subsequently, in GC cells and tissues, we performed quantitative real-time PCR (RTQ-PCR) to assess the expression of miR-335, and methylation-specific PCR (MSP) and bisulfite sequence-PCR (BSP) to evaluate the DNA methylation status in the CpG islands upstream of MiR-335. The result showed that the expression of miR-335 was significantly reduce in gastric cancer cell lines and tumor tissues compared to matched normal gastric tissues, and cell lines, and which is inverse correlation with DNA hypermethylation of miR-335 both in GC cells lines and tissues, but not in normal tissues. In addition, we found that the lower miR-335 expression induced by abnormal methylation may be mainly involved in gastric cell invasion and metastasis in GC tissues. No statistical significance was found about miR-335 expression and methylation level between healthy individuals with and without *H. pylori* (HP) infection. Finally, we carry out miRNA transfection, RTQ-PCR and western blot assay to find the RAS p21 protein activator (GTPase activating protein) 1 (RASA1) may be the possible target genes which lead to the gastric cell invasion and metastasis, furthermore, the re-expression of endogenous miR-335 by 5-Aza-dC treatment can exert effects similar to exogenous miRNAs transfection. Taken together, our results suggest that miR-335 may be silenced by promoter hypermethylation and play important roles in gastric cell invasion and metastasis through its target genes, such as RASA1. Its methylation level might be a predictive epigenetic marker of GC and remodeling on the expression by demethylation can provided a potential therapeutic strategy.

Keywords: MiR-335, methylation, gastric carcinoma, Cancer metastasis, cancer invasion, RASA1

Introduction

Gastric carcinoma remains the most common malignant tumor and causes over 723,000 deaths worldwide per year [1]. Prognosis is poor with a less than 10% five year survival rate, largely because most patients with the symptoms present with advanced disease and may have also metastasized [2]. Metastasis is defined as the formation of progressively growing secondary tumor foci at sites discontinuous from the primary lesion, and is the prime reason of mortality in patients with solid tumors [3]. If meaningful improvements in survival are to occur, then control of metastasis will be a foundation. Relatively little is known about the control of the metastatic process at the molecular level, but, fortunately, the number of genes

Table 1. Clinicopathological features of GC patients and MSP analysis of miR-335 in tumor tissues

Variable	n=81	No. of methylated (%)	X^2	*P*-value
Sex				
Male	51	38 (74.5)	0.014	0.907
Female	30	22 (73.3)		
Age (years)				
< 53	33	23 (69.7)	0.556	0.456
≥ 53	48	37 (77.1)		
Tumor size (cm)				
≤ 3.5	17	12 (70.6)	0.003	0.954
> 3.5	64	48 (75.0)		
Tumor location				
Cardia	19	13 (68.4)	0.420	0.810
Body	16	12 (75.0)		
Pyloric antrum	46	35 (76.1)		
Differentiation				
Poor	51	40 (78.4)	1.676	0.433
Moderate-poor	17	12 (70.6)		
Others	13	8 (61.5)		
Lauren's grade				
Intestinal	21	13 (80.9)	2.659	0.265
Diffuse	54	43 (79.6)		
Others	6	4 (66.6)		
pT stage				
T1	3	0 (0)	26.678	0.000*
T2	16	6 (37.5)		
T3	23	18 (78.3)		
T4	39	36 (92.3)		
Single GC	65	45 (69.2)	2.844	0.092
Multiple GC	16	15 (93.8)		
Metastasis lymph node				
Negative	21	12 (57.1)	4.232	0.040*
Positive	60	48 (80.0)		
Peritoneal metastasis				
Negative	66	45 (68.2)	6.433	0.011*
Positive	15	15 (100.0)		

*Refers to statistical significance between groups (P < 0.05).

that have been identified to functionally regulate the metastatic cascade is growing rapidly [4].

A large amount of overwhelming evidence establishes the involvement of microRNAs as essential actors in the multi-step cascade fostering a cancer cell to leave the primary tumor and form secondary tumors at distant sites [5-7]. Tavazoie and co-workers reported MiR-335 as metastasis suppressor genes. They found that restoring the expression of miR-335 in malignant breast cancer cells can suppress the metastasis to lung and bone [8]. Since then, it's role involving in metastasis had been reported in succession by other academics, Including targeting ROCK1 in osteosarcoma cells [9], targeting the formin family of actin nucleators [10], ovarian cancer omental metastases [11] and so on. To date, less study covered in gastric carcinoma (GC), only including the low expression of miR-335 targeting Bcl-w and specificity protein 1 is associated with lymph-node metastasis, poor pT stage, poor pN stage and invasion into lymphatic vessels [12], and the hsa-miR-335 has the potential to recognize the recurrence risk and relate to the prognosis of GC patients [13].

Epigenetics refers to the study of mechanisms that alter gene expression without altering the primary DNA sequence, and include DNA methylation, histone modification, chromatin remodeling, and noncoding RNAs [14]. DNA hypermethylation at gene promoter CPG islands is thought to be an alternative mechanism to down-regulate tumor suppressor genes in human cancers [15]. MiRNAs whose permanent expression silenced by DNA methylation had been reported in a few human cancers [16, 17]. To further investigate whether the downregulation of MiR-335 originates from the hypermethylation of the genomic region upstream of MiR-335, Kim J. Png [18] and OSAMU DOHI [19] had studied the epigenetic mechanisms which resulted in the low expression of miR-335 in breast cancer and hepatocellular carcinoma, respectively. They find the expression is reduced by aberrant DNA methylation. Taking account of these, we postulated that the miR-335 genes undergo similar epigenetic inactivation in GC.

Therefore, we firstly aimed to identify miRNA genes that are silenced by DNA hypermethylation in GC. We evaluate the likehood of this epigenetic mechanism that DNA promoter

Table 2. Methylation status of miR-335 family in tumor, paired non-tumor tissues and normal gastric mucosae

	N	Methylation (%)	Unmethylation (%)	X^2	*P*-value
Tumor	81	60 (74.1)	21 (25.9)		
Paired non-tumor	81	39 (48.1)	42 (51.9)	11.455	0.001*
Surgical margin tissue	76	41 (53.9)	37 (46.1)	6.922/§0.310	0.009*/§0.578
Normal gastric mucosae	39	13 (33.3)	26 (66.7)	18.399/§2.353	0.000*/§0.125
HP+	23	9 (39.1)	14 (60.9)	9.796	0.002*
HP-	16	4 (25.0)	12 (75.0)	14.335/§§0.848	0.000*/§§0.357

HP+ and HP- with and without *H. pylori* (HP) infection. Without § refers to the comparison with tumor, with one § refers to the comparison with paired non-tumor, two § shows the comparison between HP (+) and HP (-), *refers to statistical significance between groups ($P < 0.05$).

hypermethylation induce the abnormal expression of miRNAs by using large number of bioinformatics websites and software, and then carried out relative experiments which test the promoter methylation level and the correlation between the expression of miR-335, and study the possible target gene of miR-335 in GC cell lines and tissues, by which we try to testify miR-335 is downregulated by aberrant promoter hypermethylation and regulated by interacting with RASA1 in gastric cancer.

Materials and methods

The bioinformatics of MiR-335

First we search and obtain the 5 kb DNA gene sequence of 5'- upstream of miR-335 by bioinformatics genome database UCSC or NCBI and Mirbase. Second, we choose three CpG island searching websites- CpG Island Searcher, Cpgplot, Methprimer- to scan for CpG islands in this gene sequence according to limited parameters (GC%=50%, ObsCpG/ExpCpG=0.60, Length=200 bp, Gap between adjacent islands =100 bp) [20]. Third, we carry out promoter search by means of BIMAS and BDGP or Promoter 2.0 Prediction Server [21]. In addition, The predicted targets of miR-335 and their target sites were analyzed using microrna, miRBase, TargetScan, PicTar, Rnahybrid and RegRna, and the Methylation status of each DNA clone sequence was analyzed comprehensively and comparatively by CpG viewer, QUMA or Biq-analyzer. The detailed information of all bioinformatics websites mentioned above were list in Supplementary Table 1.

Tissue samples

Gastric specimens (n=81) were obtained from the tumor and an adjacent non-cancerous area that were farther than 9 cm from the tumor tissues of gastric carcinoma patients at the General surgery department of the first affiliated hospital of Nanchang University (Nanchang China), at the same time, we selected 76 surgical margin tissues. The median age of patients (30 women and 51 men) was 61 years (range 29-88). Normal gastric mucosae were obtained by endoscopic biopsy from 39 healthy individuals (23 male and 16 female; average age 56 years, ranging from 21 to 86). Helicobacter pylori infection was identified using a rapid urease test, a serum antibody test or a urea breath test. All specimens were immediately frozen in liquid nitrogen and were stored at -80°C until further use. Informed consent was obtained from all subjects, and the study was approved by the institutional review committee. The histological grade of cancers was assessed according to criteria set by the World Health Organization. The tumor-node-metastasis stage was performed using standard criteria of the seventh tumor-node-metastasis staging system.

Cell culture

Human gastric adenocarcinoma cancer cell lines SGC-7901, MKN-45, MKN-28, BGC-823, and MGC-803 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai China). The human gastric mucosa cell lines GES-1 was preserved in our central laboratory. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco), culture media was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 ug/ml streptomycin and cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from cells and frozen fresh GC tissues using a standard Trizol protocol (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse-transcribed into single-stranded cDNA using an Expand Reverse Transcriptase Kit (Takara, Danian, China) as per manufacturer's instructions. Quantitative PCR was performed using the Applied Biosystems 7500 Fast real-time PCR System with the SYBR Green PCR Master Mix (Applied Biosystems, USA) to detect RASA1 mRNAs. Gene expression was normalized using endogenous β-actin control. For the gene expression of candidate miR-335, 100 ng of total RNA was reverse transcribed by their respective stem-loop RT primers, followed by RTQ-PCR on the ABI 7900 HT Fast real-time PCR system using the SYBR Green PCR Master Mix and normalized with U6 SnRNA, Relative fold change in expression of gene transcript was determined using the comparative cycle threshold method ($2^{-\Delta\Delta ct}$). All the primers which designed by software Primer Premier 6.0 and Oligo7 were list in Supplementary Table 2.

DNA extraction, methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP)

Genomic DNA from patient sample and cell lines was isolated with Easypure Genomic DNA kit (TransGen Biotech, Beijing, China). Genomic DNA was subjected to bisulfite conversion and purification using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, CA, USA) according to the manufacturer's instructions. Then, methylation-specific PCR (MSP) was performed by simultaneous use of primers (Supplementary Table 2) for methylated and unmethylated forms. All PCR reactions were performed with negative and positive controls by using completely methylated and unmethylated human control DNA (Qiagen, Hilden, Germany), respectively, as well as water. The BSP primer (Supplementary Table 2) was designed by Methprimer or Methyl Primer Express v1.0. Amplified PCR Products were purified and cloned into pMD19-T (TaKaRa, Dalian, China). 10-15 clones of each pair of tumor and adjacent non-tumor tissue samples and 5-10 clones each cell were sequenced, respectively. Percentage of methylation was calculated comprehensively and comparatively by CpG viewer, QUMA [22], and Biq-analyzer [23].

5-Aza-2'-deoxyazacytidine demethylation treatment

For demethylation studies, all GC cells were treated with 5 μmol/l 5-Aza-2'-deoxyazacytidine (Sigma-Aldrich, St Louis, MO) for 72 h. SGC-7901 and MGC-803 cells were daily treated with 1 μmol/l, 5 μmol/l and 10 μmol/l 5-aza-CdR for 72 h, respectively. Cells were harvested for RTQ-PCR, for bisulfite sequencing and West blot.

miRNA transfection

SGC7901 and MGC-803 cells were transfected with miR mimics (Genepharma, Shanghai, China) by dint of Lipofectamine™ 2000 (Invitrogen, USA). The sequences of the primer of Hsa-miR-335 mimics and negative control (NC mimic) were used in this article list in Supplementary Table 2, cells were grown in six-well plates to 30-50% confluence before transfection. Total RNA and protein were extracted at 48 h post-transfection and subjected to qRT-PCR and western blotting.

Protein extraction and western blot analysis

Total protein was extracted from cells using a Total Protein Extraction Kit (KeyGen, Nanjing, China), Fifty micrograms of proteins of different groups were boiled for 5 min in sample buffer, separated in 10% SDS-PAGE, transferred onto PVDF membrane (Invitrogen) and then blocked for 1 hr. to overnight with 5% skim milk (blocking solution) at room temperature with agitation. Anti-RASA1 antibody (Abcam) (1:200) incubated at 4°C overnight. After washing with 0.1% Tween 20 in Tris-saline thrice, the membrane was incubated with peroxidase-labeled anti-rabbit IgG for 1 h at room temperature with agitation. The immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and a Pierce ECL Western Blotting KIT (Pierce, Rockford, IL, USA). The anti-β-actin (Santa Cruz Biotechnology) was used to normalized the amount of the analyzed samples.

Statistical analysis

Each experiment was repeated at least three times. Statistical analysis was performed by SPSS 19.0 computer software (SPSS Inc., Chicago, IL, USA) and software program GraphPad

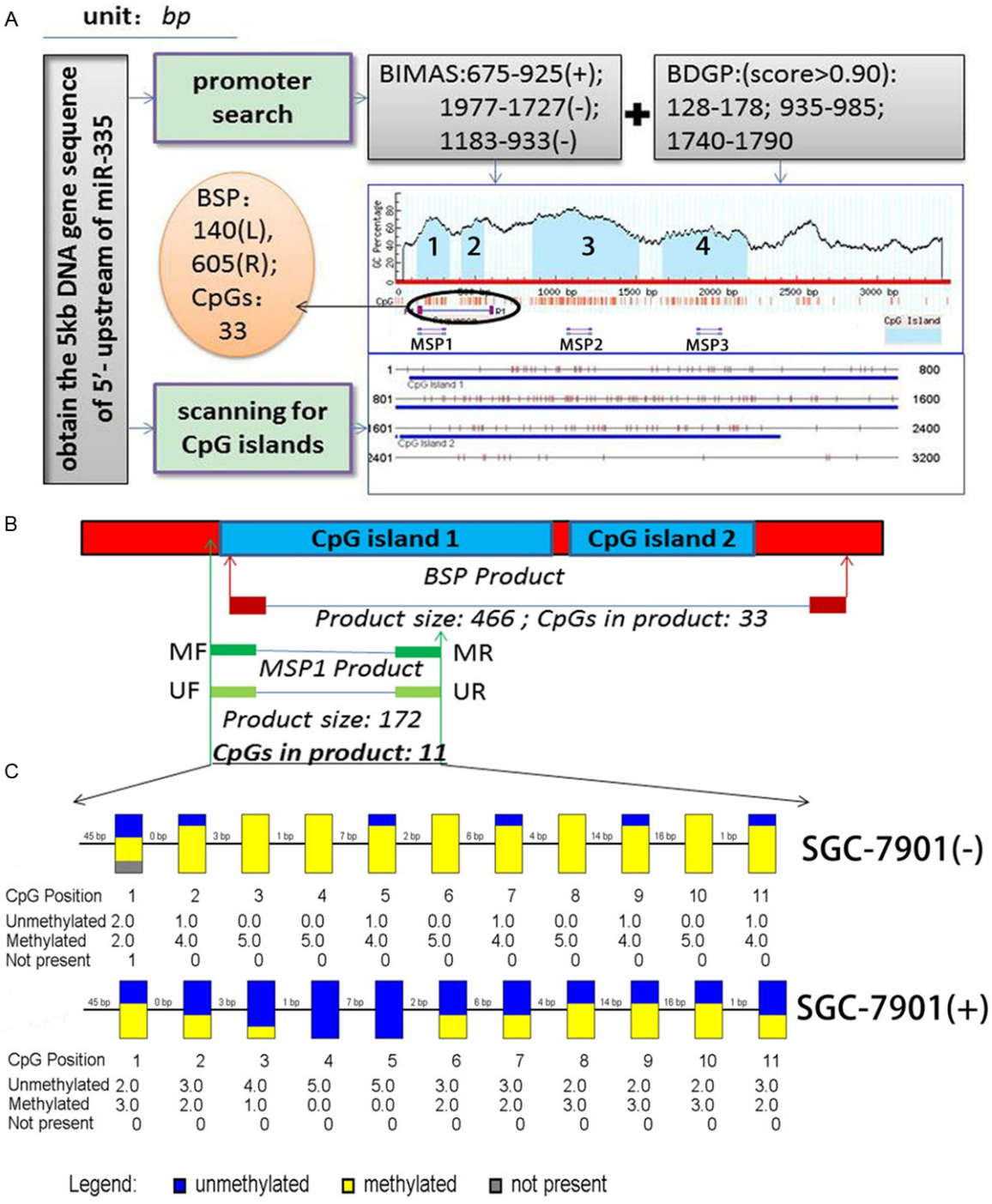

Figure 1. Bioinformatics analysis of MicroRNA-335. (A) A flow chart used for the identification of epigenetically silenced miR-335 genes by bioinformatics database. Firstly, obtaining the 5kb DNA sequence of 5'-upstream, by which we search CpG island and promoter of miR-335, the promoter, determined by comparing, is located on the island. Then, we design one BSP and three MSP primers according to the CpG cites. The black circle and (B) exhibit the specific location, PCR product and the relationship between BSP and MSP1 primer. (C) The BSP histogram result of SGC-7901 cells treated with 5-aza-dC in the region of MSP1 with 11 CpG sites, the figure come from BiQ Analyzer and detailed interpretation is shown in Supplementary Figure 2.

Prism (Version 6.02 for Windows, Graphpad Software, La Jolla, CA, USA). Methylation status of each DNA clone sequence was analyzed comprehensively and comparatively by QUMA,

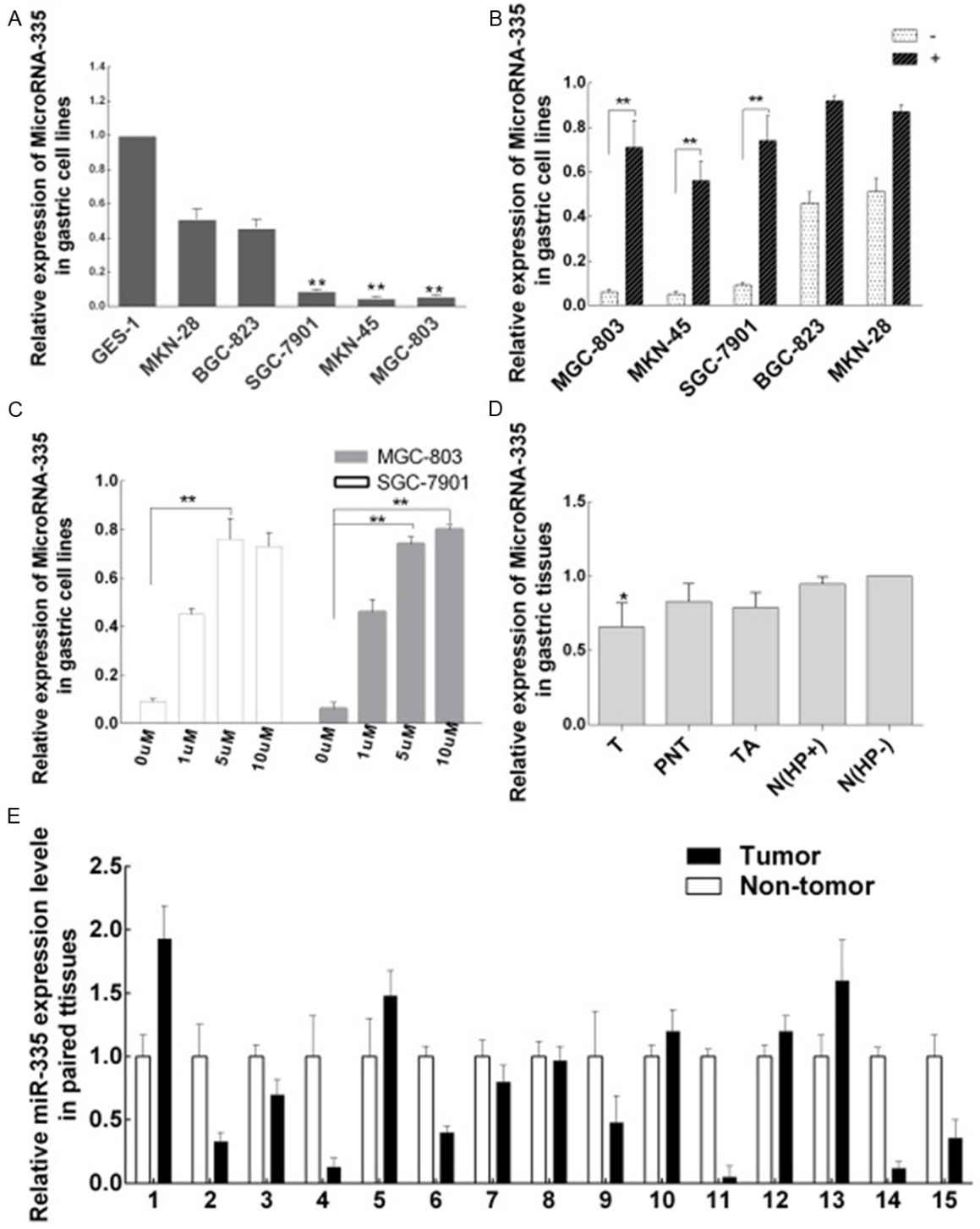

Figure 2. Analysis of miR-335 expression in GC cell lines and tissues. A. RTQ-PCR results for miR-335 in a panel of GC cell lines. Results are presented relative to normal gastric epithelial cell GES-1 (as the control) and normalized to internal U6 snRNA expression. Shown are the means of the values, error bars represent stand deviations; B. RTQ-PCR results for miR-335 in all GC cell lines, with (+) or without (-) 5-aza -CdR treatment; C. RTQ-PCR results for miR-335 in GC cell lines SGC-7901 and MGC-803 with different concentration 5-aza -CdR treatment; D. RTQ-PCR results for miR-335 in paired tumor (T) and non-tumor gastric tissues (PNT), surgical margin tissue (TA) and normal gastric mucosa from healthy individuals with (+) and without (-) *H. pylori (HP)* infection (as control); E. RTQ-PCR results for miR-335 in random selected 15 human GC paired tumor and non-tumor gastric tissues, the expression levels of tumor were independently calculated relative to those of non-tumor cases, which are normalized to 1. **refers to statistical significance between cell lines ($P < 0.01$, *$P < 0.05$).

CpG viewer and Biq-analyzer. Correlations between miRNA methylation and clinicopathological features were analyzed by chi-square test or Fisher's exact tests. The Wilcoxon signed-rank test was used to assess the expression levels between tumor and adjacent non-tumor tissues. Mann-Whitney U-test between two groups and Kruskal-Wallis test for three or more groups. Correlations between miR-335 expression and promoter CpG methylation were determined by calculating r^2 as a measure of the goodness of fit for the linear regression. All the other results presented as mean±SD, ANOVA analyses followed by Bonferroni multiple-comparison tests and Student's t-tests were carried out to detect significant differences, unless otherwise noted. $P < 0.05$ was accepted as significant.

Results

The result of bioinformatics analysis of MiR-335

Scanning for CpG islands in the submitted sequence found four, two and two CpG islands in Methprimer, CpG Island Searcher and CpG plot, respectively (the result of CpG plot is presented in Supplementary Figure 1). The overlapping rate of three was up to 88.9%. Subsequently, we confirm whether the promoter and CpG islands have conjunction. The BIMAS submitted three promoter positions, namely the sense strand 675 bp~925 bp, antisense 1977 bp~1727 bp and 1183 bp~933 bp. Meanwhile, we choose three promoter regions with score greater than 0.90 in BDGP, the result is 128~178 bp, 935~985 bp, 1740~1790 bp. By comparison, we confirmed the promoter of MiR-335 lie in the range of CpG Islands (**Figure 1A** and Supplementary Figure 1). The result is similar to previous study that the miR-335 locus resides in the second intron of the mesoderm-specific transcript (Mest) gene [24] from which its miRNA product arises. Analysis of the Mest/miR-335 promoter revealed three CpG islands upstream of the transcriptional start site. [18, 19] By comparison, we choose the CpG islands of Methprimer to design BSP and MSP primer by Methprimer or Methyl Primer Express v1.0. MSP primer(include 33 CpG site) match the CpG islands 1 and 2, MPS1, MSP2, MSP3 prime match the CpG islands 1, 3, 4 respectively (**Figure 1A**, **1B**). The bioinformatics analysis for target gene of miR-335 were described later in *"Identification of target genes of miR-335"*.

Analysis of miR-335 expression in GC cells

We used RTQ-PCR to analyze the expression of miR-335 in a panel of GC cell lines. miR-335 was downregulated in all GC cell lines, as compared with normal gastric epithelial cell line GES-1 (**Figure 2A**), It was previously reported that highly metastatic cells undergo additional promoter methylation at the remaining miR-335 locus relative to the parental lines in human breast cancer [18], and the result of bioinformatics analysis of MiR-335 discussed above have shown this possibility. So we we conducted a preliminary study that treated all GC cell lines with 5-aza -CdR (5 μmol/l) to find whether the mechanism involve in GC, which indicate that 5-aza -CdR restored expression of miR-335 in the GC cells (**Figure 2B**). We also found the optimum concentration that obviously unregulated expression of miR-335 in SGC-7901 and MGC-803 is 5 μmol/l and 10 μmol/l (**Figure 2C**). These results including the bioinformatics analysis indicate that miR-335 expression may be downregulated through DNA methylation.

Analysis of miR-335 expression in human primary GC tissues

It was reported that the low expression of miR-335 was significantly associated with lymph-node metastasis, poor pT stage, poor pN stage and invasion into lymphatic vessels in GC [12]. We confirmed it in selected tissues. In addition to lymph nodes, we find it also applicable to peritoneal metastasis. We next analyzed the expression of miR-335 in normal gastric mucosae from healthy individuals (HP-, n=16; HP+, n=23), randomly selected surgical margin tissue (n=30), randomly selected primary GC tissues and paired non-tumor tissues (n=30). Interesting, the expression levels of paired non-cancerous and cancerous tissues did not significantly differ in patients (p=0.091), cancer tissues showed a relatively higher levels of expression of miR-335 than normal gastric mucosae without *H. pylori (HP)* infection (P= 0.043). In addition, no statistical significance was found with miR-335 expression between surgical margin tissue and adjacent non-tumor tissues, and between *H. pylori*-positive and negative tissue (**Figure 2D**). The randomly selected primary GC tissues and paired non-tumor tissues (n=15) which for later bisulfite sequencing study was presented in **Figure 2E**. A significant reduction of miR-335 expression was observed in 9 (60%) of cases. All the data imply that the lower miR-335 expression in GC

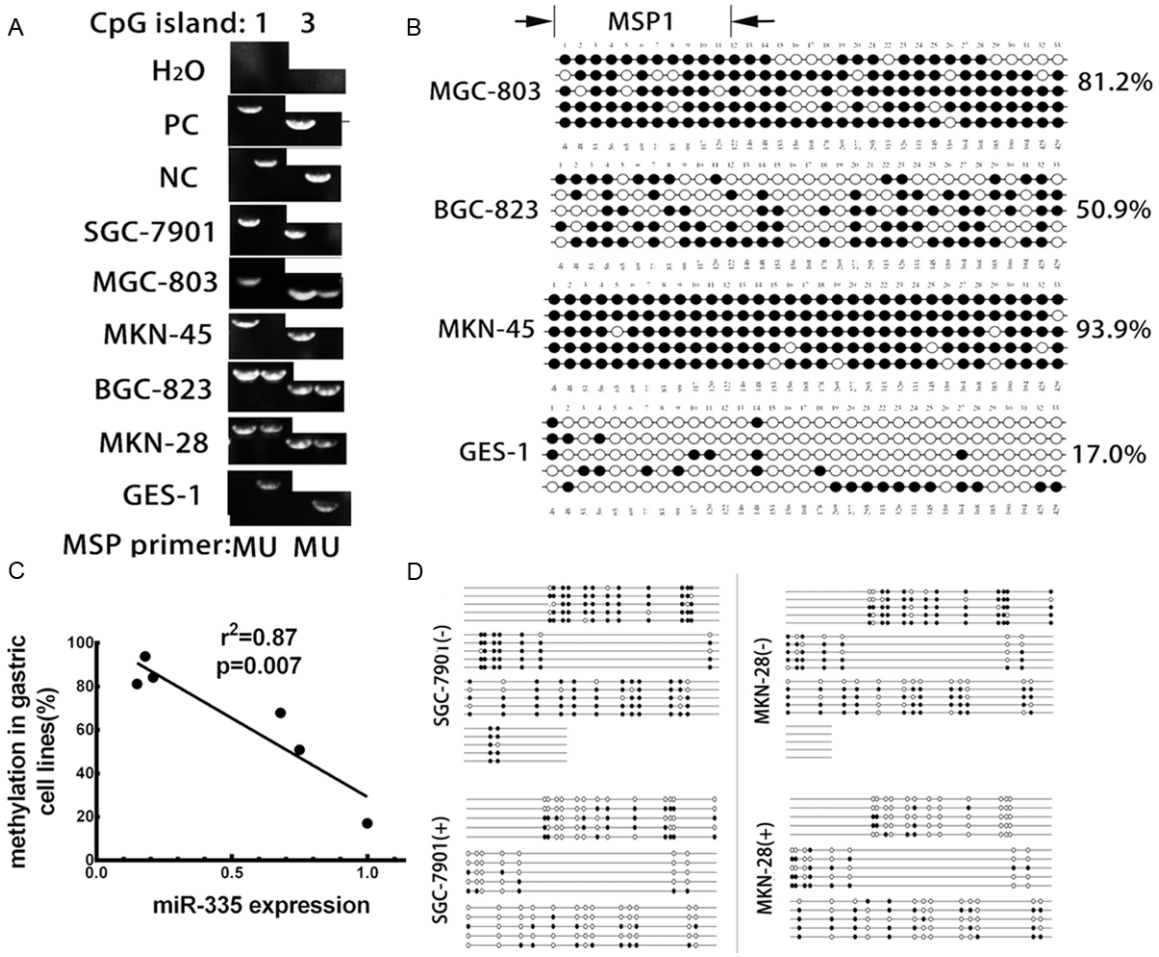

Figure 3. Analysis of miR-335 methylation status in GC cell lines and it's correlation with miR-335 expression. A. MSP of two CpG islands (1 and 3) in miR-335 promoter from bisulfite-treated DNA of various lines. PC: positive control (completely methylation) (NC) negative control (completely unmethylation); B. Bisulfite sequencing of the miR-335 CpG island (1 and 2) in GC cells, Open and filled circles represent unmethylated and methylated CpG sites, respectively. Each horizontal row represents a single clone. There are 33 CpG sites in the region. MSP1 between black arrow represent the region of methylation-specific PCR, the result of MSP1 exhibited in **Figure 3A** (island 1). The BSP histogram of SGC-7901 cell between black arrows is shown in **Figure 1C**; C. CpG methylation status in cells as a function of miR-335 expression; D. Bisulfite sequencing in GC cell lines SG-7901 and MKN-28, with (+) or without (-) 5-aza -CdR treatment. Open and filled circles represent unmethylated and methylated CpG sites, respectively. The black line between circles represent the base pairs between each CpG sites, each horizontal row represents a single clone. There are 33 CpG sites.

tissues may be mainly involved in gastric cell invasion and metastasis.

Analysis of MiR-335 methylation status in GC cell lines and the correlation with miR-335 expression

To investigate the relationship between miR-335 expression and DNA promoter methylation, we carried out Methylation-specific PCR (MSP) of these three CpG islands (we initially tried three primer sets designed in the upstream-**Figure 1A**, but CpG island 4 didn't show the complete matches with miR-335 expression, this condition also described in other article [25]). It showed that the CpG island was completely methylated in the majority of GC cell lines tested (**Figure 3A**). Furthermore, bisulfite sequencing (BSP) revealed high levels of DNA methylation in SGC-7901, MGC-803, MKN-45 and MKN-28 GC cell lines, in which miR-335 expression was downregulated accordingly. Whereas only limited methylation was found in normal gastric epithelial cell line GES-1 (**Figure 3B** and **3D**), which highly expressed miR-335. These data are consistent with MSP1 methylation signals in **Figure 3A**. All the results

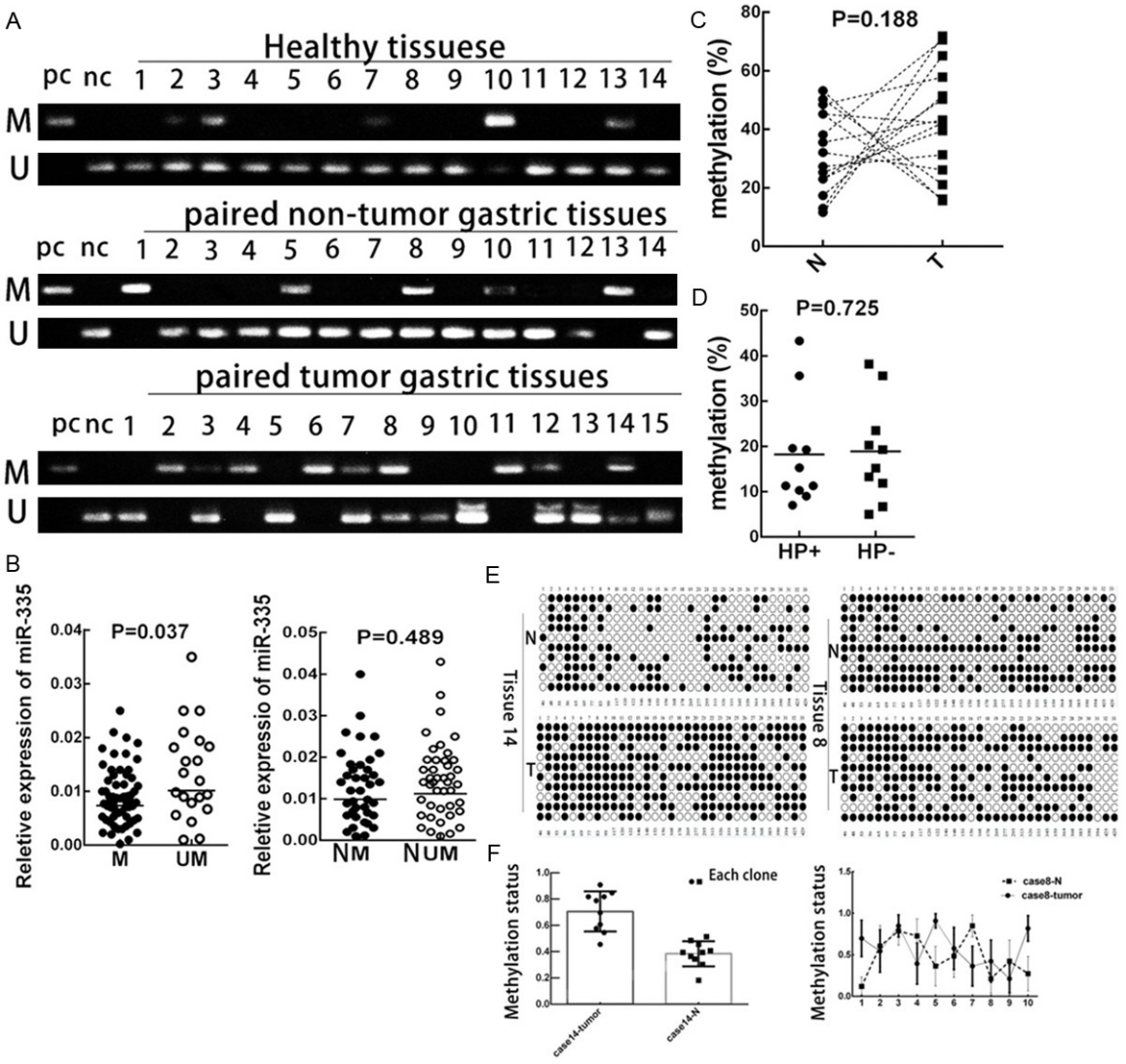

Figure 4. Analysis of miR-335 methylation status in tissues and it's correlation with miR-335 expression. A. Representative results of the MSP analysis for miR-335 in tissues; B. The expression levels of miR-335 were compared between methylated group and unmethylated group in tumor tissues and non-tumor tissues (N); C. Methylation status in selected 15 paired tumor (T) and non-tumor gastric tissues (N); D. Methylation status in selected (n=10) healthy cases with and without *H. pylori* (HP) infection; E. Bisulfite sequencing in selected paired tumor (T) and non-tumor gastric tissues (N) (Case 8 and case 14); F. Summarized BSP results of each clone exhibited in **Figure 4C**.

revealed a strong inverse correlation between methylation of island 1 and 2 (correlation coefficient r^2=0.87, P=0.007) and miR-335 expression (**Figure 3C**). The BSP result clearly confirmed all the CpG sites in promoter region of miR-335 are extensively methylated (**Figure 3B**, **3D**), as well as the single CpG site methylation condition (**Figure 1C** and Supplementary Figure 2).

Subsequently, we analyzed the methylation status in all cell lines after 5-aza -CdR (5 μmol/l and 10 μmol/, respectively) treatment by BSP, all the CpG site were extensively demethylated in all GC cells (**Figure 3D** and Supplementary Figure 3), which highly expressed miR-335. Thus, the methylation status of these CpG sites was consistent with the miR-335 expression in GC cell lines.

Promoter methylation of miR-335 in tissues and their correlation to clinicopathological features

We analyzed the promoter methylation of miR-335 in all tissues and their correlation to clini-

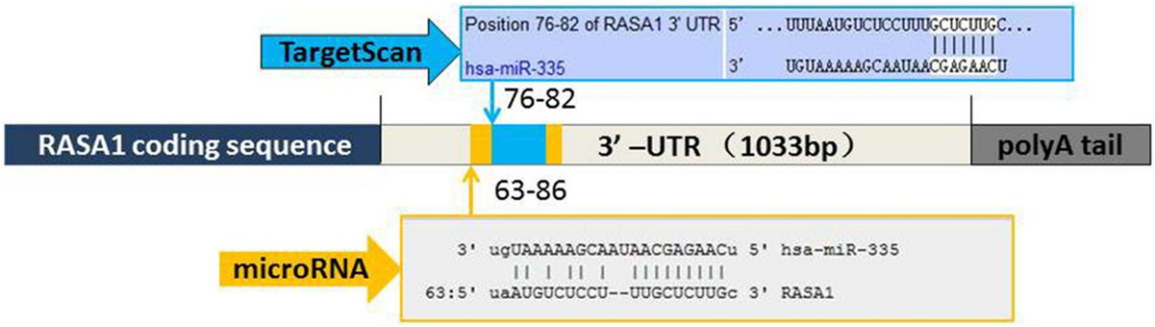

Figure 5. Schematic representations of predicted miR-335 binding sites in the RASA1 3'-UTR by two representative predicted microRNA targets website micoRNA and TargetScan 6.2. The orange and nattier blue rectangle (63-86 and 76-82) located in 3'-UTR shows the miR-335/RASA1 sequence alignment.

copathological features by means of MSP (In order to verify the possible errors by BSP, we choose the MSP1 as the primer) (**Figure 4A**). The clinical and pathological characteristics in tumor tissues are exhibited in **Table 1**. The data indicate the methylation levels didn't significantly differ in gender, age, tumor size, location, histological type and in patients with multiple GC and single GC. But the methylation level was significantly associated with lymph-node and peritoneal metastasis, poor pT stage. The result in all tissues (**Table 2**) show that cancer tissues showed significantly higher levels of methylation than non-cancerous counterparts, surgical margin tissue and normal gastric mucosae. No statistical significance was found with methylation level between paired non-tumors and surgical margin tissue and normal gastric mucosae. In addition, the methylation levels of healthy individuals did not significantly differ in cases with and without *H. pylori (HP)* infection. All the results suggest that the lower miR-335 expression induced by abnormal methylation may be mainly involved in gastric cell invasion and metastasis.

Analysis of MiR-335 methylation status in GC tissues and the correlation with miR-335 expression

To further investigate whether DNA methylation contributed to the silenced expression of miR-335 in GC, we analyzed the expression levels of miR-335 in both methylated and unmethylated groups of paired tumor tissues and non-tumor tissues, as exhibited in **Figure 4B**, the expression of miR-335 was significantly reduced in the methylated tumor group (n=60) than that in the unmethylated tumor group (n=21). However, no significant difference were observed in non-tumor tissues (Methylation=39, Unmethylation=42) (**Figure 4B**).

subsequently, we examined the methylation statuses of the CpG islands with BSP in selected primary GC tissues and corresponding non-tumor tissues (n=15), which is same to the cases that have carried out analysis of miR-335 expression (**Figure 2E**). But the methylation level presented none of statistical significance in non-tumor tissues and tumor tissues (**Figure 4C**), which may be lie in the small sample. Healthy groups also have no statistical significance in selected 10 cases (**Figure 4D**). Nevertheless, we still found paired GC cases 8 with medium miR-335 expression revealed a similar methylation status, whereas GC tissue 14 showed lower miR-335 expression than corresponding non-cancerous tissues exhibited a stronger methylation pattern than paired non-cancerous tissues. (**Figure 4E**, **4F**), the result is analogous to that showed in **Figure 4A**. These data also proved the correlation result in cell lines.

Identification of target genes of miR-335

Previous study have found that SP1 and Bcl-w that were targeted by miR-335 may contribute to the metastasis of gastric cancer [12], we confirmed it by microrna , miRBase, TargetScan, PicTar , Rnahybrid and miRNA binding site searching site RegRna. Interesting, we find another overlapping gene -RAS p21 protein activator (GTPase activating protein) 1 (RASA1) - of all targeting website in our computational searches, it has been reported contribute to the occurrence, development and metastasis of cancer [26], whose miR-335 binding site in 3'-UTR exhibit in **Figure 5**. Firstly, we analyzed the

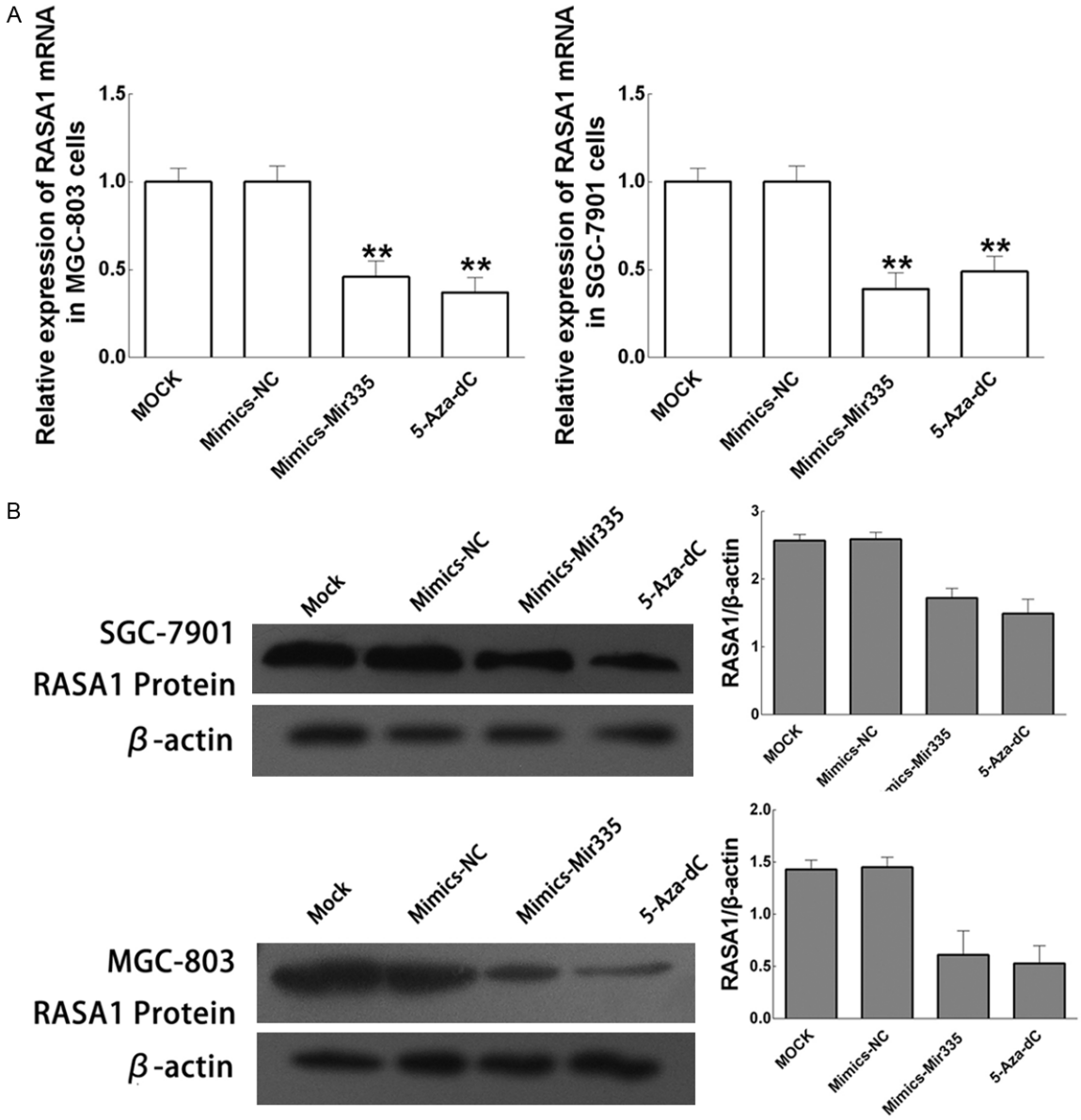

Figure 6. Targets of miR-335. A. RTQ-PCR results of candidate target genes in the indicated GC cell lines transfected with Hsa-miR-335 mimics and negative control (NC mimic) and with 5-aza -CdR treatment. Results are shown relative to a value of 1 assigned to cells without treatment (Mock), after normalization to internal β-actin RNA expression . Shown are the means of three replications, error bars represent stand deviations. **refers to statistical significance between cell lines ($p < 0.01$, *$p < 0.05$); B. West blot analysis of the protein levels downregulated by miR-335 and 5-aza -CdR in the indicated GC cell lines SGC-7901 and MGC-803. The histogram represents the gray value ratio of RASA1 and internal β-actin protein levels.

expression of RASA mRNA and protein in selected paired non-tumor tissues and tumor tissues, and metastasis tissues, the result showed that RASA protein and mRNA overexpressed in GC tissues, especially the metastasis tissues (Supplementary Figure 4). Taking account of theses, we conduct a preliminary experiment, in which we transfected GC cell lines (SGC-7901 and MGC-803) with hsa-miR-335 mimics and negative control miRNA mimics (mimics-NC) and then carried out RTQ-PCR and western blot analysis. We observed that ectopic expression of miR-335 had suppressed the expression of RASA1 mRNA after 48 h transfection (**Figure 6A**). In addition, western blot analysis showed significant suppression of RASA1 protein (**Figure 6B**). Finally, using real-time PCR, we found that the expression of

RASA1 mRNA and protein were also downregulated in SGC-7901 and MGC-803 cells treated with 5-aza -CdR, which implied that restoring the endogenous miRNAs expression can effects similar to those observed with transfection of exogenous miR mimics (**Figure 6A**, **6B**-5-aza -CdR). Because we concentrate on the methylation of miR-335 in this article, to further investigate the result and the mechanism by which cancer cell invasion and metastasis in GC, we will carry out subsequent experience. Despite of these, we tentatively put forward the RASA1 may be another target gene of miR-335 suppressed, by which inhibited the gastric cancer cell invasion and metastasis.

Discussion

MicroRNAs (miRNAs) are 20-22 nt non-coding RNAs which promote the degradation of target mRNAs or repression of the translation of mRNAs by sequence specific targeting, By which miRNAs are involved in many molecular pathways and in pivotal biological processes including cell growth, development, differentiation, proliferation and cell death [27]. Importantly, it has recently received recognition for the significant role that they play in the complex circuitry that regulates cancer metastasis [28-30]. Of these, miR-335 has been, firstly, identified as a potent suppressor of tumor cell migration and invasion in breast cancer by targeting metastasis associated gene SOX4 and TNC [8], Subsequently, more and more scholars found its role in other cancer metastasis, just as it inhibited small cell lung cancer bone metastases via IGF-IR and RANKL pathways [31], suppressed migration and invasion by targeting ROCK1 in osteosarcoma cells [9]. Xu Y [13] studied its role in gastric cancer and found the high expression of MiR-335 acts as a metastasis suppressor in gastric cancer by targeting Bcl-w and specificity protein 1. In our research, miR-335 was also found to be frequently downregulated in gastric cancer tissues and cell lines, which significantly associated with lymph node metastasis and peritoneal metastases.

What is the molecular mechanism that leads to the aberrant expression of miRNAs? Previous studies have shown that the transcriptional silencing by promoter hypermethylation has emerged as one of the important mechanisms of cancer development [14, 32]. Moreover, many miRNAs have been reported to be downregulated due to hypermethylation of the CpG islands in GC [33]. Whether miR-335 also follows this mechanism in GC is unclear, Kim J. Png [18] and OSAMU DOHI [19] analyzed its epigenetic mechanism in human breast cancer and hepatocellular carcinoma, respectively. They found that the miR-335 locus resides in the second intron on the mesoderm-specific transcript (Mest) gene and the mechanisms, namely aberrant promoter hypermethylation, that regulate the Mest gene transcript also dictate miR-335 expression. Taking account of the role of miR-335 in these cancers, we hypothesized that the miR-335 genes could also be silenced by DNA methylation in GC. Firstly, we prove the likelihood of this mechanism by means of bioinformatics data and treated 6 GC cell lines with 5-Aza-DC. We next wondered if promoter hypermethylation is sufficient to silence miR-335 expression in GC, and also whether promoter hypermethylation of these CpG islands (CGIs) and miR-335 expression are causally related. We investigated the methylation status of miR-335 in GC cell lines with and without 5-Aza-DC treatment, and tumor tissues using methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP). The result suggested the expression of miR-335 was inversely correlated to CpG island methylation in tumor tissues and cell lines. Previous researches have indicated that *H. pylori* infections induce the aberrant DNA methylation in gastric epithelial cells, and higher methylation can be seen in non-cancerous gastric mucosae from patients with multiple GC [17], but miR-335 didn't present the same result in our study, which may be suggest the lower miR-335 expression induced by abnormal methylation may be mainly involved in gastric cell invasion and metastasis rather than GC occurrence and development. In addition, our target genes preliminary study imply that the exogenous and endogenous miR-335 expression repressed expression of RASA1, which recently have been reported its role in cancer cell invasion and metastasis [26]. For the specific mechanism, detailed analysis is required in order to reach a conclusive decision.

The identification of marker that can predict the occurrence of metastases and estimate the prognosis is one of the highest priorities for translational cancer research. Previous studies have shown miRNAs could represent such long-awaited markers in several different cancer types [29, 34]. Of these, miR-335 not only cor-

related with poor metastasis-free survival, and could be used as biomarkers in metastasis risk assessment but also has the potential to recognize the recurrence risk and relate to the prognosis of cancer patients [8, 12, 13]. Our study also proved this conclusion that aberrant expression of miR-335 correlated with cancer cell invasion and metastasis. In addition to miRNAs in primary and metastatic tumor tissues just as discussed above, cell-free circulating miRNAs can be detected in plasma and serum because these miRNAs are reproducible, consistent, and resistant to RNase [35]. For instance, the positive detection rate of the serum miR-31 is much higher than that of the serum CEA (68.29% versus 21.95%) in GC patients, which indicates that miR-31 may be a novel diagnostic marker for GC.

In addition to aberrant miRNAs expression, DNA methylation changes might be useful biomarkers for diagnosis, prognosis and prediction of treatment outcome in cancer as DNA is relatively stable compared to other sources such as mRNA and proteins and can be obtained from ductal lavage fluids, cell-free DNA in blood as well as fine needle aspirates of the primary tumor [2, 36]. For example, fine needle aspirates have recently been used to diagnose early breast cancer lesions as atypical ductal hyperplasia (ADH), DCIS and LCIS [37], and hyper methylation of P16 and MGMT was detectable in sputum years before the clinical occurrence of lung cancer [38]. Similarly, miR-155 is down-regulated and methylated in GC, but up-regulated in breast cancer, pancreatic ductal adenocarcinoma [39, 40], by which we can distinguish different cancer types by comparing their miRNAs methylation profiles. We detected the DNA methylation status of miR-335 in tumor tissues by means of BSP and MSP and found the tumor tissues, especially metastatic carcinoma tissues, had aberrant hypermethylation status than normal tissues, the miRNA methylation level was positively associated with the clinical stage of GC patients. Furthermore, DNA is less prone to degradation than RNA, frequency, stability, and variability between patients, which may indicate clinical usefulness. So we believe, except the expression of miRNAs, that aberrant methylation of miR-335 promoters is another ideal epigenetic tumor marker.

Unlike genetic modifications, epigenetic changes are reversible, making them a potential therapeutic target [14]. Results in our study indicate that the expression of silenced miR-335 in GC could be restored by treating with demethylating agents 5-aza-2'-deoxycytidine, leading to inhibition of growth, invasive and metastasis of GC cells. In fact, the 5-azacytidine (azacitidine) [41] and 5-aza-2-deoxycytidine (decitabine) [42] that modify DNA methylation have been approved by the US Food and Drug Administration (FDA) for the treatment of patients with MDS. Subsequently, different DNA methylation inhibitors emerged including cytidine analogue zebularine, the antiarrhythmic procainamide and SGI-1027 [43]. But the full-scale fulfilment of these demethylating agents in the clinical setting meets several challenges. One is the not too perfect correlation between the clinical activity of these drugs and their hypomethylating effect. One possibility is that beyond a certain threshold [32]. In our research, the optimum concentration of 5-Aza-dC, with which reated GC cell lines SGC-7901 and MGC-803, were 5 μmol/l and 10 μmol/l, respectively. So how to choose agents correlating with a better clinical outcome is a problem to be solved.

In summary, our results confirmed that miR-335 as an important antimetastatic miRNA is downregulated and associated with lymph node-metastasis, peritoneal metastasis and poor pT stage in gastric cancer. We firstly described that miR-335 is downregulated in GC via aberrant promoter hypermethylation and involved in GC by target RASA1 gene. However, in normal stomachs of healthy individuals, moderate levels of miR-335 methylation aren't associated with *H. pylori* infection. These findings suggest the methylation status and the expression level of miR-335 may serve as epigenetic tumor markers of GC, Further studies on the epigenetic regulation of miRNA expression are necessary, and the regulation of miRNA expression by epigenetic drugs may has great promise for cancer prevention and therapy.

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Disclosure of conflict of interest

No potential conflicts of interest were disclosed.

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Supplementary Table 1. The bioinformatics genome database and websites in this reserch

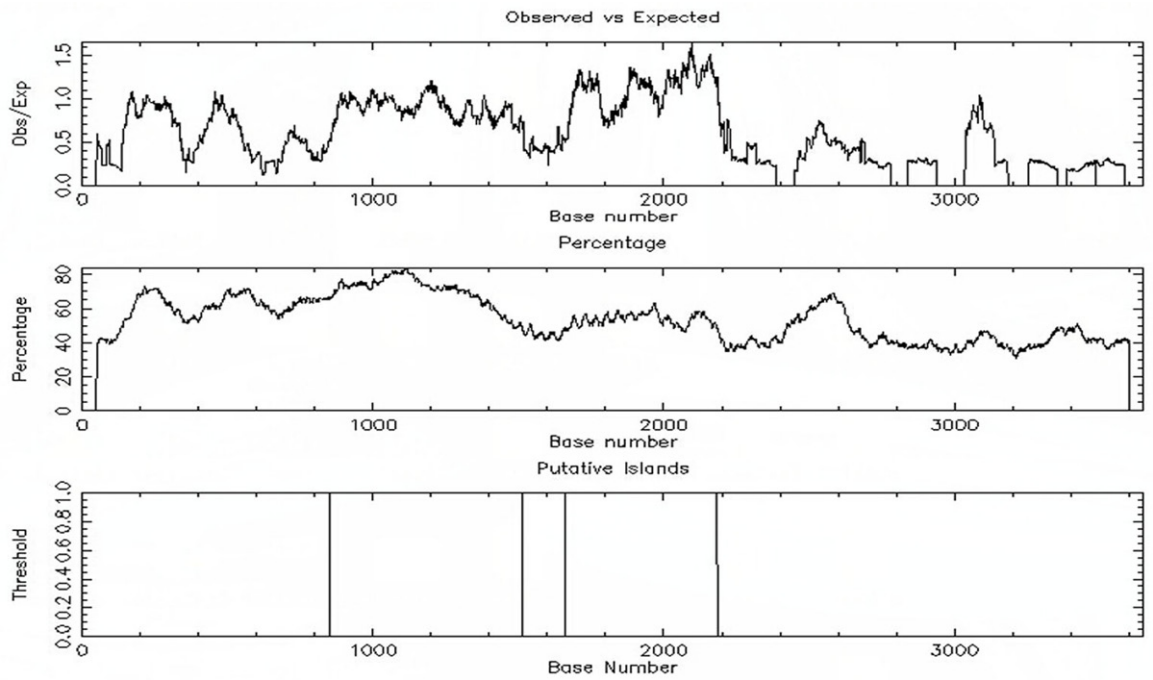
Funtion	Abbreviation in article	Uniform Resource Locator
Genome database	UCSC	http://genome.ucsc.edu/index.html
	NCBI	http://www.ncbi.nlm.nih.gov/
	Mirbase	http://www.mirbase.org/*
Promoter search	BDGP	http://fruitfly.org:9005/seq_tools/promoter.html
	BIMS	http://www-bimas.cit.nih.gov/molbio/proscan/
	Promoter 2.0 Prediction Server	http://www.cbs.dtu.dk/services/Promoter/
	TFSEARCH	http://www.cbrc.jp/research/db/TFSEARCH.html
Scanning for CpG islands	CpG Island Searcher	http://cpgislands.usc.edu/
	Cpg plot	http://www.ebi.ac.uk/Tools/emboss/
	Methprimer	http://www.urogene.org/index.html*
Design BSP primer	Methyl Primer Express v1.0	http://www.zedload.com/*
DNA methylation analysis of clone sequence	Methtools	http://genome.imb-jena.de/methtools/
	QUMA	http://quma.cdb.riken.jp/
	Biq-analyzer	http://www.mpi-inf.mpg.de/
	CpG viewer	http://dna.leeds.ac.uk/
Predicted microRNA targets	microRNA	http://www.microrna.org/microrna/home.do
	Targetscan	http://genes.mit.edu/tscan/targetscan2003.html
	PicTar	http://pictar.mdc-berlin.de/
	Rnahybrid	http://bibiserv.techfak.uni-bielefeld.de
	RegRna	http://regrna2.mbc.nctu.edu.tw/

*Part of the website can perform multiple functions, For example, Methprimer for CpG islands prediction and primer design.

Supplementary Table 2. The primer sequence for RTQ-PCR, BSP and MSP in this reasearch

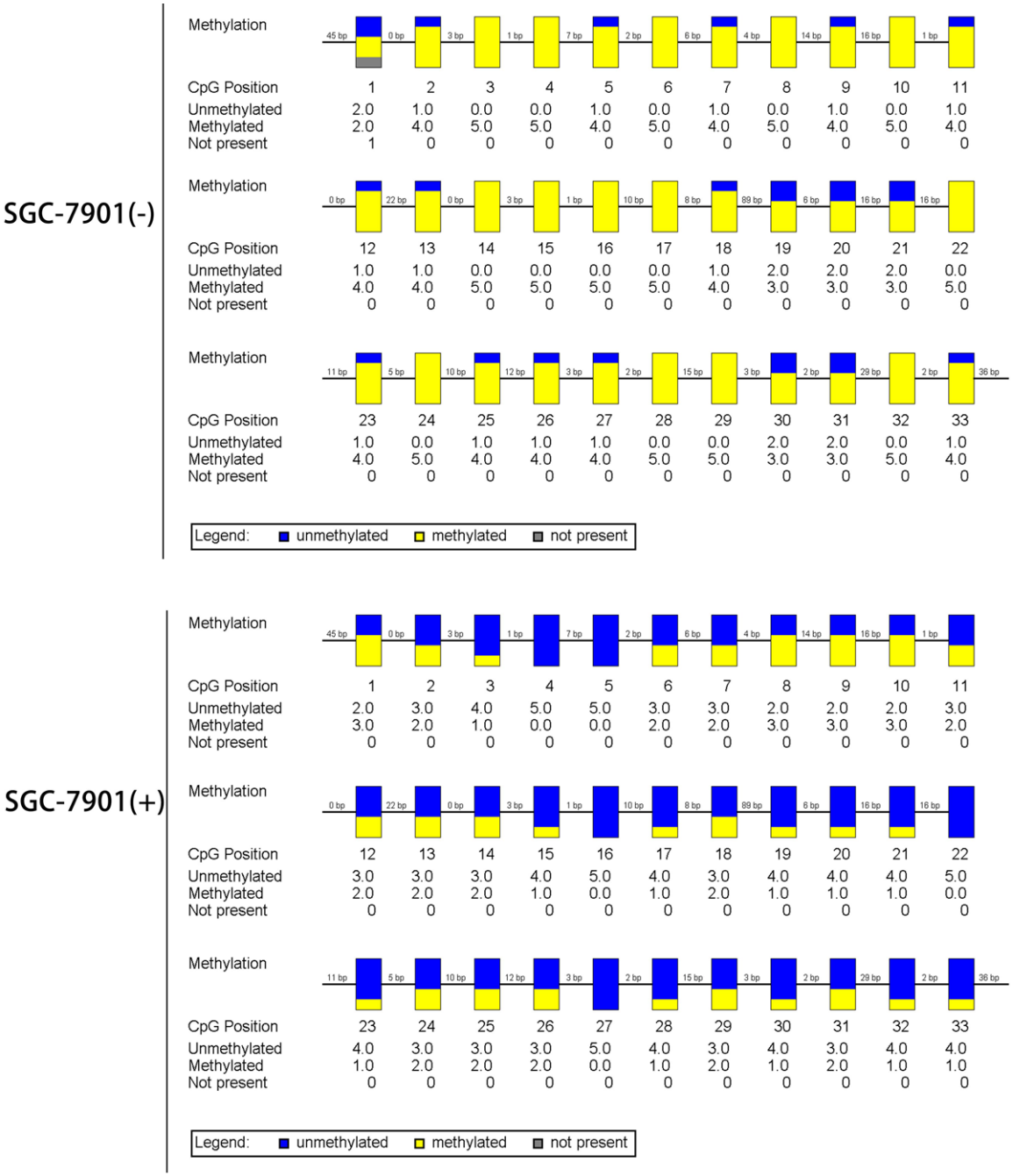
Primer	Primer sequence (5'-3')		Length (bp)
RASA1	F:	AGGCGTGTACGAGCCATTC	174
	R:	CACCTCTTCCACTAGGTCTTCAA	
U6	F:	CTCGCTTCGGCAGCACA	94
	R:	AACGCTTCACGAATTTGCGT	
MiR-335	F:	TCAAGAGCAATAACGAAAAATGT	81
	R:	GCTGTCAACGATACGCTACGT	
MiR-335 BSP primer	F:	TAAAGGGGGTTTTGTTTTTTTAATT	466*
	R:	CCCACAAACTACCCACAAAC	
MiR-335-M1	F:	TTTGTATTGTGATTTTATTTTACGT	172
	R:	AACAAATTTCCTTTACAACAACG	
MiR-335-U1	F:	TTTGTATTGTGATTTTATTTTATGT	173
	R:	AAACAAATTTCCTTTACAACAACAC	
MiR-335-M2	F:	GGTTTTAAAAGTCGGTGTTTATTC	125
	R:	AACTACAACCACTCCGACGTA	
MiR-335-U2	F:	GGGTTTTAAAAGTTGGTGTTTATTT	129
	R:	AACAACTACAACCACTCCAACATA	
MiR-335-M3	F:	TGGCGTATTTTAGGATTTTAAGAAC	141
	R:	TAAAACGACTAACGAACACTACGAA	
MiR-335-U3	F:	GTGTATTTTAGGATTTTAAGAATGG	139
	R:	TAAAACAACTAACAAACACTACAAA	
miR-335 mimics	F:	ACGCGCUGCACCAUGUUUGUUUUU	
	R:	AGCGGUGCACCAUGUUUGUUUUU	
NC mimic	F:	UUCUCCGAACGUGUCACGUTT	
	R:	ACGUGACACGUUCGGAGAATT	

RTQ quantitative real-time PCR. BSP bisulfite sequence-PCR. MSP methylation-specific PCR. F forward primer. R reverses primer. U unmethylated primer. M methylated primer. NC negative control. *this BSP product contain 33 CpG site.

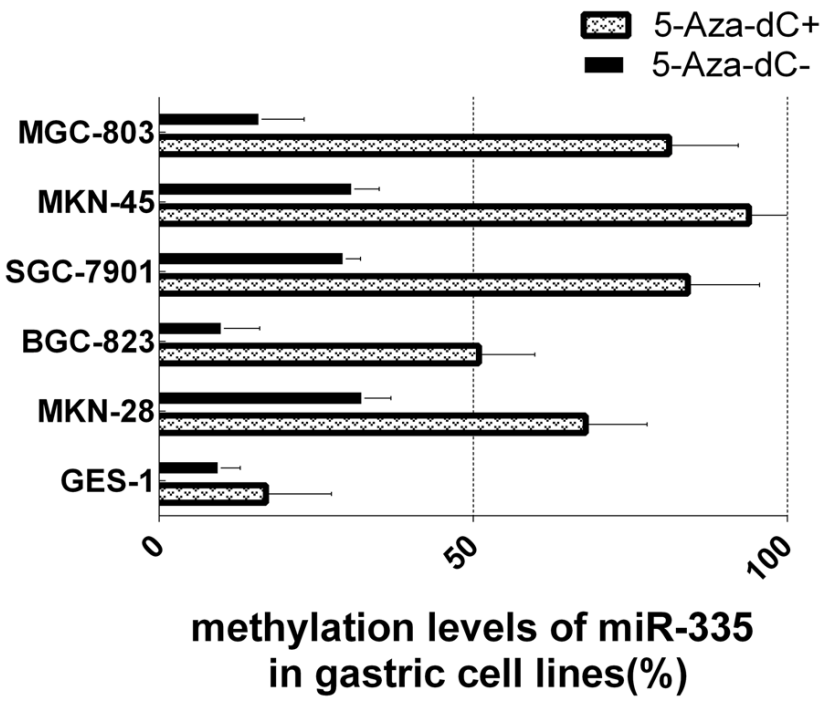



```
CPGPLOT islands of unusual CG composition
EMBOSS_001 from 1 to 3650

     Observed/Expected ratio > 0.60
     Percent C + Percent G > 50.00
     Length > 200

 Length 664  (852..1515)

 Length 522  (1664..2185)
```

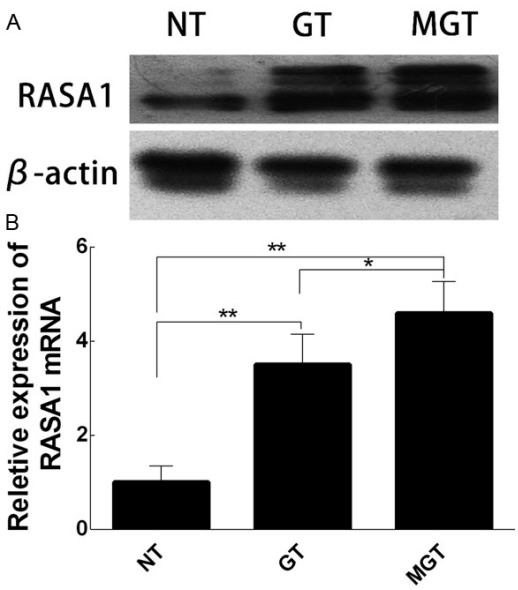
Supplementary Figure 1. Scanning for CpG islands with the submitted sequence in EMBOSS Cpg plot. Two CpG islands were shown in the Figure. Length 664 (852...1515) and length 522 (1664..2185).



Supplementary Figure 2. The bisulfite sequencing of genomic DNA analyzed by BiQ Analyzer. Each square represents a CpG site. Yellow squares represent methylated CpG dinucleotides whereas blue squares represent unmethylated CpG sites. If the methylation state of a CpG site could not be defined, it is represented as not present. The ratios of the filled area in the squares represent the methylation status in the CpG sites. The number of base pairs between each CpG dinucleotide is indicated at the top. One representative experiment in SGC-7901 with and without 5-aza -CdR treatment is shown.



Supplementary Figure 3. Methylation status in GC cell lines, with (+) or without (-) 5-aza -CdR treatment.



Supplementary Figure 4. Expression of RASA1 in different gastric tissues. A. RASA1 protein levels in the selected NT (Normal tissues), gastric cancer tissues (GT) and metastasis tissues of gastric cancer tissues; β-actin was used as an internal loading control. B. RASA1 mRNA levels in the selected NT (Normal tissues), gastric cancer tissues (GT) and metastasis tissues of gastric cancer tissues (MGT); β-actin was used as an internal loading control. **refers to statistical significance between different tissues (**$p < 0.01$,*$p < 0.05$).