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Original Article

Downregulation of microRNA-375, combined with upregulation of its target gene Janus kinase 2, predicts unfavorable prognosis in patients with gastric cancer

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Abstract: MicroRNA (miR)-375 has been reported to function as a tumor suppressor in gastric cancer via targeting Janus kinase 2 (JAK2). Until now, no clinical evidence of miR-375-JAK2 combination in gastric cancer has been documented. Here, quantitative real-time PCR was performed to detect the serum levels of miR-375 and JAK2 mRNA in 100 patients with gastric cancer and 50 healthy controls. Receiver operating characteristic (ROC) analysis was used to assess the predictive diagnostic value of serum miR-375 and/or JAK2 mRNA for gastric cancer. Associations of serum miR-375 and/or JAK2 mRNA levels with various clinicopathological features and prognosis in patients with gastric cancer were also analyzed. As a result, miR-375 expression was significantly downregulated, while JAK2 mRNA expression was dramatically upregulated, in patients' sera of gastric cancer (both $P<0.001$), compared to healthy controls. Notably, the areas under ROC curve for serum miR-375 and JAK2 discriminating gastric cancer patients from healthy controls were respectively 0.871 (sensitivity = 82.00%, specificity = 72.00%) and 0.864 (sensitivity = 55.00%, specificity = 100.00%). Moreover, miR-375 downregulation and/or JAK2 upregulation were all significantly associated with positive lymph node metastasis, high tumor-node-metastasis stage and the presence of H. pylori infection in patients with gastric cancer (all $P<0.05$). Furthermore, serum miR-375 and/or JAK2 were all independent prognostic factors for patients with gastric cancer. Importantly, the prognostic value of miR-375-JAK2 combination was more significant than considered alone. Collectively, serum miR-375 and JAK2 combination may be a potential diagnostic and prognostic predictor of gastric cancer. miR-375-JAK2 axis might be a promising candidate for a molecular targeted therapy of this malignancy.

Keywords: Gastric cancer, microRNA-375, Janus kinase 2, clinicopathological feature, prognosis

Introduction

Gastric cancer, as an aggressive and invasive malignancy, ranks the fifth most frequent cancer and the third leading cause of cancer-related deaths worldwide [1]. Due to advanced management and treatment of Helocobacter pylori, the incidence of gastric cancer has been decreasing in recent years [2]. In addition, surgical resection is an effective management for gastric cancer at the early stage. However, more than 80% of patients with gastric cancer may be diagnosed at an advanced stage because of atypical symptoms, and their outcome remains poor and unsatisfactory [3]. Thus, it is an urgent need to identify new and reliable biomarkers to diagnose the early disease and to predict prognosis for improving the management of gastric cancer.

MicroRNAs (miRNAs), a group of evolutionarily conserved, non-coding and small RNAs with 21~28 nucleotides in length, functionally regulate gene expression at a post-transcriptional level by binding to the 3' untranslated region (3' UTR) of target mRNAs [4]. miRNAs have been indicated to be crucial regulators in various physiological and pathological processes, including development, cell differentiation, proliferation, apoptosis, metabolism and signal transduction [5]. Especially, more than half of the human miRNAs have been demonstrated to be located in cancer-associated genomic regions [6]. Several miRNAs have observed to

Table 1. Clinicopathological characteristics of 100 patients with gastric cancer and 50 healthy controls

Features	Patients with gastric cancer	Healthy control
Age		
<60	45	30
≥60	55	20
Gender		
Male	68	35
Female	32	15
Tumor size (cm)		
<5	65	-
≥5	35	-
Depth of tumor		
T1~T2	30	-
T3~T4	70	-
Histological grade		
Well~moderate	32	-
Poor	68	-
Lymph node metastasis		
Absent	40	-
Present	60	-
TNM stage		
I~II	38	-
III~IV	62	-
H. pylori infection		
Negative	18	-
Positive	82	-

be dysregulated in multiple human cancers, and function as either tumor suppressors or oncogenes through regulating the corresponding target genes [7]. Moreover, miRNAs can be served as non-invasive cancer biomarkers due to their stable status in peripheral blood, serum, body fluid, and sputum. Accumulating studies have identified a number of miRNA biomarkers for improving cancer patients' diagnosis, prognosis and treatment [8].

miR-375, located in an intergenic region between the beta-A2 crystallin and undescribed coiled-coil domain-containing protein 108 genes in human chromosome 2q35 region, was originally identified as a pancreatic islet-specific miRNA from murine pancreatic β-cell line MIN6 [9]. miR-375 expression has been observed in brain, pituitary, breast, heart, lung, pancreatic islet, epididymis and skeletal muscle [10]. In recent years, the abnormal expression of miR-375 has also found in various human cancers compared to the corresponding normal counterparts, such as head and neck squamous cell carcinoma, esophageal cancer, laryngeal carcinoma, gastric cancer, pancreatic ductal adenocarcinoma and hepatocellular carcinoma [11]. To our interests, miR-375 was identified as the most downregulated miRNA in gastric cancer tissues based on miRNA microarray analysis and quantitative RT-PCR validation [12]; Zhang et al. [13] observed the highly frequent recurrence and poor survival in gastric cancer patients with high level of hsa-miR-375 and low level of hsa-miR-142-5p; miR-375 has been indicated to function as a tumor suppressor via regulating various target genes, including Janus kinase 2 (JAK2), which is a crucial component of JAK-STAT signaling pathway and plays a key role in cell proliferation, apoptosis, survival and angiogenesis [14]. Until now, no clinical evidence of miR-375-JAK2 combination in gastric cancer has been documented. To address this problem, quantitative real-time PCR was performed to detect the serum levels of miR-375 and JAK2 mRNA in 100 patients with gastric cancer and 50 healthy controls. Spearman correlation analysis was performed to evaluate the correlation between serum miR-375 and JAK2 mRNA. Receiver operating characteristic (ROC) analysis was used to assess the predictive diagnostic value of serum miR-375 and/or JAK2 mRNA for gastric cancer. Associations of serum miR-375 and/or JAK2 mRNA levels with various clinicopathological features and prognosis in patients with gastric cancer were also analyzed.

Patients and methods

Patients and ethics

In the current study, the serum cohort consisted of 100 patients with gastric cancer who underwent curative surgical resection with standard lymphadenectomy from January 2009 to December 2012 at Department of Intensive care unit of Huai'an First People's Hospital, and 50 healthy controls who received the body check from January 2012 to December 2012 at medical examination center. All patients with gastric cancer were evaluated according to the system for staging primary tumor/regional lymph nodes/distant metastasis (TNM) described in the AJCC Cancer Staging

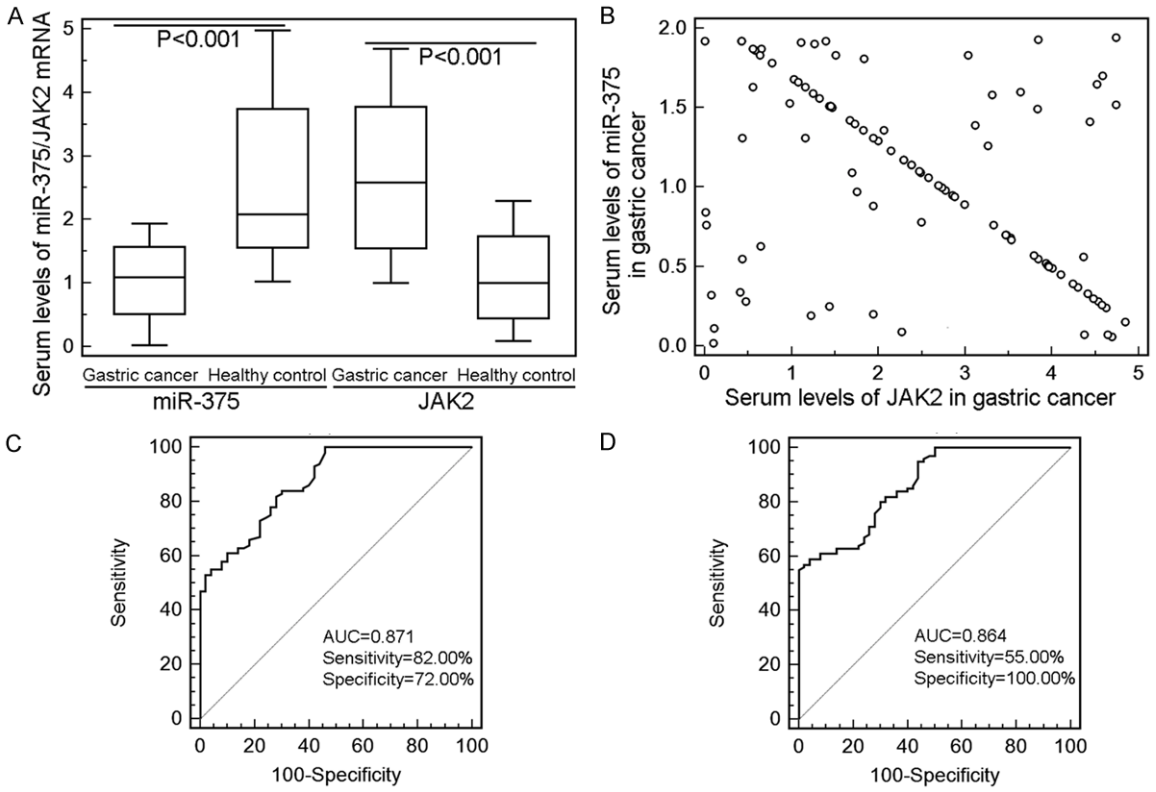

Figure 1. Diagnostic value of serum miR-375 and JAK2 mRNA for gastric cancer. A. Relative expression of miR-375 and JAK2 mRNA in serum samples collected from gastric cancer patients and healthy controls; B. Correlation between miR-375 and JAK2 mRNA expression levels in patients' sera of gastric cancer; C and D. ROC curves respectively for serum miR-375 and JAK2 discriminating gastric cancer patients from healthy controls.

Manual. None of them received neoadjuvant or adjuvant chemotherapy before the surgical resection. The clinicopathologic features of 100 gastric cancer patients were summarized in **Table 1**. Serum samples were collected from 100 patients with gastric cancer and 50 healthy controls, and then frozen at -80°C until use. Written informed consent was obtained from all patients and the study was approved by the Ethics Committee of Huai'an First People's Hospital, Nanjing Medical University, P. R. China.

All 100 patients with gastric cancer received follow up with the median duration of 36 months. Overall survival was evaluated and was defined as the duration from the initial diagnosis to the last follow-up or to mortality.

Quantitative real-time PCR assay

Total RNAs and miRNAs were extracted from serum samples respectively by Trizol reagent (Invitrogen, Carlsbad, CA, USA) and mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Serum levels of miR-375 and JAK2 mRNA in gastric cancer and healthy control groups were detected by quantitative real-time PCR assay according to the protocols of previous studies [15, 16]. U6 small nuclear RNA (U6) and GAPDH were used as the endogenous controls. The PCR primers used were as follows: miR-375 forward, 5'-CAG GGT CCG AGG TAT T-3' and reverse 5'-CTG CTT TGT TCG TTC G-3', U6 forward, 5'-CGC TTC GGC AGC ACA TAT AC-3' and reverse, 5'-CAG GGG CCA TGC TAA TCT T-3'; JAK2 forward 5'-TCT ATT TTA TTA TGG TTT CCC TTG-3' and reverse 5'-TTT TAC TTA TTT ACC TCA TTT CCC-3'; GAPDH forward 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' and reverse 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300HT real-time PCR system (Applied Biosystems, Foster City, CA, USA). Standard curves were generated, and the relative amount of miR-375

Table 2. Association of miR-375-low and/or JAK2-high with clinicopathological features of patients with gastric cancer

Features	No. of patients	miR-375-low	P	JAK2-high	P	miR-375-low/JAK2-high	P
Age							
<60	45	28 (62.22)	NS	25 (55.56)	NS	18 (40.00)	NS
≥60	55	33 (60.00)		31 (56.36)		22 (40.00)	
Gender							
Male	68	41 (60.29)	NS	38 (55.88)	NS	28 (41.18)	NS
Female	32	20 (62.50)		18 (56.25)		12 (37.50)	
Tumor size (cm)							
<5	65	40 (61.54)	NS	37 (56.92)	NS	25 (38.46)	NS
≥5	35	21 (60.00)		19 (54.29)		15 (42.86)	
Depth of tumor							
T1~T2	30	18 (60.00)	NS	16 (53.33)	NS	10 (33.33)	NS
T3~T4	70	43 (61.43)		40 (57.14)		30 (42.86)	
Histological grade							
Well~moderate	32	19 (59.38)	NS	16 (50.00)	NS	12 (37.50)	NS
Poor	68	42 (61.76)		40 (58.82)		28 (41.18)	
Lymph node metastasis							
Absent	40	19 (47.50)	0.002	16 (40.00)	0.002	5 (12.50)	<0.001
Present	60	42 (70.00)		40 (66.67)		35 (58.33)	
TNM stage							
I~II	38	16 (42.11)	0.001	13 (34.21)	0.001	4 (10.53)	<0.001
III~IV	62	45 (72.58)		43 (69.35)		36 (58.06)	
H. pylori infection							
Negative	18	6 (33.33)	0.01	5 (27.78)	0.01	4 (22.22)	0.03
Positive	82	55 (67.07)		51 (62.20)		36 (43.90)	

Note: 'NS' refers to the difference without statistical significance.

or JAK2 was normalized to the amount of U6 or GAPDH, respectively. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ method [17].

Statistical analysis

SPSS v 19.0 software (SPSS, Chicago, IL, USA) was used to perform all statistical analyses in the current study. Data were expressed as the mean ± SD of at least three independent experiments. Group differences between two groups were analyzed by Student's *t* test. The relationship between miR-375 and JAK2 mRNA expression was evaluated by Spearman's correlation. The area under the receiver operating characteristic (ROC) curve was used to assess the predictive diagnostic value of serum miR-375 and JAK2 levels for gastric cancer. Associations of serum miR-375 and/or JAK2 levels with various clinicopathological characteristics were analyzed using χ^2 test. Associations of serum miR-375 and/or JAK2 levels with overall survival were analyzed by Kaplan-Meier method with the log-rank test. Using the Cox proportional hazards model, multivariate analysis was only performed on the significance of variables in univariate analysis. *P* values <0.05 were considered to be statistically significant.

Results

Diagnostic value of serum miR-375 and JAK2 mRNA for gastric cancer

Compared to healthy controls, miR-375 expression was significantly downregulated (gastric cancer vs. Normal: 1.06 ± 0.59 vs. 2.55 ± 1.19, P<0.001, **Figure 1A**), while JAK2 mRNA expression was dramatically upregulated (gastric cancer vs. Normal: 2.69 ± 1.22 vs. 1.08 ± 0.66, P<0.001, **Figure 1A**), in patients' sera of gastric cancer. In addition, we found that there existed

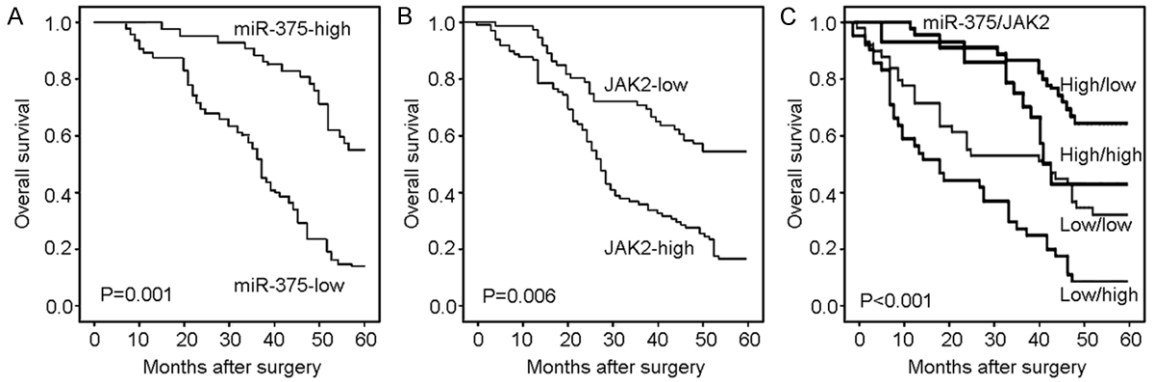

Figure 2. Kaplan-Meier survival analysis with Log-Rank test for the overall survival of 100 patients with gastric cancer. A. The overall survival of patients with miR-375-low and miR-375-high. B. The overall survival of patients with JAK2-low and JAK2-high. C. The overall survival of patients with miR-375/JAK2 combinations.

a converse relationship between miR-375 and JAK2 mRNA expression levels [Spearman's coefficient of rank correlation (rho) = -0.316, P = 0.002, **Figure 1B**]. Notably, the areas under ROC curve (AUC) for serum miR-375 and JAK2 discriminating gastric cancer patients from healthy controls were respectively 0.871 (cutoff = 1.66, P = 0.0001, sensitivity = 82.00%, specificity = 72.00%, **Figure 1C**) and 0.864 (cutoff = 2.29, P = 0.0001, sensitivity = 55.00%, specificity = 100.00%, **Figure 1D**).

MiR-375 downregulation and/or JAK2 upregulation associate with aggressive tumor progression of gastric cancer

To assess the associations of serum miR-375 and/or JAK2 mRNA levels with various clinicopathological features, all 100 patients with gastric cancer were divided into miR-375-low (n = 61), miR-375-high (n = 39), JAK2-low (n = 44) and JAK2-high (n = 56) groups using the cutoff values of ROC analysis mentioned above [miR-375 (1.66) and JAK2 (2.29)]. Of 100 patients with gastric cancer, 21 (21.00%), 40 (40.00%), 23 (23.00%), and 16 (16.00%) respectively belonged to miR-375-low/JAK2-low, miR-375-low/JAK2-high, miR-375-high/JAK2-low, miR-375-high/JAK2-high groups.

As shown in **Table 2**, miR-375-low and/or JAK2-high were all significantly associated with positive lymph node metastasis, high TNM stage and the presence of H. pylori infection in patients with gastric cancer (all P<0.05). More interestingly, the associations of combined miR-375-low/JAK2-high expression with positive lymph node metastasis and high TNM stage were both more significant than those of miR-375-low or JAK2-high alone (**Table 2**).

MiR-375 downregulation and/or JAK2 upregulation predict poor prognosis in patients with gastric cancer

Next, we investigated the associations of serum miR-375 or JAK2 with overall survival of patients with gastric cancer using Kaplan-Meier analysis. As shown in **Figure 2A**, **2B**, the patients with miR-375-low and JAK2-high expression in sera more often had short overall survival than those with miR-375-high and JAK2-low expression (all P<0.05). Then, we also evaluated the prognostic value of the miR-375 and JAK2 combination in patients with gastric cancer. The combination groups were: miR-375-low/JAK2-low, miR-375-low/JAK2-high, miR-375-high/JAK2-low, miR-375-high/JAK2-high. Kaplan-Meier analysis showed statistically differential survival patterns among these four groups (**Figure 2C**, P<0.001). Among them, the miR-375-low/JAK2-high patients had the poorest prognosis, while the miR-375-high/JAK2-low patients had the most favorable prognosis.

Moreover, the univariate COX regression analysis confirmed that poor histological grade, positive lymph node metastasis, high TNM stage, serum miR-375-low, serum JAK2-high, and serum miR-375-low/JAK2-high combination were significantly associated with overall survival (all P<0.05, **Table 3**). Further multivariate COX regression analysis identified lymph node metas-

Table 3. Univariate and multivariate analyses on the prognostic values of various clinicopathological parameters of patients with gastric cancer

Clinicopathological parameters	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Age				
<60 vs. ≥60	0.682 (0.389-1.211)	NS	-	-
Gender				
Male vs. Female	0.973 (0.568-1.376)	NS	-	-
Tumor size (cm)				
<5 vs. ≥5	0.627 (0.419-1.185)	NS	-	-
Depth of tumor				
T1~T2 vs. T3~T4	0.735 (0.428-1.162)	NS	-	-
Histological grade				
Well~moderate vs. Poor	1.698 (1.029-3.396)	0.02	1.142 (0.982-2.063)	NS
Lymph node metastasis				
Absent vs. Present	2.981 (1.299-5.671)	0.006	2.316 (1.187-4.785)	0.01
TNM stage				
I~II vs. III~IV	3.783 (1.395-7.659)	<0.001	3.203 (1.286-6.698)	0.001
H. pylori infection				
Negative vs. Positive	1.166 (0.858-2.309)	NS	-	-
Serum miR-375				
High vs. Low	3.371 (1.251-6.658)	0.001	2.923 (1.028-6.098)	0.006
Serum JAK2				
Low vs. High	3.062 (1.059-6.297)	0.006	2.631 (0.942-5.632)	0.01
miR-375/JAK2				
Other groups vs. Low/high group	3.626 (1.399-7.663)	<0.001	3.353 (1.221-7.072)	<0.001

Note: 'CI': confidence interval. 'HR': Hazard ratio. 'NS' refers to the difference without statistical significance.

tasis, TNM stage, serum miR-375, serum JAK2, and serum miR-375/JAK2 combination as independent prognostic factors for patients with gastric cancer (all P<0.05, **Table 3**).

Discussion

Owing to the lack of early diagnostic biomarkers, the incidence and mortality rate of gastric cancer are still high. miRNAs contribute to gastric carcinogenesis by altering the expression of oncogenes and tumor suppressors [18]. miRNAs also have been indicated to be served as useful biomarkers for early screening or detection of human cancers due to their stability in tissues, serum/plasma and other body fluids [19, 20]. In the present study, we confirmed the abnormal expression of miR-375 and its target gene JAK2 in patients' sera of gastric cancer compared to the healthy controls. We also evaluated the effects of serum miR-375 and/or JAK2 levels on tumor progression and patients' prognosis, and the results indicated that combined expression of serum miR-375 and JAK2 may be a more reliable predictor of gastric cancer progression and prognosis, than miR-375 expression or JAK2 expression alone.

Accumulating evidence has showed that miR-375 may be widely expressed in various tissues and organs, and is dramatically reduced in malignant cells [21]. Functionally, miR-375 may play a tumor suppressor in various human cancers. For example, enforced expression of miR-375 could decrease liver cancer cell growth, invasion and induces G1 arrest and apoptosis [22]; miR-375, as one of the most frequently downregulated miRNAs in gastric cancer tissues, was able to distinctly suppress gastric cancer cell proliferation *in vitro* and *in vivo* [23]; Kong et al. [24] elucidated a tumor suppressive role of miR-375 through inhibiting cell proliferation of esophageal squamous cell carcinoma, colony formation ability and metastasis *in vitro* and *in vivo*. miRNAs exert their functions via regulating the expression of their target genes.

JAK2 has been identified as one of target genes of miR-375 [12, 25, 26]. JAK2, together with JAK1, JAK3 and TYK2, belongs to a family of intracellular, non-receptor protein tyrosine kinases [27, 28]. It functions as a component of the JAK-signal transducers and activators of transcription (STAT) signaling pathway, which plays a crucial role in numerous aspects of cell development, proliferation, apoptosis, survival, and immune system regulation [29]. During the signal transduction of this pathway, JAK2 is activated via phosphorylation at two adjacent tyrosine residues in response to various hormones or cytokines, and results in the phosphorylation of STAT proteins, which dimerize and translocate to the nucleus to regulate transcription of various target genes, including MYC, BCL2 and cyclin D1 [30]. In gastric cancer, either inhibition of JAK2 activity by a small chemical inhibitor or downregulation of JAK2 by RNAi dramatically inhibited the proliferation of malignant cells [12]; Ectopic expression of JAK2 partially reversed the inhibition of cell proliferation, invasion and migration caused by miR-375 [12, 25]; Miao et al. [26] found that the JAK2-STAT3 pathway regulated by miR-375 might be involved in H. pylori-induced inflammation. These previous data suggest that the tumor suppressive role of miR-375 in malignant phenotypes of gastric cancer cells via targeting the JAK2 oncogene. In the current study, our results revealed the negative correlation between miR-375 and JAK2 expression in patients' sera of gastric cancer. ROC analysis confirmed the efficient diagnostic values of serum miR-375 and JAK2 levels in patients with gastric cancer. Moreover, miR-375 downregulation and/or JAK2 upregulation were all demonstrated to be independent prognostic factors of this cancer.

In conclusion, our data suggest that serum miR-375 and JAK2 combination may be a potential diagnostic and prognostic predictor of gastric cancer. Therefore, miR-375-JAK2 axis might be a promising candidate for a molecular targeted therapy of this malignancy.

Disclosure of conflict of interest

None.

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