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Genetic polymorphism at miR-181a binding site contributes to gastric cancer susceptibility

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Recent evidences show that genetic polymorphisms falling in miRNA binding sites can alter the strength of miRNA binding and disturb miRNA-mediated posttranscriptional regulation. Our study aimed to investigate the role of single-nucleotide polymorphisms (SNPs) in putative miRNA binding sites in gastric cancer (GC). Based on microarray and quantitative reverse transcription PCR analyses, we found that miR-181a was significantly upregulated in GC tissues. Bioinformatics survey was used to explore SNPs within miR-181a binding sites. Three SNPs were genotyped in a case–control study (500 cases and 502 controls). The T allele genotypes (rs12537CT and TT) of *MTMR3* were found associated with significantly increased GC risk [adjusted odds ratio 1.72, 95% confidence interval (CI) 1.36–2.16, $P = 3.99 \times 10^{-5}$] and poor overall survival [hazard ratio (HR) 1.38, 95% CI 1.03–1.83, $P = 0.029$], although they were not an independent prognostic factor in multivariate Cox regression analysis (HR 1.28, 95% CI 0.95–1.72, $P = 0.11$). We further demonstrated that the rs12537CT genotype carriers had lower *MTMR3* mRNA expression levels than CC genotype carriers in GC tissues ($P = 0.013$), whereas no significant difference in miR-181a expression levels was found ($P = 0.135$). Luciferase assay revealed that miR-181a directly targeted *MTMR3*, and its suppressive effect was enhanced when the rs12537C allele was substituted by T variant, although the difference was not significant ($P = 0.055$). Our study suggested that rs12537 is associated with susceptibility and prognosis of GC in southern Han Chinese, and miR-181a and its target gene *MTMR3* play important roles in GC.

Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide, with an estimated 989 600 new cases and 738 000 deaths occurred in 2008, accounting for 8% of the total cancer cases and 10% of total cancer deaths (1). Contributing factors for GC include environmental factors and genetic background. Hence, searching for susceptibility genes is currently under intense investigation.

MicroRNAs (miRNAs) play key roles in posttranscriptional regulation mainly by pairing to the 3′ untranslated regions (UTRs) of target mRNA. It has been demonstrated that perfect complementarity to the seed region—nucleotides 2 through 8 from the 5′ end of the mature miRNA—is essential for miRNA–mRNA interactions, whereas incomplete complementarity in seed region can disturb miRNA-mediated repression of target gene expression (2,3). Recently, a growing number of studies suggest that single-nucleotide polymorphisms (SNPs) can create, destroy or modify the efficiency of miRNA binding to the 3′UTR of target gene, and ultimately affect individual's cancer risk (4–6) and prognosis (7,8). For instance, SNP rs61764370 (T>G) in the 3′UTR of *KRAS* is associated with a 2.3-fold increased risk of developing lung cancer in moderate smokers by disrupting *let-7* binding (4). SNP rs17281995 (G>C) in the 3′UTR of *CD86* was predicted to alter the binding sites for five miRNAs, and was found associated with a 2.74-fold increased risk of colorectal cancer (5). Similarly, G-containing genotype of rs1044129 (A>G) in the 3′UTR of *RYR3* was related to breast cancer calcification and poor survival, due to the change of miR-367 binding sequence (6). Nevertheless, the extent to which SNPs interfere with miRNAs regulation and affect GC susceptibility remain largely unknown.

In this study, firstly, microarrays were used to examine the expression profiles of miRNAs in human GC tissues. Then we validated the aberrantly expressed miRNAs using quantitative reverse transcription PCR (qRT–PCR), and confirmed that miR-181a was significantly upregulated. Secondly, we performed a bioinformatics survey and found five SNPs within miR-181a target sites. Thirdly, a case–control study was conducted and showed that rs12537 of myotubularin-related protein 3 (MTMR3) in miR-181a target site contributed to increased risk and poor overall survival of GC. Finally, we investigated miR-181a and *MTMR3* expression in GC tissues from patients and in different cell lines. Furthermore, functional assays were performed using site-directed mutagenesis and luciferase assays and validated the interaction between miR-181a and *MTMR3*. Our finding indicates that genetic variants affect cancer risk by altering the interactions between miRNA and its target gene, which is an important molecular mechanism of carcinogenesis.

Abbreviations: CI, confidence interval; GC, gastric cancer; GCT, gastric cancer tissues; MAF, minor allele frequency; MFE, minimum free energy; miRNA, MicroRNA; MTMR3, myotubularin-related protein 3; NC, negative control; NCT, non-cancerous tissues; NT, normal tissues; OR, odds ratio; RFLP, restriction fragment length polymorphism; SNP, single-nucleotide polymorphism; TNM, tumor-node-metastasis; UTR, untranslated region.

Materials and methods

Patients and tissue samples

The study was approved by the Clinical Research Ethics Committee of the Guangzhou First Municipal People's Hospital. Written informed consent was obtained from each participant. All recruited subjects were unrelated Chinese Han ethnic, inhabiting southern China. All cases enrolled were primary gastric adenocarcinoma.

For the miRNAs study, 50 GC patients underwent surgery between April 2009 and December 2010 at the Guangzhou First Municipal People's Hospital; they were consecutively recruited. The patients who received chemotherapy and/or radiotherapy before surgery were excluded. Fresh gastric cancer tissues (GCT) and corresponding adjacent non-cancerous tissues (NCT) (at least 5 cm away from the tumor margin) were collected from each patient. Meanwhile, normal gastric mucosa tissues (NT) were collected by endoscopy from 50 age- and gender-matched cancer-free controls. All tissue samples were snap-frozen in liquid nitrogen and stored at –80°C.

A hospital-based case–control study, consisting of 500 GC cases and 502 cancer-free controls, was conducted to investigate the relationship between SNPs and GC risk. Cases were consecutively enrolled from July 1999 to December 2008 at the same hospital. Formalin-fixed, paraffin-embedded NCT of GC patients were collected from Department of Pathology. Controls were consecutively recruited from patients hospitalized in the Department of Gastroenterology with non-malignant diseases from January 2009 to October 2010. The control group mainly comprised patients with irritable bowel syndrome, cholecystitis, pancreatitis, duodenal ulcers, etc. Exclusion criteria included gastrointestinal polyps, adenomas, gastric ulcer, diseases related to or causing predisposition to cancer (e.g. ulcerative colitis, Crohn's disease and chronic atrophic gastritis), or a family history of GC. DNA samples of controls were isolated either from peripheral blood (from 162 patients) or from gastroscopic biopsy specimens of NT (from 340 patients), depending on the accessibility of peripheral blood or biopsy samples. Clinical data and pathological characteristics were collected from patient medical records.

The tumor stage was determined based on the surgical and imaging findings according to the tumor-node-metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) (9). The histological

diagnosis of each case was double confirmed by two experienced pathologists independently. The pathohistological classification was based on Lauren's system (10), and the differentiation grade was also assessed according to the AJCC (9).

MiRNA microarray analysis

Microarray hybridization was performed on seven matched-pairs of GCT and NCT, which were randomly selected from the 50 matched-pair specimens of GC. The array contained 1146 human miRNAs to detect >97% of the miRNAs described in the miRBase (Release 12). Total RNA was isolated from frozen tissues with Trizol (Invitrogen). Then, 200 ng of total RNA was labeled and hybridized on Human v2 MicroRNA Expression BeadChips (Illumina, San Diego, CA). BeadChips were scanned with Illumina Scanner and data were analyzed by BeadStudio software (Illumina). The aberrantly expressed miRNAs were determined using quantile normalization and the Illumina custom error model. The BeadStudio software uses diffscores to describe expression differences between groups. Diffscore of >13 or <–13 correspond to a P value of 0.05. Diffscore of >20 or <–20 correspond to a P value of 0.01. A miRNA expression was considered significantly different if the P value was <0.05. We selected miRNA with a P value <0.01 for further analysis. The miRNA microarray was performed by Shanghai Biotechnology Co., Ltd (Shanghai, China).

Quantitative reverse transcription PCR analysis

Total RNA was extracted from tissues and cell lines using Trizol (Invitrogen). Reverse transcription was done using miRCURY LNA Universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark) for miRNA, and using GoScript Reverse Transcription System (Promega, Madison, WI) for mRNA.

Mature miRNA expression was quantified using SYBR Green master mix (Exiqon) and specific LNA PCR primer sets (Exiqon). Small nuclear RNA U6 snRNA (Exiqon) was chosen as the normalization control. Quantitative PCR (qPCR) was performed using Brilliant II SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). GAPDH was used as an internal control. Primers and qPCR reaction conditions are listed in Supplementary Table I, available at *Carcinogenesis* Online.

qPCR was performed using Opticon 2 Fluorescence Detection System (MJ Research, Waltham, MA). Each qPCR was performed in triplicate. Relative quantification was based on the threshold cycle (Ct) values generated by the Opticon Monitor Analysis Software 2.02 (MJ Research). Since the distributions of *MTMR3* and miR-181a expressions in tissue samples were highly skewed, a log transformation was used. The relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method (11).

DNA extraction

Genomic DNA of the 500 GC patients was isolated from formalin-fixed, paraffin-embedded tissues using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). DNA of the fresh frozen tissue samples and cell lines were extracted by QIAamp DNA Mini Kit (Qiagen). The germline DNA was extracted from 200 µl of peripheral blood using QIAamp DNA Blood Kit (Qiagen).

Genotyping

PCR-restriction fragment length polymorphism (PCR-RFLP) was used to identify polymorphisms. See also Supplementary Methods, available at *Carcinogenesis* Online. The RFLP-PCR analyses were performed by two staffs independently without knowledge of the subjects' case–control status. To confirm the genotyping results, 10% of the samples (randomly selected) and all the cell lines were verified by direct sequencing (Invitrogen, Guangzhou, China). PCR-RFLP genotyping results and sequence validation of SNPs are shown in Supplementary Figure 1, available at *Carcinogenesis* Online.

Bioinformatics

Firstly, to search for potential target genes of miR-181a, four online algorithms, including TargetScan 5.2 (12), miRanda (13), PITA (14) and PicTar (15), were used. Genes predicted by at least two target prediction algorithms were selected. Secondly, we performed a PubMed literature search to choose genes related to GC. The search strategies included the names of the predicted genes and "Gastric Cancer". Next, we searched PubMed and three online databases, including Tarbase (16), miRecords (17) and miRTarBase (18), to retrieve all experimentally validated hsa-miR-181a target genes. Thirdly, after selection of validated miR-181a target genes and unvalidated target genes related to GC, SNPs residing in the 3′UTRs of these genes were retrieved from dbSNP Build 129 (http://www.ncbi.nlm.nih.gov/projects/SNP). Only SNPs with the minor allele frequency (MAF) ≥0.10 (for Chinese) were chosen for further study. For each SNP, we retrieved two sequences, centering on each allele with 25 nucleotides flanking on both sides. Finally, we used miRanda software (version 3.3a) (19) with the default parameters and cutoffs [Score ≥140 and minimum free energy (MFE) ≤−7 kcal/mol] to assess the base-pairing interaction between miR-181a and SNP-centered sequences for both common and variant alleles. Only the SNP within the putative binding site of miR-181a seed region was selected.

Cell lines and miRNA transfections

Human gastric cancer cell line SGC-7901, MGC-803, BGC-823, AGS, and human embryonic kidney cell line HEK-293T were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. The human gastric epithelial cell line GES-1, human gastric cancer cell lines HGC-27 and MKN-28 were donated by Prof. Yiguo Jiang (Guangzhou Medical University, China) (20). All the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin (both from Gibco, Carlsbad, CA,). All cell lines were incubated at 37°C in humidified 5% CO_2 atmosphere.

All the RNA oligoribonucleotides were synthesized and purchased from GenePharma (Shanghai, China). See Supplementary Table I, available at *Carcinogenesis* Online, for the oligoribonucleotides sequences. SGC-7901 cells were cultured in 24-well plates at a density of 20 000 cells per well the day before transfections. Cells (~50% confluent) were transfected with miR-181a mimic (50 nmol/l), miR-181a inhibitor (100 nmol/l), miRNA negative control (NC) (50 nmol/l) or NC inhibitor (100 nmol/l) using Lipofectamine™ RNAiMAX (Invitrogen). After 5 h incubation, medium in each well was replaced by serum-containing medium. Total RNA and protein were extracted 48 h after transfection. A blank control only treated with the transfection reagent was used.

Western blot analysis

Total proteins were extracted from SGC-7901 cells using Cell Lysis Reagents (Pierce, Rockford, IL) and quantified by the bicinchoninic acid assay method (Pierce). A 30 µg sample of protein was separated on 10% sodium dodecyl sulfate-polyacrylamide gel, and then transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Chicago, IL). The membrane was blocked and incubated with an mouse anti-MTMR3 polyclonal antibody (dilution, 1:400, Novus, Littleton, CO) or rabbit anti-GAPDH polyclonal antibody (dilution, 1:2500, Abcam, Cambridge, UK) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse 1:2000; goat anti-rabbit 1:4000, Dako, Glostrup, Denmark) for 40 min at room temperature. Signals were detected by chemiluminescence using Luminol Reagent (Santa Cruz Biotechnology). The GAPDH gene was used as an internal control. The band intensity was analyzed using Gel-Pro Analyzer software (Version 4.0, Media Cybernetics, LP).

Luciferase reporter assay

Homo sapiens MTMR3 have three transcript variants, and they share the same 3′UTR sequences of 5065 bp. The full-length of *MTMR3* 3′UTR was too long to be cloned into psiCHECK2 plasmid (Promega). To reduce the length, we deleted 1381 bp sequences, which did not contain the predicted binding sites of miR-181a, and chose the remaining 3684 bp (from 1382 to 5065 bp of 3′UTR), containing allele. The remaining 3′UTR of *MTMR3* sequences was amplified from a homozygous human genomic DNA sample. The PCR products were separated in 1% agarose gel and extracted, purified and cloned into the *Not*I and *Xho*I sites in a psiCHECK2 plasmid. A mutant plasmid with T allele at the site of rs12537 was constructed using site-directed mutagenesis primers based on rs12537C psiCHECK2-MTMR3 3′UTR plasmid. Primers and PCR reaction conditions are provided in Supplementary Table I, available at *Carcinogenesis* Online. All vectors were confirmed by direct sequencing (Invitrogen). The generated reporter vectors were named psiCHECK2:rs12537C and psiCHECK2:rs12537T, respectively.

To conduct the luciferase reporter assay, HEK-293T cells were cultured in 24-well plates at 20 000 cells per well. After an overnight incubation, each well was treated with a transfection mixture consisting of 300 µl of Opti-MEM (Invitrogen), 1 µl of Lipofectamine™ RNAiMAX (Invitrogen), 0.5 µg of psiCHECK2 vector (psiCHECK2:rs12537C or psiCHECK2:rs12537T), and with or without 20 pmol of miRNA (miR-181a mimic, miR-181a inhibitor, NC or NC inhibitor). After 5 h incubation, medium in each well was replaced by serum-containing medium. At 48 h after transfection, the Renilla and firefly luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega). Each experiment was performed in triplicate. Relative luciferase activity was normalized to firefly luciferase activity and then compared with the psiCHECK2 vector (psiCHECK2:rs12537C or psiCHECK2:rs12537T) blank control.

Statistical analysis

Hardy–Weinberg equilibrium test was performed for the genotype distribution. Discrete variables were evaluated by Pearson's chi-square test. Unconditional logistic regression, adjusted by gender, age, smoking and alcohol status, was used to analyze odds ratios (ORs) and 95% confidence intervals (CIs) for the association of genotypes with GC risk. Major allele homozygotes were set as the reference genotype. As the genetic inheritance model is unknown, analyses

were done under a codominant model and an additive model. Bonferroni correction was applied for multiple comparisons. Overall survival time was calculated from the date of operation until death or the last follow-up contact. The Kaplan–Meier survival curve and the log-rank test were used to evaluate the relationship between genotypes and survival. Hazard ratio (HR) and 95% CI for each factor in univariate analysis was calculated from the Cox proportional hazard regression model. The multivariate Cox regression method was used to determine the independent prognostic factors influencing overall survival. Multivariate Cox analysis was performed using variables with a $P < 0.05$ in univariate analysis. Differences between two groups were analyzed by Student's *t*-test or the non-parametric test. The statistical power was calculated using PASS 11.0 (NCSS, Kaysville, UT). Statistical tests were two-sided, and a $P < 0.05$ was considered statistically significant using SPSS 17.0 (SPSS, Chicago, IL) and STATA 12.0 (StataCorp, TX).

Results

Microarray results and real-time PCR validation

In seven matched-pairs of NCT and GCT analyzed by microarray, 30 miRNAs were aberrantly expressed ($P < 0.05$). Among them, six miRNAs were more significantly upregulated, and three were downregulated in GCT ($P < 0.01$) (Supplementary Table II, available at *Carcinogenesis* Online). Because the three downregulated miRNAs (HS_138, HS_153 and HS_157), originally identified by deep sequencing of small RNAs(21), were not included in miRBase (Release 18) (22), and two upregulated miRNAs (miR-21 and miR-21*) were found significantly overexpressed in GC in published literatures (23,24), they were excluded from current analysis. Therefore, the remaining four upregulated miRNAs (miR-181a, miR-27a, miR-584 and miR-93) were validated with qRT–PCR in 50 matched pairs of GCT and NCT. The overexpression rates in GCT for miR-181a, miR-27a, miR-584 and miR-93 were 40/50 (80%), 35/50 (70%), 28/50 (56%) and 30/50 (60%), respectively. qRT–PCR analysis showed that miR-181a ($P = 7.72 \times 10^{-5}$) and miR-27a ($P = 0.022$) were significantly overexpressed, whereas the other two miRNAs did not reach significance (miR-584, $P = 0.073$; miR-93, $P = 0.304$). We further evaluated the associations of clinicopathologic factors of GC with miR-181a and miR-27a. The high expression of miR-181a in GCT was associated with positive lymph node metastasis ($P = 0.042$), but not with age, gender, Lauren classification, tumor differentiation or TNM tumor stage. However, no significant associations were observed between expressions of miR-27a and clinicopathologic factors.

SNPs selection

We focused our study on miR-181a this time, since miR-27a has been reported previously (25). Five SNPs of target genes within miR-181a binding sites were selected using bioinformatics approach. Because rs13035 of *RECQL* failed genotyping by PCR-RFLP, thus was not investigated further. Another SNP, rs9266 of *KRAS*, has little MFE change (1.70%) between wild-type and variant alleles, which implied that it may have very limited impact on miR-181a binding. Therefore, we excluded it. Finally, three SNPs were chosen for further analysis (Table I and Supplementary Figure 2, available at *Carcinogenesis* Online).

Case–control study of SNPs with GC

The SNP analysis comprised 500 GC cases and 502 controls. Age and gender were adequately matched between two groups. There were no statistically significant differences between the cases and control subjects in terms of smoking status and alcohol consumption (Table II). All SNPs were in Hardy–Weinberg equilibrium in both controls and cases. As presented in Table III, adjusted logistic regression of the three SNPs showed that only rs12537 was associated with elevated GC risk. After adjustment for age, gender, smoking status and alcohol consumption, the rs12537CT genotype was associated with a 1.86-fold increased risk for GC (95% CI: 1.42–2.45, $P = 7.78 \times 10^{-6}$, Bonferroni-corrected $P = 7.0 \times 10^{-5}$), compared with the CC genotype. Subjects carrying the TT genotype were associated with higher risk of GC (adjusted OR = 2.17, 95% CI: 1.07–4.39, $P = 0.032$). However, the association was not significant after Bonferroni correction (Bonferroni-corrected $P = 0.288$). Meanwhile, the additive genetic effect model showed that T allele genotypes (CT and TT) carriers were at high risk of GC than CC carriers (adjusted OR = 1.72, 95% CI: 1.36–2.16, Bonferroni-corrected $P = 3.99 \times 10^{-5}$). Moreover, a study power of 92.1% (two-sided test, $\alpha = 0.05$) has been achieved to detect an OR of 1.72 for the T allele genotypes (rs12537CT and TT) in an additive model with a MAF of 0.154.

Subsequently, whether the rs12537 genotypes could be used as a survival predictive factor was determined. Among the 500 GC patients, 281 cases with complete follow-up data were chosen. Demographic characteristics of the subpopulation are summarized in Supplementary Table III, available at *Carcinogenesis* Online. The median follow-up was 41 months (range: 1–141 months). Kaplan–Meier survival curves showed that there was no association between three genotypes of rs12537 and overall survival ($x^2 = 5.042$, $P = 0.080$). However, compared with the CC genotype, the T (CT+TT) allele genotypes were significantly associated with poor overall survival ($x^2 = 4.932$, $P = 0.026$) (Figure 1). Univariate Cox regression analysis indicated that depth of tumor invasion, positive lymph node metastasis, TNM tumor stage and rs12537CT or CT+TT genotypes were significantly associated with decreased overall survival ($P < 0.05$ or $P < 0.01$). When those risk factors were entered together into the multivariate Cox regression analysis, only TNM tumor stage IV was found to be an independent prognostic factor for GC (Supplementary Table III, available at *Carcinogenesis* Online).

Association of rs12537 genotypes with MTMR3 mRNA expressions and miR-181a levels

To further evaluate the influence of rs12537 polymorphism on the expression of *MTMR3* and miR-181a, we examined the expressions of *MTMR3* mRNA and miR-181a in GCT and NCT from 50 GC cases, as well as in NT from 50 cancer-free controls (Figure 2). Among GC cases, 29 were CC genotype and 21 were CT genotype, whereas in the controls, 35 were CC genotype and 15 were CT genotype. The TT genotype was not found in either group.

In inter-group comparison, *MTMR3* expression levels were significantly higher in NT than in GCT and NCT from subjects both with

Table I. Candidate gastric cancer-associated genes having an SNP in the predicted miR-181a binding sites with MAF ≥ 0.10 (for Chinese)

Gene, dbSNP ID and allele substitution	MAF[a]	SC_w[b]	SC_v[b]	|ΔSC|	MFE_w[b]	MFE_v[b]	|ΔMFE|	MFE change (%)
MTMR3 rs12537 C>T	0.232	128	160	32	−10.85	−15.76	4.91	45.25
EREG rs1460008 G>A	0.329	140	108	32	−17.07	−10.73	6.34	37.14
IRF5 rs10954213 A>G	0.383	108	140	32	−6.31	−10.74	4.43	70.21
RECQL rs13035 A>C	0.455	146	114	32	−15.73	−12.40	3.33	21.17
KRAS rs9266 T>C	0.220	149	120	29	−12.97	−12.75	0.22	1.70

SC, seed region sequence complementarity score; w, wild-type allele; v, variant allele.
$\Delta SC = SC_w - SC_v$.
$\Delta MFE = MFE_w - MFE_v$.
[a]MAF in HAPMAP-CHB (retrieved from dbSNP Build 129)
[b]Detected by miRanda software (version 3.3a).

Table II. Demographic characteristics of gastric cancer cases and cancer-free controls

Characteristics	Case, *n* (%)	Controls, *n* (%)	*P*
All subjects	500 (100.0)	502 (100.0)	
Age (years)			
Range	20–92	18–91	
Mean	60 ± 13	60 ± 15	0.853[a]
≤60	249 (49.8)	248 (49.4)	0.900[b]
>60	251 (50.2)	254 (50.6)	
Gender			
Males	314 (62.8)	319 (63.5)	0.807[b]
Female	186 (37.2)	183 (36.5)	
Smoking status			
Ex-/current smoker	142 (28.4)	130 (25.9)	0.373[b]
Non-smoker	358 (71.6)	372 (74.1)	
Alcohol consumption			
Habitual	71 (14.2)	75 (14.9)	0.740[b]
Non	429 (85.8)	427 (85.1)	

[a]Two-side two-sample *t*-test.
[b]Two-side chi-square test.

CC genotype and CT genotype (all $P < 0.001$). However, no significant differences of *MTMR3* levels were found between GCT and NCT ($P = 0.071$), regardless of genotypes (CC genotype, $P = 0.619$; CT genotype, $P = 0.056$). Notably, the overexpression of miR-181a was highest in GCT, followed by NCT, and lowest in NT (all $P < 0.001$), for both CC genotype and CT genotype (all $P < 0.01$).

In the within-group comparison, the results suggested that subjects with CT genotype had lower levels of *MTMR3* expression than those with CC genotype. The difference, however, was statistically significant in GCT group ($P = 0.013$), but not in NCT group ($P = 0.254$) or NT group ($P = 0.110$). Nevertheless, we did not find a significant difference in miR-181a expression levels between patients with CC or CT genotypes among GCT ($P = 0.135$), NCT ($P = 0.116$) and NT group ($P = 0.060$).

Rs12537 genotypes affect MTMR3 expression by regulating the miR-181a binding

Based on miRanda software analysis (19), the 3′UTR of *MTMR3* had a predicted binding site (at 2899–2922) for miR-181a, and a substitution of T for C allele of rs12537 would create another miR-181a binding site (at 1653–1673) (Figure 3A). Generally, the inhibition of translation will be more effective and robust, when more than one binding site is present at the 3′UTR (2). Therefore, miR-181a would exert more inhibition on *MTMR3* expression when the C allele of rs12537 was substituted by T variant.

To test this hypothesis, a luciferase assay was performed in HEK-293T cells. The dual-luciferase assays demonstrated that both the psiCHECK2:rs12537C and psiCHECK2:rs12537T, cotransfected with miR-181a mimics, could lead to significant suppression of the luciferase activity, when compared with the control, miR-181a inhibitor, NC and NC inhibitor (all $P < 0.05$) (Figure 3B). Furthermore, the luciferase activities of psiCHECK2:rs12537T were lower than psiCHECK2:rs12537C after transfection with miR-181 mimics, although the difference was not significant ($P = 0.055$). However, elevation of luciferase activities in miR-181a inhibitor group was not observed. In spite of this, our experiments suggested that miR-181a could suppress luciferase expression by targeting the 3′UTR of *MTMR3*.

To further confirm our findings, we intended to detect the expression levels of *MTMR3* and miR-181a in GC cell lines carrying different rs12537 genotypes. Among the six different GC cell lines and gastric epithelial cell line GES-1 we screened, only CC genotype of rs12537 was identified. The data indicated that the expression of *MTMR3* was elevated when the miR-181a levels decreased among five GC cell lines, except for SGC-7901 (Figure 3C). Since SGC-7901 did not show inverse relationship between miR-181a and *MTMR3* expressions, we selected it to test the MTMR3 protein levels by transfecting with the miR-181a mimic and inhibitor. The results of qRT–PCR confirmed that miR-181a levels effectively decreased in cells transfected with miR-181a inhibitor, and significantly elevated when transfected with miR-181a mimic (Figure 3D). Similarly, artificial overexpression of miR-181a effectively reduced the levels of *MTMR3* protein, whereas inhibition of endogenous miR-181a could not increase the levels of *MTMR3* protein, suggesting a more complicated regulation mechanism (Figure 3E). Taken together, these results revealed that miR-181a suppressed MTMR3 expression by direct targeting of the 3′UTR of *MTMR3*.

Discussion

To our knowledge, this is the first study to demonstrate the effects of GC-associated gene 3′UTR SNPs within putative miR-181a binding sites on GC susceptibility and prognosis. We found that the T allele genotypes (rs12537CT and TT) in the 3′UTR of *MTMR3* were associated with increased risk and poor prognosis of GC in southern Chinese. Functional assays revealed that miR-181a suppressed *MTMR3* expressions by targeting the 3′UTR of *MTMR3*.

Aberrant miRNAs expression patterns have been investigated in GC. For instance, overexpressions of miR-192, miR-214 and miR-223,

Table III. Genotype distribution of SNPs among gastric cancer cases and cancer-free controls, and their association with gastric cancer risk

Gene, dbSNP ID and genotype	Case, *n* (%)	Controls, *n* (%)	Adjusted OR[a] (95% CI)	*P*	Bonferroni-corrected *P*
All subjects	500 (100.0)	502 (100.0)			
MTMR3 rs12537					
CC	287 (57.40)	360 (71.71)	1.00		
CT	191 (38.20)	129 (25.70)	1.86 (1.42–2.45)	7.78×10^{-6}	7.0×10^{-5}
TT	22 (4.40)	13 (2.59)	2.17 (1.07–4.39)	0.032	0.288
CC versus CT versus TT[b]			1.72 (1.36–2.16)	4.43×10^{-6}	3.99×10^{-5}
EREG rs1460008					
AA	237 (47.40)	261 (51.99)	1.00		
AG	214 (42.80)	192 (38.25)	1.23 (0.95–1.60)	0.122	1.098
GG	49 (9.80)	49 (9.76)	1.12 (0.72–1.72)	0.623	5.607
AA versus AG versus GG[b]			1.12 (0.93–1.35)	0.247	2.223
IRF5 rs10954213					
GG	139 (27.80)	127 (25.30)	1.00		
GA	266 (53.20)	256 (50.99)	0.95 (0.71–1.28)	0.734	6.606
AA	95 (19.00)	119 (23.71)	0.73 (0.51–1.04)	0.084	0.756
GG versus GA versus AA[b]			0.86 (0.72–1.03)	0.096	0.864

[a]Adjusted for age, gender, smoking status and alcohol consumption in the unconditional logistic regression model.
[b]The logistic regression was under an additive model, and the major allele homozygotes were set as the reference genotype.

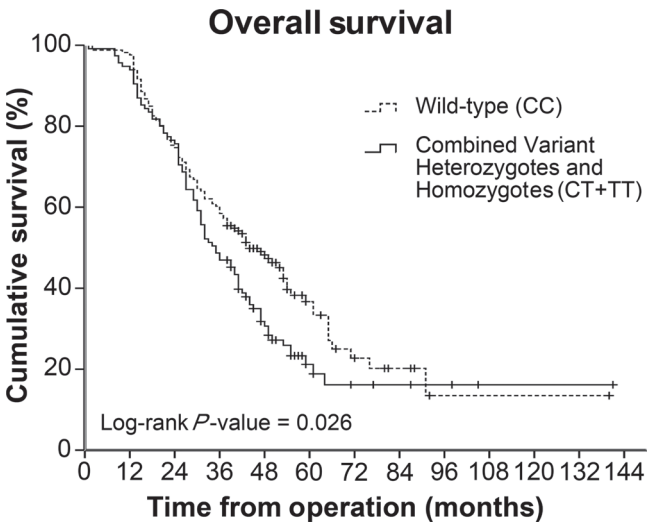

Fig. 1. Kaplan–Meier survival curves of overall survival for 281 gastric cancer patients after surgical operation in combined variant heterozygotes and homozygotes of *MTMR3* rs12537 (CT+TT) versus wild-type (AA).

and downexpressions of let-7, miR-126 and miR-433 were associated with GC development (23,24,26). In this study, miRNA expression microarray analysis identified nine significantly dysregulated miRNAs. To our knowledge, in GC, the HS_138, HS_153, HS_157 and miR-584 have not been reported, whereas overexpression of miR-27a, miR-21, miR-21* and miR-93 were reported to act as oncogenes (23–25). MiR-181a was found to be upregulated by microarray analysis (26,27), but its role in GC remains to be determined.

Family members of miR-181 include miR-181a-1, miR-181a-2, miR-181b-1, miR-181b-2, miR-181c and miR-181d. miR-181a was the mature form of miR-181a-1 and miR-181a-2 (28). The roles of miR-181 in human carcinogenesis have been explored recently. miR-181a demonstrated overexpression in breast cancer (29), hepatocellular carcinoma (28) and oral squamous cell carcinoma (30), and functions as oncogene by direct targeting of tumor suppressive genes such as ATM (31), RASSF1A (32) and CDX2 (28). Meanwhile, miR-181a was also reported to act as tumor suppressor by targeting KRAS (33), Bcl-2 (34) and PLAG1 (35), and lower levels of miR-181a were observed in some cancers, such as lung cancer (36), leukemia (35,37) and glioblastoma (38). The apparent opposing roles of miR-181a may be due to different target genes, depending on the tissue or cellular environment. We identified that miR-181a was highly overexpressed in GC tissues than surrounding normal tissues, which was consistent with the evidence from

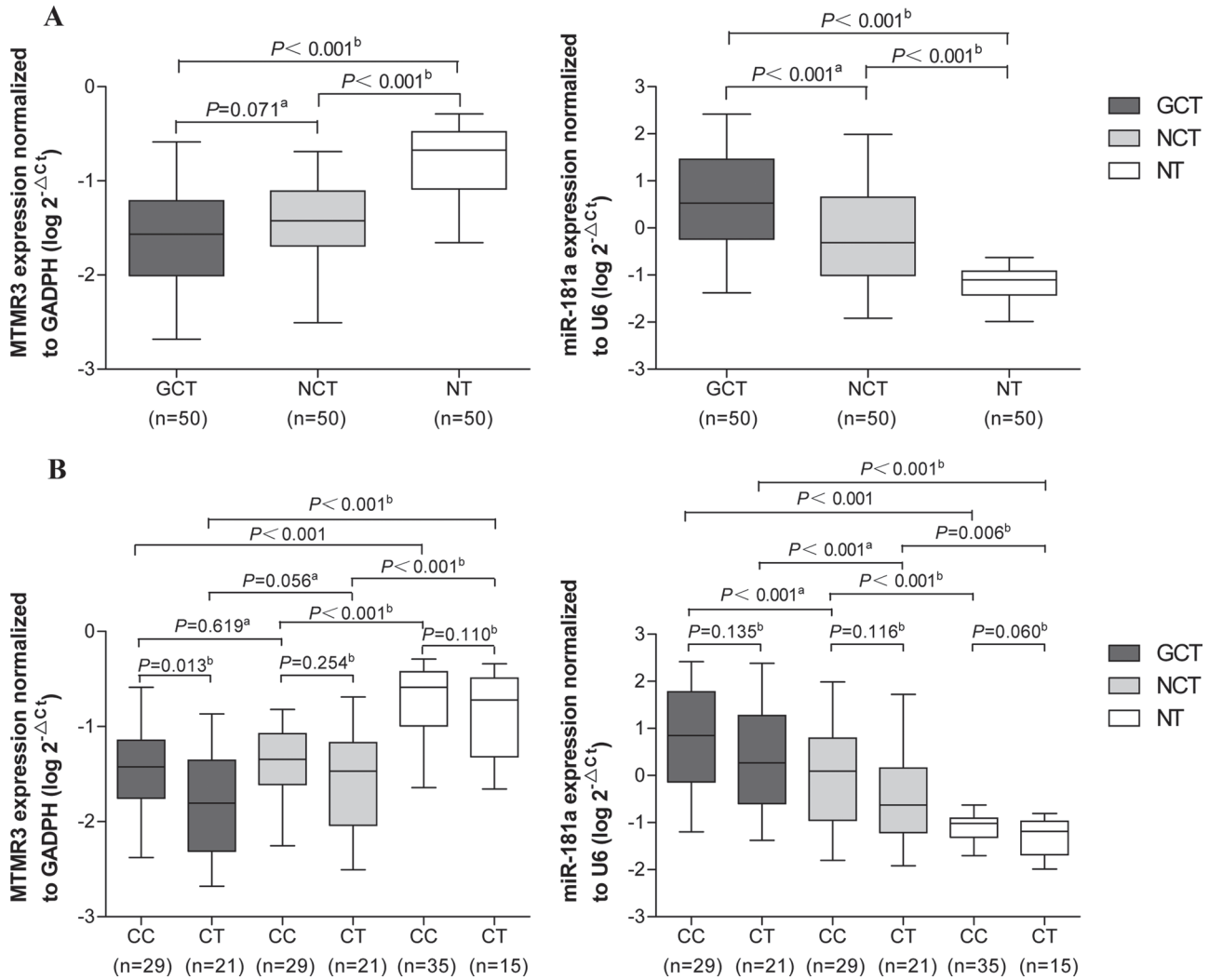

Fig. 2. qRT–PCR analysis of *MTMR3* mRNA and miR-181a expressions in GCT, NCT and NT groups with different genotypes. The data are shown as box and whisker plots (box median with 25th and 75th percentile, and whiskers are minimum and maximum values). (**A**) Expression of *MTMR3* mRNA and miR-181a among three groups. *MTMR3* and miR-181a were normalized to GADPH and U6, respectively. (**B**) Expressions of *MTMR3* mRNA and miR-181a in each group with different genotypes (CC versus CT). *P* values were obtained by [a]Wilcoxon signed-rank test or [b]Mann–Whitney *U*-test. .

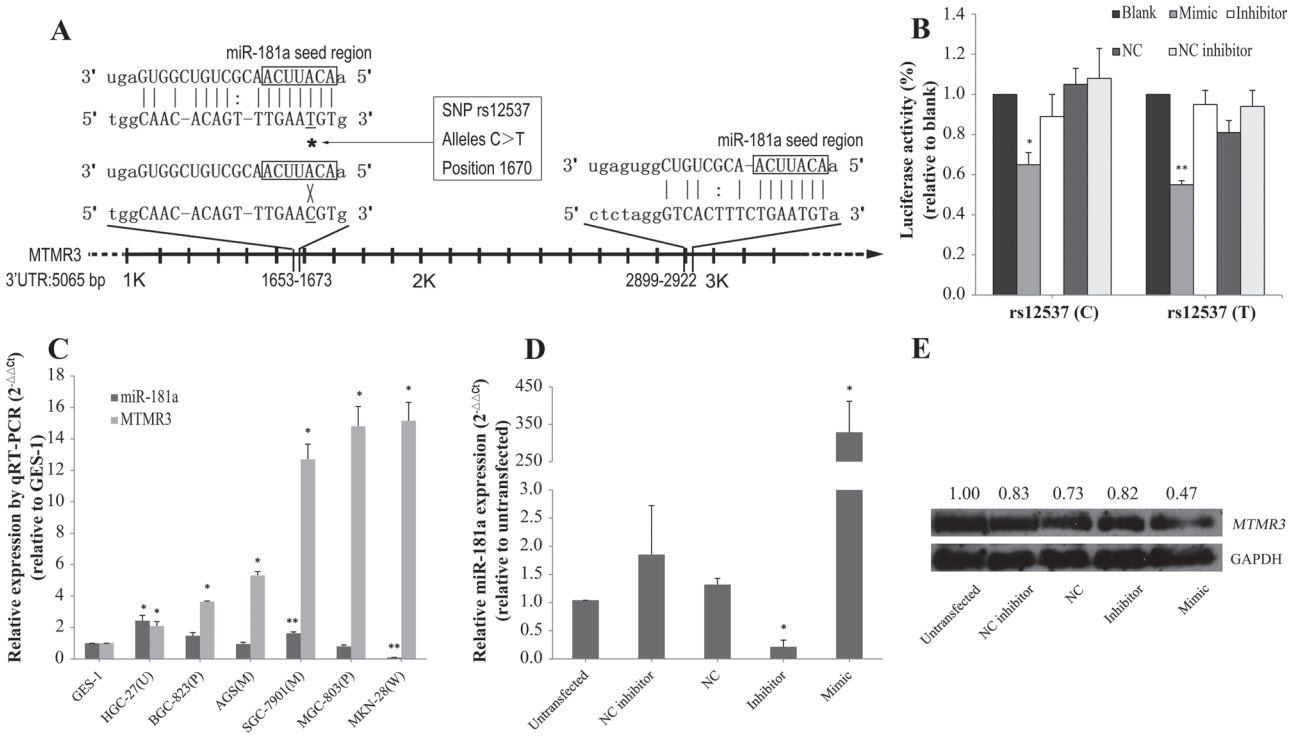

Fig. 3. miR-181a negatively regulates *MTMR3* by binding to the MTMR3 3′UTR. (**A**) Schematic representation of the interaction of miR-181a and the 3′UTR of *MTMR3*. Based on miRanda software prediction, one target site (at 2899–2922) in the 3′UTR of *MTMR3* was complementary to miR-181a seed region, and the replacement of the C allele by the variant T allele of rs12537 will create another miR-181a binding site (at 1653–1673). The alleles of rs12537 are underlined. (**B**) Luciferase reporter assays. Overexpression of miR-181a significantly suppressed the luciferase activity of psiCHECK2: rs12537C and psiCHECK2:rs12537T in HEK-293T cells, when compared with blank control, miR-181a inhibitor, NC and NC inhibitor. (**C**) Levels of miR-181a and *MTMR3* mRNA in six gastric cancer cell lines compared with the normal gastric cell line GES-1. All the cell lines carry CC genotype of rs12537. U, undifferentiated cancer cell line; P, poorly differentiated cancer cell line; M, moderately differentiated cancer cell line; W, well differentiated cancer cell line. (**D** and **E**) The change of miR-181a expression and *MTMR3* protein levels at 48 h after transfection of miR-181a mimic, miR-181a inhibitor, NC or NC inhibitor in SGC-7901 cells. NC, miRNA negative control. *$P < 0.05$, ** $P < 0.01$. P values were obtained by two-sided Student's t-test.

miRNAs microarray previously (26,39,40). In addition, patient with high level of miR-181a was more likely to have lymph node metastases. This observation was consistent with the finding in oral squamous cell carcinoma that higher level of miR-181a was associated with advanced lymph node metastasis (30). Further investigation of six GC cell lines found that miR-181a overexpressed in HCG-27, SGC-7901 and BGC-823 cells, and underexpressed in MKN-28, MGC-803 and AGS cells, when comparing to GES-1. We speculated that some other mechanisms, such as epigenetic modification, may contribute to the low expressions of miR-181a in some GC cell lines. Likewise, it was observed that miR-181c was expressed at low levels in KATO-III and MKN45 cells, and can be silenced through methylation (41). These results suggested that miR-181a may be an important contributor to GC.

To our knowledge, 28 target genes of hsa-miR-181a have been validated. Among them, *TIMP3* and *KRAS* each have a SNP located in miR-181a binding sites, according to the bioinformatics analysis. However, there is no frequency data of rs1143552 of *TIMP3* in dbSNP database. As to rs9266 of *KRAS*, we excluded it due to little MFE change between different alleles. We chose three SNPs to investigate their relationship with GC, and showed that rs12537 of *MTMR3* was associated with GC risk, whereas rs1460008 of *EREG* and rs10954213 of *IRF5* were not. rs1460008 has not been reported previously, whereas rs10954213 has been found associated with susceptibility of rheumatoid arthritis (42) and systemic lupus erythematosus (43). A genome-wide association study in Han Chinese identified significant associations of rs12537 with the susceptibility to IgA nephropathy; it also found that the risk allele T of rs12537 was associated with severe proteinuria (44). Here, we showed that rs12537 can affect GC risk, probable by influencing the interaction between miR-181a and *MTMR3*.

MTMR3, encoding myotubularin-related protein 3, is ubiquitously expressed and has been demonstrated to negatively regulate autophagy (45,46). Autophagy is a cellular degradation pathway for the clearance of damaged or superfluous proteins and organelles. In tumor cells, autophagy serves as a protective mechanism to limit tumor necrosis and inflammation, and to mitigate genome damage in tumor cells in response to metabolic stress, and thus prolongs survival (47). Previous evidence showed that, compared with controls, MTMR3 expression was significantly reduced in colonic biopsies from ulcerative colitis patients (48). Another study revealed that mutations in MTMR3 could be detected in GC tissues with high microsatellite instability, but not in those with low or stable microsatellite instability, suggesting that alterations in MTMR3 may play a role in the development of GC with high microsatellite instability (49). However, to date, there is little knowledge on cancer-related functions of MTMR3. Our results showed that the *MTMR3* levels dramatically declined not only in tumor tissues but also in surrounding non-cancerous tissues of GC patients, compared with cancer-free controls. The fact that miR-181a increased the autophagic activity of cancer cells by inhibition of MTMR3 may be one possible explanation for its contribution to GC.

Potential limitations of this present study should be considered. Firstly, although the luciferase reporter assay showed that miR-181a exert more inhibition on *MTMR3* in T allele than C allele, the difference was not significant. We planned to confirm this finding in GC. But we failed to collect fresh tissue samples of GC cases with TT genotype, since the TT genotype frequency was rare (0.044 for GC in our study). We also failed to obtain GC cell line carrying T allele. Thus, additional studies are needed to identify the function of the variant T allele. Secondly, the expected elevation of *MTMR3* levels after inhibition of endogenous miR-181a was not observed. This implied that the interaction between *MTMR3* and miR-181a may also involve other underlying regulatory mechanisms which we did not identify. Thirdly, potential confounding factors should be considered. The

details of antacid treatment of cases and controls were both unknown, which would change the cellular environment of tissues and influence the gene expression. Additionally, compared with blood samples, tissue samples used for genotyping might have a risk of accumulation of genetic alterations, therefore interpreting and generalizing these findings should be cautious. Finally, the case–control study based on hospital data and recruited participants from southern China may bring selection bias. More studies are needed to replicate our findings, especially in other ethnic populations.

In summary, this is the first study to provide evidence that T allele genotypes (rs12537CT and TT) are associated with higher GC risk and poor prognosis. Our work showed that miR-181a and its target gene *MTMR3* play important roles in GC. In addition, miRNA-related SNPs, especially those in miRNAs binding sites, are a new source of human cancer susceptibility loci.

Supplementary materials

Supplementary Methods, Tables I–III and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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