MiR-27a rs895819 is involved in increased atrophic gastritis risk, improved gastric cancer prognosis and negative interaction with Helicobacter pylori

Qian Xu1, Tie-jun Chen1, Cai-yun He2, Li-ping Sun3, Jing-wei Liu1 & Yuan Yuan1

MiR-27a rs895819 is a loop-stem structure single nucleotide polymorphism affecting mature miR-27a function. In this study, we performed a comprehensive analysis about the association of rs895819 with gastric cancer risk and prognosis, atrophic gastritis risk, as well as the interactions with environmental factors. A total of 939 gastric cancer patients, 1,067 atrophic gastritis patients and 1,166 healthy controls were screened by direct sequencing and MALDI-TOF-MS. The association of rs895819 with clinical pathological parameters and prognostic survival in 357 gastric cancer patients was also been analyzed. The rs895819 variant genotype increased the risk for atrophic gastritis (1.58-fold) and gastric cancer (1.24-fold). While in stratified analysis, the risk effect was demonstrated more significantly in the female, age >60y, Helicobacter pylori (H. pylori) negative and non-drinker subgroups. Rs895819 and H. pylori showed an interaction effect for atrophic gastritis risk. In the survival analysis, the rs895819 AG heterozygosis was associated with better survival than the AA wild-type in the TNM stage I–II subgroup. In vitro study by overexpressing miR-27a, cells carrying polymorphic-type G allele expressed lower miR-27a than wild-type A allele. In conclusion, miR-27a rs895819 is implicated as a biomarker for gastric cancer and atrophic gastritis risk, and interacts with H. pylori in gastric carcinogenesis.

Single nucleotide polymorphisms (SNPs) are common variations of the hereditary factors thought to be associated with several forms of cancers1,2. MicroRNAs (miRNAs) SNPs are located in the pre-/pri- or mature miRNAs, which could change the binding site with transcriptional factors or Drosha/Dicer enzyme and thus affect the quality and quantity of mature miRNAs3,4. MiR-27a contains two SNPs, rs895819 and rs11671784, with rs895819 being located in the 6bp downstream of rs11671784 on chromosome 19p13.13,2. The miR-27a rs895819 polymorphism is an unusual miRNA-SNP due to its location in the coding region of the pre-mir-27a hairpin in the stem-loop, which could be cut by Dicer in the process of pre-miRNA maturation5. Recent studies speculated that the A → G change of rs895819 could shorten the stem-loop structure and affect the processing of miR-27a6,7, suggesting that rs895819 was a functional SNP. The results of previous investigations concerning the association between this polymorphism and cancer risk are controversial. For example, several studies reported the variant allele could decrease the cancer risk7–9, while another study found the AG and GG genotypes increased the risk of gastric cancer10, and the others suggested that the association between the variant G allele and cancer risk was statistically insignificant11–13. However, for the most important of all, it was reported that direct sequencing or MALDI-TOF Mass-ARRAY using primer extension from one direction (not overlapping with any SNP) is essential for investigations of rs895819 and rs1167178414.

1Tumor Etiology and Screening Department of Cancer Institute and General Surgery, the First Affiliated Hospital of China Medical University, and Key Laboratory of Cancer Etiology and Prevention (China Medical University), Liaoning Provincial Education Department, Shenyang 110001, China. 2Department of Molecular Diagnostics, Sun Yat-Sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou, China. Correspondence and requests for materials should be addressed to Y.Y. (email: yuanyuan@cmu.edu.cn)
Gastric carcinogenesis is influenced by multiple hereditary and environmental factors, and epidemiological studies have suggested that individual hereditary susceptibility affects the incidence of gastric cancer. Recently, accumulating evidences have demonstrated environmental factors such as smoking, drinking and Helicobacter pylori (H. pylori) infection, are all critical risk factors for gastric carcinogenesis. In addition, environmental factors and gene polymorphisms may also be involved in gastric carcinogenesis. Helicobacter pylori (H. pylori), as one of the important factors for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma, are able to result in chronic infection. A synergistic effect of H. pylori infection with gene polymorphisms could contribute to the development of gastric cancer. However, it is still unclear whether an interaction effect exists between miR-27a rs895819 SNP and H. pylori for gastric carcinogenesis, of which the exploration will help to the comprehension of the carcinogenic biology.

In this study, sequencing and MALDI-TOF-MS were used as the accepted detection technology to perform a comprehensive analysis for this special SNP, including the association with risk and prognosis, cancerous and precancerous status, and interaction effects with the environmental factor H. pylori.

Results

Patient characteristics. Two-stage study was conducted: in stage 1, the samples came from 724 retrospectively recruited individuals, including 215 gastric cancer patients, 205 atrophic gastritis cases and 304 controls, which attended the First Affiliated Hospital of China Medical University between 2005 and 2010. In stage 2, a total of 2,448 cases were included, consisting of 724 gastric cancer cases, 862 atrophic gastritis cases and 862 matched healthy controls from the Zhuanghe Gastric Diseases Screening Program or from patients who attended the First Affiliated Hospital of China Medical University between 2002 and 2013.

The baseline characteristics of the subjects in the control, atrophic gastritis and gastric cancer groups were listed in Supplementary Table S1. No significant difference was found in terms of age and sex between the case and control groups. In stage 2, the subjects were selected from two different sources, then we analyzed the baseline of the subjects and the distribution frequency of rs895819 and no significant difference was discovered between the two groups (Supplementary Table S2). The characteristics of the subjects in the intestinal-type and diffuse-type gastric cancer subgroups were listed in Supplementary Table S3.

Frequencies of miR-27a rs895819 and rs11671784 determined by direct sequencing. Direct sequencing of miR-27a rs895819 in the 724 samples revealed 392 cases of the AA genotype, 291 cases of the AG genotype and 41 cases of the GG genotype. Among the 724 samples in the control group, only one individual carried the rs11671784 AG genotype, while the other 723 samples all carried GG genotype.

Association of miR-27a rs895819 with gastric disease risk. The miR-27a rs895819 SNP was found to meet with Hardy–Weinberg equilibrium (P > 0.05, Table 1). Rs895819 was associated with an increased risk for atrophic gastritis in stage 2 (GG vs. AA: P = 0.045, OR = 1.58; GG vs. AG + AA: P = 0.045, OR = 1.56). We also found rs895819 was associated with an increased risk of gastric cancer in the analysis of merge data (AG vs. AA: P = 0.027, OR = 1.24; AG + GG vs. AA: P = 0.043, OR = 1.21; Table 1). In addition, the method suggested by Thakkinstian et al. was employed to select the optimal genetic models for rs895819 SNP including dominant model, recessive model, codominant model and complete overdominant model. In the merged data, for the atrophic gastritis risk, OR1, OR2 and OR3 were 1.30 (P = 0.174), 1.02 (P = 0.797) and 1.25 (P = 0.248), respectively, which was in accord with the principle of OR1 > OR2 > 1 and OR1 > OR3 > 1, indicating a codominant model effect (Table 1). For the gastric cancer risk, OR1, OR2 and OR3 were 1.03 (P = 0.906), 1.24 (P = 0.027) and 0.81 (P = 0.318), respectively, which was in accord with the principle of OR2 = 1/OR3 ≠ 1 and OR1 = 1, indicating a complete overdominant model effect (Table 1). Thus, for the gastric cancer risk, in the complete overdominant model, AG heterozygote was associated with an increased risk of gastric cancer compared with GG + AA genotypes (P = 0.025, OR = 1.24, Table 1).

When the gastric cancer group was divided into intestinal-type and diffuse-type according to Lauren classification, no association was found between diffuse-type subgroup and controls, which was also fit for intestinal-type subgroup though the dominant model closely reaching statistical significance (GG vs. AA, P = 0.050, OR = 1.32; Supplementary Table 4).

In the stratified analysis, the heterogeneity test was performed for the host’s characteristics, and the results were shown in Supplementary Table S5. Both the age factor for atrophic gastritis vs. control group and the H. pylori infection factor for gastric cancer vs. control group almost reached statistical significance (Pheqogeneity = 0.095 and 0.078, respectively). Thus, the stratified analysis for all the host’s characteristics was conducted. In the female subgroup, rs895819 was found to be associated with an increased risk of atrophic gastritis and gastric cancer (atrophic gastritis risk: GG vs. AA, P = 0.030, OR = 1.86; gastric cancer risk: AG vs. AA, P = 0.032, OR = 1.44). In the age >60y subgroup, individuals carrying with the AG heterozygosis were associated with an increased gastric cancer risk (AG vs. AA, P = 0.001, OR = 1.74). In the H. pylori-negative subgroup, the GG genotype was associated with an increased risk when compared with the AA wild-type (P = 0.047, OR = 1.62). In the non-drinking subgroup, individuals with the GG variant genotype were associated with increased atrophic gastritis risk (GG vs. AA, P = 0.037, OR = 2.08, Table 2).

Interaction of rs895819 and H. pylori in the risk of gastric cancer/atrophic gastritis. More significant association was found in the H. pylori subgroup with the risk of gastric cancer and atrophic gastritis compared with that of the total samples. Therefore, we analyzed the interaction effect between rs895819 and H. pylori infection status. The result showed a negative interaction for atrophic gastritis risk (Piinteraction = 0.013, OR = 0.54; Table 3).
Table 1. The association of miR-27a rs895819 polymorphisms and gastric cancer/atrophic gastritis risk.  
Note: a Using Logistic Regression adjusted by sex, age and H. pylori infection; b Means Hardy–Weinberg Equilibrium in population; c A codominant model was indicated for rs895819 in the comparison of atrophic gastritis vs. control group, and GG was compared with AG and with AA. d A complete overdominant model was implied for rs895819 in the comparison of gastric cancer vs. control group. The original grouping was collapsed and the new group of AG heterozygote was compared with GG and with AA. CON: controls; AG: atrophic gastritis; GC: gastric cancer; NCBI Ref: the reference frequencies of these polymorphisms in Beijing Han, China in NCBI database.

| Genotype | NCBI Ref (%) | AG (%) | GC (%) | OR (95% CI) | P  |
|----------|--------------|--------|--------|-------------|----|
| Stage 1  |              |        |        |             |    |
| AA       | 21 (48.8)    | 110 (53.7) | (Ref) | 1.16 (0.59–2.29) | 0.174 |
| AG       | 17 (39.5)    | 83 (40.5) | (Ref) | 1.01 (0.81–1.26) | 0.97 |
| GG       | 5 (11.6)     | 12 (5.9) | (Ref) | 0.84 (0.44–1.64) | 0.666 |
| GG vs AG | 0.80 (0.38–1.64) | 0.559 | 0.41 (0.17–0.99) | 0.047 |
| GG vs CON |             |        |        |             |    |
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| Meta-analysis | n = 1166 | n = 1067 | n = 1033 | n = 939 |
| AA       |              |        |        |             |    |
| AG       | 451 (38.7)   | 408 (34.5) | (Ref) | 1 (Ref) | 1 |
| GG       | 62 (5.3)     | 51 (4.4) | (Ref) | 1 (Ref) | 1 |
| GG vs AG | 1.24 (1.03–1.50) | 0.027 | 0.24–0.32; Table 4.  

We further analyzed the cumulative effect of these two risk factors for atrophic gastritis and also found a significantly negative effect (P<0.001, 95%CI = 0.24–0.32; Table 4).

Rs895819 genotype and clinical pathological characteristics. To investigate the association of genotype with phenotype, we analyzed the association between rs895819 and the clinical pathological characteristics of gastric cancer patients. No significant association was found between age and sex, macroscopic type, Lauren grade, TNM stage, depth of invasion, lymphatic metastasis and rs895819 genotypes (Supplementary Table 6).

Association of miR-27a rs895819 with cancer survival prognosis. To analyze the relationship between rs895819 and survival time, univariate and multivariate Cox proportional hazard analysis were performed adjusted by adding all SNP variables to the clinicopathological parameters with P<0.05 (TNM stage, lymphatic metastasis and depth of invasion; Supplementary Table 7). No significant difference was discovered between rs895819 and survival, while the result was totally contrary in the subgroups. In the TNM stage I–II subgroup, the overall survival in the subjects carrying with rs895819 AG heterozygosis was better than the AA wild-type (univariate: P = 0.036, HR = 0.11; multivariate: P = 0.041, HR = 0.12, Fig. 1-A,B). In the lymphatic metastasis subgroup, individuals carrying with rs895819 AG + GG genotype had better survival compared with AA wild-type (univariate: P = 0.040, HR = 0.20, Table 5, Fig. 1-C).

The effect of rs895819 on miR-27a expression. We first analyzed the differential expression of miR-27a between cancer patients and control groups, as well as between tumor tissues and paired non-cancer tissues. miR-27a expression in cancer patients was significantly higher than that of the control groups in serum expression level (P<0.001, Table 6). And the same tendency was revealed in tissue expression level rather than reaching the statistical significance (P = 0.063).

To preliminarily explore the effect of rs895819 on mature miR-27a expression, we analyzed the mature miRNA expression based on different SNP genotypes both in tissue and in serum or in canerous and non-cancerous groups. No significant effect of different rs895819 genotypes on mature miR-27a expression in tissues or in serum was observed (Table 6).
| Variables          | Genotype | AG vs CON | OR (95% CI) | P    | GC vs CON | OR (95% CI) | P    |
|--------------------|----------|-----------|-------------|------|-----------|-------------|------|
| Gender*            | n = 1067 vs 1166 | n = 939 vs 1033 |
| Male               |          |           |             |      |           |             |      |
| AA                 | 343/377  | 1 (Ref)   | 0.97 (0.76–1.23) | 0.803 | 334/377   | 1 (Ref)     | 1.15 (0.92–1.45) | 0.227 |
| AG                 | 238/255  | 0.93 (0.56–1.55) | 0.787 | 33/38 | 0.96 (0.58–1.58) | 0.875 |
| GG                 | 33/38    | 1.00 (0.77–1.22) | 0.772 | 0.95 (0.57–1.56) | 0.829 | 0.89 (0.55–1.46) | 0.648 |
| AG + GG VS. AA     | 1.05 (0.84–1.32) | 0.609 | 0.87 (0.26–2.93) | 1.39 (1.01–1.91) | 0.045 |
| GG VS. AG + AA     | 1.77 (1.03–3.06) | 0.040 | 0.98 (0.50–1.95) | 0.959 |
| n = 1067 vs 1166   | n = 939 vs 1033 |
| Age*               |          |           |             |      |           |             |      |
| ≤60                |          |           |             |      |           |             |      |
| AA                 | 283/324  | 1.02 (0.82–1.26) | 0.886 | 232/266 | 1.06 (0.84–1.33) | 0.655 |
| GG                 | 46/39    | 1.39 (0.87–2.21) | 0.168 | 32/55 | 1.02 (0.61–1.70) | 0.948 |
| AG + GG VS. AA     | 1.06 (0.86–1.30) | 0.609 | 1.05 (0.84–1.32) | 0.667 |
| GG VS. AG + AA     | 1.37 (0.87–2.16) | 0.173 | 1.01 (0.61–1.66) | 0.983 |
| >60                |          |           |             |      |           |             |      |
| AA                 | 171/176  | 1.07 (0.76–1.49) | 0.712 | 176/217 | 1.74 (1.25–2.42) | 0.001 |
| GG                 | 25/23    | 1.14 (0.61–2.14) | 0.682 | 19/23 | 1.10 (0.56–2.14) | 0.785 |
| AG + GG VS. AA     | 1.08 (0.78–1.48) | 0.659 | 1.63 (1.18–2.24) | 0.003 |
| GG VS. AG + AA     | 1.11 (0.60–2.07) | 0.731 | 0.85 (0.44–1.62) | 0.614 |
| H. pylori*         | n = 1067 vs 1166 | n = 939 vs 1033 |
| negative           |          |           |             |      |           |             |      |
| AA                 | 250/469  | 1 (Ref)   | 0.98 (0.84–1.15) | 0.629 | 240/165   | 1 (Ref)     | 1.27 (1.00–1.62) | 0.055 |
| GG                 | 179/315  | 1.06 (0.84–1.35) | 0.629 | 204/117 | 1.27 (1.00–1.62) | 0.055 |
| AG + GG VS. AA     | 1.12 (0.89–1.41) | 0.319 | 1.24 (0.98–1.57) | 0.069 |
| GG VS. AG + AA     | 1.58 (0.99–2.51) | 0.054 | 0.94 (0.55–1.58) | 0.803 |
| positive           |          |           |             |      |           |             |      |
| AA                 | 327/184  | 1 (Ref)   | 0.98 (0.74–1.30) | 0.899 | 240/136   | 1 (Ref)     | 1.19 (0.88–1.61) | 0.262 |
| GG                 | 35/21    | 0.92 (0.52–1.64) | 0.785 | 24/39 | 0.98 (0.52–1.82) | 0.936 |
| AG + GG VS. AA     | 0.97 (0.74–1.27) | 0.820 | 1.15 (0.86–1.55) | 0.335 |
| GG VS. AG + AA     | 0.93 (0.53–1.64) | 0.810 | 0.90 (0.49–1.64) | 0.720 |
| Smoking*           | n = 548 vs 586 | n = 533 vs 500 |
| Never smoker       |          |           |             |      |           |             |      |
| AA                 | 204/224  | 1 (Ref)   | 0.98 (0.78–1.50) | 0.642 | 69/105    | 1.17 (0.78–1.77) | 0.444 |
| GG                 | 26/18    | 1.95 (0.99–3.85) | 0.055 | 14/16 | 1.64 (0.74–3.67) | 0.225 |
| AG + GG VS. AA     | 1.17 (0.85–1.60) | 0.330 | 1.23 (0.83–1.82) | 0.295 |
| GG VS. AG + AA     | 1.87 (0.96–3.64) | 0.066 | 1.53 (0.70–3.34) | 0.288 |
| Ever smoker        |          |           |             |      |           |             |      |
| AA                 | 94/109   | 1 (Ref)   | 0.95 (0.60–1.50) | 0.828 | 68/81     | 1.13 (0.71–1.82) | 0.606 |
| GG                 | 11/11    | 1.21 (0.48–3.09) | 0.685 | 6/11 | 0.60 (0.20–1.84) | 0.376 |
| AG + GG VS. AA     | 0.98 (0.64–1.52) | 0.936 | 1.06 (0.67–1.67) | 0.819 |
| GG VS. AG + AA     | 1.23 (0.50–3.07) | 0.653 | 0.54 (0.18–1.64) | 0.277 |
| Alcohol drinking*  | n = 547 vs 585 | n = 296 vs 499 |
| Nondrinker         |          |           |             |      |           |             |      |
| AA                 | 239/256  | 1 (Ref)   | 0.98 (0.60–1.50) | 0.828 | 71/127    | 1.10 (0.74–1.64) | 0.641 |
| GG                 | 27/17    | 1.15 (0.85–1.55) | 0.358 | 1.13 (0.77–1.66) | 0.521 |
| AG + GG VS. AA     | 1.99 (1.01–3.90) | 0.047 | 1.35 (0.59–3.08) | 0.484 |
| Drinker            |          |           |             |      |           |             |      |
| AA                 | 60/76    | 1 (Ref)   | 0.99 (0.58–1.67) | 0.959 | 50/60     | 1.20 (0.70–2.06) | 0.503 |
| GG                 | 11/11    | 1.03 (0.49–3.05) | 0.677 | 5/11 | 0.73 (0.23–2.30) | 0.587 |
| AG + GG VS. AA     | 1.03 (0.63–1.70) | 0.902 | 1.14 (0.68–1.93) | 0.620 |
| GG VS. AG + AA     | 1.33 (0.54–3.27) | 0.542 | 0.71 (0.23–2.21) | 0.555 |

Table 2. Association of miR-27a rs895819 polymorphism with the risk of atrophy gastritis and gastric cancer stratified by host characteristics. Note: *Using Logistic Regression adjusted by the other two factors of gender, age and H. pylori infection status. †Using Logistic Regression adjusted by gender, age and H. pylori infection status. CON: controls; AG: atrophic gastritis; GC: gastric cancer.
In the *vitro* level, AGS cell line was selected by the screening of miR-27a expression. And the AGS cells were transinfected into two plasmids, pCMV-miR-27a-rs895819-A and pCMV-miR-27a-rs895819-G. After 24 hours, the miR-27a expression level of variant G allele was significantly lower compared with the ancient A allele (*P* = 0.05, Fig. 1-D).

**Discussion**

We comprehensively and systematically conducted the risk and survival study for the special miR-27a rs895819 SNP and further performed the expression study in tissue and in serum or *in vitro* as well as *in vivo*. We found that the rs895819 SNP was associated with an increased risk of atrophic gastritis and gastric cancer. An interaction between rs895819 and *H. pylori* for atrophic gastritis risk was found. Individuals carrying with the rs895819 AG heterozygosis had better survival in the TNM stage I–II subgroup. And cells carrying with polymorphic-type G allele expressed lower miR-27a level than wild-type A allele.

In this study, we found that rs895819 AG genotype was associated with an increased risk of gastric cancer. It has been reported that rs895819 increased the gastric cancer risk in Chinese population, and could affect transcription proceeding from pri-miRNA to pre-miRNA resulting in the changes in the expression of mature miRNA. It suggested that rs895819 was a functional SNP, and our result was consistent with the previously published study. Only one study has shown negative association of this SNP with atrophic gastritis risk. It has been suggested by Lauren that gastric cancer, especially intestinal-type gastric cancer, develops from atrophic gastritis, which is known to be an important precancerous disease. Our analysis of a second set of samples by MALDI-TOF-MS revealed that the rs895819 SNP was associated with a 1.56-fold increase in the risk of atrophic gastritis, suggesting that this variant is involved in the progression of gastric carcinogenesis, and furthermore, implicating this SNP could be a biomarker of the risk of precancerous atrophic gastritis.

Environmental factors consist of the host's natural and social environment. The natural environment contains geographical and occupational factors as well as bacterial or viral infection. *H. pylori* infection is an important environmental factor that influences the risk of gastric cancer. In this study, when stratified by the *H. pylori* infection status, the OR value of the *H. pylori*-negative subgroup was found to be higher than that of the overall population (1.62 vs. 1.58, respectively). This suggests that the role of rs895819 SNP will be more apparent when the *H. pylori* factor is removed from the analysis. Further analysis based on this finding showed that rs895819 and *H. pylori* infection status could exert a negative interaction effect. Surprisingly, linear regression analysis showed that accumulation of the risk factors including the rs895819 risk genotype and *H. pylori* infection was associated with a protective effect against carcinogenesis. This accumulation effect revealed by linear regression analysis was consistent with the interaction analysis, which suggested that the association of this rs895819 SNP with *H. pylori* infection was based on antagonism. Both rs895819 SNP and *H. pylori* infection are risk factors for carcinogenesis, as *H. pylori* is a tumour accelerator and the rs895819 SNP is a polymorphism associated with increased gastric cancer risk. However, it can be speculated that rs895819 SNP weakens the carcinogenic role of *H. pylori* in individuals carrying with both two risk factors. Similar situations were observed in the associations of *H. pylori* infection and microsatellite instability with gastric cancer prognosis. A recent meta-analysis of 2,434 gastric cancer patients suggested a protective role for infection in prognosis, and also that *H. pylori*-induced inflammation might modulate antitumor immunity. Microsatellite instability is a hallmark of the DNA-mismatch repair deficiency, which is one of the pathways of gastric carcinogenesis although microsatellite alterations are related to better post-operative survival. Therefore, it is plausible that the rs895819 SNP has a negative interaction with *H. pylori* infection in gastric carcinogenesis. In our research, we also found that miR-27a rs895819 GG genotype was associated with an increased risk of atrophic gastritis in stage 2. Some scholars have also reported that miRNA expression changes have already been detectable in early stages of gastric carcinogenesis including *H. pylori* induced atrophic gastritis. But whether the variations in miRNAs, especially the SNP in miR-27a, were associated with *H. pylori*-related atrophic gastritis was still not clear. Several investigators were concerned with the association between rs895819 and atrophic gastritis risk, but no significant association was shown. Thus, large-scales and multi-central studies are needed in the near future.

We also found that in the TNM stage I–II or non-lymphatic metastasis subgroups, the patients carrying with *miR-27a* rs895819 AG + GG genotypes had better prognosis. It has been reported that *miR-27a* rs895819 G is associated with better survival, which is consistent with the results of our study. In the risk study, this rs895819 variant genotype showed a risk function, although in the prognosis study, the same variant genotype showed a protective function. These conflicting results are consistent with the observation that the rs895819 SNP has a negative interaction with *H. pylori* infection in gastric carcinogenesis. In our research, we also found that *miR-27a* rs895819 GG genotype was associated with an increased risk of gastric cancer in stage 2. *H. pylori* infection was associated with better survival in the TNM stage I–II subgroup. And cells carrying with polymorphic-type G allele expressed lower miR-27a level than wild-type A allele. Therefore, it is plausible that the rs895819 SNP has a protective function against carcinogenesis. This accumulation effect revealed by linear regression analysis was consistent with the interaction analysis, which suggested that the association of this rs895819 SNP with *H. pylori* infection was based on antagonism. Both rs895819 SNP and *H. pylori* infection are risk factors for carcinogenesis, as *H. pylori* is a tumour accelerator and the rs895819 SNP is a polymorphism associated with increased gastric cancer risk. However, it can be speculated that rs895819 SNP weakens the carcinogenic role of *H. pylori* in individuals carrying with both two risk factors. Similar situations were observed in the associations of *H. pylori* infection and microsatellite instability with gastric cancer prognosis. A recent meta-analysis of 2,434 gastric cancer patients suggested a protective role for infection in prognosis, and also that *H. pylori*-induced inflammation might modulate antitumor immunity. Microsatellite instability is a hallmark of the DNA-mismatch repair deficiency, which is one of the pathways of gastric carcinogenesis although microsatellite alterations are related to better post-operative survival. Therefore, it is plausible that the rs895819 SNP has a negative interaction with *H. pylori* infection in gastric carcinogenesis. In our research, we also found that *miR-27a* rs895819 GG genotype was associated with an increased risk of atrophic gastritis in stage 2. Some scholars have also reported that miRNA expression changes have already been detectable in early stages of gastric carcinogenesis including *H. pylori* induced atrophic gastritis. But whether the variations in miRNAs, especially the SNP in miR-27a, were associated with *H. pylori*-related atrophic gastritis was still not clear. Several investigators were concerned with the association between rs895819 and atrophic gastritis risk, but no significant association was shown. Thus, large-scales and multi-central studies are needed in the near future.

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This phenomenon is very puzzling and interesting. Other scholars also reported controversial results for the miR-27a function, for example, Zhou L et al. found that miR-27a could promote cancer cell proliferation\(^33\); while Wang X et al. reported that miR-27a acted as a tumor suppressor by suppressing oncogene MAP2K4 in vivo experiment\(^34\). Accordingly, some thought it was an “oncogene”\(^33,35\) but some believed it could induce apoptosis\(^36\). Thus, it is still unclear whether the mature miR-27a acts as an “oncogene” or “tumor suppressor” during carcinogenesis. Maybe miR-27a play a role as double-edged sword, in that way, it is also still unclear that when miR-27a acts as an “oncogene” or “tumor suppressor”. The real function of miR-27a still needs to be investigated in the future.

In 2012, Yang reported that rs895819 polymorphism is associated with an unusual structure making primer extension possible from only one direction (not overlapping with any SNP). The use of direct sequencing or MALDI-TOF Mass-ARRAY is essential for miR-27a rs895819 and rs11671784 genotyping because qPCR and PCR-RFLP are associated with unacceptable risk of false-positive genotyping\(^37\). Later in 2013, other researchers noted that only the direct sequencing or MALDI-TOF Mass-ARRAY was acceptable for genotyping this rs895819 polymorphism\(^38\). Subsequently, however, the special structure of rs895819 was rarely considered and Taqman or PCR-RFLP technologies were still used, rendering the association of this polymorphism and disease risk being inconsistent. To make the unusual structure of this rs895819 polymorphism being compatible, we used direct sequencing and Mass-ARRAY technology to increase the credibility of our study. Furthermore, we found only one case of the rs11671784 AG genotype among 724 samples was analyzed by direct sequencing. This distribution frequency is in accordance with the NCBI databases (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=11671784). Several studies\(^3,38\) investigated this rs11671784 polymorphism using PCR-RFLP or Taqman technology and the distribution frequency of the variant allele was higher than 50% both in the control and cancerous groups, which increases the risk of false genotyping.

There are some limitations of our study. First, the small sample size limited the subgroup analysis of rare genotypes. Second, complete information for other environmental factors is required to analyze polymorphism interaction effects.

In summary, we found that the rs895819 variant genotype was associated with an increased risk of atrophic gastritis and gastric cancer. In the stratified analysis, these effects were more significant in the female, age >60, \(H.\ pylori\)-negative and non-drinkers subgroups. The negative interaction between rs895819 and \(H.\ pylori\) in atrophic gastritis risk should be noted. For the survival analysis, individuals carrying with the rs895819 AG heterozygosity had better survival compared with individuals with the AA wild-type genotype in the TNM stage I–II subgroup. And it was confirmed by the \textit{vitro} study that cells carrying polymorphic-type G allele expressed lower miR-27a than wild-type A allele. In conclusion, \(miR-27a\) rs895819 is implicated as a biomarker for gastric cancer and atrophic gastritis risk, and interacts with \(H.\ pylori\) in gastric carcinogenesis.

### Table 3. The interaction of miR-27a rs895819 polymorphism and \(H.\ pylori\) infection status in the risk of gastric cancer/atrophic gastritis.

| Genotype | AG vs CON | GC vs CON |
|----------|-----------|-----------|
|          | \(H.\ pylori\) | \(H.\ pylori\) |
|          | \((-\)\) | \((+)\) | \((-\)\) | \((+)\) |
| AG = AA  | Case/Control 385/709 | 528/284 | 395/618 | 396/246 |
|          | OR (95%CI) 1 (Ref) | 3.44 (2.84–4.16) | 1 (Ref) | 2.53 (2.07–3.10) |
| GG       | Case/Control 80/116 | 74/57 | 73/114 | 75/55 |
|          | OR (95%CI) 1.27 (0.93–1.73) | 2.38 (1.65–3.44) | 1.00 (0.73–1.38) | 2.13 (1.47–3.08) |
|              | \(P_{interaction} = 0.013\) | \(P_{interaction} = 0.505\) |

| No. of interacting risk factors | Total population |
|--------------------------------|-----------------|
|                                 | Cases/controls | \(P^a\) | OR (95% CI) |
| 0                               | 329/592 | 1 (ref) |
| 1                               | 502/259 | <0.001 | 3.53 (2.89–4.33) |
| 2                               | 31/11   | <0.001 | 5.06 (2.51–10.21) |
| \(P_{trend} < 0.001, 95\% CI = 0.24–0.32\) |

### Table 4. Cumulative effect of the interacting risk factors of miR-27a rs895819-\(H.\ pylori\) infection on the atrophic gastritis risk.

| No. of interacting risk factors | Total population |
|--------------------------------|-----------------|
|                                 | Cases/controls | \(P^a\) | OR (95% CI) |
| 0                               | 329/592 | 1 (ref) |
| 1                               | 502/259 | <0.001 | 3.53 (2.89–4.33) |
| 2                               | 31/11   | <0.001 | 5.06 (2.51–10.21) |
| \(P_{trend} < 0.001, 95\% CI = 0.24–0.32\) |

Note: \(^a\)Adjusted by sex and age.
Methods

Study design. This study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University and the methods were carried out in accordance with the relevant guidelines and regulations. All participants were non-consanguineous ethnic Han Chinese. This study was divided into three parts focusing on risk, prognosis and miRNA expression. For the evaluation of risk, the study design comprised two stages using two different detection methods (direct sequencing and Sequenom Mass-ARRAY analysis) to investigate a total of 939 gastric cancer patients, 1,067 atrophic gastritis patients and 1,166 controls. To explore the association between miR-27a rs895819 with the risk of gastric cancer and atrophic gastritis, the first stage of the study involved direct sequencing of the samples from 724 retrospectively recruited individuals, consisting of 215 gastric cancer patients, 205 atrophic gastritis cases and 304 controls who attended the First Affiliated Hospital of China Medical University between 2005 and 2010. In the second stage of the study, the Sequenom Mass-ARRAY platform was used to investigate a total of 2,448 cases, consisting of 724 gastric cancer cases, 862 atrophic gastritis cases and 862 matched healthy controls from the Zhuanghe Gastric Diseases Screening Program or from patients who attended the First Affiliated Hospital of China Medical University between 2002 and 2013. All the subjects in this study were endoscopically and histologically confirmed. The classification of gastric cancer was based on Lauren’s classification, which was divided into intestinal-type and diffuse-type for subgroup analysis. The classification and grading of gastritis were based on the Updated Sydney System. Subjects who were endoscopically and histologically confirmed with normal mucosa or only minimal gastritis without other systemic disease and stomach diseases served as controls. Written informed consent was obtained from all participants. Medical histories (including details of age, sex, smoking, and alcohol consumption) were obtained by questionnaire and the records were computerized.

To further investigate the correlation of risk-associated polymorphisms with clinicopathologic parameters and survival in gastric cancer patients, we analyzed the data of 357 gastric cancer cases, which were all suffered surgical resection and the information of death or survival was available. The tumour histological grade was evaluated according to the World Health Organization criteria and tumours were staged using the 7th edition of the

Figure 1. The effect of miR-27a rs895819 A/G SNP on the mature miR-27a expression in vivo and vitro. (A) Kaplan–Meier survival curve analysis with the different genotypes of miR-27a rs895819 (GG + AG vs. AA) for the subgroup for TNM I-II stage. (B) Kaplan–Meier survival curve analysis with the different genotypes of miR-27a rs895819 (AG vs. AA) for the subgroup for TNM I-II stage. (C) Kaplan–Meier survival curve analysis with the different genotypes of miR-27a rs895819 (GG + AG vs. AA) for the subgroup for lymph node metastasis (−). (D) The cell mature miR-27a expression transinfected by different miR-27a rs895819 plasmid. *P = 0.05.
Table 5. Univariate and multivariate cox proportional hazard analysis for miR-27a rs895819 polymorphism. Note: HR, hazard ratio; CI, confidence interval; MST, median survival time (months). aWhen MST could not be calculated, mean survival time was provided. bMultivariate survival analysis was carried out by adding all the SNP variables to the clinicopathological parameters with \( P < 0.05 \) including TNM stage, lymphatic metastasis and depth of invasion. NA, not available.

| Genotype | All GC | Death | MST (M) | Univariate | Multivariate |
|----------|--------|-------|---------|------------|--------------|
|          | n = 357 | n = 89 |         | HR (95% CI) | \( P \) | HR (95% CI) | \( P \) |
| AA       | 184     | 48    | 58.4\(^a\) | 1 (Ref) | 1 (Ref) | 1 (Ref) |
| AG       | 148     | 36    | 58.8\(^a\) | 0.89 (0.58–1.37) | 0.597 | 0.84 (0.54–1.29) | 0.415 |
| GG       | 25      | 5     | 45.4\(^a\) | 0.75 (0.30–1.88) | 0.535 | 0.62 (0.25–1.57) | 0.316 |
| AG + GG VS. AA | 35     | 11    | 66.1\(^a\) | 0.68 (0.33–1.42) | 0.276 | 0.52 (0.25–1.08) | 0.063 |
| GG VS. AG + AA |       |       |         | 0.62 (0.37–1.05) | 0.065 |

Subgroup analysis for TNM stage I–II

| Genotype | All GC | Death | MST (M) | Univariate | Multivariate |
|----------|--------|-------|---------|------------|--------------|
| I–II     | n = 171 | n = 12 |         | HR (95% CI) | \( P \) | HR (95% CI) | \( P \) |
| AA       | 91     | 11    | 67.0\(^a\) | 1 (Ref) | 1 (Ref) | 1 (Ref) |
| AG       | 68     | 1     | 79.0\(^a\) | 0.11 (0.01–0.87) | 0.036 | 0.12 (0.02–0.91) | 0.041 |
| GG       | 12     | 0     | NA      | NA | NA | NA |
| AG + GG VS. AA | 123    | 12    | 68.7\(^a\) | 0.69 (0.35–1.39) | 0.289 | 0.60 (0.32–1.14) | 0.115 |
| GG VS. AG + AA |       |       |         | 0.62 (0.36–1.05) | 0.071 |

Subgroup analysis for Lymphatic metastasis (–)

| Genotype | All GC | Death | MST (M) | Univariate | Multivariate |
|----------|--------|-------|---------|------------|--------------|
| Negative | n = 141 | n = 12 |         | HR (95% CI) | \( P \) | HR (95% CI) | \( P \) |
| AA       | 74     | 10    | 64.7\(^a\) | 1 (Ref) | 1 (Ref) | 1 (Ref) |
| AG       | 58     | 2     | 76.9\(^a\) | 0.22 (0.05–1.02) | 0.054 | 0.25 (0.06–1.15) | 0.076 |
| GG       | 9      | 0     | NA      | NA | NA | NA |
| AG + GG VS. AA | 86    | 12    | 65.6\(^a\) | 0.78 (0.40–1.52) | 0.468 | 0.65 (0.34–1.25) | 0.221 |
| GG VS. AG + AA |       |       |         | 0.73 (0.39–1.38) | 0.373 |

Table 6. The effect of miR-27a rs895819 polymorphism to its mature miR-27a expression. Note: aMeans the variance analysis was used to calculate the difference between the expressions of three genotypes.

| Serum expression | Tissue expression in situ |
|------------------|--------------------------|
| Cancer | Controls | Cancer Tissue | Noncancer Tissues |
| n | Mean ± SD | n | Mean ± SD | n | Mean ± SD |
| Total | 87 | 2.12 ± 1.14 | 83 | 1.46 ± 0.73 | 31 | 8.71 ± 0.37 | 32 | 8.55 ± 0.31 |
| \( P \) | <0.001 | 0.565 |

| Genotype | Cancer | Controls | Cancer Tissue | Noncancer Tissues |
|---------|--------|----------|---------------|------------------|
| AA      | 38     | 2.25 ± 1.13 | 44 | 1.57 ± 0.78 | 14 | 8.84 ± 0.40 | 16 | 8.53 ± 0.32 |
| AG      | 47     | 2.01 ± 1.14 | 34 | 1.33 ± 0.71 | 16 | 8.64 ± 0.30 | 16 | 8.56 ± 0.30 |
| GG      | 2      | 2.02 ± 1.90 | 5  | 1.35 ± 0.18 | 1  | 8.18 | 0  | NA |
| \( P \) | 0.630 | 0.340 | 0.103 | 0.744 |
using the primers F: 5′-AACCTAGCCACTGTAACG-3′ and R: 5′-AGTTGCTGACCCCTCTGGTC-3′ with an annealing temperature of 59 °C. The genotyping in stage 2 assay was performed using the Sequenom Mass-ARRAY platform (Sequenom, San Diego, CA, USA) by Biomiao (Beijing, China) as described previously. 5% samples in the same groups were random chosen to be detected in both the direct sequencing and MALDI-TOF Mass-ARRAY methods to test the consistency of these two methods, with a concordance rate of 100%. Another 5% samples were random chosen to be detected in the same method, and the consistency also reached 100%.

**Serum H. pylori-IgG titer determination.** Serum H. pylori-IgG titers were determined by enzyme linked immunosorbent assay (ELISA, Helicobacter pylori-IgG kit; Biohit, Helsinki, Finland) according to a previously described method. H. pylori-IgG titers >35 was judged to be positive.

**RNA Extraction and real-time PCR analysis of miRNA expression in vivo.** MiRNA was extracted from serum and tissue samples as described previously with some modifications. The One Step Prime Script miRNA cDNA (Perfect Real-Time) Kit (TAKARA Biotechnology Co., Ltd, Dalian, China) was used to generate cDNA and the miRcute miRNA qPCR detection kit (SYBR) (TIANGEN Biotech Co., Ltd, Beijing, China) was used for real-time PCR analysis with the primer 5′-CGCGTTCACCCTGCGTTAAGTCC-3′. The methods used have been described previously.

**Transient transfection and Real-time PCR reaction for miRNA expression in vitro.** The commercial expression plasmid pCMV-miR-27a-rs895819-A was purchased from Genechem Company (Genechem Biotech Co., Ltd, Shanghai, China). And the polymorphic plasmid was conducted for site-specific mutagenesis from A to G (pCMV-miR-27a-rs895819-G) by Genechem Company and confirmed by sequencing. Then, the lowest miR-27a expression cell line, AGS, was selected for transfection (More details see Supplementary Methods and Supplementary Figure 1). After 24 hours, the total RNA of cells was extracted and Real-time PCR was used to detect miR-27a expression after reverse transcription in order to compare the mature miR-27a produced by pCMV-miR-27a-rs895819-G vs. pCMV-miR-27a-rs895819-A.

**Statistical analysis.** To determine the genetic models for rs895819 SNP, we employed the method suggested by Thakkinstian et al. The strength of association between genotype and disease risk was assessed by odds ratio (OR) and 95% confidence interval (CI) measures, adjusted by sex, age and status of H. pylori infection. Genetic effects for three pairwise comparisons were calculated to determine the optimal genetic model. The One Step Prime Script miRNA cDNA (Perfect Real-Time) Kit (TAKARA Biotechnology Co., Ltd, Dalian, China) was used to generate cDNA and the miRcute miRNA qPCR detection kit (SYBR) (TIANGEN Biotech Co., Ltd, Beijing, China) was used for real-time PCR analysis with the primer 5′-CGCGTTCACCCTGCGTTAAGTCC-3′. The methods used have been described previously.

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Author Contributions
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