PIK3C2A is a gene-specific target of microRNA-518a-5p in imatinib mesylate-resistant gastrointestinal stromal tumor

Yuan Shi[1,3], Xiaodong Gao[2,3], Qin Hu[1], Xiaojing Li[1], Jianfang Xu[1], Shaohua Lu[1], Yalan Liu[1], Chen Xu[1], Dongxian Jiang[1], Jiaqian Lin[2], Anwei Xue[2], Yunshan Tan[1], Kuntang Shen[2] and Yingyong Hou[1]

Imatinib mesylate resistance occurs in some patients with gastrointestinal stromal tumors (GISTs) during the course of treatment. In this study, we investigated the relationship between microRNAs (miRNAs) and imatinib-resistant GISTs, and the effect of miR-518a-5p on PIK3C2A in imatinib-resistant GISTs. A total of 20 matched-pair GIST samples from imatinib-resistant patients were included in the study. Each of the paired tumor specimens were from the same patient who had surgical removal of GISTs preimatinib and postimatinib treatment. Seven pairs of tissues were resected for microarray analysis, and the remaining 13 pairs were utilized for miRNAs analysis. Target genes were selected based on bioinformatics from multiple biological databases. Luciferase reporter assays were used to confirm the binding of miR-518a-5p to PIK3C2A 3′UTR. GIST882R-NC, 882R-miR-518a-5p-OE, and 882R-miR-518a-5p-KD cell lines were constructed using lentiviral vectors. miR-518a-5p and PIK3C2A expression in 882R-NC, 882R-OE, and 882R-KD cells was assessed by real-time PCR and western blotting. A cell counting kit was used to detect the influence of miR-518a-5p to cell proliferation. TUNEL staining was applied to detect the influence of miR-518a-5p to cell apoptosis. Microarray analysis showed that miR-518a-5p was downregulated in imatinib-resistant GISTs, and the expression of miR-518a-5p was confirmed with good concordance between real-time PCR and miRNA microarray results. Luciferase reporter assays indicated that miR-518a-5p bound to the PIK3C2A 3′UTR. Compared with 882R-OE, PIK3C2A expression was significantly increased in 882R-KD cells. MiR-518a-5p reduced 882R proliferation and promoted 882R apoptosis. In conclusion, PIK3C2A is a gene-specific target of miR-518a-5p in imatinib mesylate-resistant GISTs. Low expression of miR-518a-5p is likely to upregulate PIK3C2A and affect the cellular response to the drug, causing resistance to imatinib in GISTs.
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Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors in the gastrointestinal (GI) tract. To date, approximately 85% of GISTs are reported to harbor activating mutations in KIT or the homologous receptor tyrosine kinase PDGFRA gene.[1] Imatinib mesylate (formerly STI571, now referred to as Gleevec in the United States and Glivec in Europe (Novartis)) is a selective inhibitor of certain protein tyrosine kinases: the intracellular ABL kinase, the chimeric BCR-ABL fusion oncoprotein of chronic myeloid leukemia, the trans-membrane receptor KIT, and the platelet-derived growth factor receptors.[2–5] Studies have proved that imatinib is effective for many patients with advanced GISTs.[6] GISTs are therefore another molecular target therapy model in addition to chronic myoleukemia (CML). However, increasing numbers of studies

indicate that primary and secondary resistance may occur during the process of imatinib treatment in GISTs. Correlation between microRNAs (miRNAs) and drug resistance has recently begun to attract the researcher's attention and may be a new mechanism of molecular-targeted drug resistance.

A microRNA is a short RNA molecule found in eukaryotic cells. An miRNA molecule has very few nucleotides (at an average of 22) compared with other RNAs. miRNAs are posttranscriptional regulators that bind to complementary sequences on target mRNA transcripts (mRNAs), usually resulting in translational repression or target degradation and gene silencing.[7,8] miRNAs have a variety of biological functions, being closely related to growth, development, physiological function, and tumor development. miRNAs

[1]Department of Pathology, Zhongshan Hospital, Fudan University, Shanghai, China and [2]Department of General Surgery, Zhongshan Hospital, Fudan University, Shanghai, China
Correspondence: Dr Y Hou or Dr K Shen, Department of Pathology, Zhongshan Hospital, Fudan University, Fenglin Road #180, Shanghai 200032, China.
E-mail: houyingyong@hotmail.com or shen.kuntang@zs-hospital.sh.cn
[3]These authors contributed equally to this work.
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may have roles similar to oncogenes or tumor-suppressor genes in tumors regulating signaling pathways through the direct effect on target genes, thereby affecting the biological behavior of cells. Research to discover the association between miRNAs and drug resistance is just at the beginning stage. San *et al*[9] found 19 miRNAs correlated with imatinib resistance in CML. Our study explored the relationship between selected miRNAs and imatinib-resistant GISTs. miR-518a-5p was found downregulated in imatinib-resistant GISTs by miRNA microarray and real-time PCR. PIK3C2A was confirmed to be a gene-specific target of miR-518a-5p. Results suggested that miR-518a-5p is likely to regulate PIK3C2A and subsequently affect the cellular response to the imatinib in GIST.

MATERIALS AND METHODS
Cell Lines and Antibodies
Human 293T (Cat. No. 632180) was purchased from Clontech. Human GIST882 cell line was provided by Professor Christopher Fletcher, Brigham and Women's Hospital, Harvard Medical School, USA. Imatinib-resistant cell line GIST882R was induced from GIST882 cell line by the intermittent concentration gradient doubling method.

Patients and Specimens
Formalin-fixed paraffin-embedded matched-pair specimens from 20 GIST patients (1998–2013, Zhongshan Hospital, Fudan University, Shanghai, China) were obtained, the clinical and pathological information being listed in Table 1. All patients in the study had undergone surgery followed by imatinib treatment for a duration of 2–11 years. After the patients developed imatinib resistance, surgical resections were performed again to remove their resistant tumors. Both the preimatinib treatment and imatinib-resistant tissues were included in this study. The gene status of KIT, PDGFRA, and BRAF were detected in all of the above tissues. KIT mutations are listed in Table 1, while neither PDGFRA nor BRAF mutation was found in any of the tissues. Among the 20 patients, 7 pairs of preimatinib treatment and imatinib-resistant tissues were utilized for microarray analysis and the remaining 13 pairs were used to test the miRNAs. Clinical information was obtained from the GIST database. This study was approved by the Review Board of the Institute, Zhongshan Hospital, Fudan University.

Microarray Analysis
Total RNA was harvested using Trizol (Invitrogen) and miRNeasy mini kit (QIAGEN) according to the manufacturer's instructions. After passing RNA quantity measurement using the NanoDrop 1000, the samples were labeled with the miRCURY Hy3/Hy5 Power Labeling Kit and hybridized on the miRCURY LNA Array (v.18.0), containing 3100 capture probes. After rinsing, the slides were scanned using the Axon GenePix 4000B microarray scanner. Scanned images were then imported into the GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. miRNAs replicates were

Table 1 Clinical and pathological information of the 20 GIST patients (Nos.1–7 were utilized for microarray analysis and Nos.8–20 were used to test the miRNAs)

No.	Gender	Age	Primary site	KIT mutation of primary GIST	KIT mutation(s) of imatinib-resistant GIST
1	Male	56	Gastric	Exon 11	Exon 11
2	Male	60	Intestinal	Exon 11	Exon 11
3	Male	33	Gastric	Wild	Wild
4	Male	66	Gastric	Exon 11	Exons 11, 13
5	Male	55	Intestinal	Wild	Exon 17
6	Male	52	Intestinal	Exon 11	Exon 11
7	Male	65	Intestinal	Wild	Exon 11
8	Male	47	Abdominal cavity	Exon 11	Exon 11
9	Male	53	Intestinal	Exon 9	Exons 9, 17
10	Female	64	Intestinal	Exon 11	Exon 11
11	Male	72	Intestinal	Exon 11	Exon 11
12	Male	46	Intestinal	Exon 11	Exons 11, 17
13	Male	33	Abdominal cavity	Wild	Exons 9, 13
14	Female	62	Intestinal	Exon 11	Exons 11, 13
15	Male	62	Gastric	Exon 11	Exons 11, 13
16	Male	48	Gastric	Exon 11	Exons 11, 13
17	Male	57	Intestinal	Exon 11	Exons 11, 13
18	Female	46	Gastric	Exon 11	Exons 11, 13
19	Female	48	Abdominal cavity	Exon 11	Exon 11
20	Female	40	Intestinal	Exon 9	Exon 9

averaged, and all samples with miRNA intensity ≥ 30 were chosen for calculating normalization factor. Expressed data were normalized using the Median normalization. miRNAs expression with significant differences were identified through Volcano Plot filtering. Hierarchical clustering was performed to show distinguishable miRNA expression profiling among samples.

Quantitative Reverse Transcription-PCR
miR-518a-5p and PIK3C2A expression was analyzed by using SYBR Premix Ex Taq (TAKARA). In all, 1 μg of total RNA was reverse-transcribed by using specific stem-loop reverse transcription (RT) primers and then amplified and detected by using PCR with specific primers and ABI PRISM 7500 Sequence Detection System. U6 was used as an endogenous control for miR-518a-5p and RPL13A was used for PIK3C2A. The miR-518a-5p real-time PCR primers were 5′-CTGCAAAGGGAAGCCCTTTC-3′ and 5′-GTGCAGGGTCCGAGGT-3′. The PIK3C2A real-time PCR primers were 5′-CTTACTCATTGCTTCACCAGTGG-3′ and 5′-GCCTCAATCCAGGTCACAGCTA-3′. The U6 real-time PCR primers were 5′-CTCGCTTCGGCAGCACA-3′ and 5′-AACGCTTCACGAATTTGCGT-3′. The RPL13A real-time PCR primers were

Table 2 Oligonucleotides used for psiCHECK-2 luciferase reporter vector construction

Name	Sequences (5′ → 3′)
PIK3C2AXhoIF	CCGCTCGAGACTAGTGAATGTTTGAGCTTTGGAAGC
PIK3C2ANotIR	ATAAGAATGCGGCCGCTTTATTCCAGAAATATTTTAATACAATCGTAG
mutPIK3C2A-1F	ACATTTCCTTAAAGTATGTAAGTACGAAACGTAATGCAAATGTACTAGTAATTTTCA
mutPIK3C2A-1R	TGAAAATTACTAGTACATTTGCATTACGTTTCGTACTTACATACTTTAAGGAAATGT
mutPIK3C2A-2F	CGTACCAAAAAGTAGAGTGGAGCCTGAAACGTCTACTACTATCAATAAATTTTAAAT
mutPIK3C2A-2R	ATTTAAAATTTATTGATAGTAGTAGACGTTTCAGGCTCCACTCTACTTTTTGGTACG

5′-CTCAAGGTGTTTGACGGCATCC-3′ and 5′-TACTTC
CAGCCAACCTCGTGAG-3′. Relative quantification analysis
was performed using the comparative C T ($2^{-\Delta\Delta CT}$) method.

miRNAs Target Predictions
Predicted targets of miR-518a-5p and their sites were
analyzed by searching the databases miRDB (http://mirdb.
org/miRDB/), microRNA.org (http://www.microrna.org/),
TargetScan (http://www.targetscan.org/), miRWalk (http://
www.umm.uniheidelberg.de/apps/zmf/mirwalk/), RNAhybrid
(http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), PICTAR
(http://pictar.mdc-berlin.de/), PITA (http://genie.weizmann.
ac.il/pubs/mir07/mir07_data.html), and RNA22 (http://
cbcsrv.Watson.ibm.com/rna22.html).

Construction of 882R-NC (Negative Control), 882R-OE (882R-miR-518a-5p-Overexpression), and 882R-KD (882R-miR-518a-5p-Knockdown) Cell Lines
Lentiviral vectors (pHY-LV-KD5.1, pHY-LV-miR1.2) for
overexpression and knockdown of miR-518a-5p (negative
control, miR-518a-5p-OE, and miR-518a-5p-KD) were
purchased from Hanyin Biotechnology (Shanghai, China).
Lentiviral particles were produced via transfection of the
lentiviral vectors into 293T cells according to the manufac-
turer's protocol. Packed lentivirus- and negative control-
infected 882R cells and subsequent stable cell lines were
established.

Luciferase Reporter Assay
DNA fragments of miR-518a-5p-binding sites (wild type and
mutant) in the PIK3C2A 3′UTR (predicted by microrna.org
and TargetScan Human 5.1) were synthesized and cloned into
the multiple cloning sites (XhoI-NotI) of psiCHECK-2 vector
(Promega). All constructions were confirmed by DNA
sequencing. The constructions were transfected into 882R-
NC, 882R-OE, and 882R-KD cells. Luciferase activity was
measured 48 h after transfection using the Dual-luciferase
reporter assay system (Promega) and Glomax luminometer
(Promega). All experimental protocols were performed
according to the manufacturer's instructions. DNA fragment
sequences are listed in Table 2.

Western Blotting Analysis
Total cell lysate was prepared in RIPA buffer supplemented
with 1 mM PMSF. Protein concentrations were determined
by the Bradford assay. Equal amount of proteins were loaded
and separated using 10% SDS-polyacrylamide gel electro-
phoresis and transferred onto polyvinylidene fluoride mem-
branes. Membranes were blocked in TBS-0.1% Tween 20
(TBST)/5% (w/v) milk for 2 h at room temperature. The
membranes were then incubated with anti-PIK3C2A or anti-
β-actin (Cell Signaling Technology, USA). Proteins on the
membranes were detected using the ECL Kit (Thermo
Scientific, USA) after washing in TBST, and the blots were
quantified with Bio-Rad Gel system (Bio-Rad, USA).

Cell Counting Kit Assay
882R-NC, 882R-OE, and 882R-KD cells were cultured in a
96-well plate under regular conditions. At 24, 48, 72, and
96 h, 10 μl cell counting kit-8 (CCK8, Dojindo Laboratories,
Japan) was added and incubated for 2 h at 37 °C in a
humidified incubator. Optical density value was measured at
450 nm. IC50 were calculated by Logit method.

TUNEL Staining
882R-NC, 882R-OE, and 882R-KD cells were cultured on
coverslips. Cells were harvested and fixed with 4% parafor-
maldehyde. Cell apoptosis analysis was performed using a
TUNEL Staining Kit (Abcam, USA). Cell nuclei were stained
with DAPI. All fluorescent images were examined using a
Leica DM3000 microscope and photographed using a DFC
420 camera (Leica, Germany).

Statistical Analysis
All the statistical analyses were performed with the SPSS
v.19.0. Microarray data were analyzed by the paired *t*-test. The
data according to normal distribution were analyzed by
Student's *t*-test, whereas not according to normal distribution
were analyzed by the Wilcoxon signed-rank test, and the
expression was represented by M (P25–P75). *P*-value < 0.05
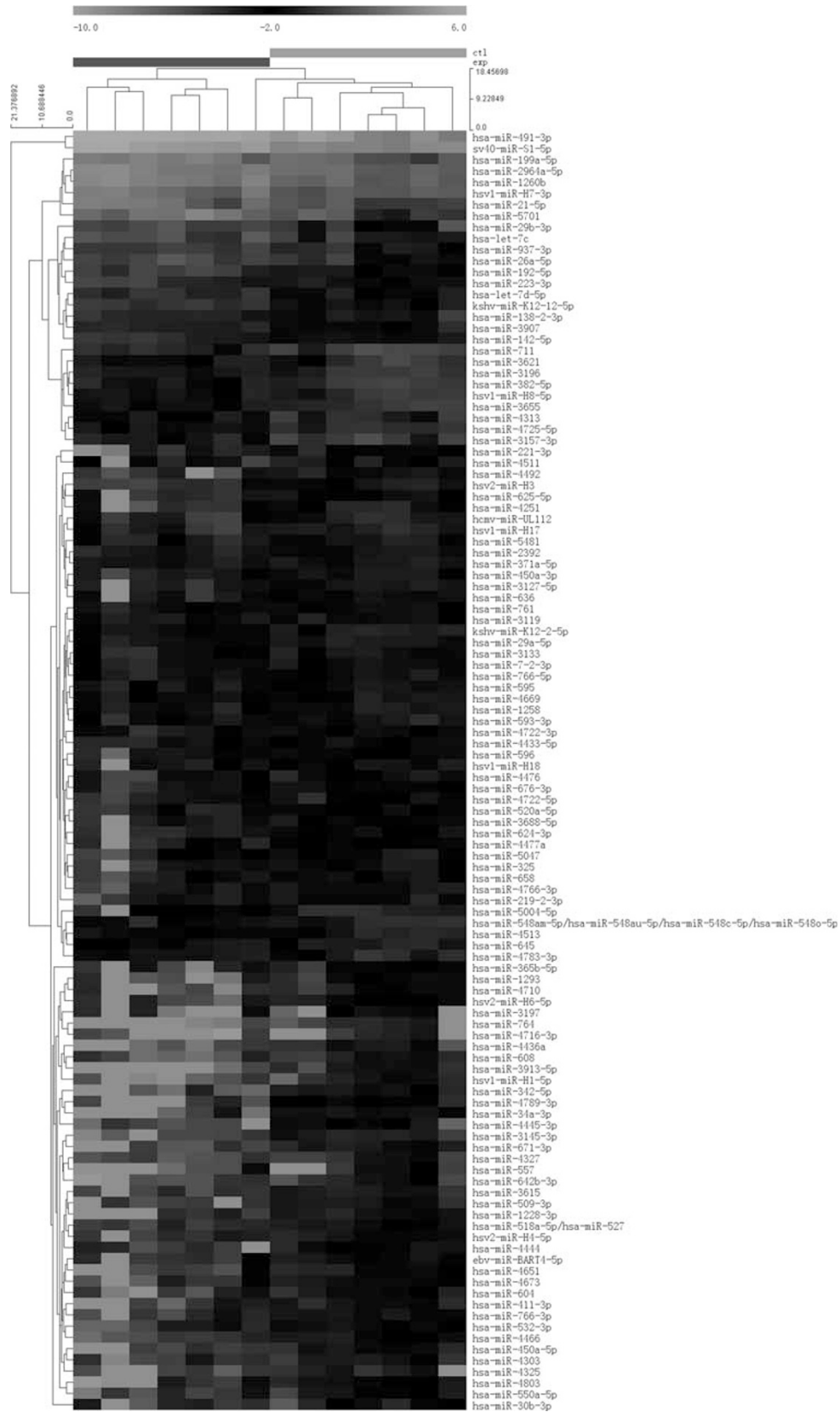was considered statistically significant.

Figure 1 All differentially expressed genes between preimatinib and imatinib-resistant GIST.

RESULTS

Microarray Analysis of Preimatinib and Imatinib-resistant GISTs

Among the 3100 genes analyzed, 114 genes were differentially expressed between preimatinib and imatinib-resistant GIST (Figure 1), and 10 genes were most differentially expressed by at least two folds (fold change >2 or <0.5; $P<0.05$). Compared with the preimatinib GISTs, the expression levels of four miRNAs (hsa-miR-1260b, hsa-miR-2964a-5p, hsa-miR-3907, and hsa-miR-491-3p) were upregulated, and the levels of six miRNAs (hsa-miR-518a-5p, hsa-miR-4466, hsa-miR-595, hsa-miR-221-3p, hsa-miR-3145-3p, and hsa-miR--3655) were significantly downregulated in imatinib-resistant GISTs (Table 3).

qRT-PCR Analysis of miR-518a-5p in Tissues

Rubie et al[10] in their study found that transfection of colorectal cancer (CRC) cells with miR-518a-5p leads to significant CCR6 downregulation. As CCR6 was found to be upregulated in CRC and involved in CRC pathology, miR-518a-5p might be the tumor suppressor of miRNA. Former microarray analyses showed that miR-518a-5p was downregulated in imatinib-resistant GISTs, so miR-518a-5p was chosen to examine the expression level by qRT-PCR in 13 pairs of preimatinib and imatinib-resistant tissues of GIST. In consistency with microarray data, qRT-PCR results showed that miR-518a-5p expression was reduced in imatinib-resistant samples. miR-518a-5p was differentially expressed between the two groups significantly, preimatinib 126306.260 (19230.355–9061297.280) and imatinib-resistant 10.410 (1.735–2216.285) ($P=0.011$). These data suggested that the downregulation of miR-518a-5p in GISTs might be responsible for the tumor acquiring drug-resistant potential, resulting in imatinib resistance.

The PIK3C2A 3'UTR Contains Binding Sites for miR-518a-5p

We had previously established the miRNA profile of GISTs by deep sequencing and had discovered that miR-518a-5p was downregulated 2.94-fold in imatinib-resistant GISTs ($P=0.0037$). These findings had been confirmed by qRT-PCR analyses by using a set of 13 corresponding pairs of preimatinib and imatinib-resistant tissues of GISTs. As the mRNA of PIK3C2A (phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 alpha) has been predicted to be a target for miR-518a-5p by a bioinformatics search, we therefore performed a luciferase reporter assay to determine whether miR-518a-5p can directly regulate PIK3C2A expression. In the PIK3C2A 3′UTR, there are two potential binding sites (PBS) for miR-518a-5p, including positions 1999–2005 (PBS1) and 2425–2431(PBS2) (Figure 2). The miR-518a-5p-binding sites were then inserted into the reporter vector and co-transfected with or without miR-518a-5p into GIST882R cells. Compared with 882R-NC, the luciferase activity was significantly decreased in 882R-OE (fold change: 0.755,

Figure 2 Predicted miRNA target sites on the PIK3C2A 3′UTR. There are two potential binding sites for miR-518a-5p, including positions 1999–2005 and 2425–2431. Paired sequence alignment is marked by continuous lines.
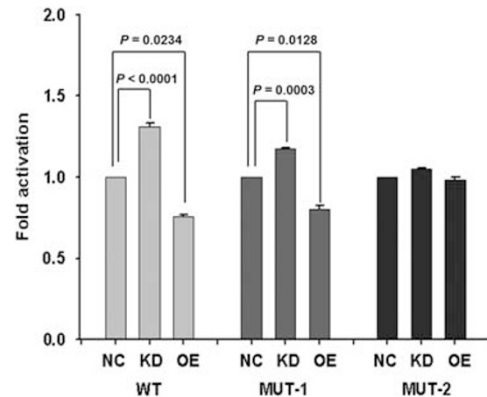

Figure 3 Luciferase activity of WT (wild type, no mutagenesis), MUT-1 (mutagenesis of PBS1), and MUT-2 (mutagenesis of PBS2) in 882R-NC, 882R-OE, and 882R-KD cells.

$P=0.0234$), while the luciferase activity was significantly increased in 882R-KD (fold change: 1.311, $P<0.0001$). Site-directed mutagenesis (MUT-1 and MUT-2, respectively) was used to remove PBS1 and PBS2. Compared with 882R-NC-MUT-1, the luciferase activity was significantly decreased in 882R-OE-MUT-1 (fold change 0.804, $P=0.0128$), while the luciferase activity was significantly increased in 882R-KD-MUT-1 (fold change: 1.173, $P=0.0003$). Compared with 882R-NC-MUT-2, the luciferase activity was no different in 882R-OE-MUT-2 and 882R-KD-MUT-2. This indicated that PBS2 is the target for miR-518a-5p. Taken together, these results suggested that the PIK3C2A 3′UTR is an independent target for miR-518a-5p (Figure 3).

Relationship Between miR-518a-5p and PIK3C2A Expression

miR-518a-5p and PIK3C2A expression in 882S (imatinib-sensitive), 882R, 882R-NC, 882R-OE, and 882R-KD cells were determined using qRT-PCR and western blotting. The result of qRT-PCR showed that, compared with 882S, miR-518a-5p expression was significantly decreased in 882R (fold change 0.626, $P=0.0007$), whereas PIK3C2A was significantly increased (fold change 1.427, $P=0.0003$). Compared with 882R-NC, PIK3C2A expression was significantly decreased in 882R-OE (fold change 0.416, $P<0.0001$) and significantly increased in 882R-KD cells (fold change 1.234, $P<0.0001$). The result of western blotting showed that, compared with 882S, PIK3C2A was significantly increased in

Table 3 Ten most differentially expressed genes between preimatinib and imatinib-resistant GIST

	P-value	Fold change	Upregulated or downregulated
hsa-miR-1260b	0.000645	2.753673	Upregulated
hsa-miR-2964a-5p	0.000545	2.354872	Upregulated
hsa-miR-3907	0.000101	3.238698	Upregulated
hsa-miR-491-3p	5.57E-05	2.708252	Upregulated
hsa-miR-518a-5p	0.003665375	0.34043	Downregulated
hsa-miR-4466	0.000669118	0.238874	Downregulated
hsa-miR-595	0.001009732	0.365527	Downregulated
hsa-miR-221-3p	0.00163104	0.235397	Downregulated
hsa-miR-3145-3p	0.008019024	0.253782	Downregulated
hsa-miR-3655	0.000160236	0.305696	Downregulated

Table 4 Gene expression of miR-518a-5p and PIK3C2A in 882S, 882R, 882R-NC, 882R-OE, and 882R-KD cells

	miR-518a-5p (fold change)	PIK3C2A (fold change)
882S	1	1
882R	0.626	1.427
882R-NC	1	1
882R-OE	1579	0.416
882R-KD	0.73	1.234

882R, and compared with 882R-OE, PIK3C2A expression was significantly increased in 882R-KD cells (Table 4, Figure 4).

miR-518a-5p Reduced 882R Proliferation

The effect of miR-518a-5p on the dynamics of 882R growth was determined using a CCK8 assay. Compared with the NC group (27.41 ± 0.055), the IC50 of 882R-OE (21.27 ± 0.065) and 882R-KD (30.69 ± 0.039) were significantly reduced or promoted, respectively ($P = 0.0015$ and $P = 0.016$, respectively, Figure 5).

miR-518a-5p Promoted 882R Apoptosis

To examine the effect of miR-518a-5p on 882R apoptosis, TUNEL staining was performed. TUNEL staining showed that the proportion of apoptotic cells in 882R-OE was increased (108 ± 1.65) compared with NC group (70 ± 1.59) ($P < 0.0001$), whereas 882R-KD (47 ± 1.39) cells decreased compared with the NC group ($P < 0.0001$) (Figure 6). These above described results revealed that miR-518a-5p might regulate 882R proliferation and apoptosis by directly targeting PIK3C2A.

DISCUSSION

Hirota *et al*[11] reported gain-of-function mutation in KIT gene in GIST in 1998. Heinrich *et al*[12] found *PDGFRA* gene mutation in GIST in 2003. Later, imatinib mesylate was used in GIST treatment.[6]

However, owing to the extended treatment time, drug resistance occurs in some patients, and the median progression-free survival is in the range of 20–24 months.[13] Although the second-line drug sunitinib and third-line drug sorafenib were introduced into treatment protocols, some patients showed drug resistance after temporary relief, and ultimately treatment failed.

Studies found that miRNAs are closely related to tumorigenesis, invasion, and drug resistance and may have a role as oncogenes or tumor-suppressor genes. Those studies provide us new ideas and methods for research in GISTs. miRNAs are non-coding, single-stranded RNAs that function in gene regulation. An miRNA is complementary to a part of one or more mRNAs. Animal miRNAs are usually complementary to a site in the 3′UTR. The match-ups are imperfect. They regulate gene expression by promoting degradation of the mRNA or repressing its translation.[9] miRNAs are involved in cell proliferation, apoptosis, differentiation, metabolism, and development of physiological processes, as well as the pathological process of cardiovascular disease, neurological diseases, and cancer metastasis. It is estimated that up to more than one-third of human genes have conserved targets for miRNAs. The roles of miRNAs in human disease, especially in tumors, is an area of active investigation.

In recent years, a large number of studies have shown that miRNAs are involved in tumor development and invasion through regulating the cell cycle, apoptosis, cell migration, angiogenesis, and other tumor-associated gene expression. The relationship between the miRNA and the tumor was first discovered in leukemia. Calin *et al*[14] speculated that miRNA may be associated with chronic lymphocytic leukemia. Hereafter, Calin *et al*[15] also found that >50% of the miRNAs located at fragile sites and genomic regions are involved in cancers. Therefore, miRNAs may have an important role in cancers.

Zhu *et al*[16] first discovered the correlation between miRNAs and drug resistance. They showed that expression of miR-27a and miR-451 were upregulated in multidrug-resistant (MDR) cancer cell lines A2780DX5 and KB-V1, as compared with their parental lines A2780 and KB-3-1. They also reported that miRNAs miR-27a and miR-451 are involved in activating the expression of P-glycoprotein, the MDR1 gene product that confers cancer cell resistance to a broad range of chemotherapeutics. About the same time, Xia *et al*[17] found that expression of miR-15b and miR-16 were downregulated in the MDR gastric cancer cell line SGC7901/VCR compared with its parental SGC7901 cell line. Their findings also suggested that miR-15b and miR-16 could have a role in the development of MDR in gastric cancer cells at least in part by modulation of apoptosis via targeting bcl-2. Inspired by the work of Zhu *et al*[16] and Xia *et al*,[17] San *et al*[9] have identified a group of 19 miRNAs that may predict clinical resistance to imatinib in patients with newly diagnosed CML. Among all the possible targets for these
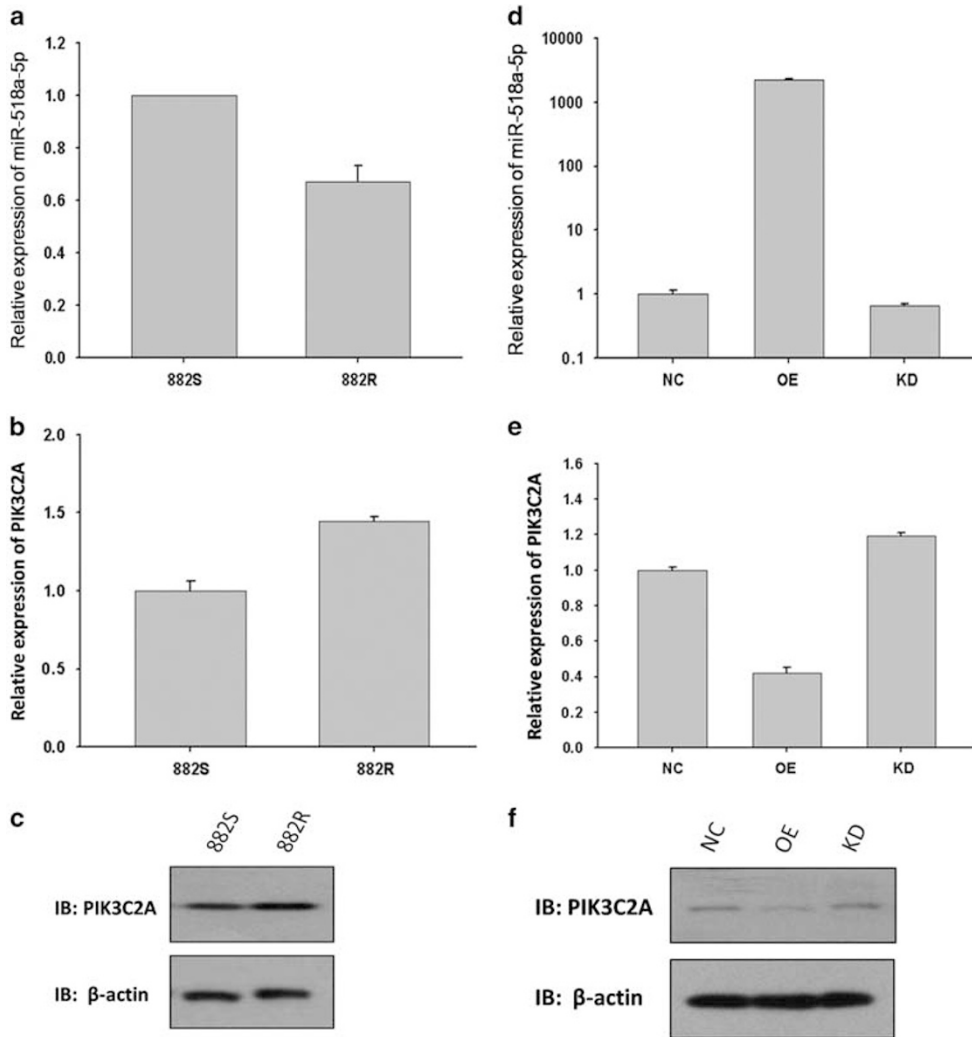
Figure 4 Gene expression of miR-518a-5p and PIK3C2A in 882S, 882R, 882R-NC, 882R-OE, and 882R-KD cells. (**a**) miR-518a-5p gene expression in 882S and 882R cells. (**b**) PIK3C2A gene expression in 882S and 882R cells. (**c**) PIK3C2A protein expression in 882S and 882R cells. (**d**) miR-518a-5p gene expression in 882R-NC, 882R-OE, and 882R-KD cells. (**e**) PIK3C2A gene expression in 882R-NC, 882R-OE, and 882R-KD cells. (**f**) PIK3C2A protein expression in 882R-NC, 882R-OE, and 882R-KD cells.

miRNAs, they found several membrane transporters that belong to the ATP-binding cassette superfamily of transmembrane transporters have been implicated in resistance to chemotherapy.

Our research team reported previously that miR-320a downregulation is associated with imatinib resistance in GISTs.[18] However, unmatched-pair samples were used to make microarray analysis, which may cause bias. Therefore, in this study, we used matched-pair samples for microarray and real-time PCR analysis. We found that miR-518a-5p was downregulated in imatinib-resistant GISTs and confirmed that PIK3C2A is the target of miR-518a-5p. Functional interactions between miR-518a-5p and PIK3C2A were investigated.

PIK3C2A is also known as CPK, PI3-K-C2A and PI3-K-C2 (ALPHA). The protein encoded by this gene belongs to the phosphoinositide 3-kinase (PI3K) family. The PI3K pathway
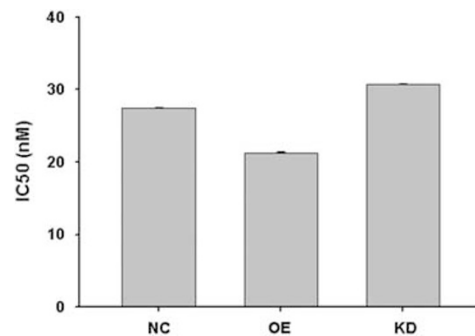

Figure 5 IC50 of 882R-NC, 882R-OE, and 882R-KD cells.

regulates various cellular processes, such as cell growth, proliferation, apoptosis, motility, differentiation, survival, and intracellular trafficking.[19] PI3 kinases have roles in signaling pathways involved in cell proliferation, oncogenic
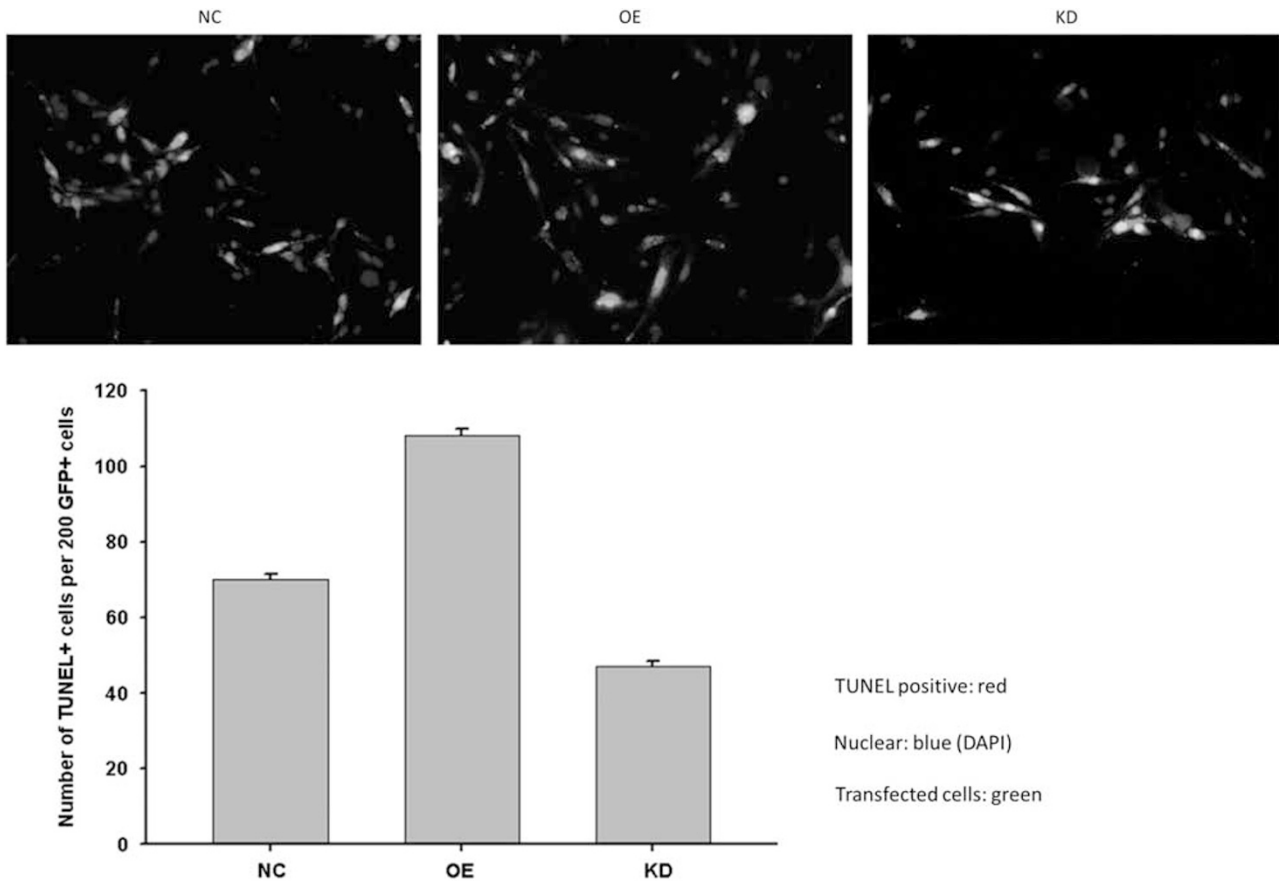
Figure 6 TUNEL staining of 882R-NC, 882R-OE and 882R-KD cells.

transformation, cell survival, cell migration, and intracellular protein trafficking. This protein contains a lipid kinase catalytic domain as well as a C-terminal C2 domain, a characteristic of class II PI3 kinases. C2 domains act as calcium-dependent phospholipid-binding motifs that mediate translocation of proteins to membranes and may also mediate protein–protein interactions. This protein was shown to be able to be activated by insulin and may be involved in integrin-dependent signaling. The insulin receptor (INSR), via which insulin initiates its signaling, and insulin receptor substrates, which serve as docking proteins binding the PI3K complex, a key regulatory messenger in insulin signaling.[20,21] PI3K, via its downstream effector RAC-alpha serine/threonine protein kinase (AKT, otherwise known as protein kinase B or PKB), activates several signaling molecules, including mammalian target of rapamycin (MTOR), a promoter of protein synthesis.[20–22] Rapamycin, an MTOR inhibitor and autophagy inducer, was shown to reduce total and phospho-KIT expression levels and enhance apoptosis in imatinib-resistant GIST cells.[23] PI3Ks are heterodimeric lipid kinases that are composed of regulatory and catalytic subunits that catalyze the production of several phosphoinositides critical for the signal transduction in these multiple cellular processes.[24] Mutation, amplification, and rearrangement in the PI3K pathway and its downstream targets have been observed in several cancer sites.[25] Because of the role of PI3Ks in cell proliferation, much of the research on this pathway concerns its potential as a target for anticancer therapies.[26] By using our research result for reference,[27] Fan et al[28] in their study found that the expression of miR-218 is downregulated in an imatinib mesylate-resistant GIST cell line (GIST430), whereas miR-218 overexpression can improve the sensitivity of GIST cells to imatinib mesylate, with PI3K/AKT signaling pathway possibly involved in the mechanism.

There are few studies on miR-518a-5p. To our knowledge, no other investigator has seen miR-518a-5p expression in GIST and we are the first to investigate the relationship between miR-518a-5p and GIST. Our results provide evidence that miR-518a-5p functionally interacts with the 3′UTR of PIK3C2A. It is further documented that miR-518a-5p reduced 882R proliferation and promoted 882R apoptosis, and transfection of GIST cells with miR-518a-5p leads to significant PIK3C2A downregulation at the mRNA and protein level. PIK3C2A has been reported to promote tumor survival,[29,30] therefore, low expression of miR-518a-5p is likely to upregulate PIK3C2A and affect the cellular response to the drug, causing resistance to imatinib in GIST. Consequently, our findings may aid in the understanding of miRNA gene regulation with

respect to PIK3C2A and their interaction in GIST cells, emphasizing the importance of further studies of the regulative mechanism underlying the miR-518a-5p/PIK3C2A interaction.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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