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Serum miR-518e-5p is a potential biomarker for secondary imatinib-resistant gastrointestinal stromal tumor

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Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumor of the intestinal tract. Imatinib is used as first-line therapy for GIST patients; however, secondary imatinib resistance poses a significant clinical challenge. Here, we analyzed serum miRNA expression profiles to identify specific serum miRNAs that could be used as early diagnostic markers. Candidate miRNAs were validated using Taqman quantitative PCR with serum samples from secondary imatinib-resistant GIST patients (n = 39), imatinib-sensitive GIST patients (n = 37), and healthy controls (n = 28). Serum miR-518e-5p and miR-548e levels were higher in secondary imatinib-resistant GIST than imatinib-sensitive GIST patients or healthy controls ($P < 0.0001$). However, ROC analysis indicated that only miR-518e-5p could distinguish imatinib-resistant GIST. To discriminate imatinib-resistant from imatinib-sensitive GIST patients, the AUC for serum miR-518e-5p was 0.9938, with 99.8% sensitivity and 82.1% specificity. Serum miR-518e-5p could also discriminate imatinib-resistant GIST patients from healthy controls with 99.9% sensitivity and 97.4% specificity. These data indicate that serum miR-518e-5p is a potentially promising non-invasive biomarker for early detection and diagnosis of secondary imatinib-resistant GIST.

Keywords. Biomarker; GIST; miR-518e-5p; secondary imatinib resistance; serum

1. Introduction

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors with an annual worldwide incidence of 10–20 cases per million people (Saleem *et al*. 2009). GISTs are frequently characterized by expression of the receptor tyrosine kinase KIT (Homick *et al*. 2007; Antonescu 2011). About 85% of advanced GISTs have activating mutations in *c-kit*, and another 5% harbor mutations in the platelet-derived growth factor receptor alpha (PDGFRA) gene (Hirota *et al.* 1998). Imatinib is a small-molecule tyrosine kinase inhibitor initially developed for the treatment of chronic myelogenous leukemia. It was later discovered that imatinib also inhibited oncogenic KIT signaling, leading to its clinical use in the treatment of GIST (Heinrich *et al*. 2000; Tuveson *et al*. 2001; Mol *et al.* 2004). Before the use of imatinib, less than 5% of GIST cases responded to conventional chemotherapy, and patients with advanced disease had a median survival of 18 months. Imatinib treatment of GIST patients increased the median survival to 5 years (Dematteo *et al*. 2002), and GISTs became one of the earliest examples of solid tumors responding to targeted therapy.

Unfortunately, most GIST patients eventually develop secondary resistance to imatinib, owing to certain mechanisms that cause GIST cells to acquire resistance to imatinib and allow KIT signaling to remain constitutively active (Corless *et al*. 2011). This imatinib secondary resistance poses a significant challenge for the clinical management of GIST. Thus, novel and specific biomarkers for early diagnosis of secondary imatinib-resistant GIST are urgently needed.

MicroRNAs (miRNAs) are a class of small RNAs that control gene expression by targeting messenger RNAs and triggering either post-translational repression or RNA degradation (Lee *et al.* 1993; Jackson *et al*. 2007). Aberrant miRNA expression underpins a variety of pathological processes, including carcinogenesis (Calin *et al.* 2002; Michael *et al.* 2003). The potential of miRNAs as novel tumor biomarkers has recently been of great interest, because of their tissue specificity and unique ability to predict tumor behavior with accuracy superior to that of mRNA expression profiling (Lu *et al.* 2005). These characteristics led to investigations of miRNAs as potential non-invasive serum biomarkers that could be used in cancer diagnosis.

In this study, we aimed to identify serum miRNAs specific for imatinib-resistant GIST. Our results show that serum miR-518e-5p is significantly increased in secondary

imatinib-resistant GIST patients compared to imatinib-sensitive patients or healthy controls, indicating that it might serve as a non-invasive biomarker for early detection of secondary imatinib-resistant GIST.

2. Materials and methods

2.1 *Ethics statement*

The Shengjing Hospital Research Ethics Board approved this study, and all study participants signed a consent form prior to their surgical procedure.

2.2 *Patient selection*

A total of 76 GIST patients from the Gastrointestinal Surgery Department of Shengjing Hospital were enrolled since 2015. All patients underwent surgery before treatment with imatinib; tumors were diagnosed by histopathology.

2.3 *Pathology, immunohistochemistry (IHC), and DNA sequencing*

Slides of formalin-fixed paraffin-embedded GISTs collected in our study were analyzed. Nuclear division count was evaluated under 50 high power fields. IHC was performed using anti-CD117 and anti-DOG1 antibodies (Abcam, ab32363 for anti-CD117 antibody and ab53212 for anti-DOG1 antibody). DNA was extracted from tumor areas containing at least 80% of tumor cells after histologic review. Mutations of KIT exons 9, 11, 13 and 17 and PDGFRA exons 12 and 18 were analyzed by sequencing polymerase chain reaction (PCR) products.

2.4 *Collection of serum samples*

All patients were treated with imatinib. Peripheral blood specimens were collected from all patients before starting chemotherapy with imatinib. Blood samples from age-/gender-matched healthy individuals (n = 28) were obtained from the Medical Examination Centre of Shengjing Hospital. Healthy individuals had no personal or family history of cancer and had not been hospitalized in the 6 months prior to collection. Whole blood was collected in standard 10-ml lavender-top glass vacutainer tubes containing EDTA as anticoagulant. Samples containing 600 µl of serum in 1000 µl of Trizol reagent (Thermo Fisher Scientific Inc.; Waltham, MA USA) were stored at −80°C until use.

2.5 *Total RNA extraction*

Total RNA was extracted from 600 µl of serum using mirVana PARIS Kit (Thermo Fisher Scientific Inc.; Waltham, MA USA) after addition of 25 fmol of synthetic miRNA cel-miR-39 (Thermo Fisher Scientific Inc.; Waltham, MA USA), according to the manufacture's protocol. Because humans do not possess the cel-miR-39 sequence, it can be used to normalize RNA isolation and reverse transcription efficiency. Total RNA was eluted in RNase-free H_2O. The quantity and quality of total RNA was analyzed using the NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.; Waltham, MA USA), according to the manufacturer's instructions. Each 600 µL of serum sample yielded 50 µL of total RNA, with yields in the range of 100–300 ng/µl of small RNAs. The extracted RNA samples were transferred to tubes and stored at −80°C until further analysis.

2.6 *Serum miRNA expression profiling*

The analysis of miRNA expression profiles was performed for 3 secondary imatinib-resistant GIST cases and 3 imatinib-sensitive GIST cases. Samples were analyzed using the Human miRNA OneArray Microarray Profiling Service (Agilent Technologies; California, USA), containing 6,153 miRNAs and 144 control probes. The samples were then hybridized following the manufacturer's protocol. Arrays were scanned using an Agilent microarray scanner and images were quantified using Agilent Feature Extraction Software, version 10.5.1.1.

Complete miRNA microarray data were initially normalized to the 75th percentile and then averaged for each group of samples using GeneSpring GX (Agilent Technologies; California, USA) software. The Kruskall-Wallis test was used for comparisons between groups and the Benjamini-Hochberg False Discovery Rate correction was applied to adjust for multiple comparisons. Unsupervised hierarchical clustering was performed using Genespring GX software.

2.7 *Taqman PCR of candidate reference genes and biomarker miRNAs*

Expression of candidate reference genes (RNU1-4, RNU6-2, SNORD43, SNORD44 and SNORD48) and biomarker miRNAs (miR-518e-5p, miR-548e, miR-615-3p, miR-3136, miR-4695, and miR-4777-3p) was measured using TaqMan miRNA assays (Thermo Fisher Scientific Inc.; Waltham, MA USA) according to the manufacturer's protocol. The level of cel-miR-39 in each sample was measured to evaluate the efficiency of RNA extraction and reverse transcription.

Total RNA was reverse transcribed using the MultiScribe-based High-Capacity cDNA Archive kit (Thermo Fisher

Scientific Inc.; Waltham, MA USA). Reverse transcriptase-negative controls were included in each batch of reactions. PCR reactions were carried out in final volumes of 10 µl using an ABI 7300 HT Fast Real-Time PCR System (Thermo Fisher Scientific Inc.; Waltham, MA USA). Briefly, reactions consisted of 0.7 µl of cDNA, 5 µl of TaqMan Universal PCR Fast Master Mix, and 0.2 µM TaqMan primer-probe mix (Thermo Fisher Scientific Inc.; Waltham, MA USA). Reactions were initiated with a 10-min incubation at 95°C followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Melting curve analysis was used to confirm specificity of the PCR products. Fold differences in miRNA expression were calculated using $2^{-\Delta Ct}$ values. Three replicate samples of each specimen were measured, and the results were calculated as means ±S.D.

2.8 *Statistical analysis*

The results are presented as the mean ± S.D. GraphPad Prism, version 6.0 and SPSS software were used for analysis. The Fisher's exact test and Student's *t*-test were used to determine statistical significance, using a cut-off value of $P \leq 0.05$. Receiver-operating characteristic analysis was performed to determine the $-\Delta Ct$ value that could efficiently discriminate secondary imatinib-resistant GIST patients from imatinib-sensitive GIST patients and healthy individuals. AUC curve was used as a measurement.

3. Results

3.1 *Characteristics of GIST patients*

In total, 76 GIST patients ranging in age from 27 to 83 years were followed up for 108 months, or until the time of death, after starting administration of imatinib. Clinical imatinib response was evaluated by flurodeoxyglucose positron emission tomography and/or contrast-enhanced computed tomography. The progression of GIST was documented by 'choi' standard (Choi 2008). 39 GIST patients who received imatinib and developed GIST progression after an initial response or stable disease were defined as secondary imatinib-resistant GIST patients. The other 37 GIST patients whose tumors shrunk or showed significant cystic changes or necrosis were defined as imatinib-sensitive GIST patients.

The clinical, histopathological, and follow-up details of the patients are listed in table 1. GIST characteristics were compared between secondary imatinib-resistant patients and imatinib-sensitive patients, including nuclear division level, and expression of CCD17 and DOG1. Exons in KIT and PDGFRA transcripts were sequenced and analyzed. No significant differences in the above markers were found between secondary imatinib-resistant patients and imatinib-sensitive patients.

Table 1. Clinical and demographic characteristics of the GIST patients

Characteristics	Number of patients N (%)	
	Imatinib-sensitive GIST (N = 37)	Imatinib-resistant GIST (N = 39)
Age, median (range)	66.5 (27–78)	61.2 (45–83)
Gender, N (%)		
Male	22 (59.5)	27 (69.2)
Female	15 (40.5)	12 (30.8)
WHO performance status at baseline, N (%)		
0	13 (35.1)	15 (38.5)
1	21 (56.8)	20 (51.3)
2	3 (8.1)	4 (10.2)
Primary site of lesion, N (%)		
Small intestine	15 (40.1)	19 (48.7)
Stomach	15 (40.1)	17 (43.6)
Rectum	4 (10.8)	2 (5.1)
Other	3 (9)	1 (2.6)
Metastatic sites, N (%)		
Liver	22 (59.5)	25 (64.1)
Peritoneum/omentum	4 (10.8)	4 (10.3)
Intra-abdominal	6 (16.2)	7 (17.9)
Other/unknown	5 (13.5)	3 (7.7)
Nuclear division (/50 hpf), N (%)		
≥ 5	28 (75.7)	32 (82.1)
< 5	9 (24.3)	7 (17.9)
CCD17, N (%)		
Positive	36 (97.3)	37 (94.9)
Negative	1 (2.7)	2 (5.1)
DOG1, N (%)		
Positive	32 (86.5)	34 (87.2)
Negative	5 (13.5)	5 (12.8)
Mutation status, N (%)		
KIT exon9	5 (13.5)	4 (12.8)
KIT exon11	28 (75.7)	30 (74.3)
KIT exon13	0 (0)	1 (2.6)
KIT exon17	0 (0)	0 (0)
PDGFRA exon12	0 (0)	0 (0)
PDGFRA exon18	1 (2.7)	0 (0)
Wild type	3 (8.1)	4 (10.3)
Highest prior imatinib dose, mg per day		
400	33 (89.2)	12 (30.8)
600	4 (10.8)	27 (69.2)

Evaluated parameters did not differ between groups ($P > 0.05$ by Fisher's exact test and *t*-test)

3.2 *Comparison of serum miRNA profiles between imatinib-sensitive and secondary imatinib-resistant GIST patients*

Analysis of cel-miR-39 allowed the efficiency estimation of RNA isolation and cDNA synthesis. The recovery of cel-miR-39 in all samples was higher than 2% (figure 1),

indicating the success of RNA isolation and cDNA synthesis in all serum samples. The mean recovery of cel-miR-39 was 15.8% (range 2.3–34.3%) in secondary imatinib-resistant GIST patients, 12.4% (range 1.9–30.7%) in imatinib-sensitive GIST patients, and 16.7% (range 4.6–35.8%) in control group. The recovery of cel-miR-39 was not statistically different between the groups.

Serum samples from 3 secondary imatinib-resistant GIST patients and 3 imatinib-sensitive GIST patients were selected randomly and their miRNA expression profiles were analyzed using microarrays. Serum miRNA levels from secondary imatinib-resistant GIST patients and imatinib-sensitive GIST patients were compared using the $\log_2$-fold change ≥ 0.585 and a non-stringent cutoff $P < 0.05$ (figure 2A). Six serum miRNAs (miR-518e-5p, miR-548e, miR-615-3p, miR-3136, miR-4695, and miR-4777-3p) were significantly increased in secondary imatinib-resistant GIST patients, while no serum miRNA was decreased (figure 2B). Therefore, the 6 dysregulated serum miRNAs were further evaluated as candidate biomarkers.

3.3 *Selection of suitable serum reference gene*

Small nuclear RNAs, such as RNU1-4, RNU6-2, SNORD43, SNORD44, and SNORD48, have been widely used as reference genes for circulating miRNA studies in tumors; however, no report was found for serum miRNAs in GISTs.

To select a suitable reference gene for serum miRNAs in our study, we used Taqman quantitative polymerase chain reaction (qPCR) to analyze the gene expression of RNU1-4, RNU6-2, SNORD43, SNORD44, and SNORD48 in all samples. Because SNORD44 was present at very low levels or undetectable in most samples (92 out of 104, 88.46%), it was excluded from further analyses. For the other 4 genes, we compared their ΔCt values in each sample, and analyzed their stability according to repeatability of gene expression difference. As shown in Table 2, the comparative ΔCt results showed that RNU6-2 was the most stable reference gene in all samples and showed no significant differences between the groups. Thus, RNU6-2 was selected as a serum reference gene to normalize the miRNAs levels in the samples.

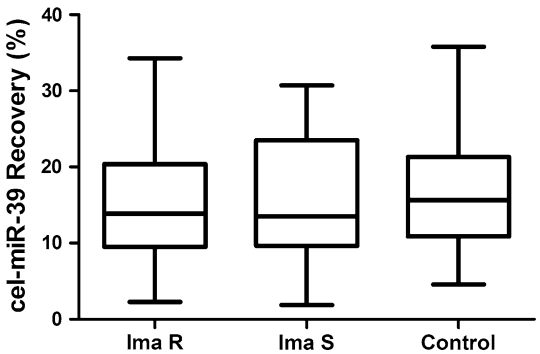

Figure 1. Cel-miR-39 recovery results. Non-human miRNA cel-miR-39 was added to all human serum samples before RNA extraction, to standardize miRNA recovery. 'Ima R' indicates secondary imatinib-resistant GIST patients; 'Ima S' indicates imatinib-sensitive GIST patients; 'Control' indicates healthy individuals.

3.4 *Validation of serum miRNAs specific for secondary imatinib-resistant GISTs*

To validate the expression of serum miRNAs in secondary imatinib-resistant GIST patients, we analyzed serum levels of the six identified miRNAs using Taqman qPCR in peripheral blood samples of 39 secondary imatinib-resistant GIST patients and 37 imatinib-sensitive GIST patients.

As shown in figure 3A, although the relative levels for serum miR-615-3p, miR-3136, miR-4695, and miR-4777-3p differed between imatinib-sensitive and secondary imatinib-resistant GIST patients, the differences were not statistically significant ($P = 0.0730$, 0.5523, 0.0969 and 0.2673, respectively).

Importantly, the serum levels of miR-518e-5p and miR-548e were significantly increased in secondary imatinib-resistant GIST patients ($P < 0.0001$), and the $2^{-\Delta Ct}$ values differed between secondary imatinib-resistant GIST patients and imatinib-sensitive GIST patients. The mean serum levels of miR-518e-5p ($2^{-\Delta Ct}$ value of 6.19×10^{-5} compared to 0.0970×10^{-5}) and miR-548e ($2^{-\Delta Ct}$ value of 0.0063 compared to 0.0019) in secondary imatinib-resistant GIST patients were significantly increased compared to imatinib-sensitive GIST patients.

To investigate the potential of miR-518e-5p and miR-548e as non-invasive biomarkers, their serum levels were measured in 28 control subjects. As shown in figure 3B, both miR-518e-5p and miR-548e could be detected in control individuals; however, their serum levels in control subjects were significantly lower than in secondary imatinib-resistant GIST patients ($P < 0.0001$). The high serum level of miR-518e-5p was more specific for secondary imatinib-resistant GIST than miR-548e. There was no significant difference in serum miR-518e-5p levels between imatinib-sensitive GIST patients and control subjects ($P = 0.7001$).

3.5 *Evaluation of serum miR-518e-5p and miR-548e as potential biomarkers of secondary imatinib-resistant GIST*

Receiver operating characteristic (ROC) curves were plotted to evaluate the diagnostic accuracy of serum miR-518e-5p and miR-548e. As shown in figure 4A, serum miR-518e-5p

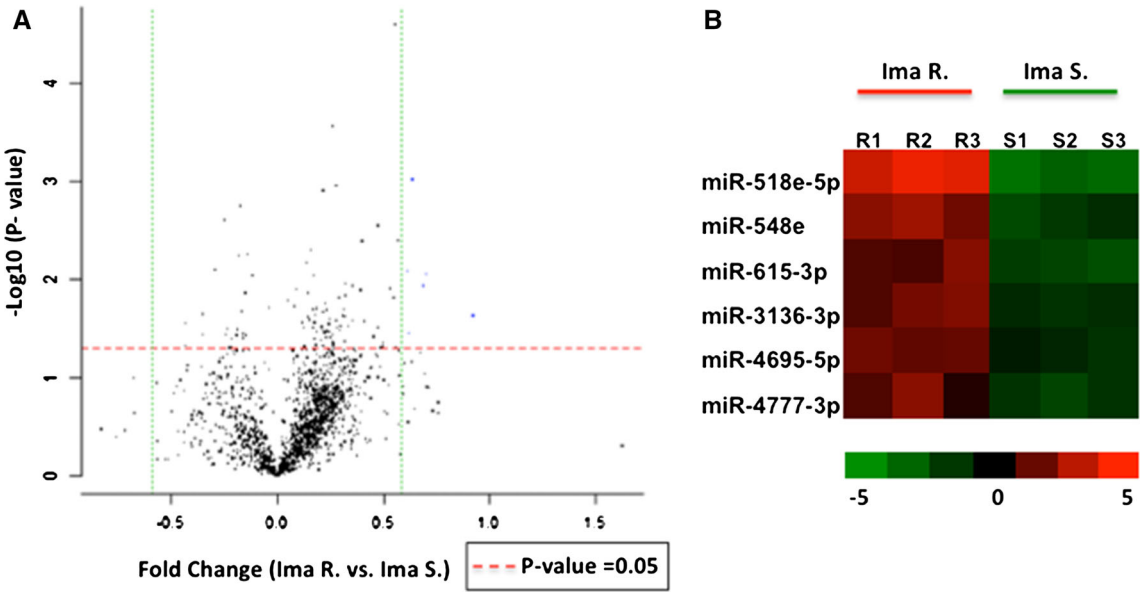

Figure 2. MiRNA microarray results. (**A**) The volcano plot of miRNA profiles in serum samples from GIST patients. Standard selection criteria to identify differentially expressed miRNAs are established as |fold change|≥ 0.585 and $P < 0.05$. (**B**) Hot map of 6 dysregulated miRNAs based on clustering of miRNA expression profiles by microarray. 'Ima R' indicates secondary imatinib-resistant GIST patients; 'Ima S' indicates imatinib-sensitive GIST patients; 'Control' indicates healthy individuals.

Table 2. Comparison of delta-Ct values between candidate reference genes

	Mean delta-Ct	SD	Mean SD
RNU1-4 versus RNU1-4	–/–	–/–	1.34
RNU1-4 versus RNU6-2	− 6.34	0.82	
RNU1-4 versus SNORD43	− 7.68	1.12	
RNU1-4 versus SNORD48	− 10.20	2.07	
RNU6-2 versus RNU1-4	6.34	0.82	0.99
RNU6-2 versus RNU6-2	–/–	–/–	
RNU6-2 versus SNORD43	− 2.75	0.94	
RNU6-2 versus SNORD48	− 4.31	1.21	
SNORD43 versus RNU1-4	7.68	1.12	1.27
SNORD43 versus RNU6-2	2.75	0.94	
SNORD43 versus SNORD43	–/–	–/–	
SNORD43 versus SNORD48	− 3.59	1.76	
SNORD48 versus RNU1-4	10.20	2.07	1.68
SNORD48 versus RNU6-2	4.31	1.21	
SNORD48 versus SNORD43	3.59	1.76	
SNORD48 versus SNORD48	–/–	–/–	

could well discriminate secondary imatinib-resistant GIST patients from imatinib-sensitive GIST patients; the area under the curve (AUC) value was 0.9938 (95% CI = 0.9825–1.005, $P < 0.0001$). The sensitivity and specificity at cutoff value of 16.2096 were 99.8% and 82.1%, respectively. Serum miR-518e-5p could also discriminate secondary imatinib-resistant GIST patients from healthy controls with AUC value of 0.9982 (95% CI = 0.9932–1.003, $P < 0.0001$). The sensitivity and specificity at cutoff value of 17.4613 were 99.9% and 97.4%, respectively. These data suggest that serum miR-518e-5p has a high discriminating ability to distinguish secondary imatinib-resistant GIST patients from both imatinib-sensitive GIST patients and healthy controls.

As shown in Figure 4B, the AUC value of serum miR-518e-5p to discriminate secondary imatinib-resistant GIST patients from healthy controls was 0.9753 (95% CI = 0.9420–1.009, $P < 0.0001$). To discriminate secondary imatinib-resistant GIST patients from imatinib-sensitive GIST patients, the AUC value of miR-548e was 0.8870 (95% CI = 0.8079–0.9662, $P < 0.0001$). However, the sensitivity and specificity at cutoff value of 8.6672 dropped to 83.8% and 92.3%, respectively (figure 4B). These data indicate that serum miR-548e is not as accurate as serum miR-518e-5p for imatinib-resistant GIST diagnosis.

4. Discussion

Currently, imatinib is used as the standard first-line therapy for GIST patients. About 20% of GIST patients do not primarily respond to this treatment, partly due to the c-KIT/PDGFRA mutations (Heinrich *et al*. 2003; Debiec-Rychter *et al*. 2004). However, most imatinib sensitive GIST patients will develop a secondary resistance to imatinib after initially achieving a clinical response (Erinn *et al*. 2011). The mechanisms of secondary imatinib resistance still remain unclear. Delay of the diagnosis of secondary imatinib resistance will seriously affect the clinical treatment result.

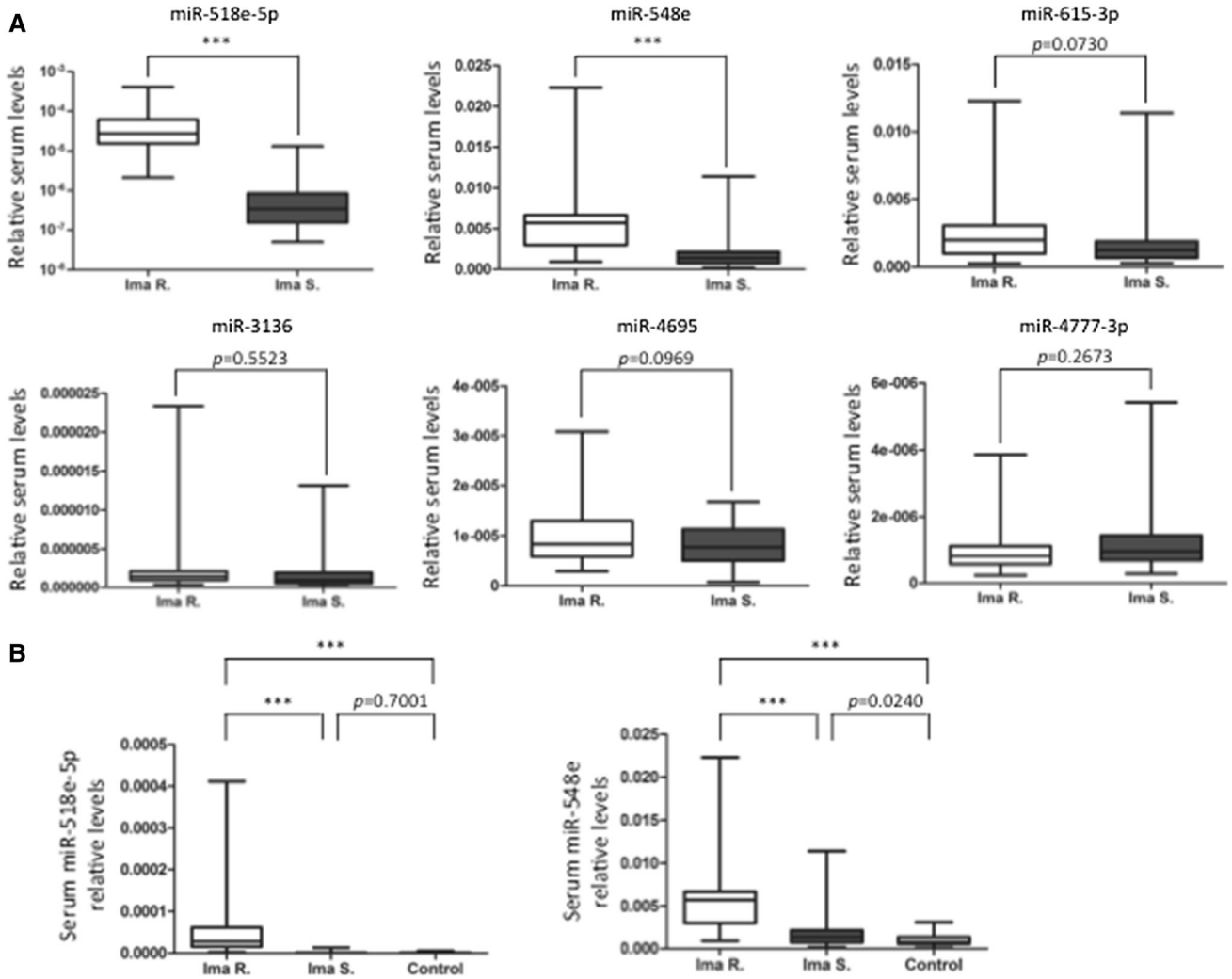

Figure 3. Expression of miR-518e-5p and miR-548e in serum samples. (**A**) Box plots illustrate the relative levels of 6 candidate miRNAs in serum samples. (**B**) Box plots illustrate the relative levels of miR-518e-5p and miR-548e in 39 imatinib-resistant GIST patients, 37 imatinib-sensitive GIST patients, and 28 healthy control individuals. 'Ima R' indicates imatinib-resistant GIST patients; 'Ima S' indicates imatinib-sensitive GIST patients; ***$P < 0.0001$.

Thus, it is urgent to distinguish secondary imatinib-resistant GIST patients from imatinib-sensitive GIST patients to help clinicians make treatment decisions.

MiRNAs have been investigated as novel biomarkers and therapeutic targets for various diseases. Some miRNAs have been associated with GISTs, and GIST location, mutation status, tumor risk, and chromosomal changes (Kang *et al.* 2005; Yao *et al.* 2009; Chun-Zhi *et al.* 2010; Haller *et al.* 2010; Xie *et al.* 2010; Kim *et al.* 2011; Panarelli *et al.* 2011; Valladares-Ayerbes *et al.* 2011; Song *et al.* 2011). Recent studies have indicated that miRNAs are associated with imatinib resistance in GISTs (Akcakava *et al.* 2014; Gao *et al.* 2014; Shi *et al.* 2016); however, since the miRNAs were identified from tissue specimens of GIST patients, they were not suitable as prognostic markers of imatinib resistance.

In our study, we aimed to identify serum miRNAs specific for imatinib-resistant GIST, to monitor the occurrence of secondary imatinib resistance. To exclude the disturbance by imatinib treatment, serum samples were collected before imatinib treatment. All GIST patients involved in our study were sensitive to imatinib at the primary time. During the follow up period, about 50% of GIST patients exhibited secondary imatinib resistance and were categorized into the secondary imatinib-resistant group. Serum miRNA expression profile analysis identified six miRNAs (miR-518e-5p, miR-548e, miR-615-3p, miR-3136, miR-4695, and miR-4777-3p) that were upregulated in secondary imatinib-resistant GIST patients compared with imatinib-sensitive GIST patients.

Some miRNAs and small nucleolar RNAs (snoRNAs) have been used as serum reference genes to measure target

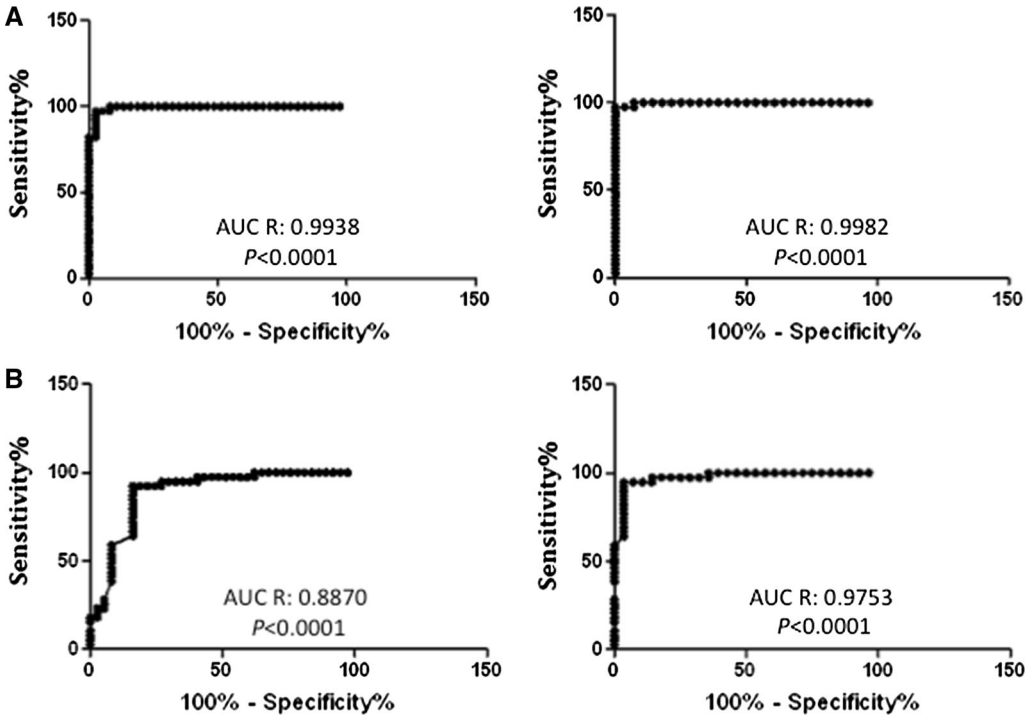

Figure 4. Diagnostic performance of serum miR-518e-5p and miR-548e for imatinib-resistant GIST. (**A**) ROC curves were plotted for serum miR-518e-5p to discriminate imatinib-resistant GIST from imatinib-sensitive GIST and healthy controls, respectively. (**B**) ROC curves were plotted for serum miR-548e to discriminate imatinib-resistant GIST from imatinib-sensitive GIST and healthy controls, respectively.

serum miRNA levels. However, no reference gene was reported for serum samples of GIST patients. To validate the serum levels of the candidate miRNAs, we first needed to identify a suitable reference gene present in GIST patients' serum samples. Five candidate reference genes (RNU1-4, RNU6-2, SNORD43, SNORD44, and SNORD48) were amplified and analyzed by paired comparison. RNU6-2 was found to be the most stable gene in all samples, and its expression showed no significant differences between the groups. Thus, we used serum RNU6-2 as a reference gene to normalize expression of target serum genes in GIST patients.

All 6 miRNAs were validated using Taqman qPCR to be present in serum of GIST patients and healthy individuals. Expression of serum miR-518e-5p and miR-548e was significantly increased in secondary imatinib-resistant GIST patients compared to imatinib-sensitive GIST patients or healthy controls. ROC analysis showed that AUC of serum miR-518e-5p was 0.9938, with a high sensitivity of 99.8% and specificity of 82.1% for discriminating imatinib-resistant GIST patients from imatinib-sensitive GIST patients. In addition, serum miR-518e-5p could discriminate imatinib-resistant GIST patients from healthy controls. By contrast, serum miR-548e showed a lower sensitivity of 83.8% for identifying imatinib-resistant GIST. These data suggested that only serum miR-518e-5p could be used for diagnosis of imatinib-resistant GISTs.

Surprisingly, neither miR-518e-5p nor miR-548e was associated with imatinib resistance in previous miRNA profile studies using GIST tissue specimens (Akcakaya *et al*. 2014; Fan *et al*. 2014), suggesting that miR-518e-5p and miR-548e are present mainly in serum samples. Are they specifically released into peripheral blood from imatinib-resistant GIST cells? To investigate the mechanism of miR-518e-55 involved in the occurence of secondary imatinib-resistant GIST, clinical characteristics were compared between secondary imatinib-resistant patients and imatinib-sensitive patients, including nuclear division level and metastatic sites. No significant differences in the above markers were found between secondary imatinib-resistant patients and imatinib-sensitive patients. There was no correlation between miR-518e-5p or miR-548e and clinical characteristics. The distribution and function of miR-518e-5p are unknown. It has been reported that expression of miR-548e is decreased in joint tissues in rheumatoid arthritis mice, suggesting that it might regulate NF-kB signaling (Yan *et al*. 2016). Future studies should examine the function of miR-518e-5p and miR-548e in serum of secondary imatinib-resistant GIST patients.

In conclusion, our study indicates that serum miR-518e-5p might serve as a non-invasive biomarker for early detection and clinical diagnosis of imatinib-resistant GISTs.

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