APOBEC3B and SPAG5, the Target Genes of miR-539, Involved into Imatinib-Resistant of Gastrointestinal Stromal Tumors

Qianqian Zhang

Yinfeng Gene Technology Co.Ltd.

Haiyun Liu

Yinfeng Gene Technology Co. Ltd.

Xingmin Li

Yinfeng Gene Technology Co. Ltd.

Hongxing Wang (✉ wanghongxing_122@mail.sdu.edu.cn)

Shandong University <https://orcid.org/0000-0002-0292-4966>

Research article

Keywords: miR-539, SPAG5, APOBEC3B, CD4+ T cells, Gastrointestinal Stromal Tumors, weighted co-expression network

Posted Date: October 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-72201/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Although imatinib can effectively treat gastrointestinal stromal tumor (GIST), some patients are still resistant to it or cannot tolerate the adverse reactions of the drug. This study aimed to investigate potential miRNAs, mRNA and tumor-infiltrating lymphocytes (TILs) associated with the prognosis of imatinib-resistant GIST.

Methods: mRNA, miRNA sequencing data and patient clinic traits of primary (imatinib-naive) and imatinib-resistant GIST were obtained from GEO (Gene Expression Omnibus) database. A systems biology approach combining Weighted co-expression network analysis (WGCNA) and differential expression analysis were utilized to detect the imatinib-resistant-related miRNA and gene modules and construct a miRNA-gene network. Tumor-infiltrating immune cells were analyzed by Estimating the Proportion of Immune and Cancer cells (EPIC) and Tumor-Immune System Interactions (TISIDB). miR-539 was measured by qRT-PCR. SPAG5 and APOBEC3B was measured by qRT-PCR and WB. Transforming growth factor (TGF)- β and interleukin (IL)-10 were assessed with enzyme-linked immunosorbent assay (ELISA). The proportions of CD4⁺ T cells, CD8⁺ T cells, NK cells and B cells in tumor-infiltrating lymphocytes were analyzed via flow cytometry (FCM).

Results: Two gene modules (brown and yellow) and one miRNA module were associated with the imatinib-resistant. Two hub genes (APOBEC3B and SPAG5) were associated with the imatinib-resistant. Three hub miRNAs were identified to be related to imatinib-resistant (miR-539, miR-376b and miR-18b). G1/S transition of mitotic cell cycle, G2/M transition of mitotic cell cycle, and cell proliferation were common pathways of the gene modules and miRNA module. apolipoprotein B mRNA editing catalytic polypeptide-like 3B (APOBEC3B) and Sperm-associated antigen 5 (SPAG5), which were both target genes of miR-539 was located at the core of miRNA-gene network. APOBEC3B ($\rho=0.509$, $p < 2.2e-16$) and SPAG5 ($\rho=0.468$, $p < 2.2e-16$) was positive correlated with the infiltration levels of activated CD4⁺ T cells. The proportions of CD4⁺ T cells, and the mRNA and protein relative expression of APOBEC3B and SPAG5 in imatinib-resistant tumor samples significantly increased as compared with tumor samples in imatinib-naive group. Imatinib-resistant tumor samples exhibited significantly downregulation of miR-539 and TGF- β 1. Over-expression of miR-539 sensitized imatinib resistant GIST48 cells and increased the secretion of TGF- β 1 by inhibiting APOBEC3B and SPAG5.

Conclusion: APOBEC3B and SPAG5, the target genes of miR-539 may play as key factor for imatinib-resistant GIST by increasing the proportion of tumor-infiltrated activated CD4⁺ T cells via TGF- β 1. These findings help to advance the understanding of imatinib-resistant GIST and provide potential therapeutic targets.

Background

Gastrointestinal stromal tumour (GIST) is mainly initialised by mutations of the receptor tyrosine kinase genes KIT or PDGFRA [1]. The advent of a small-molecule inhibitor imatinib mesylate, which targets

several receptor tyrosine kinases, including both KIT and PDGFRA, dramatically improved patient outcome [2]. However, Still, 10% of GISTs have primary resistance to imatinib and 40–50% of GISTs have secondary resistance within 2 years [3]. MicroRNAs have been found to play a role in imatinib resistance of GIST [3].

Microarrays of mRNA and microRNAs have been applied to research into GIST imatinib-resistant [4-6]. MicroRNAs (miRNAs) are short, noncoding regulatory RNAs. By binding to the target mRNAs, miRNAs lead to mRNA degradation and/or translational repression. Accumulated evidence has shown that miRNAs are associated with imatinib response in GISTs [7]. Although distinct mRNA and miRNA expression signatures have been found in GISTs imatinib-resistant, miRNA–target relationships are not well considered.

Weighted gene co-expression network analysis (WGCNA) is a novel gene co-expression network-based approach focusing on gene or miRNA sets other than individual genes or miRNA from different group. WGCNA was used to explore molecular interaction mechanism analysis and drug resistant correlation networks resolving based on RNA sequencing or microarray data [8].

In this study, weighted co-expression network analysis was performed on the mRNA and miRNA to identify the potential key miRNAs and genes associated with the GIST imatinib-resistant. We further investigated the expression and functional role of miR-539 and its potential targets APOBEC3B and SPAG5 in imatinib resistance of GIST and GIST48, a imatinib-resistant GIST cell line. The results may provide novel information for the study of GIST imatinib-resistant, and provide novel potential biomarkers for the clinical therapy of GIST.

Methods

Data collection and preprocessing

The genes or miRNAs estimated with verbose less than 3 were removed using Weighted Gene Co-expression Network Analysis (WGCNA) package of R (Version 3.6.1). The outlier samples with verbose more than 40% were removed detecting by cluster diagram. abnormally low expressed genes and miRNAs with reads per kilobase of exon per million reads mapped (RPKM) less than 10 were removed. The microRNA and mRNA expression profiles datasets GSE and clinical information of primary (imatinib-naive) and imatinib-resistant with GIST (n=126) were obtained from from Gene Expression Omnibus database (GEO; <http://www.ncbi.nlm.nih.gov/geo/>). Datasets GSE132542, GSE69465, GSE51697, and GSE45901 were chosen for further study. Data processing was performed using the R programming language (v3.6.1).

Weighted Gene co-expression network analysis (WGCNA)

Briefly, correlation coefficient all pairs of genes or miRNA was calculated based on their expression values. A soft-thresholding power β was selected and the co-expression similarity was transformed into a weighted undirected network and topological overlap matrix (TOM). Then, genes or miRNA were clustered into modules by using dynamic tree cut with the following parameters: maxBlockSize of 6000, TOMType of unsigned, and minModuleSize of 30. Next, The module eigengene (ME) were identified based on the first principal component (PC) of gene or miRNAs expression in each module.

Identification of differentially expressed genes (DEGs) and miRNAs (DEMs)

The differentially expressed genes (DEGs) and differentially expressed miRNAs (DEMs) between imatinib-resistant and imatinib-naïve group were screened by the R package, edgeR. The thresholds for DEGs were set as the values of $p < 0.05$ and false discovery rate (FDR) < 0.1 . The $p < 0.05$ and false discovery rate (FDR) < 0.2 were set as the cut-off criteria for DEMs.

Relating modules to clinical parameters and identifying characteristic genes

Hub genes were identified based on gene significance (GS) and module membership (MM). The gene significance (GS) defined as the correlation between the gene and four clinical factors of GIST (c-KIT mutation, location, malignancy risk, and imatinib-resistant). The module membership (MM) defined as the correlation between the module eigengene (ME) and the gene expression profile. The module significance (MS) defined as the average miRNA significance or gene significance (GS). Hub Genes with high GS and MM were common in the module significance (MS) and differentially expressed genes (DEGs) profile defined as characteristic genes. characteristic miRNAs were also identified by this method.

Functional enrichment analysis of genes and miRNAs

The characteristic genes were submitted to Database for Annotation, Visualization and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/>) to conduct Gene Ontology (GO) functional enrichment analysis. $P < 0.05$ were considered significant by Fisher's Exact Test. The miRNA pathway analysis was conducted using mirPath v.3 (<http://snf-515788.vm.okeanos.grnet.gr/>).

Construction of disease-related gene co-expression network containing small-molecule drugs

Imatinib-resistant characteristic genes were submitted to the Connectivity Map (Cmap) to screen small molecules [9].

Construction of the miRNA–gene interaction network

The miRNA–gene interaction network was constructed based on the Imatinib-resistant characteristic miRNAs and Imatinib-resistant characteristic genes. Target genes of characteristic miRNAs were predicted based on the information in microT-CDS (v5.0) and TargetScan (<http://www.targetscan.org/>). Only the interactions that were common in the two databases with false discovery rate (FDR) < 0.05 were used to construct the miRNA the interaction network. Cytoscape 3.6.1 was used to visualize the network.

Evaluation of Tumor Microenvironment

Differentially expressed genes (DEGs) were uploaded to Estimating the Proportion of Immune and Cancer cells (EPIC), a method for characterizing tumor infiltrating lymphocytes (TILs) of solid tumor tissues from their gene expression profiles (<http://epic.gfellerlab.org>) [10]. We cross-check the role of APOBEC3B and SPAG5 in tumor-immune interactions in sarcoma using the Tumor-Immune System Interactions (TISIDB) (<http://cis.hku.hk/TISIDB>) [11].

Flow cytometry (FCM) assay

The study protocol was approved by the Medical Ethics Committee of Shandong Provincial Qianfoshan Hospital (Approval number: 20191002). The detailed characteristics of GIST patients are shown in Table 5. Imatinib-naïve (n=14) and imatinib-resistant GIST (n=15) tumor samples were chopped into small pieces and dissociated with collagenase-IV (1 mg/ml, Sigma-Aldrich) and DNase I (10 mg/ml, Sigma-Aldrich) for 0.5-1h at 37°C in Hank's buffered salt solution. Single-cell suspensions were prepared with falcon cell filternylon mesh (100µm, corning) and human lymphocyte separation solution (solarbio, China). CD45⁺ cells, CD4⁺ T cells, CD8⁺ T cells, CD3⁻ CD56⁺CD16⁺ NK cells, and CD3⁻ CD19⁺ B cells were detected by flow cytometry (American ACEA BIO, NovoCyte D2040R) using lymphocytes detection kit (ACEA biosciences). The data were analyzed by FlowJo software (Tree Star Inc.).

ELISA assay

Tumor samples or GIST48 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer. Transforming growth factor (TGF)-β1 and interleukin (IL)-10 levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocols (beyotime).

CCK8 assay

Control mimic, miR-539 mimic (0.1µM), combination of imatinib (0.1µM) and control mimic, or combination of imatinib and miR-539 mimic was transfected into GIST48 cells. Cell proliferation were

measured at 0, 0.05, 0.1, 0.2 μ M by cholecystokinin octapeptide (CCK8) according to the manufacturer's protocols (MedChem Express).

miRNAs and mRNAs detection by quantitative Real time PCR

The miR-539 expression was measured by TaqMan Small RNA primer and probe sets (Applied Biosystems, USA) according to the manufacturers' instruction. Total RNA was isolated from GIST48 cells or tumor of patient clinic traits of primary (imatinib-naive) and imatinib-resistant GIST using TRIzol. U6-snrRNA was used as an endogenous control. APOBEC3B and SPAG5 mRNA expression level was investigated by SYBR green-based quantitative real-time polymerase chain reaction (qPCR) assay. β -actin was used as the internal control. The formula $2^{-\Delta\Delta Ct}$ was used for calculating relative mRNA or miRNA expression. The primers were: APOBEC3B (forward: 5'-ACCCATCCTCTATGGTCGGA-3'; reverse: 5'-GCTTGAAATACACCTGGCCTC-3'); SPAG5 (forward: 5'-CATCTCACAGTGGGATAACTAATAAAC-3'; reverse: 5'-CAGGGA TAGGTGAAGCAAGGATA-3'); β -actin (forward: 5'-AGTCATTCCAAATATGAGATGCGTT-3'; reverse: 5'-TGCTATCACCTCCCCTGTGT-3').

Western blots

The protein was resolved by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was probed with anti-Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B (APOBEC3B) (AV46114, Sigma-Aldrich) and anti-Sperm-associated antigen 5 (SPAG5) (WH0010615M1, Sigma-Aldrich). β -actin (ab8226, abcam) was used as the internal control.

Statistical analysis

The comparison between the two groups were evaluated by Student's t-test using Prism 5.0 software. $P < 0.05$ was considered to be statistically different. Cell viability assay were conducted by 2-way ANOVA and Tukey's post-hoc test.

Results

mRNA expression module analysis of WGCNA

After data preprocessing, for the mRNA sequencing data, the samples ($n=29$) were well clustered and 5880 of 23520 mRNAs were remained for further analysis (**Fig. 1a**). Using the discovery set (GSE132542 dataset), 17 mRNA network modules were identified using WGCNA. The modules were assigned a color label (black, brown, yellow, midnightblue, pink, salmon, red, turquoise, purple, tan, cyan, lightcyan,

magenta, greenyellow, blue, green, and grey) (**Fig. 1b**). Correlations between these gene network modules and clinical data were analyzed. Module brown was found to be markedly positive correlated with imatinib-resistant ($r = 0.54$, $p = 0.002$); Module yellow was found to be markedly positive correlated with imatinib-resistant ($r = 0.43$, $p = 0.02$) (**Fig. 1c**). To reveal the functions of the mRNAs of the modules correlated with imatinib-resistant of GIST, GO enrichment analysis was performed. According to the results, the mRNAs in the brown module ($n=786$) and yellow module ($n=582$), they were involved with cell division, mitotic nuclear division, chromosome segregation, G1/S transition of mitotic cell cycle, sister chromatid cohesion, DNA replication, DNA replication initiation, spindle organization, G2/M transition of mitotic cell cycle, cell proliferation, mitotic sister chromatid segregation, mitotic cytokinesis, and mitotic spindle organization. (**Fig. 1d and Table1**).

Weighted Gene co-expression network analysis (WGCNA) of miRNA

After data preprocessing, for the miRNA sequencing data, the samples ($n=53$) were well clustered and 722 miRNAs were remained for further analysis (**Fig. 2a**). According to the module analysis of miRNA coexpression networks, 7 miRNA network modules were identified, and assigned the color labels red, blue, green yellow, brown, turquoise, and grey. The correlation analysis of modules and clinical prognostic data revealed that module red was significantly negative associated with imatinib-resistant of GIST ($r = -0.29$, $p = 0.04$) (**Fig. 2b**). Cytoscape 3.6.1 was used to visualize the miRNA network in the red module ($n=23$) (**Fig. 2c**). To reveal the functions of the miRNAs of the modules correlated with imatinib-resistant of GIST, GO enrichment analysis was performed using mirPath v.3. According to the results, 210 GO terms were totally enriched in biological process (BP). G1/S transition of mitotic cell cycle, G2/M transition of mitotic cell cycle, and cell proliferation were common pathways of the gene modules and miRNA module. (**Fig. 2d**).

Associations of miRNA target genes and gene network modules

The thresholds for the differentially expressed genes (DEGs) were set as the values of $p < 0.05$ and false discovery rate (FDR) < 0.1 between Imatinib-Naive and Imatinib-Resistant GIST Samples. 436 significantly differentially expressed genes were detected between the two groups. 102 genes were found in module brown and 16 genes were found in module yellow of WGCNA such as CDK1, AURKB, AURKA, CD44, HMMR, BIRC5 and CCNB1 (**Fig. 3a and Table 2**). The Connectivity Map (Cmap) was used to predict small-molecule drugs against GIST. We found 28 small-molecule drugs could target CDK1 such as JNJ-7706621, Terameprocol, 1-Azakenpaullone, and Kenpaullone. We found 24 small-molecule drugs could target AURKB such as JNJ-7706621, tozasertib and orantinib. We found 25 small-molecule drugs could target AURKA such as JNJ-7706621, tozasertib and orantinib. We found 1 small-molecule drugs could target CD44 such as hyaluronic-acid. We found 2 small-molecule drugs could target BIRC5 such as

Terameprocol and YM-15. We found 2 small-molecule drugs could target CCNB1 such as Kenpaullone and 1-Azakenpaullone. These small-molecule drugs might have a therapeutic effect against imatinib-resistant GIST. (**Table 3**). A two-sample t-test ($p < 0.05$) Benjamini-Hochberg false discovery rate (FDR) ($FDR < 20\%$) was used to determine differentially expressed microRNAs between Imatinib-Naive and Imatinib-Resistant GIST Samples. 35 significantly differentially expressed miRNAs (DEMs) were detected between the two groups (**Table 4**). Three miRNAs (miR-539, miR-376b and miR-18b) were found in both in module red of WGCNA and DEMs. The results are shown in the Venn diagram (**Fig. 3b**).

Three miRNAs (miR-539, miR-376b and miR-18b) contained in the module red and DEMs were chosen for target genes. A total of 265 target genes were screened out based on the information in TargetScan, miRanda, and miRwalk. Three critical miRNAs target gene regulatory network (miR-539, miR-376b and miR-18b) and the regulatory network of 120 mRNA gene in module brown and yellow were then constructed. Interestingly, Two target gene APOBEC3B and SPAG5 of miR-539 are also in network of mRNA gene in module brown (**Fig. 3c**).

Relationship between the expression of APOBEC3B/SPAG5 and immune infiltration

To investigate the association between differentially expressed genes (DEGs) and the tumor microenvironment, the 8 immune cell fraction was calculated by EPIC. We found that imatinib-naive group has a lower density of CD4⁺ T cells and endothelial cells compared to the imatinib-resistant group (**Fig. 4a**). The correlation between APOBEC3B/SPAG5 expression and immune invasion in sarcoma using the Tumor-Immune System Interactions (TISIDB) (**Fig. 4b**). We found that both the expression of APOBEC3B ($\rho = 0.509$, $p < 2.2 \times 10^{-16}$) and SPAG5 ($\rho = 0.468$, $p < 2.2 \times 10^{-16}$) was positive correlated with the infiltration levels of activated CD4⁺ T cells (**Fig. 4c**).

Clinical correlation between miR-539, TGF- β 1 and infiltrating CD4⁺ T cells in imatinib-naive and imatinib-resistant GIST.

We compared the expressions of miR-539, APOBEC3B, SPAG5 and TGF- β in imatinib-naive ($n = 14$) and imatinib-resistant tumor samples ($n = 15$). Consistent with bioinformatics results, imatinib-resistant tumor samples exhibited significantly downregulation of miR-539 ($p = 0.0005$) (**Fig. 5a**). We found that the mRNA relative expression of APOBEC3B and SPAG5 in imatinib-resistant tumor samples significantly increased as compared with tumor samples in imatinib-naive group ($p = 0.0051$ and 0.0035 , respectively) (**Fig. 5b**). As expected, the protein relative expression of APOBEC3B and SPAG5 in imatinib-resistant tumor samples were significantly increased ($p = 0.0051$ and 0.0085 , respectively) (**Fig. 5c**). We detected Transforming growth factor (TGF)- β 1 and interleukin (IL)-10, known as important immunosuppressive cytokines, by ELISA. We found that TGF- β 1 secretion of imatinib-resistant tumor samples were significantly decreased

compared with tumor samples in imatinib-naive group ($p=0.0051$) (**Fig.5d**). The proportions of CD4⁺ T cells, CD8⁺ T cells, NK cells and B cells in tumor-infiltrating lymphocytes from imatinib-naive and imatinib-resistant patients were analyzed via flow cytometry (FCM). We found that the proportions of CD4⁺ T cells of imatinib-resistant tumor samples were significantly increased compared with tumor samples in imatinib-naive group ($p=0.003$) (**Fig.5e**). We analyzed the correlation of miR-539 expressions and CD4⁺ T cells proportions in 29 GIST patients. We observed a significantly negative correlation between miR-539 expressions and CD4⁺ T cells proportions in GIST patients ($P < 0.001$, $R^2 = 0.5353$) (**Fig.5f**).

Overexpression of miR-539 sensitized imatinib resistant GIST48 cells and increased the secretion of TGF- β 1 by inhibiting APOBEC3B and SPAG5.

We investigated whether miR-539 can change the effects of imatinib on GIST48, a imatinib-resistant GIST cell line. We found that miR-539 can significantly sensitized the inhibitory effects of GIST48 cells proliferation induced by imatinib (**Fig.6a**). ELISA was employed to measure the concentration of TGF- β 1 and IL-10 secreted by in the supernatants of GIST48 cells. We found that both miR-539 and imatinib can significantly rise the TGF- β 1 secretion of GIST48 cells, compared with GIST48 cells treated with imatinib only ($p=0.0231$) (**Fig.6b**). miR-539 targeted a site at 3'-UTR of APOBEC3B and SPAG5 mRNA analyzed by bioinformatics. (**Fig.6c**). We identified that APOBEC3B and SPAG5 protein expression was significantly downregulated in miR-539 and imatinib as compared with cells GIST48 cells treated with imatinib only ($p=0.001$) (**Fig.6d**).

Discussion

In the present study, 17 mRNA network modules were identified based on the WGCNA of sequencing data of GIST samples, of which four modules were significantly related to imatinib-resistant. Meanwhile, seven miRNA network modules were identified, of which one module was associated with tumor stage of imatinib-resistant. We found that G1/S transition of mitotic cell cycle, G2/M transition of mitotic cell cycle, and cell proliferation were common pathways of the gene modules and miRNA module. 118 genes such as CDK1, AURKB, AURKA, CD44, HMMR, BIRC5, CCNB1, APOBEC3B, and SPAG5 were both involved in the differentially expressed genes (DEGs) and the markedly positive correlated with imatinib-resistant module.

Previous studies have shown that the specific activities of Cyclin-dependent kinase 1 (CDK1) significantly correlated with recurrence of GIST [12]. Altered expression of AURKB has been observed previously GIST [13]. Aurora kinase A (AURKA) could be a poor risk marker and potential treatment target for metastatic GIST [14, 15]. CD44 cleavage activity was significantly associated with disease progression and poor survival in GIST [16, 17]. Survivin (BIRC5) was associated with high risk and a poor prognosis in GIST [18,

19]. Increased expression cyclin B1 (CCNB1) could predict the proliferative activity of GIST [20, 21]. The Connectivity Map (Cmap) was used to predict small-molecule drugs targeting these gene. JNJ-7706621 had three target genes of CDK1, AURKB and AURKA. Hyaluronic-acid had two target genes of CD44 and HMMR. Terameprocol had two target genes of CDK1 and BIRC5. 1-Azakenpaullone had two target genes of CDK1 and CCNB1. Kenpaullone had two target genes of CDK1 and CCNB. So, these five small-molecule drugs JNJ-7706621, Hyaluronic-acid, Terameprocol, 1-Azakenpaullone, kenpaullone were predicted to have a therapeutic effect against imatinib-resistant GIST.

microRNA-539 (miR-539) might act as a tumor suppressor in various types of human cancers such as bladder cancer [22], renal cell carcinoma [23], pancreatic ductal adenocarcinoma (PDAC) [24] and gastric cancer [25] by targeting high mobility group AT-hook 2(HMGA2), insulin-like growth factor 1 receptor (IGF-1R) or SRY-box 5 (SOX5). microRNA-376b (miR-376b) might act as a tumor promotor in breast cancer [26] and as a tumor suppressor in clinical nonfunctioning pituitary adenomas (CNFPAs) [27] by targeting Hoxd10. microRNA-18b (miR-18b) might act as a tumor promotor in Ovarian cancer (OC) [28], hepatoma [29] and Colorectal Cancer [30] and as a tumor suppressor in Ovarian cancer (OC) [31] and breast cancer [32] by targeting PTEN, NUSAP1,CDKN2B, VMA21, LOXL1-AS1 or MDM2. In the present study, Three hub miRNAs (miR-539, miR-376b and miR-18b) were identified to be involved into imatinib-resistant. Although the role of this three miRNAs (miR-539, miR-376b and miR-18b) in GIST has not been reported yet, the following research supports our results. miRNA-539 overcame arsenic trioxide resistance in hepatocellular carcinoma[33]. The decreased expression of miR-376 family might be related to the tumorigenesis of human glioma[34]. miR-18b played a role as a tumor suppressor in melanoma [35]. In the present study, we found that imatinib-resistant tumor samples exhibited significantly downregulation of miR-539, and overexpression of miR-539 sensitized imatinib resistant GIST48 cells, a imatinib-resistant GIST cell line. These results showed that miR-539 might be involved into imatinib-resistant.

Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3B (APOBEC3B), an antiviral cytidine deaminase that induces DNA mutations, was considered as a medium for cancer development and therapeutic resistance [36]. Recent study have shown that the overexpression of APOBEC3B in tumor increased the resistance to chemotherapy and triggered anti-tumor T cells [37]. Sperm-associated antigen 5 (SPAG5), a new amplification gene of Ch17q11.2, could have clinical utility as a prognostic biomarker and chemosensitivity predictor for breast cancer [38]. In the present study, we found that two hub genes (APOBEC3B and SPAG5) were associated with the imatinib-resistant. Both APOBEC3B [39] and SPAG5 [40] expression were related to drug resistance. We found that mRNA and protein relative expression of APOBEC3B and SPAG5 in imatinib-resistant tumor samples significantly increased as compared with tumor samples in imatinib-naive group. Interestingly, We also found that miR-539 can directly target APOBEC3B and SPAG5, and the previous studies supports our results [41] [42]. However, up to now, the roles of miRNA-539, APOBEC3B and SPAG5 in GIST are still unclear. We found that overexpression of miR-539 sensitized imatinib resistant GIST48 cells by inhibiting APOBEC3B and SPAG5.

Recent studies have shown that infiltrating CD4⁺ T cells attenuate chemotherapy sensitivity in prostate cancer [43]. APOBEC3 family expression correlated with T-Cell infiltration in high-grade serous ovarian

carcinoma [44]. SPAG5 expression level was positively correlated with the infiltration levels of CD8⁺ T cells [45]. In the present study, we found that both the expression of APOBEC3B and SPAG5 was positive correlated with the infiltration levels of activated CD4⁺ T cells, and miRNA-539 was negative correlated with the infiltration levels of activated CD4⁺ T cells. Tumor-derived TGF- β suppresses antitumor function of CD4⁺ T cells [46]. We found that transforming growth factor (TGF)- β 1, known as important immunosuppressive cytokines, were significantly decreased in imatinib-resistant tumor samples. Overexpression of miR-539 sensitized imatinib resistant GIST48 cells and increased the secretion of TGF- β 1 by inhibiting APOBEC3B and SPAG5.

Conclusions

In conclusion, APOBEC3B and SPAG5, the target genes of miR-539, might play as key factor for imatinib-resistant GIST based on the WGCNA of sequencing data. We inferred that low expression of miR-539 in imatinib-resistant GIST might increase the expression of APOBEC3B and SPAG5. And then, APOBEC3B and SPAG5 might increase imatinib resistance in GIST patients by increasing the proportion of tumor-infiltrated activated CD4⁺ T cells.

Abbreviations

APOBEC3B: Apolipoprotein B mRNA editing catalytic polypeptide-like 3B; DEGs: Differentially expressed genes; DEMs: Differentially expressed miRNAs; ELISA: Enzyme-linked immunosorbent assay; EPIC: Estimating the proportion of immune and Cancer cells; FCM: Flow cytometry; GEO: Gene expression omnibus; GIST: Gastrointestinal stromal tumor; SPAG5: Sperm-associated antigen 5; TGF: Transforming growth factor; TILs: Tumor-infiltrating lymphocytes; TISIDB: Tumor-Immune System Interactions; TOM: Topological overlap matrix.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Medical Ethics Committee of Shandong Provincial Qianfoshan Hospital (Approval number: 20191211, China).

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

The authors confirm that all data underlying the findings are fully available without restriction. The original data used in this study was mentioned in the section “Data collection and preprocessing” [GSE132542, GSE69465, GSE51697, and GSE45901]. The secondary datasets used or generated by analysis in this study are available from online supplementary.

Competing interests

The authors declare that they have no competing interests.

Funding

None.

Authors' contributions

Q.Q. Z. participated in the statistical analysis and drafted the manuscript. H. X. W. participated in the design of this study. H. Y. L. and X. M. L. participated in the experiment. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by Yinfeng Gene Technology Co. Ltd. We thank the GEO database for providing data for us in this report.

Authors' Information

1. PM Laboratory, Yinfeng Gene Technology Co. Ltd., 1109 Gangxing 3 Road, Jinan, Shandong Province, 250102, People's Republic of China.
2. Shandong Provincial Qianfoshan Hospital, Shandong University, 16766 Jingshi Road, Jinan 250014, Shandong Province, People's Republic of China.
3. Department of Clinical Laboratory, Qilu Hospital, Shandong University, 107 Wenhua West Road, Jinan 250012, Shandong Province, People's Republic of China.

References

1. Wozniak A, Rutkowski P, Schoffski P, Ray-Coquard I, Hostein I, Schildhaus HU, Le Cesne A, Bylina E, Limon J, Blay JY *et al*. Tumor genotype is an independent prognostic factor in primary

- gastrointestinal stromal tumors of gastric origin: a european multicenter analysis based on ConticaGIST. Clin Cancer Res. 2014;20(23):6105-16.
2. Corless CL, Barnett CM, Heinrich MC. Gastrointestinal stromal tumours: origin and molecular oncology. Nat Rev Cancer. 2011;11(12):865-78.
 3. Gramza AW, Corless CL, Heinrich MC. Resistance to Tyrosine Kinase Inhibitors in Gastrointestinal Stromal Tumors. Clin Cancer Res. 2009;15(24):7510-18.
 4. Amirnasr A, Gits CMM, van Kuijk PF, Smid M, Vriends ALM, Rutkowski P, Sciort R, Schoffski P, Debiec-Rychter M, Sleijfer S *et al*. Molecular Comparison of Imatinib-Naive and Resistant Gastrointestinal Stromal Tumors: Differentially Expressed microRNAs and mRNAs. Cancers (Basel). 2019;11(6).
 5. Zhang JQ, Zeng S, Vitiello GA, Seifert AM, Medina BD, Beckman MJ, Loo JK, Santamaria-Barria J, Maltbaek JH, Param NJ *et al*. Macrophages and CD8(+) T Cells Mediate the Antitumor Efficacy of Combined CD40 Ligation and Imatinib Therapy in Gastrointestinal Stromal Tumors. Cancer Immunol Res. 2018;6(4):434-47.
 6. Yan J, Chen D, Chen X, Sun X, Dong Q, Du Z, Wang T. Identification of imatinib-resistant long non-coding RNAs in gastrointestinal stromal tumors. Oncol Lett. 2019;17(2):2283-95.
 7. Akcakaya P, Caramuta S, Ahlen J, Ghaderi M, Berglund E, Ostman A, Branstrom R, Larsson C, Lui WO. microRNA expression signatures of gastrointestinal stromal tumours: associations with imatinib resistance and patient outcome. Br J Cancer. 2014;111(11):2091-102.
 8. Zeleznik OA, Eliassen AH, Kraft P, Poole EM, Rosner BA, Jeanfavre S, Deik AA, Bullock K, Hitchcock DS, Avila-Pacheco J *et al*. A prospective analysis of circulating plasma metabolites associated with ovarian cancer risk. Cancer Res. 2020.
 9. Michnick SW. The connectivity map. Nat Chem Biol. 2006;2(12):663-4.
 10. Racle J, de Jonge K, Baumgaertner P, Speiser DE, Gfeller D. Simultaneous enumeration of cancer and immune cell types from bulk tumor gene expression data. Elife. 2017;6.
 11. Ru B, Wong CN, Tong Y, Zhong JY, Zhong SSW, Wu WC, Chu KC, Wong CY, Lau CY, Chen I *et al*. TISIDB: an integrated repository portal for tumor-immune system interactions. Bioinformatics. 2019;35(20):4200-02.
 12. Nishida T, Matsushima T, Tsujimoto M, Takahashi T, Kawasaki Y, Nakayama S, Omori T, Yamamura M, Cho H, Hirota S *et al*. Cyclin-Dependent Kinase Activity Correlates with the Prognosis of Patients Who Have Gastrointestinal Stromal Tumors. Ann Surg Oncol. 2015;22(11):3565-73.
 13. Meza-Zepeda LA, Kresse SH, Barragan-Polania AH, Bjerkehagen B, Ohnstad HO, Namlos HM, Wang J, Kristiansen BE, Myklebost O. Array comparative genomic hybridization reveals distinct DNA copy number differences between gastrointestinal stromal tumors and leiomyosarcomas. Cancer Res. 2006;66(18):8984-93.
 14. Yeh CN, Yen CC, Chen YY, Cheng CT, Huang SC, Chang TW, Yao FY, Lin YC, Wen YS, Chiang KC *et al*. Identification of aurora kinase A as an unfavorable prognostic factor and potential treatment target for metastatic gastrointestinal stromal tumors. Oncotarget. 2014;5(12):4071-86.

15. Yen CC, Yeh CN, Cheng CT, Jung SM, Huang SC, Chang TW, Jan YY, Tzeng CH, Chao TC, Chen YY *et al*. Integrating bioinformatics and clinicopathological research of gastrointestinal stromal tumors: identification of aurora kinase A as a poor risk marker. *Ann Surg Oncol*. 2012;19(11):3491-9.
16. Hsu KH, Tsai HW, Shan YS, Lin PW. Significance of CD44 expression in gastrointestinal stromal tumors in relation to disease progression and survival. *World J Surg*. 2007;31(7):1438-44.
17. Hsu KH, Tsai HW, Lin PW, Hsu YS, Shan YS, Lu PJ. Clinical implication and mitotic effect of CD44 cleavage in relation to osteopontin/CD44 interaction and dysregulated cell cycle protein in gastrointestinal stromal tumor. *Ann Surg Oncol*. 2010;17(8):2199-212.
18. Yun S, Kim WK, Kwon Y, Jang M, Bauer S, Kim H. Survivin is a novel transcription regulator of KIT and is downregulated by miRNA-494 in gastrointestinal stromal tumors. *Int J Cancer*. 2018;142(10):2080-93.
19. Chen WB, Cheng XB, Ding W, Wang YJ, Chen D, Wang JH, Fei RS. Centromere protein F and survivin are associated with high risk and a poor prognosis in colorectal gastrointestinal stromal tumours. *J Clin Pathol*. 2011;64(9):751-5.
20. Fujita A, Yamamoto H, Imamura M, Nakamura N, Maehara Y, Tsuneyoshi M, Oda Y. Expression level of the mitotic checkpoint protein and G2-M cell cycle regulators and prognosis in gastrointestinal stromal tumors in the stomach. *Virchows Arch*. 2012;460(2):163-9.
21. Nakamura N, Yamamoto H, Yao T, Oda Y, Nishiyama K, Imamura M, Yamada T, Nawata H, Tsuneyoshi M. Prognostic significance of expressions of cell-cycle regulatory proteins in gastrointestinal stromal tumor and the relevance of the risk grade. *Hum Pathol*. 2005;36(7):828-37.
22. Liao G, Chen F, Zhong J, Jiang X. MicroRNA539 inhibits the proliferation and invasion of bladder cancer cells by regulating IGF1R. *Mol Med Rep*. 2018;17(4):4917-24.
23. Ye ZH, Gui DW. miR539 suppresses proliferation and induces apoptosis in renal cell carcinoma by targeting high mobility group A2. *Mol Med Rep*. 2018;17(4):5611-18.
24. Lin Y, Rong L, Zhao J, Lin R, Li S. MicroRNA539 inhibits cell proliferation, colony formation and invasion in pancreatic ductal adenocarcinoma by directly targeting IGF1R. *Mol Med Rep*. 2018;18(2):1804-11.
25. Ding S, Zhang Y. MicroRNA539 inhibits the proliferation and migration of gastric cancer cells by targeting SRYbox 5 gene. *Mol Med Rep*. 2019;20(3):2533-40.
26. An N, Luo X, Zhang M, Yu R. MicroRNA-376b promotes breast cancer metastasis by targeting Hoxd10 directly. *Exp Ther Med*. 2017;13(1):79-84.
27. Zhu D, Xiao Z, Wang Z, Hu B, Duan C, Zhu Z, Gao N, Zhu Y, Wang H. MEG3/MIR-376B-3P/HMGA2 axis is involved in pituitary tumor invasiveness. *J Neurosurg*. 2020:1-13.
28. Han X, Zhang Y, Wang D, Fu X, Li M, Wang A. Upregulation of microRNA-18b induces phosphatase and tensin homolog to accelerate the migration and invasion abilities of ovarian cancer. *Oncol Lett*. 2017;14(5):5631-37.
29. Yang Z, Li J, Feng G, Wang Y, Yang G, Liu Y, Zhang S, Feng J, Zhang X. Hepatitis B virus X protein enhances hepatocarcinogenesis by depressing the targeting of NUSAP1 mRNA by miR-18b. *Cancer*

30. Li Y, Chen M, Liu J, Li L, Yang X, Zhao J, Wu M, Ye M. Upregulation of MicroRNA 18b Contributes to the Development of Colorectal Cancer by Inhibiting CDKN2B. *Mol Cell Biol.* 2017;37(22).
31. Xue F, Xu YH, Shen CC, Qin ZL, Zhou HB. Non-coding RNA LOXL1-AS1 exhibits oncogenic activity in ovarian cancer via regulation of miR-18b-5p/VMA21 axis. *Biomed Pharmacother.* 2020;125:109568.
32. Li H, Wang Z, Jiang M, Fang RP, Shi H, Shen Y, Cai XL, Liu Q, Ye K, Fan SJ *et al.* The oncoprotein HBXIP promotes human breast cancer growth through down-regulating p53 via miR-18b/MDM2 and pAKT/MDM2 pathways. *Acta Pharmacol Sin.* 2018;39(11):1787-96.
33. Zhu C, Zhou R, Zhou Q, Chang Y, Jiang M. microRNA-539 suppresses tumor growth and tumorigenesis and overcomes arsenic trioxide resistance in hepatocellular carcinoma. *Life Sci.* 2016;166:34-40.
34. Huang Q, Wang C, Hou Z, Wang G, Lv J, Wang H, Yang J, Zhang Z, Zhang H. Serum microRNA-376 family as diagnostic and prognostic markers in human gliomas. *Cancer Biomark.* 2017;19(2):137-44.
35. Dar AA, Majid S, Rittsteuer C, de Semir D, Bezrookove V, Tong S, Nosrati M, Sagebiel R, Miller JR, 3rd, Kashani-Sabet M. The role of miR-18b in MDM2-p53 pathway signaling and melanoma progression. *J Natl Cancer Inst.* 2013;105(6):433-42.
36. Serebrenik AA, Starrett GJ, Leenen S, Jarvis MC, Shaban NM, Salamango DJ, Nilsen H, Brown WL, Harris RS. The deaminase APOBEC3B triggers the death of cells lacking uracil DNA glycosylase. *Proc Natl Acad Sci U S A.* 2019;116(44):22158-63.
37. Driscoll CB, Schuelke MR, Kottke T, Thompson JM, Wongthida P, Tonne JM, Huff AL, Miller A, Shim KG, Molan A *et al.* APOBEC3B-mediated corruption of the tumor cell immunopeptidome induces heteroclitic neoepitopes for cancer immunotherapy. *Nat Commun.* 2020;11(1):790.
38. Abdel-Fatah TMA, Agarwal D, Liu DX, Russell R, Rueda OM, Liu K, Xu B, Moseley PM, Green AR, Pockley AG *et al.* SPAG5 as a prognostic biomarker and chemotherapy sensitivity predictor in breast cancer: a retrospective, integrated genomic, transcriptomic, and protein analysis. *Lancet Oncol.* 2016;17(7):1004-18.
39. Onguru O, Yalcin S, Rosembli C, Zhang PJ, Kilic S, Gunduz U. APOBEC3B expression in drug resistant MCF-7 breast cancer cell lines. *Biomed Pharmacother.* 2016;79:87-92.
40. Yuan LJ, Li JD, Zhang L, Wang JH, Wan T, Zhou Y, Tu H, Yun JP, Luo RZ, Jia WH *et al.* SPAG5 upregulation predicts poor prognosis in cervical cancer patients and alters sensitivity to taxol treatment via the mTOR signaling pathway. *Cell Death Dis.* 2014;5:e1247.
41. Liu Y, Feng J, Sun M, Yang G, Yuan H, Wang Y, Bu Y, Zhao M, Zhang S, Zhang X. Long non-coding RNA HULC activates HBV by modulating HBx/STAT3/miR-539/APOBEC3B signaling in HBV-related hepatocellular carcinoma. *Cancer Lett.* 2019;454:158-70.
42. Zhang H, Li S, Yang X, Qiao B, Zhang Z, Xu Y. miR-539 inhibits prostate cancer progression by directly targeting SPAG5. *J Exp Clin Cancer Res.* 2016;35:60.
43. Xiang P, Jin S, Yang Y, Sheng J, He Q, Song Y, Yu W, Hu S, Jin J. Infiltrating CD4+ T cells attenuate chemotherapy sensitivity in prostate cancer via CCL5 signaling. *Prostate.* 2019;79(9):1018-31.

- ## Figures

Figure 2

Weighted Gene co-expression network analysis (WGCNA) of miRNA. (a) Sample clustering to detect outliers. (b) Correlations between these gene network modules and clinical data were analyzed. (c) The regulatory network of differentially expressed miRNAs (DEMs). (d) The Gene Ontology (GO) functional annotation diagram of target genes of miRNAs.

Figure 3

Associations of miRNA target genes and gene network modules. (a) The Venn diagrams of differentially expressed genes (DEGs) and mRNA in module brown and module yellow of WGCNA. (b) The Venn diagrams of miRNAs in module red and differentially expressed miRNAs (DEMs). (c) Three critical miRNAs target gene regulatory network (miR-539, miR-376b and miR-18b) and the regulatory network of 120 mRNA gene in module brown and yellow were then constructed.

Figure 4

Relationship between the expression of SPAG5/APOBEC3B and immune infiltration. (a) Relative density of 8 types of infiltrated immune cell in imatinib-naive and imatinib-resistant group by EPIC. (b) Correlation of SPAG5/APOBEC3B expression with 28 types of infiltrated immune cell by TISIDB. (c) Correlation of SPAG5/APOBEC3B expression with activated CD4+ T cells by TISIDB.

Figure 5

Clinical correlation between miR-539, TGF- β and infiltrating CD4+ T cells in imatinib-resistant GIST. (a) The comparison of miR-539 expressions in imatinib-naive and imatinib-resistant tumor samples. miR-539 was measured by qRT-PCR. (b) The comparison of SPAG5 and APOBEC3B mRNA expressions in imatinib-naive and imatinib-resistant tumor samples. SPAG5 and APOBEC3B was measured by qRT-PCR. (c) The comparison of SPAG5 and APOBEC3B protein expressions in imatinib-naive and imatinib-resistant tumor samples. SPAG5 and APOBEC3B was measured by WB. Full-length gels are presented in Supplementary Figure 6. A gel imaging system (GelDoc XR+, Bio-Rad, USA) was used to expose and develop the blots. The images was cropped using Adobe Photoshop CC 1.0. The expression normalization was performed with ImageJ software (NIH, Bethesda, MD, USA). (d) Transforming growth factor (TGF)- β and interleukin (IL)-10 of tumor tissue lysates from imatinib-naive and imatinib-resistant tumor patients were assessed with enzyme-linked immunosorbent assay (ELISA). (e) The proportions of CD4+ T cells, CD8+ T cells , NK cells and B cells in tumor-infiltrating lymphocytes from imatinib-naive and imatinib-resistant patients were analyzed via flow cytometry (FCM). (f) The invert expression between miR-539 and infiltrating CD4+ T cells in GIST tumor samples. miR-539 was measured by qRT-PCR. CD4+ T cells was measured by FCM.

Figure 6

Overexpression of miR-539 sensitized imatinib resistant GIST48 cells and increased the secretion of TGF- β 1 by inhibiting APOBEC3B and SPAG5. Control mimic, miR-539 mimic (0.1 μ M), combination of imatinib (0.1 μ M) and control mimic, or combination of imatinib and miR-539 mimic was transfected into GIST48 cells for 48 hours. (a) Cell proliferation were measured at 0, 0.05, 0.1, 0.2 μ M by cholecystokinin octapeptide (CCK8). (b) ELISA was employed to measure the concentration of TGF- β 1 and IL-10 secreted by in the supernatants of GIST48 cells. (c) The predicted miR-539 binding site at 3'UTR of SPAG5 and APOBEC3B analyzed from microRNA.org. (d) The expression of APOBEC3B and SPAG5 was measured by Western blot. Full-length gels are presented in Supplementary Figure 6. A gel imaging system (GelDoc XR+, Bio-Rad, USA) was used to expose and develop the blots. The images was cropped using Adobe Photoshop CC 1.0. The expression normalization was performed with ImageJ software (NIH, Bethesda, MD, USA).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1Table1.xls](#)
- [Additionalfile2Table2.xls](#)
- [Additionalfile3Table3.xls](#)
- [Additionalfile4Table4.xls](#)
- [Additionalfile5Table5.xls](#)
- [Additionalfile6.ppt](#)