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ORIGINAL RESEARCH

Pri-let-7a-1 rs10739971 polymorphism is associated with gastric cancer prognosis and might affect mature let-7a expression

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Abstract: The relationship between the pri-let-7a-1 rs10739971 polymorphism and gastric cancer (GC) risk has been reported. However, the role of this polymorphism in the prognosis of GC remains largely elusive. Sequenom MassARRAY platform method and the polymerase chain reaction (PCR)-restriction fragment length polymorphism were used to investigate pri-let-7a-1 rs10739971 G→A in 334 GC patients. Real-time PCR detected expression of mature let-7a in serum and tissue. Patients with AA or GA+AA genotypes of the pri-let-7a-1 rs10739971 polymorphism demonstrated significantly longer survival time than those with the wild GG genotype. Stratified analysis indicated that survival time was significantly longer in women with AA or GA+AA genotypes and in Borrmann type I/II patients with GA heterozygote or GA+AA genotypes. AA genotype was more frequent in the lymphatic-metastasis-negative subgroup. Serum mature let-7a expression in healthy people with the GA heterozygote and the GA+AA genotype was higher than in those with the GG genotype, and the difference remained significant in the female healthy subgroup. Pri-let-7a-1 rs10739971 polymorphism might be a biomarker for GC prognosis, especially for female and Borrmann type I/II patients. The pri-let-7a-1 rs10739971 polymorphism might affect serum mature let-7a expression, and partly explain the mechanism of the relationship between the pri-let-7a-1 rs10739971 polymorphism and GC survival.

Keywords: miRNA, let-7a, polymorphism, gastric cancer, prognosis, expression

Introduction

microRNAs (miRNAs) are involved in a variety of important biological behaviors including tumorigenesis, differentiation, apoptosis, and proliferation.[1,2] It is widely accepted that miRNA polymorphisms can be used as biomarkers of tumor prognosis. For example, the miR-196a-2 rs11614913 polymorphism can predict the survival of several tumors, including gastric cancer (GC),[3,4] non-small-cell lung cancer,[5,6] esophageal squamous cell carcinoma,[7] oral squamous cell carcinoma,[8–10] kidney cancer,[11] and breast cancer.[12] The miR-27a rs895819 polymorphism can be used as a prognostic factor for advanced GC[4] and non-small-cell lung cancer.[13]

The let-7 plays a critical role in cell proliferation and differentiation; it is the earliest discovered miRNA and has continued to gain recognition in recent years. Several studies have proved that let-7 miRNA participates in the tumorigenesis and metastasis of different types of cancer, such as breast and lung cancers.[14–16] The let-7 family has ten members: let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let7i, miR-98, and miR-202. Recent research has linked polymorphisms of some let-7 family members to cancer, for example, the interaction of the pri-let-7a-1 rs10739971 polymorphism and the *ERCC6* rs1917799 polymorphism is significantly correlated

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with the risk of GC.[17] The rs10877887 polymorphism in the promoter region of let-7 is significantly associated with prognosis of hepatocellular carcinoma[18] and the pri-let-7a-2 rs629367 polymorphism is significantly related to poor survival of GC.[19] The relationship between the pri-let-7a-1 rs10739971 polymorphism and GC risk has been reported; however, few studies have investigated the association of the pri-let-7a-1 polymorphism and cancer prognosis. The relationship between the pri-let-7a-1 rs10739971 polymorphism and GC prognosis has not been reported.

In this study, systematic clinical data were collected for 334 cases of GC and the relationship between the pri-let-7a-1 rs10739971 polymorphism and cancer prognosis was investigated. We analyzed the effect of this polymorphism on mature let-7a expression both in serum and tissue. The aim of this study was to establish the relationship between this polymorphism and GC prognosis, and its possible mechanism.

Materials and methods

Patients and clinicopathological data

This study was approved by the Human Ethics Committee of the First Affiliated Hospital of China Medical University, and conformed to Chinese laws and the Helsinki Declaration. Written informed consent was obtained from the patients, and baseline characteristics (including age, sex, smoking, alcohol drinking, and family history) were obtained by questionnaire and the records above were computerized. Subjects were recruited who underwent gastric surgery in the First Affiliated Hospital of China Medical University, Liaoning Province, People's Republic of China between 2010 and 2013. Fasting venous blood was collected. Serum and blood coagulation were separated and stored at –20°C. All GC patients were diagnosed based on the histopathological confirmation according to Borrmann's as well as Lauren's classification, and the tumors were staged according to the 7th edition of the tumor node metastasis (TNM) staging system of the International Union Against Cancer/American Joint Committee on Cancer (2010), based on postoperative pathological examination. Exclusion criteria were 1) patients with distant metastasis found preoperatively; 2) patients who underwent preoperative radiotherapy or chemotherapy; or 3) patients with incomplete pathological data. Follow-up was completed by August 2013. The basic features of 334 GC patients enrolled in survival analysis are shown in Table S1.

To assess the effect of polymorphism on mature miRNA expression at the tissue level, 131 GC specimens from patients who underwent subtotal gastrectomy in the First Affiliated Hospital of China Medical University between 2010 and 2013 were included. At the serum level, we matched 99 GC patients with 99 healthy individuals. Patients who were endoscopically and histologically diagnosed with normal mucosa or only mild gastritis without other systemic diseases or other gastropathy served as controls. The basic information of all subjects is summarized in Table S2.

Single nucleotide polymorphism genotyping

The genomic DNA was extracted using the method stated previously with proteinase K digestion and phenol/chloroform extraction.[20] Sequenom MassARRAY platform method (Sequenom, San Diego, CA, USA) and polymerase chain reaction (PCR) restriction-fragment length polymorphism were used to detect the polymorphism of pri-let-7a-1 rs10739971 G→A. To assess the quality of the genotyping, 5% samples of all were genotyped repeatedly and the results were 100% consistent.

RNA extraction and real-time quantitative PCR reaction for miRNA expression in vivo

miRNAs were extracted from the serum and tissues as described previously,[21] with some modifications. One Step Prime Script miRNA cDNA (Perfect Real Time) Kit (TaKaRa Biotechnology, Dalian, People's Republic of China) was used in the reverse transcription reaction and miRcute miRNA qPCR detection kit SuperReal PreMix Plus (SYBR) (Tiangen Biotech, Beijing, People's Republic of China) was used for real-time quantitative PCR. Details of the materials are shown in the Supplementary materials.

Statistical analysis

All the statistical analyses were carried out using SPSS Version 16.0 software (SPSS Inc., Chicago, IL, USA). Correlation between the pri-let-7a-1 rs10739971 polymorphism and clinicopathological parameters of GC was estimated by χ^2 test. Survival curves were estimated by the Kaplan–Meier method, and the association between the single nucleotide polymorphism (SNP) and different survival times was assessed by the log-rank test. The median survival time was calculated; the hazard ratios (HRs) and their 95% confidence intervals (CIs) were estimated by univariate and multivariate Cox proportional hazards regression models. The log value of copies of miRNA were used to reach a normal distribution, and the effect of miRNA polymorphisms on the expression level was tested by the independent-samples *t*-test, with the mean and standard deviation calculated.

Results

Characteristics and clinical features of the study subjects

The demographic and clinical features of the 334 patients enrolled in this study are shown in Table S1, including age, sex, tumor size, Borrmann type, Lauren classification, TNM stage, growth pattern, lymphatic metastasis, smoking, alcohol consumption, and family history. Tumor size, Lauren grade, TNM stage, growth pattern, and lymphatic metastasis significantly affected survival time ($P<0.05$). Therefore, we used these as adjustment factors for a multivariate Cox proportional hazards regression model.

Correlation between the pri-let-7a-1 rs10739971 polymorphism and clinicopathological parameters of GC

The correlation between the pri-let-7a-1 rs10739971 polymorphism and clinicopathological parameters of GC was analyzed, including age, sex, tumor size, Borrmann type, Lauren classification, TNM stage, growth pattern, lymphatic metastasis, smoking, alcohol consumption, and family history. AA genotype was more frequent in the lymphatic-metastasis-negative subgroup than in the subgroup with lymphatic metastasis (52.5% vs 31.6%, P=0.009; 25.2% vs 14.6%, P=0.015). There was no significant correlation between the pri-let-7a-1 rs10739971 polymorphism and other clinicopathological parameters of GC (Table 1).

Association of the pri-let-7a-1 rs10739971 polymorphism with survival time of GC patients

We analyzed the association of the pri-let-7a-1 rs10739971 polymorphism with prognosis of GC using a univariate Cox proportional hazards regression model. The log-rank test showed that survival time in patients with the AA or GA+AA genotype was significantly longer than in those with GG genotype (P=0.099, P=0.092, respectively; Table 2). Multivariate Cox proportional hazards regression model analysis showed that survival time was longer in patients with the variant AA genotype polymorphism (HR =0.75,

Table 1 Correlation between pri-let-7a-1 rs10739971 polymorphism and clinicopathological parameters in gastric cancer

Variables	GG	GA	$P_{adjustment}$[a]	AA	$P_{adjustment}$[a]	$P_{adjustment}$[a] GA+AA vs GG	$P_{adjustment}$[a] AA vs GA+GG
Size (cm)			0.690		0.980	0.762	0.775
≤4	15	25		8			
>4	16	32		8			
Borrmann type			0.228		0.636	0.444	0.203
Borrmann I–II	29	42		20			
Borrmann III–IV	48	100		28			
Lauren classification			0.872		0.428	0.870	0.294
Intestinal	32	61		17			
Diffuse	60	109		45			
TNM stage			0.737		0.872	0.819	0.803
I–III	86	158		56			
IV	9	19		6			
Growth pattern			0.547		0.632	0.513	0.733
Massive and nested	33	60		17			
Diffused	38	81		26			
Lymphatic metastasis			0.298		0.010[b]	0.095	0.023[b]
Negative	29	111		32			
Positive	65	66		30			
Smoking			0.964		0.782	0.981	0.915
Nonsmoker	45	86		26			
Smoker	26	55		17			
Alcohol drinking			0.251		0.141	0.169	0.307
Nondrinker	53	92		25			
Drinker	18	49		18			
Family history			0.495		0.271	0.358	0.396
No	58	120		38			
Yes	13	21		5			

Notes: [a]$P_{adjustment}$: using logistic regression adjusted by two factors of sex and age; [b]P=0.010, OR (95% CI) =0.41 (0.21–0.81); P=0.023, OR (95% CI) =0.52(0.30–0.91).
Abbreviations: CI, confidence interval; OR, odds ratio.

Table 2 Univariate and multivariate Cox proportional hazard analysis for pri-let-7a-1 rs10739971 polymorphism

Variables	All GC	Death	MST (M)	Univariate		Multivariate	
				P-value	HR (95% CI)	*P*-value	HR (95% CI)
rs10739971							
GG	95	31	44		1 (Ref)		1 (Ref)
GA	177	46	62.0[a]	0.169	0.73 (0.46–1.15)	0.563	1.48 (0.39–5.65)
AA	62	15	51.0[a]	0.099	0.59 (0.32–1.10)	0.762	0.75 (1.12–4.69)
GA+AA vs GG				0.092	0.69 (0.45–1.06)	0.677	1.32 (0.36–4.80)
AA vs GG+GA				0.269	0.73 (0.42–1.27)	0.580	0.66 (0.15–2.88)

Note: [a]Mean survival time was provided when MST could not be calculated.
Abbreviations: CI, confidence interval; GC, gastric cancer; HR, hazard ratio; MST, median survival time; M, month; Ref, reference.

P=0.762; Table 2), with adjustment for potential confounding factors, such as tumor size, Lauren classification, TNM stage, growth pattern, and lymphatic metastasis.

Stratified analysis of the effect of the pri-let-7a-1 rs10739971 polymorphism on survival time of GC patients

To evaluate the influence of different factors on the relationship between the pri-let-7a-1 rs10739971 polymorphism and GC patients' survival, we performed stratification analysis. This polymorphism was significantly associated with prognosis of GC in certain subgroups: survival time of subjects with AA or GA+AA genotype was significantly longer compared with those with wild-type GG genotype, especially in female patients (HR =0.24, 95% CI =0.07–0.86; HR =0.40, 95% CI =0.19–0.83; Table 3, Figure 1A–D) and Borrmann type I/II patients (HR =0.44, 95% CI =0.20–0.94; HR =0.42, 95% CI =0.21–0.84; Table 3, Figure 1E–H).

Effect of the pri-let-7a-1 rs10739971 polymorphism on mature let-7a expression

To study the possible mechanism of pri-let-7a-1 rs10739971 polymorphism in the survival time of GC patients, we further analyzed the effect of this polymorphism on its mature miRNA expression in serum and tissue. There was no significant difference for age and sex in all subjects (including GC patients and healthy controls, Tables S2 and S3). In serum, mature let-7a expression of this polymorphism in healthy controls with the GA heterozygote and GA+AA genotype showed a significant increase compared with healthy people with the wild-type GG genotype (GA vs GG, *P*=0.028; GA+AA vs GG, *P*=0.018; Table 4 and Figure 2A). Mature let-7a expression in healthy women with this polymorphism (GA+AA) was significantly higher than those with GG genotype (GA+AA vs GG, *P*=0.043; Table 4 and Figure 2B). In GC patients, however, this polymorphism had no significant effect on its mature miRNA expression in serum or tissue (Tables 4 and 5).

Discussion

We reported the relationship of the pri-let-7a-1 rs10739971 polymorphism with the prognosis of GC patients in Northern China. Cox proportional hazards regression model analysis showed that AA mutation genotype and GA+AA genotype correlated with GC prognosis ($P<0.10$), especially in female patients or Borrmann type I/II patients. Further analysis found that AA mutation genotype was significantly correlated with lymphatic metastasis. After analyzing the effect of this polymorphism on its mature miRNA expression in serum and tissue, we found that mature miRNA expression of GA and GA+AA genotype was significantly higher than wild-type GG miRNA expression in the serum of all healthy people and the female healthy controls.

The miRNA polymorphism can act directly (including primary, secondary precursor, and mature polymorphism, which can affect maturation or function of miRNA) or indirectly (promoter region polymorphism affects the transcription and polymorphism-binding sites of the target gene). The pri-let-7a-1 rs10739971 polymorphism is located in –599 bp of mature let-7a-1, which is the primary precursor area of let-7a-1 and may also be the promoter region. We found that carriers of the variant genotype of this polymorphism demonstrated favorable prognosis compared with those with the wild genotype. The relationship between pri-let-7a-1 rs10739971 polymorphism and GC prognosis might arise from that this polymorphism is located at the promoter region. We adopted RNAfold software to predict secondary structure of the let-7a-1 gene near ±600 bp. The minimum free energy of those who carry the rs10739971 variant A allele was lower than that of those who carry the rs10739971 wild G allele. Furthermore, the secondary structure of miRNA with rs10739971 variant A allele tended to be more stable.

Table 3 Stratified analysis for the association of pri-let-7a-1 rs10739971 genotypes and gastric cancer survival

Variables	Death/ patients	GA vs GG		AA vs GG		GA+AA vs GG		AA vs GA+GG	
		P-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)
Age (years)									
≤59	54/184	0.122	0.61 (0.33–1.14)	0.429	0.75 (0.36–1.54)	0.144	0.65 (0.37–1.16)	0.933	1.03 (0.55–1.92)
>59	38/150	0.676	0.87 (0.44–1.70)	0.052	0.23 (0.05–1.01)	0.315	0.71 (0.37–1.38)	0.065	0.26 (0.06–1.09)
Sex									
Male	63/244	0.779	0.92 (0.52–1.64)	0.686	0.86 (0.41–1.79)	0.724	0.91 (0.52–1.57)	0.771	0.91 (0.49–1.71)
Female	29/90	0.056	0.47 (0.22–1.02)	**0.028**	**0.24 (0.07–0.86)**	**0.014**	**0.40 (0.19–0.83)**	0.121	0.39 (0.12–1.29)
Size (cm)									
≤4	1/48	0.595	NA	0.650	NA	0.581	NA	0.737	NA
>4	15/56	0.514	1.55 (0.42–5.78)	0.904	1.12 (0.19–6.70)	0.602	1.41 (0.39–5.06)	0.678	0.73 (0.16–3.28)
Borrmann type									
Borrmann I–II	45/176	**0.033**	**0.44 (0.20–0.94)**	0.065	0.41 (0.16–1.06)	**0.015**	**0.42 (0.21–0.84)**	0.310	0.63 (0.26–1.53)
Borrmann III–IV	33/91	0.334	0.72 (0.36–1.41)	0.690	0.84 (0.35–2.02)	0.356	0.74 (0.39–1.41)	0.872	1.07 (0.50–2.29)
Lauren classification									
Intestinal	20/110	0.687	0.83 (0.33–2.10)	0.099	0.17 (0.02–1.39)	0.346	0.64 (0.26–1.61)	0.108	0.19 (0.03–1.44)
Diffuse	66/214	0.162	0.68 (0.39–1.17)	0.373	0.74 (0.38–1.44)	0.159	0.69 (0.42–1.16)	0.864	0.95 (0.53–1.71)
TNM stage									
I–III	72/300	0.108	0.65 (0.39–1.10)	0.298	0.71 (0.37–1.36)	0.102	0.67 (0.41–1.08)	0.778	0.92 (0.51–1.65)
IV	20/34	0.881	0.93 (0.35–2.46)	0.080	0.15 (0.02–1.26)	0.497	0.72 (0.27–1.88)	0.098	0.18 (0.02–1.37)
Growth pattern									
Massive and nested	15/110	0.063	0.49 (0.23–1.04)	0.743	0.76 (0.15–3.94)	0.782	0.86 (0.29–2.52)	0.748	0.78 (0.18–3.48)
Diffused	44/145	0.624	0.84 (0.42–1.68)	0.404	0.671 (0.26–1.71)	0.489	0.79 (0.41–1.54)	0.475	0.75 (0.33–1.67)
Lymphatic metastasis									
Negative	14/128	0.588	0.71 (0.21–2.43)	0.479	0.58 (0.13–2.60)	0.495	0.67 (0.21–2.13)	0.636	0.74 (0.21–2.63)
Positive	78/206	0.327	0.78 (0.48–1.28)	0.469	0.78 (0.39–1.54)	0.297	0.78 (0.49–1.25)	0.761	0.91 (0.49–1.68)
Smoking									
Nonsmoker	35/158	0.447	0.76 (0.38–1.54)	0.108	0.36 (0.10–1.25)	0.225	0.65 (0.33–1.30)	0.138	0.41 (0.13–1.33)
Smoker	24/96	0.528	1.43 (0.47–4.36)	0.313	1.92 (0.54–6.81)	0.420	1.56 (0.53–4.46)	0.398	1.49 (0.59–3.75)
Alcohol drinking									
Nondrinker	39/164	0.948	0.98 (0.50–1.93)	0.344	0.55 (0.16–1.92)	0.744	0.90 (0.46–1.74)	0.332	0.56 (0.17–1.81)
Drinker	20/91	0.714	0.80 (0.25–2.58)	0.876	0.90 (0.25–3.22)	0.759	0.842 (0.28–2.53)	0.876	1.08 (0.41–2.82)
Family history									
No	54/216	0.671	0.88 (0.48–1.60)	0.192	0.55 (0.23–1.35)	0.422	0.79 (0.44–1.41)	0.192	0.59 (0.27–1.30)
Yes	5/39	0.971	1.05 (0.10–11.55)	0.178	5.25 (0.47–58.60)	0.641	1.69 (0.19–15.08)	0.076	5.09 (0.84–30.78)

Note: The significant results marked in bold.

Abbreviations: CI, confidence interval; HR, hazard ratio; NA, not available.

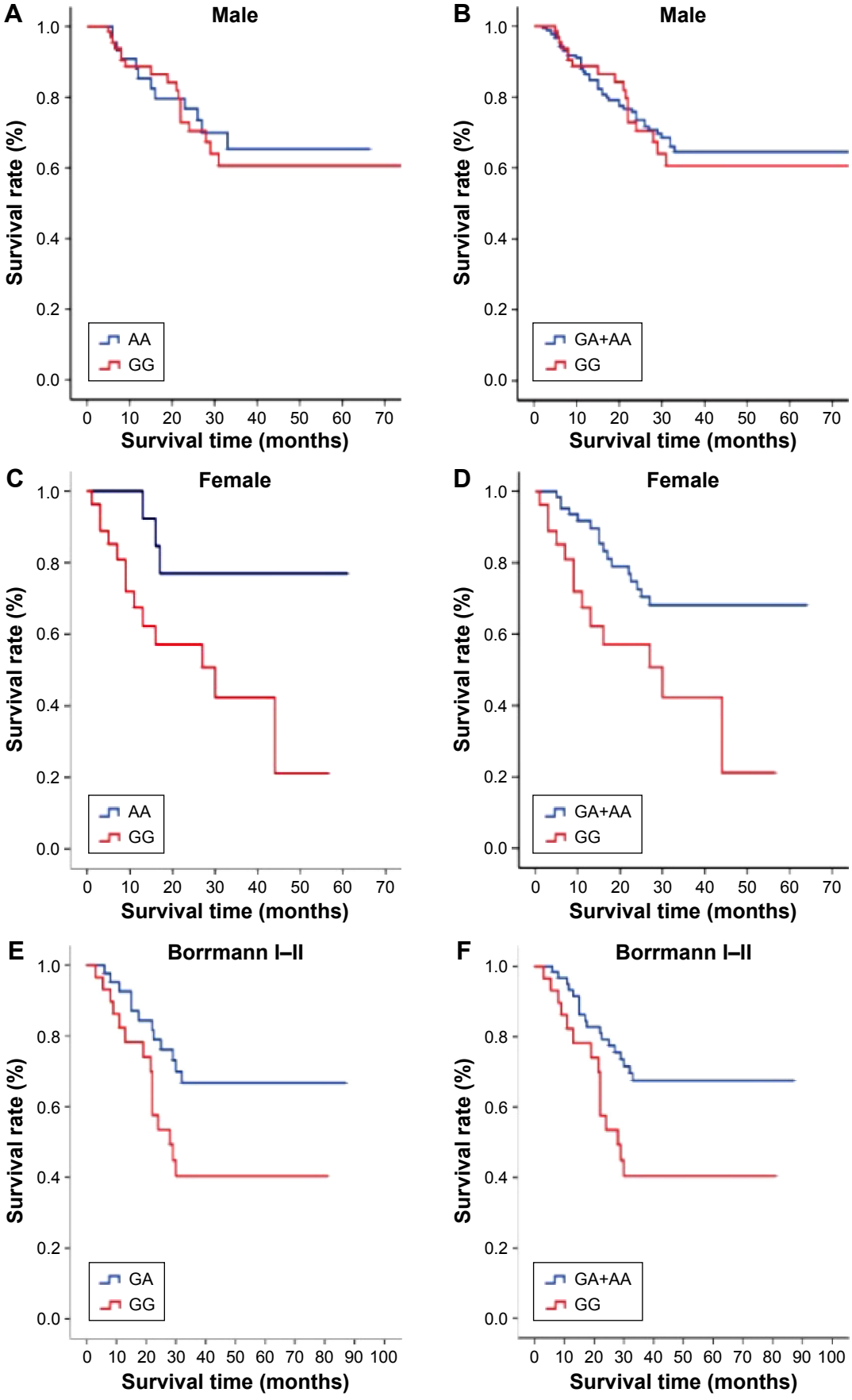

Figure 1 (*Continued*)

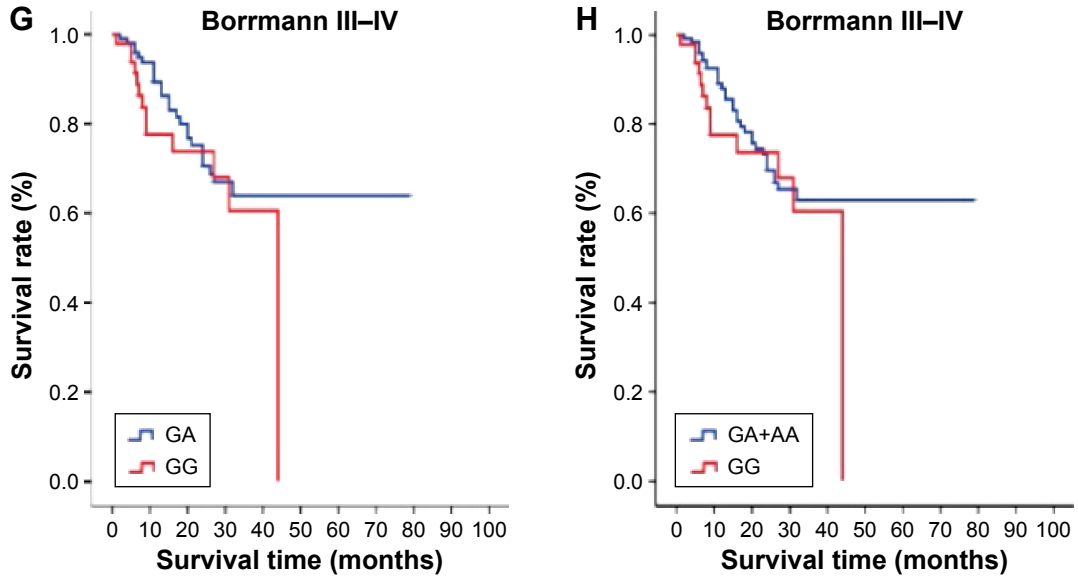

Figure 1 Kaplan–Meier survival curve analysis with the different genotypes of pri-let-7a-1 rs10739971 in different subgroups.
Notes: (**A**) AA vs GG in male subgroup. (**B**) GA+AA vs GG in male subgroup. (**C**) AA vs GG in female subgroup. (**D**) GA+AA vs GG in female subgroup. (**E**) GA vs GG in Borrmann I–II subgroup. (**F**) GA+AA vs GG in Borrmann I–II subgroup. (**G**) GA vs GG in Borrmann III–IV subgroup. (**H**) GA+AA vs GG in Borrmann III–IV subgroup.

Therefore, we speculated that the different structure of the rs10739971 G/A polymorphism may lead to a difference in function, because the variant A allele had a more stable secondary structure, which may be more conducive to the maturation process of miRNA. In addition, let-7a is recognized as a tumor suppressor, which might have a positive effect on tumor prognosis. GC patients who carry the variant A allele demonstrated longer survival. The wild-type G allele has a loose secondary structure, which may not be conductive to miRNA maturation, leading to a shorter survival time of GC patients who carry the wild-type G allele. We also found that the rate of lymphatic metastasis in GC patients with the AA mutation genotype was significantly lower than in those with wild GG genotype. This was consistent with our finding that patients with the AA mutation genotype exhibited significantly longer survival time than those with the wild GG genotype. Lymphatic metastasis is a risk factor for GC; thus, we consider that the significantly longer survival time observed for carriers of the AA mutation genotype may be related to the absence of lymphatic metastasis.

Table 4 Independent-samples *t*-test for the effect of pri-let-7a-1 rs10739971 polymorphism to its mature miRNA expression in serum level

Variables	CON			GC		
	N	Mean ± SD	*P*-value	N	Mean ± SD	*P*-value
Total						
GG	28	3.18±0.57	1 (Ref)	31	3.33±0.64	1 (Ref)
GA	54	3.46±0.53	**0.028**	54	3.39±0.50	0.627
AA	17	3.45±0.43	0.094	14	3.27±0.53	0.745
GA+AA vs GG			**0.018**			0.769
AA vs GA+GG			0.541			0.519
Male						
GG	17	3.22±0.61	1 (Ref)	19	3.32±0.64	1 (Ref)
GA	35	3.49±0.59	0.137	37	3.45±0.53	0.435
AA	11	3.43±0.51	0.354	9	3.28±0.59	0.860
GA+AA vs GG			0.129			0.553
AA vs GA+GG			0.875			0.532
Female						
GG	11	3.11±0.51	1 (Ref)	12	3.35±0.66	1 (Ref)
GA	19	3.41±0.41	0.086	17	3.28±0.44	0.724
AA	6	3.49±0.28	0.115	5	3.25±0.49	0.776
GA+AA vs GG			**0.043**			0.683
AA vs GA+GG			0.345			0.837

Note: The significant results marked in bold.
Abbreviations: CON, control; GC, gastric cancer; SD, standard deviation; Ref, reference.

Stratified analysis suggested that female patients who carry this polymorphism AA or GA+AA genotype had significantly longer survival. The let-7 is a direct estrogen receptor (ER)-α target gene, and its expression correlates with ER-α in breast, ovarian, and gastric cancer, and it inhibits cancer cell proliferation and promotes cancer cell apoptosis.[15,22,23] Previous studies showed that let-7 is an important regulator of ER-α target genes and it shares many binding sites with ER-α. Notably, at shared sites, let-7 inhibits the ER-α signaling pathway, which decreases the survival of cancer cells and increases their rate of apoptosis.[14,15] As a result, in female patients with more ER-α, more let-7 polymorphisms, which act as a protective factor, contribute to better prognosis. We speculated that

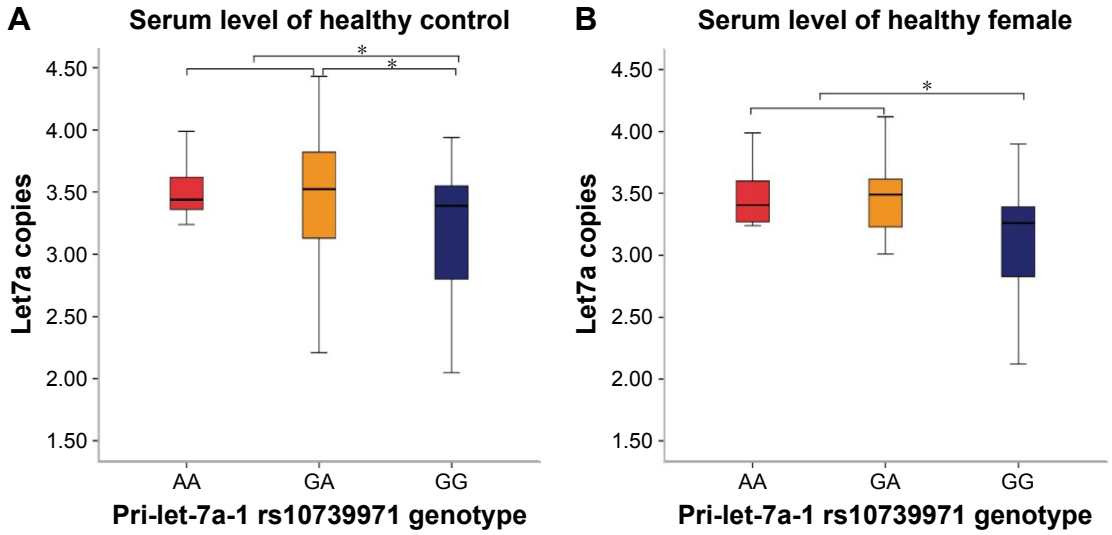

Figure 2 The effect of pri-let-7a-1 rs10739971 G/A SNP on the mature let-7a expression.
Notes: (**A**) The let-7a expression of different genotype of pri-let-7a-1 rs10739971 in healthy people serum. (**B**) The let-7a expression of different genotype of pri-let-7a-1 rs10739971 in healthy female serum.

female GC patients who carried variant A allele had increased expression of mature let-7a compared with G allele carriers, which could more efficiently inhibit tumor cell proliferation and induce tumor cell apoptosis, thereby leading to longer survival time. However, the result was not what we expected: expression of mature let-7a was not increased significantly in GC patients with the variant A allele. The binding of mature let-7a and ER-α is due to the variant A allele, which may lead to the result above. In stratified analysis, we also found that Borrmann type I/II patients who carry this polymorphism of GA heterozygous or (GA+AA) genotype demonstrated more favorable prognosis than GG genotype subjects. Yang found that the Borrmann type is an independent prognostic factor only in stage IV GC,[24] which showed that it has an important role in GC prognosis. rs10739971 is located in the promoter region of let-7a miRNA, which might affect the post-transcriptional regulation of let-7a miRNA, through the binding of different transcription factors. We conjectured that rs10739971 could affect the Borrmann type through its effect on the expression of mature let-7a. Moreover, the phenomenon that the A allele of this polymorphism was related to favorable prognosis may be because the effect of the variant A allele is great in the case of healthy individuals and patients with primary stage GC. Besides, we found that survival time was not significantly longer in men with AA or GA+AA genotypes and in Borrmann type III/IV patients with GA heterozygote of GA+AA genotypes, which suggest that sex and Borrmann classification might influence the effect of SNP on GC survival.

Table 5 Independent-samples *t*-test for the effect of pri-let-7a-1 rs10739971 polymorphism to its mature miRNA expression in tissue level

Variables	CON			GC		
	N	Mean ± SD	*P*-value	N	Mean ± SD	*P*-value
Total						
GG	33	5.48±0.51	1 (Ref)	40	5.43±0.69	1 (Ref)
GA	40	5.38±0.82	0.542	46	5.41±0.72	0.890
AA	16	5.20±0.73	0.129	18	5.14±0.59	0.137
GA+AA vs GG			0.329			0.496
AA vs GA+GG			0.253			0.127
Male						
GG	21	5.55±0.44	1 (Ref)	25	5.34±0.67	1 (Ref)
GA	28	5.46±0.77	0.637	34	5.42±0.75	0.698
AA	11	5.24±0.84	0.178	13	5.19±0.66	0.517
GA+AA vs GG			0.419			0.946
AA vs GA+GG			0.262			0.377
Female						
GG	12	5.36±0.63	1 (Ref)	15	5.57±0.73	1 (Ref)
GA	12	5.19±0.92	0.608	12	5.37±0.65	0.485
AA	5	5.12±0.42	0.455	5	5.01±0.40	0.125
GA+AA vs GG			0.500			0.244
AA vs GA+GG			0.674			0.152

Abbreviations: CON, control; GC, gastric cancer; SD, standard deviation; Ref, reference.

Mature miRNA is part of the regulatory mechanisms controlling gene expression at both transcriptional and post-transcriptional levels. According to recent research,[25] one miRNA can regulate the expression of multiple genes, while a single gene is regulated by a series of different miRNAs, and ~60% of protein-coding genes are regulated by miRNAs. At present, research about the pathogenic mechanism of the miRNA promoter region polymorphism has mainly focused

on the functional miR-34b/c rs4938723 polymorphism. This polymorphism might affect the binding ability of transcription factor GATA and further influence mature pri-miR-34b/c expression.[26] Xu et al thought it may serve as biomarker of liver cancer prognosis. We speculated that the significant association of the pri-let-7a-1 rs10739971 polymorphism with the prognosis of GC may arise from the influence of mature let-7a expression, like the miR-34b/c rs4938723 polymorphism. Therefore, we explored further the effect of this polymorphism on its mature miRNA expression level. In serum, we found that mature let-7a expression in healthy individuals with the GA heterozygote and GA+AA genotype showed a significant increase, and mature let-7a expression in healthy women with the GA+AA genotype was significantly higher. This indicated that the variant A allele was beneficial to let-7a miRNA maturation, illustrating that the miRNA polymorphism acts as a tumor suppressor by increasing the expression of mature miRNA, which is consistent with the effect of the miR-34b/c rs4938723 polymorphism on the expression of its maturity.[26] In tissue, we found that mature let-7a expression in individuals with different genotypes showed no significant difference between the GC and healthy groups, which may be because the tissue of the healthy group was taken from distal GC patients instead of really healthy people. In GC patients, no significant effect of this polymorphism on its mature miRNA expression, either in serum or tissue, was observed. The following reasons may explain the results. First, the state of disease (cancerous) disturbs multiple cellular functions and regulations, which probably weaken the effect of the miRNA SNP on mature miRNA expression. Second, the sample size of expression study was relatively small, which might restrict the results and should be confirmed in a larger sample of future investigation. In addition, it should be further researched that whether other cofactors also affect let-7a expression together with SNP.

In summary, this study analyzed the association of the pri-let-7a-1 rs10739971 polymorphism and GC prognosis stratified by clinicopathological parameters on the basis of overall analysis. We preliminarily studied the effect of the pri-let-7a-1 rs10739971 polymorphism on the expression level of miRNA to explore the possible mechanism of the effect of miRNA polymorphism on GC prognosis. This is believed to be the first study exploring the relationship between the pri-let-7a-1 rs10739971 G/A polymorphism and GC prognosis in Northern China. There were some limitations to our study. First, the sample size was insufficient, and our results need validation with a larger sample of different ethnicities. Second, our study only investigated overall survival time, and other relevant indicators of prognosis research need to be explored, such as disease-free survival time. Third, subsequent experiments concerning function of miRNA are still needed.

Conclusion

The pri-let-7a-1 rs10739971 polymorphism has potential as a prognostic biomarker of GC, especially for female and Borrmann type I/II patients. The pri-let-7a-1 rs10739971 polymorphism might affect serum mature let-7a expression, which might partly explain the mechanism of the pri-let-7a-1 rs10739971 polymorphism in GC survival. Future large-sample studies and mechanism experiments are needed to prove our findings.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Serum RNA extraction and real-time quantitative PCR reaction for miRNA expression in vivo

Approximately 6 mL venous blood was collected from each subject. Serum total RNA was extracted using a method described by Liu et al[1] with some modifications. Serum was separated by centrifugation at 3,000× *g* for 10 minutes, followed by a 15 minutes high-speed centrifugation at 12,000× *g* to completely remove cell debris. The supernatant serum was recovered and stored at −80°C until further processing. Total RNA was extracted from 200 μL of serum by acid phenol/chloroform purification and centrifugation in isopropyl alcohol. Every sample was all in 20 μL RNA-free ddH_2O solution and 6 μL of total RNA was reverse-transcribed to cDNA using One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa Biotechnology, Dalian, People's Republic of China). Real-time PCR was performed using a miRcute miRNA qPCR detection kit (Tiangen Biotech, Beijing, People's Republic of China) on the Thermal Cycler Dice Real Time System (TaKaRa Biotechnology). To calculate the levels of the miRNAs, synthetic hsa-let-7a at known concentrations (TaKaRa Biotechnology) were also reverse-transcribed and amplified. The concentration of each miRNA was then calculated according to the standard curve. All reactions, including no-template controls, were run in duplicate.

Table S1 Clinical features of gastric cancer patients

Variables	GC patients N=334 (%)	Death N=92	MST (M)	Log-rank	
				P-value	HR (95% CI)
Age (years)					
≤59	184 (55.1)	54	55.8[a]		1 (Ref)
>59	150 (44.9)	38	59.1[a]	0.852	0.96 (0.64–1.46)
Sex					
Male	244 (73.1)	63	61.7[a]		1 (Ref)
Female	90 (26.9)	29	43.3[a]	0.339	0.81 (0.52–1.25)
Size (cm)					
≤4	48 (14.4)	1	27.3[a]		1 (Ref)
>4	56 (16.8)	15	13.0	**0.009**	**15.24 (1.99–116.80)**
Borrmann type					
Borrmann I–II	176 (65.9)	45	44.0		1 (Ref)
Borrmann III–IV	91 (34.1)	33	59.3[a]	0.810	1.06 (0.67–1.67)
Lauren classification					
Intestinal	110 (32.9)	20	63.3[a]		1 (Ref)
Diffuse	214 (64.1)	66	57.3[a]	**0.031**	**1.74 (1.05–2.86)**
Unclassified	10 (3.0)	2			
TNM stage					
I–III	300 (89.8)	72	58.9[a]		1 (Ref)
IV	34 (10.2)	20	20.0	**1×10^{-6}**	**3.46 (2.10–5.69)**
Growth pattern					
Massive and Nested	110 (32.9)	15	34.7[a]		1 (Ref)
Diffused	145 (43.4)	44	26.6[a]	**1×10^{-4}**	**3.21 (1.78–5.80)**
Lymphatic metastasis					
Negative	128 (38.3)	14	70.7[a]		1 (Ref)
Positive	206 (61.7)	78	44.0	**1×10^{-6}**	**4.06 (2.30–7.18)**
Smoking					
Nonsmoker	158 (47.3)	35	30.5[a]		1 (Ref)
Smoker	96 (28.7)	24	29.9[a]	0.843	1.05 (0.63–1.77)
Alcohol drinking					
Nondrinker	164 (49.1)	39	30.2[a]		1 (Ref)
Drinker	91 (27.2)	20	30.6[a]	0.547	0.81 (0.40–1.63)
Family history					
No	216 (64.7)	54	29.7[a]		1 (Ref)
Yes	39 (11.7)	5	34.9[a]	0.584	0.86 (0.50–1.48)

Notes: [a]Mean survival time was provided when MST could not be calculated. The significant results marked in bold.
Abbreviations: CI, confidence interval; HR, hazard ratio; GC, gastric cancer; MST, median survival time; M, month; Ref, reference.

Table S2 The basic characteristics of the subjects included to investigate the association of pri-let-7a-1 rs10739971 polymorphism with its mature miRNA expression in serum or tissue of gastric cancer

Variables	Serum		Tissue
	CON (%) N=99	GC (%) N=99	GC (%) N=131
Sex	*P*=0.766		
Male	63 (63.64)	65 (65.66)	46 (35.11)
Female	36 (36.36)	34 (34.34)	85 (64.89)
Age (years)	*P*=0.657		
Mean ± SD	59.56±11.19	60.67±11.14	59.18±11.57
Median	60	62	59
Range	23–81	27–80	30–87

Abbreviations: CON, control; GC, gastric cancer; miRNA, microRNA; SD, standard deviation.

Table S3 Independent-samples *t*-test for the effect of pri-let-7a-1 rs10739971 polymorphism to its mature miRNA expression in serum level and in tissue level

Variables	GG			GA			AA		
	N	Mean ± SD	*P*-value	N	Mean ± SD	*P*-value	N	Mean ± SD	*P*-value
Serum expression									
Total									
CON	28	3.18±0.57	1 (Ref)	54	3.64±0.53	1 (Ref)	17	3.45±0.43	1 (Ref)
GC	31	3.33±0.64	0.325	54	3.39±0.50	0.881	14	3.27±0.53	0.303
Male									
CON	17	3.22±0.61	1 (Ref)	35	3.49±0.59	1 (Ref)	11	3.43±0.51	1 (Ref)
GC	19	3.32±0.64	0.636	37	3.45±0.53	0.757	9	3.28±0.59	0.535
Female									
CON	11	3.11±0.51	1 (Ref)	35	3.41±0.41	1 (Ref)	11	3.49±0.28	1 (Ref)
GC	12	3.35±0.66	0.336	37	3.28±0.44	0.368	9	3.25±0.49	0.35
Tissue expression in situ									
Total									
CON	33	5.48±0.51	1 (Ref)	40	5.38±0.82	1 (Ref)	16	5.20±0.72	1 (Ref)
GC	40	5.43±0.69	0.709	46	5.41±0.72	0.881	18	5.14±0.59	0.787
Male									
CON	21	5.55±0.44	1 (Ref)	28	5.46±0.77	1 (Ref)	11	5.24±0.84	1 (Ref)
GC	25	5.34±0.67	0.226	34	5.42±0.75	0.814	13	5.19±0.66	0.877
Female									
CON	12	5.36±0.63	1 (Ref)	12	5.19±0.92	1 (Ref)	5	5.12±0.42	1 (Ref)
GC	15	5.57±0.73	0.439	12	5.37±0.65	0.577	5	5.01±0.40	0.677

Abbreviations: CON, control; GC, gastric cancer; SD, standard deviation; Ref, reference.

Reference

1. Liu R, Zhang C, Hu Z, et al. A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis. *Eur J Cancer*. 2011;47:784–791.