A germline mutation in the miR-125a coding region reduces miR-125a expression and is associated with human gastric cancer

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Abstract. MicroRNAs (miRNAs) are small non-coding RNAs that inhibit the expression of target protein-coding genes, most often at the post-transcriptional level. miRNAs are often found to be misregulated in human cancer and they can act as potent oncogenes or tumor suppressor genes. In this study, we found that a germline mutation in the miR-125a coding region is associated with human gastric cancer. This mutation reduced the expression of mature miR-125a and alleviated its inhibitory effect on erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*) gene expression and on gastric tumor cell proliferation. Thus, the data of this study suggested that this germline mutation in pri-miR-125a likely contributes to the genetic predisposition to gastric cancer by reducing the production of miR-125a, thereby interfering with the expression of miR-125a target genes.

Introduction

MicroRNAs (miRNA) are a type of short, non-coding RNAs that suppress the expression of protein-coding genes by partial complementary binding, especially to the 3' untranslated regions (UTRs), of messenger RNAs (mRNAs). Alterations in miRNA expression are involved in the initiation, progression and metastasis of human cancer, and it is believed that miRNAs function as tumor suppressors and oncogenes in cancer development (1,2).

A number of studies have shown that miR-125a is an important tumor suppressor gene, and reduced miR-125a expression has been detected in many types of human cancer, including breast (3,4), lung (5,6) and ovarian cancer (7), as well as glioblastoma (8). Nishida *et al* (9) reported that a reduced expression of miR-125a-5p is associated with enhanced potential to develop gastric cancer. Furthermore, Hashiguchi *et al* (10) reported that miR-125a-3p, commonly ignored by investigators, has almost the same function in gastric cancer as miR-125a-5p. There are also reports that germline mutations in the miR-125a coding region can reduce miR-125a expression and are associated with human breast cancer (3,11). These findings strongly suggest that miR-125a-5p and -3p variants can act as tumor suppressors and reduce miR-125a expression, thereby serving as genetic markers for gastric cancer diagnosis and treatment.

In this study, we first examined the expression level of miR-125a-5p and -3p in gastric cancer tissue and adjacent healthy gastric tissue. We then genotyped the miR-125a coding region in gastric cancer patients and healthy controls. We found a germline mutation in the pri-miR-125a coding region that was associated with gastric cancer and the reduction of miR-125a expression, suggesting that miR-125a is likely to function as a tumor suppressor gene in human gastric cancer.

Materials and methods

Study population, tissue samples and cell lines. A total of 75 pairs of histopathologically confirmed gastric cancer and adjacent non-cancer tissue samples were obtained from patients in the Department of Gastroenterology, Qilu Hospital of Shangdong University, China. Informed consent was obtained from all patients and the procedure was approved by the Medical Ethics Committee of the hospital. Control samples from a total of 287 healthy Han-Chinese individuals were also collected at the Central Hospital of Zibo, Shandong, China. Human gastric adenoma cell lines (MGC-803 and BGC-823) were purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37˚C in a humidified atmosphere with 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression level of miR-125a-5p and -3p was

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assessed by TaqMan miRNA real-time (quantitative) RT-PCR. Total RNA was extracted from tissues and cells using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and then amplified using the TaqMan Universal PCR Master mix together with miRNA-specific TaqMan minor groove binder (MGB) probes for miR-125a-5p and -3p (all from Applied Biosystems). The small nuclear (sn) RNA for U6 was used for normalization. Each sample of a group was measured in triplicate and the experiment was repeated at least three times.

DNA collection and genotyping. DNA from tumor and adjacent healthy tissues of the gastic cancer cohort was isolated using the TIANamp Genomic DNA kit (Tiangen Biotech, Beijing, China). DNA from blood samples was extracted using the TIANamp Blood DNA kit (Tiangen Biotech). DNA samples were amplified using standard PCR protocols. The PCR products were sequenced in the forward direction on an ABI 3730xl sequencing platform. The sequencing results were analyzed using the open-source software DNAMAN and Chromas Lite. The PCR primers used for miR-125a sequencing were: 5'-TGT GTC TCT TTC ACA GTG GAT C-3' and 5'-CCA TCG TGT GGG TCT CAA G-3'.

Secondary structure prediction. The secondary structure of the 217-bp pri-miR-125a sequence, which includes the mutation site, was predicted using the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

miR-125a expression vectors. To construct miR-125a expression vectors, 1,016-nt fragments corresponding to pri-miR-125a and its flanking regions (previously confirmed to bear the two alleles) were amplified from cDNA and cloned into the pcDNA3.1 vector (Invitrogen Life Technologies). The sequences of the two resulting vectors were confirmed by direct sequencing, with the only difference being in the mutation site. The primers used were: miR125a-F/*Xho*I, 5'-CCG CTC GAG GGT AGG AGG TTG TAT AGT TGA GGA GG-3' and miR-125a-R/*Xba*I, 5'-GCT CTA GAC CTC TGG GCC TCT CCT GC-3'.

Dual luciferase assay. The full-length region (618 bp) of the 3' UTR of the erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*) gene was cloned downstream of the coding region of the firefly luciferase gene within the pmirGLO Dual-Luciferase miRNA Target vector (Promega Corp., Madison, WI, USA) to generate the luciferase reporter vector. For luciferase reporter assays, MGC-803 and BGC-823 cells were seeded into 48-well plates. The miR-125a expression and the luciferase reporter vectors were co-transfected using Lipofectamine 2000 (Invitrogen Life Technologies). Two days later, the cells were harvested and assayed with the Dual-Luciferase Assay system (Promega Corp.). Each assay was performed in triplicate, in three independent experiments. The results were expressed as relative luciferase (LUC) activity (firefly LUC/*Renilla* LUC).

Cell proliferation assay. MGC-803 and BGC-823 cells were seeded into 96-well plates at low density ($5x10^3$) in Dulbecco's modified Eagle's medium (DMEM) and were allowed to attach overnight. The cells were then transfected with different haplotype miR-125a expression vectors, with the empty pcDNA3.1 vector used as the control. Twenty microliters MTT (5 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) were added into each well 48 h following transfection, and the cells were incubated for an additional 4 h. Following the addition of dimethylsulfoxide (DMSO) to the samples, their absorbance was recorded at 570 nm on a 96-well plate reader.

Statistical analysis. Data were analyzed using the SPSS statistical package version 16 (SPSS, Inc. Chicago, IL, USA). Comparisons between two independent groups were performed with the Student's t-test. Expression data for miR-125a were compared using the Mann-Whitney U test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Increased miR-125a-5p and -3p in gastric cancer tissues. In order to explore the role of miR-125a in gastric carcinogenesis, the expression patterns of miR-125a were analyzed in 75 pairs of human gastric cancer and adjacent healthy gastric tissues using RT-qPCR (Fig. 1). Each sample consisted of pooled RNAs from cancer tissues of three patients (25 samples for 75 pairs). The levels of miR-125a-5p and -3p were significantly increased in 92 (23/25) and 80% (20/25 samples) of gastric cancer tissues, respectively (Fig. 1).

A germline mutation was detected in the pri-miR-125a coding region. Since nucleotide variants can alter miRNA expression and are associated with many types of human diseases, we genotyped the coding region of pri-miR-125a by sequencing the DNA extracted from gastric cancer tissues. We found five patients carrying the minor G allele (Table I), which existed in the +43 relative to the mature miR-125a-5p and +29 relative to pre-miR-125a. Furthermore, we sequenced genomic DNAs isolated from adjacent healthy gastric tissues and found the same genotypes as the ones from gastric cancer tissues (data not shown).

The frequency of the minor G allele was examined in the population of 287 healthy individuals (control group) collected in the same region. We found no individual carrying the G allele in this population, while this allele was also not found in the 1000 Genomes database (http://www.1000genomes.org/), suggesting that the T allele is a mutation. Furthermore, since no individual in the control group from the same area carried the G allele, the presence of the G allele among gastric cancer patients is unlikely to be due to a founder effect. Together, these results suggest that a germline mutation in pri-miR-125a is associated with gastric cancer tumorigenesis.

The G mutation can enhance the predicted stability of pri-miR-125a and reduce miR-125a expression. To explore the function of the mutation (Fig. 2A), we first compared the predicted secondary structure of pri-miR-125a molecules bearing or not the minor allele. As shown in Fig. 2B, the minor allele G causes an apparent change in loop size (from 8 nucleotides loop to 6 nucleotides loop and from 10 paired nucleotides stem to 12 paired nucleotides stem) and

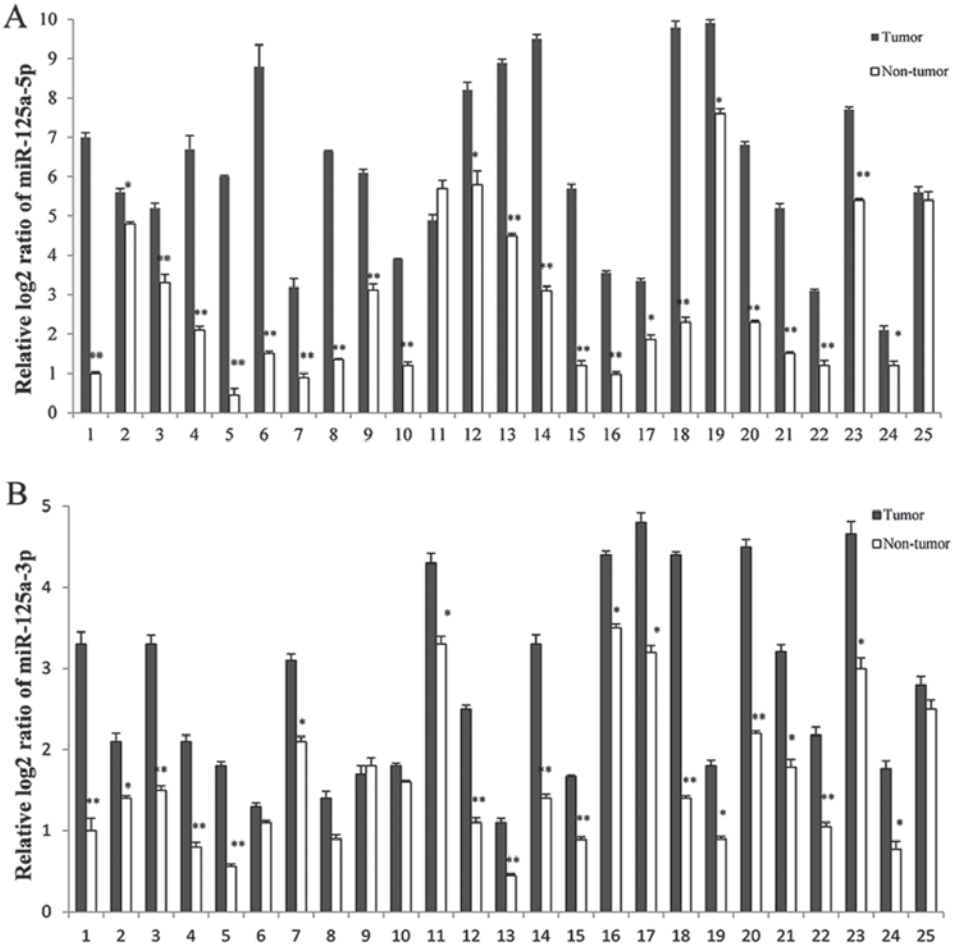

Figure 1. Increased mature miR-125a levels in gastric cancer (tumor) tissues. We pooled RNAs from cancer tissues of three patients for each sample. The expression level of (A) miR-125a-5p and (B) miR-125a-3p in each sample was detected by reverse transcription-quantitative polymerase chain reaction. Statistical analyses were performed to analyze the overall trend of miR-125a expression in gastric cancer and adjacent healthy (non-tumor) gastric tissues. U6 small nuclear (sn) RNA served as an internal control, allowing to normalize for differences in constitutive expression among samples (x-axis). The y-axis shows the $\log_2$ ratio of miR-125a-5p or -3p to U6 levels. *P<0.05; **P<0.01.

Table I. The minor G allele of pri-miR-125a is detected in gastric cancer patients.

	Genotype distribution		
Samples	AA	AG	GG
Gastric cancer (Qilu Hospital)	70 (93.3%)	5 (6.7%)	0 (0%)
Healthy (Zibo Central Hospital)	287 (100%)	0 (0%)	0 (0%)

a reduction of the predicted ΔG from -74.30 to -77.08 kcal/mol. Using RT-qPCR and two different expression vectors carrying the alternative miR-125a-5p alleles, we quantified the expression level of the mature miR-125a-5p in the two gastric cancer cell lines MGC-803 and BGC-823. The presence of the mutation was associated with an ~40% reduction in mature miR-125a-5p expression (Fig. 2C), in agreement with RT-qPCR analyses on gastric cancer tissue samples (Fig. 3B).

Altered miR-125a expression attenuates the inhibitory effect of miR-125a on ERBB2 expression and gastric cancer cell proliferation. The *ERBB2* gene, a confirmed target of miR-125a-5p (12,13), is a member of the epidermal growth factor receptor (EGFR/ERBB) family. The protein is commonly overexpressed in numerous types of cancer cells and has been suggested to promote cell proliferation (14,15). We used the *ERBB2* 3' UTR reporter system to study the effect of the identified mutation on *ERBB2* gene expression. As shown in Fig. 3A, the reduction in *ERBB2* expression caused by transfection with the miR-125a vector was significantly attenuated in cells bearing the G compared to those bearing the A allele. To explore the *in vivo* expression pattern of *ERBB2*, we measured the *ERBB2* mRNA level by using RT-qPCR in gastric cancer and adjacent healthy gastric tissues. The *ERBB2* expression level was significantly higher (Fig. 3C) in cancer tissues compared to healthy controls, which had reduced miR-125a expression (Fig. 1).

Based on the predicted function of ERBB2, the reduction of miR-125a expression is expected to promote cell proliferation. Therefore, a proliferation assay was carried out to explore potential differences in antitumor activity caused by different miR-125a genotypes in gastric cancer cells. MGC-803 and

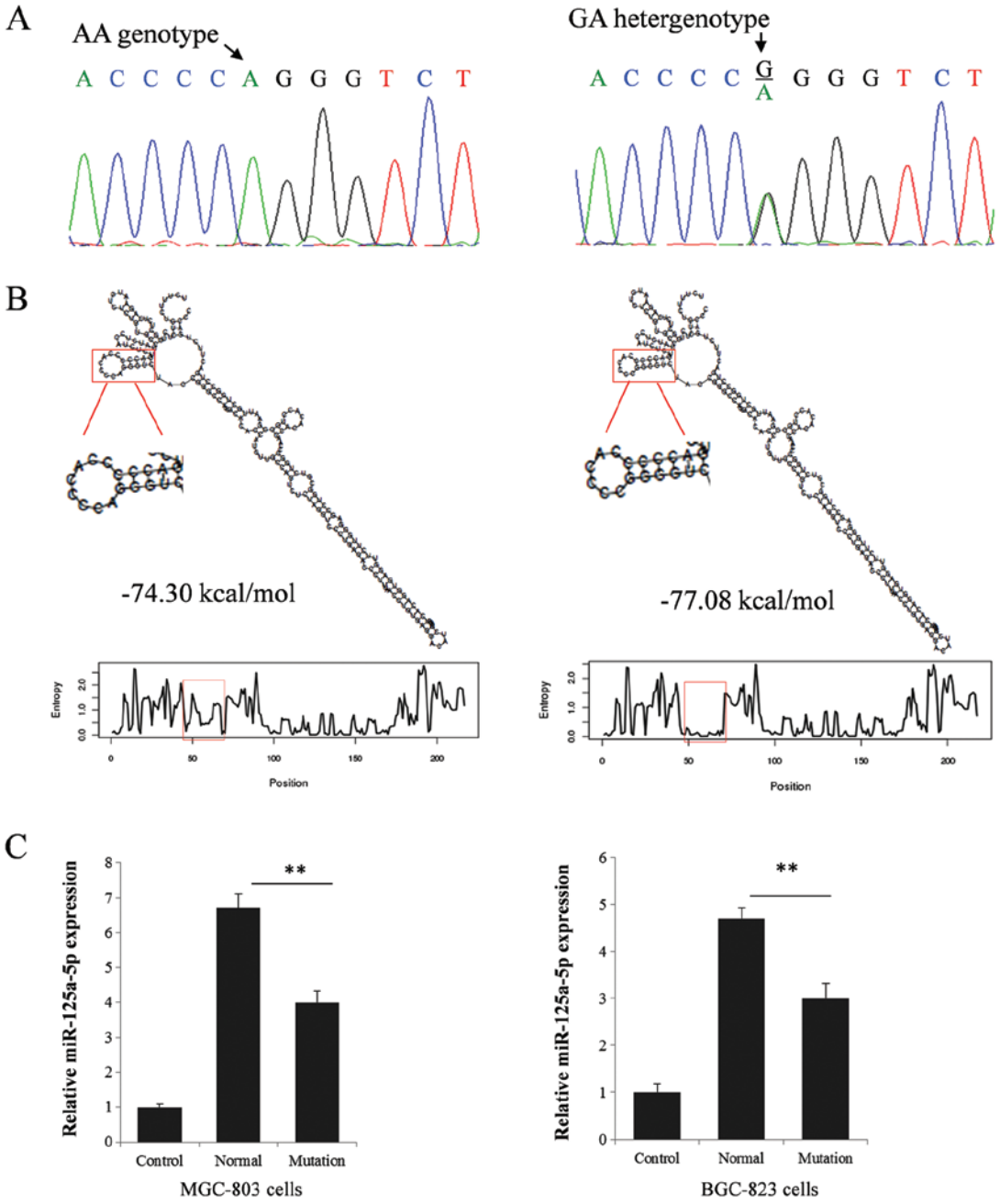

Figure 2. The mutation site modifies the predicted secondary structure of pri-miR-125a and reduces mature miR-125a expression. (A) Representative examples from DNA sequencing in a collection of 75 gastric cancer patients are shown. (B) Predicted structure of the 217-nt pri-miR-125a. The size of one loop and the stem structure are changed when the +29 nucleotide is G. (C) Expression level of mature miR-125a-5p resulting from different genotypes of pri-miR-125a, as assessed by reverse transcription-quantitative polymerase chain reaction. The U6 small nuclear (sn) RNA served as an internal control, allowing to normalize for differences in constitutive expression among samples. The y-axis displays the expression level of miR-125a relative to that of U6. *P<0.05; **P<0.01.

BGC-823 cells were transfected with different pri-miR-125a expression vectors, and, as expected, the proliferation of MGC-803 cells was significantly reduced (P=0.0008 and 0.0067 compared to the control) in pri-miR-125a genotypic backgrounds (bearing the A and the mutant G allele), while the A to G mutation attenuated this effect by almost 20% (P=0.023 compared to the A allelic background). In BGC-823 cells, proliferation was also reduced in the pri-miR-125a genotype bearing the A allele (P=0.0075), but this effect was not significantly attenuated by the G mutation (Fig. 4).

Discussion

Growing evidence indicates that altered patterns of miRNA expression correlate with various human diseases, and especially with numerous cancer types (16,17). The effects of miRNAs are complex, owing to the fact that these molecules regulate hundreds of targets, resulting in the downregulation of numerous genes, including oncogenes and tumor suppressors. Therefore, exploring the clinical potential of miRNAs is of particular value.

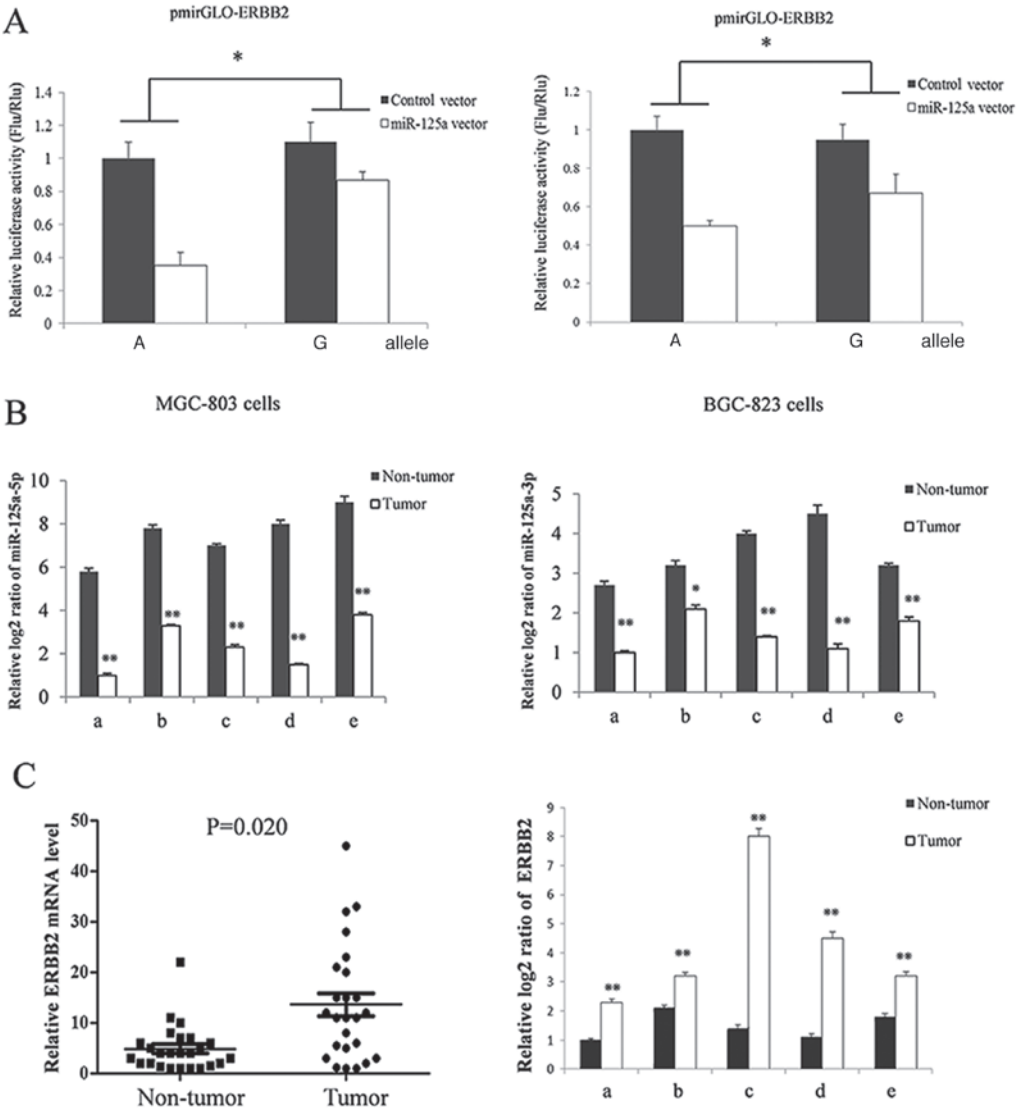

Figure 3. The G mutation attenuates the inhibitory effect of miR-125a on *ERBB2* expression, reduces miR-125a expression and upregulates *ERBB2* in gastric cancer tissues. (A) The relative luciferase activity (LUC) of the reporter vector bearing the 3' UTR of *ERBB2* was measured in the presence of the two genotypes of pri-miR-125a and is expressed as firefly LUC/*Renilla* LUC (Flu/Rlu). The mutation reduced the inhibitory effect of miR-125a on *ERBB2* expression by 67.8 and 41% in MGC-803 and BGC-823 cells, respectively. (B) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect the miR-125a-5p and -3p expression levels. (C) RT-qPCR was used to detect the *ERBB2* mRNA level in 25 (left panel; 3 random total RNA samples from 75 gastric cancer patients were mixed and form 25 group) and 5 pairs (right panel) of gastric tissue, tumorous and adjacent non-tumorous. All tissues on the right panel bear the G allele. *P<0.05; **P<0.01.

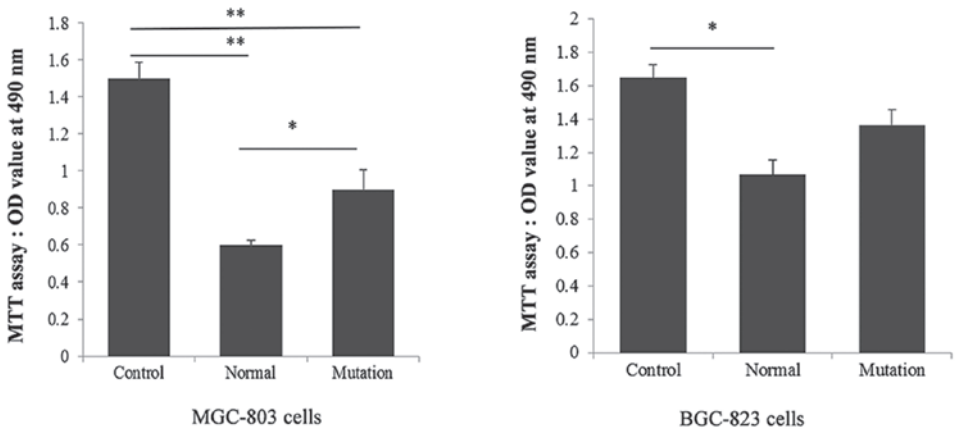

Figure 4. The G mutation attenuates the suppressive effect of miR-125a on gastric cancer cell line proliferation. MGC-803 and BGC-823 cells were transfected with the pcDNA3.1 (control), normal (bearing the A allele) and mutant (bearing the G allele) miR-125a expression vectors. After 48 h of transfection, cell proliferation was determined by the MTT assay. Experiments were performed at least three times. *P<0.05, **P<0.01.

In this study, we first detected a link between high miR-125a expression and gastric carcinogenesis. Nucleotide variants in coding regions can disturb the expression of miRNAs and relate to many types of human disease, especially cancer (3,11,18-20). We therefore sequenced the coding region of miR-125a in 75 gastric cancer patients. To exclude the possibility of false negatives caused by a founder effect, we sequenced the same region in a population of 287 healthy individuals collected in the same region. The 1000 Genomes database was used to exclude known variants and false negatives in the two different populations. We identified a nucleotide mutation (+29A>G, position relative to the pri-miR-125a start codon) that was only detected in gastric cancer patients. The G mutation at this site attenuated the inhibitory effect of miR-125a on *ERBB2* gene expression by reducing the expression of the mature miR-125a both *in vitro* and *in vivo*. Reduced miR-125a expression further attenuated the suppressive effect of miR-125a on gastric cancer cell proliferation, especially in MGC-803 cells. This result indicated that miR-125a acts as a tumor suppressor and that the identified germline mutation in pri-miR-125a is associated with gastric cancer tumorigenesis.

Our data indicate that miR-125a is likely to function as a tumor suppressor gene in human gastric cancer. The reduction in the miR-125a expression level can lead to upregulation of miR-125a target genes, such as *ERBB2*, and predispose individuals carrying the G allele to gastric cancer. To the best of our knowledge, this is the first mutation in the miR-125a coding region reported to associate with human gastric cancer. Systematic sequencing of known miRNAs in a high number of distinct populations is expected to provide valuable insights in the regulation of various diseases.

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