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ORIGINAL ARTICLE

Identifying Secondary Mutations in Chinese Patients with Imatinib-Resistant Gastrointestinal Stromal Tumors (GISTs) by Next Generation Sequencing (NGS)

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Abstract

The aim of this study was to characterize secondary kinase mutations in Chinese patients with imatinib-resistant gastrointestinal stromal tumors (GISTs). Mutations in receptor tyrosine kinase (*KIT*; exons 9, 11, 13, 14, 17, and 18) and platelet-derived growth factor-alpha (*PDGFRA*; exons 12, 14, and 18) were analyzed by direct sequencing. After imatinib treatment, 425 cancer-related target genes were analyzed by next generation sequencing (NGS) in imatinib-resistant patients. Correlation of sequencing results with clinicopathologic features were analyzed. We identified 320 patients with secondary acquired resistance. We determined that 65.63% (210/320) of resistant patients had secondary *KIT* mutations in exon 13 ($n = 134$), exon 14 ($n = 10$), or exon 17 ($n = 66$), and 4.38% (14/320) had additional *PDGFRA* mutations in exon 14 ($n = 3$) or exon 18 ($n = 11$). All secondary *KIT* mutations were missense mutations and were mostly located in kinase domains. Ninety-six imatinib-resistant GIST patients did not have secondary *KIT* or *PDGFR*A mutations. Common independent mutation events were found in retinoblastoma protein 1 (*RB1*) (18/96 cases), SWI/SNF-related matrix associated actin-dependent regulator of chromatin subfamily B member 1 (*SMARCB1)* (16/96 cases), and myc-associated factor X (*MAX)* (10/96 cases). *RB1* or *SMARCB1* mutations coexisted with activation of other oncogenes in 6 or 15 cases, respectively. Multiple mutations were also seen in cases with *MAX* mutations. These mutations are frequently associated with clinicopathological factors. Secondary mutations of *KIT*/*PDGFRA* were the most important contributors in GISTs developing resistance to imatinib treatment. Additional genetic events including *RB1*, *SMARCB1*, and *MAX* except secondary KIT/PDGFRA mutations are the most common for GISTs to evolve into resistant disease. Clinical assessment of the effect of these mutations may benefit existing risk assessment models and selection of adjuvant therapies in GIST patients.

Keywords Gastrointestinal stromal tumors · Secondary mutation · Acquired resistance · Next generation sequencing

Introduction

Gastrointestinal stromal tumors (GISTs) are the most common primary mesenchymal tumors of the gastrointestinal tract [1–3]. Most GISTs are associated with mutations in receptor tyrosine kinases (*KIT)* or platelet-derived growth factor receptor alpha (*PDGFRA*). In primary GISTs, approximately 75%–90% mutations are found in *KIT*, with most mutations observed in exon 11, followed by exon 9. Less than 10% of GISTs have *PDGFRA* mutations, and the mutations usually occur in exons 12 and 18 [4–8]. Primary, untreated GISTs harbor only a single mutation either in *KIT* or *PDGFRA* [9–11].

Targeted therapies for GISTs have been developed with tyrosine kinase inhibitors (TKIs). Imatinib, a TKI, has revolutionized the treatment of unresectable, metastatic, and/or recurrent GISTs [2, 12, 13]. Although imatinib improves the prognosis of patients with advanced GISTs, with a median progression-free survival of 2 years and overall survival of 5 years, most patients eventually acquire resistance to the drug. Several mechanisms for acquired imatinib resistance have been proposed, including the evolvement of secondary mutations. Secondary mutations have been found mainly in patients who initially had primary *KIT* mutations, and rarely in those with primary *PDGFRA* mutations [14–16].

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However, in imatinib-resistant patients, there are genomic mutations besides secondary *KIT* or *PDGFRA* mutations. Understanding the biology of how resistance is acquired during the complex progression of GISTs may help researchers find ways to improve life expectancy. New strategies are being investigated to overcome resistance, including new molecules, drug combinations, and the integration of locoregional treatments [17–20].

Over the last decade, advances in next generation sequencing (NGS) technology have enabled simultaneous examination of numerous genes. With the rapid increase in clinical biomarkers available, testing of specific gene mutations in patients is becoming more widespread. The pursuit of identifying additional genetic events involved in GIST progression stems from the heterogeneity of clinical outcomes in patients, suggesting that molecular events other than secondary mutations in *KIT* and *PDGFRA* are also involved in tumor resistance.

In this study, we identified the characteristics of the population with secondary kinase mutations in Chinese patients with imatinib-resistant GISTs. We provide a better understanding of additional aberrations occurring in each tumor, and identify simultaneous molecular events that are responsible for tumor progression that may be new potential drug target candidates.

Material and Methods

Imatinib-Resistant GIST Patient Demographics and Specimens

Surgical or biopsy samples of GIST patients were observed at the First Affiliated Hospital of China Medical University. The pathologists, according to the spindle cells, diagnosed all the cases and described whether epithelioid tumor cells were positive for CD117 and/or DOG-1 by immunohistochemistry (IHC) (Fig. 1). An enhanced computerize tomography (CT) scan was performed every 3 months in the patients, where clinical responses were assessed after the initial imatinib treatment and throughout disease progression or until death. The follow-up period from initial treatment with imatinib ranged between 6.32 to 67.28 months, with a median period of 40.31 months. This study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University and was performed according to the principles of the Declaration of Helsinki. Written informed consents were obtained from the patients in this study.

Immunohistochemistry

IHC analysis was performed using the SP immunohistochemical Kit (Maixin® Biotechnology Co. Ltd., Fuzhou, China) according to the manufacturer's instructions. Serial paraffin-embedded sections (5 μm) were stained with the following primary antibodies: mouse anti-CD117 (1:10; Maixin® Biotechnology Co. Ltd., Fuzhou, China), mouse anti-DOG-1 (1:10; Maixin® Biotechnology Co. Ltd., Fuzhou, China), mouse anti-SMA (1:10; Maixin® Biotechnology Co. Ltd., Fuzhou, China), rabbit anti-S-100 (1:10; Maixin® Biotechnology Co. Ltd., Fuzhou, China), and rabbit anti-Desmin (1:10; Maixin® Biotechnology Co. Ltd., Fuzhou, China). Phosphate buffer saline (PBS) was used as the negative control for each experiment. On the second day, the sections were washed 3 times for 5 min with PBS, and then incubated with the secondary antibody, goat anti-rabbit/mouse IgG (1:100; Maixin® Biotechnology Co. Ltd., Fuzhou, USA) for 1 h at room temperature. Freshly prepared 3,3′-diaminobenzidine (DAB) was applied for 5 min after rinsing off the secondary antibody with PBS.

Evaluation of Imatinib Resistance

Tumor responses and progression were assessed according to the response of GISTs to imatinib by the criteria proposed by Choi et al. [21, 22]. Radiological appearance of resistance was classified into three categories; a nodule within a mass, enlargement or regrowth of a pre-existing mass, or appearance of a new lesion. Primary resistance to imatinib was defined as clinical progression developing within 6 months of initial imatinib treatment. Secondary resistance was defined as patients receiving imatinib treatment for more than 6 months with an initial response of remission or stability, followed by further progression. After imatinib treatment, the lesions in the patients were excised or a biopsy was performed. Patients who were receiving ongoing adjuvant imatinib treatment were excluded from our analyses. Overall, 320 patients with GISTs, who presented disease progression after initial imatinib treatment and presented resistance to imatinib, were enrolled in the study.

KIT and PDGFRA Mutation Analysis

Paraffin-embedded primary GIST tissues from patients before imatinib therapy, with progressed lesions, and after resistance to imatinib were used for genomic DNA analyses. In Eppendorf tubes, 5 to 10 tissue sections (5 μm) were combined, and genomic DNA was extracted using a commercial kit (Tiangen® Biotech Co. Ltd., Beijing, China). Sequencing of *KIT* (exons 9, 11, 12, 13, 14, 17, and 18) and *PDGFRA* (exons 12, 14, and 18) was performed by Sino-MD Gene® Technology Co. Ltd. (Beijing, China). PCR amplification products were purified and sequenced. Gene mutations were determined by comparing wild-type (WT) sequences. Samples that contained mutations were further examined for the presence of WT *KIT* by subcloning the purified PCR products using a TA cloning vector system (Stratagene®, La Jolla, CA, USA). Six independent subclones from each PCR were sequenced using the 3500 Dx Series Genetic Analyzer (Applied Biosystems B.V.®, Singapore).

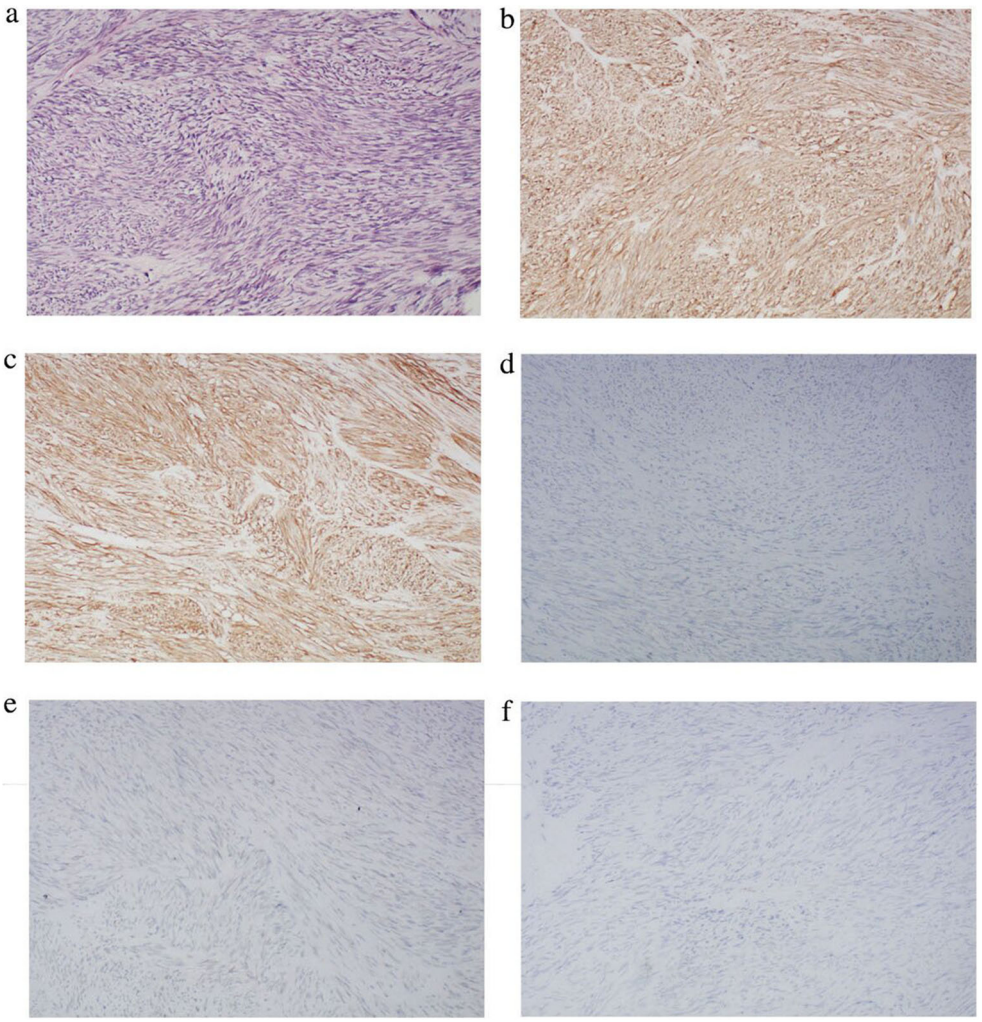

Fig. 1 Histologic and immunohistochemical features of gastric GIST (SP*200) **a** Results of HE staining showing that the neoplastic spindle cells were clustered or swirled. The nucleus was fusiform, both sides of the nuclei were pointed, the cytoplasm was weakly eosinophilic, and the nucleoli were not obvious. The intercellular mass was dominated by inflammatory cell infiltration. Immunohistochemical results showed that CD117 (**b**) and DOG-1 (**c**) were positive in the cell plasma, and SMA (**d**), Desmin (**e**), and S-100 (**f**) proteins were negative

DNA Extraction and Library Construction

DNA was extracted from GIST tissues using the QIAmp DNA Micro kit (Qiagen Inc., Valencia, CA), and quantified with a Picogreen fluorescence assay using the provided lambda DNA standards (Invitrogen). A DNA library was constructed with the KAPA Hyper DNA Library Prep Kit, containing pre-made solutions for end repair, dA addition, and ligation. Experiments were performed in 96-well plates (Eppendorf), and dual-indexed sequencing libraries were amplified by PCR for 4–7 cycles.

Hybrid Selection and Ultra-Deep Next Generation Sequencing of DNA

The 5′-biotinylated probe solution was provided as capture probes, and the target 425 cancer-related genes were used as baits. Mixtures of 5 μg of each DNA-fragment sequencing library, 5 μg of human Cot-1 DNA, 5 μg of salmon sperm DNA, and 1 U adaptor-specific blocker DNA in hybridization buffer were prepared. Samples were heated for 10 min at 95 °C and then for 5 min at 65 °C in the thermocycler. Within 5 min, the capture probes were added to the mixture, and the hybridization step was performed for 16–18 h at 65 °C. After hybridization was complete, the captured targets were selected by pulling down the biotinylated probe/target hybrids using streptavidin-coated magnetic beads. Off-target library components were removed by washing with wash buffer. The PCR master mix was added to directly amplify the captured library from the washed beads (6–8 cycles). After amplification, the samples were purified by AMPure XP beads, quantified by qPCR (Kapa), and sized with a bioanalyzer 2100 (Agilent). Libraries were pooled and normalized to 2.5 nM. Deep sequencing was performed on an Illumina HiSeq 4000 machine using a PE75 V1 Kit. Cluster generation and sequencing was performed according to manufacturer's protocol.

Sequence Alignment and Processing

Base calling was performed using bcl2fastq v2.16.0.10 (Illumina, San Diego, CA) to generate sequence reads in FASTQ format (Illumina 1.8+ encoding). Quality control (QC) was performed with Trimmomatic [23]. High quality

reads were mapped to the human genome (*HG19*, *GRCh37*-Genome Reference Consortium Human Reference 36) using a modified Burrows-Wheeler aligner (BWA) 0.7.12 [24] with BWA-MEM algorithms and default parameters to create sequence alignment map (SAM) files. Picard 1.119 (http://picard.sourceforge.net/) was used to convert SAM files to compressed binary SAM (BAM) files, which were then sorted according to chromosome coordinates. The Genome Analysis Toolkit [25] (GATK, version 3.4–0) was modified and used to locally realign BAM files at intervals with insertion/deletion (indel) mismatches, and to recalibrate base quality scores of reads in BAM files [26].

Detection of SNVs, Indels, and CNVs

Single nucleotide variants (SNVs) and short indels were identified using VarScan2 2.3.9 [22] with the minimum variant allele frequency threshold set at 0.01, and *p* value threshold for calling variants set at 0.05 to generate variant call format (VCF) files. All SNVs and indels were annotated with ANNOVAR, and were each manually verified with Integrative Genomics Viewer (IGV) [27]. Copy number variations (CNVs) were identified using ADTEx 1.0.4 [28].

Statistical Analysis

Statistical analysis was performed using SPSS13.0 (IBM®, New York, USA). Chi-square tests were performed, where *p*-values <0.05 were considered statistically significant.

Results

Clinical Characteristics of Patients with GISTs Exhibiting Imatinib-Resistance

Characteristics of 320 patients with resistant GISTs are summarized in Table 1. There were 152 male and 168 female patients. The median age was 55 years-old, with the ages ranging between 27 to 69 years-old. The tumor sizes were divided into 4 groups; less than 2 cm (81 cases), 2 to 5 cm (90 cases), 5 to 10 cm (105 cases), and more than 10 cm (44 cases). There were 175 cases that showed increased mitosis (>5/50 high power fields, HPF). Radiological examinations revealed that the tumors primarily appeared as nodules in 92 patients, as an enlargement or regrowth of the pre-existing mass in 104 patients, and as new lesions in 124 patients.

Genotypes of Secondary KIT and PDGFRA Mutations in Imatinib-Resistant GISTs

Among the 320 patients with imatinib-resistant GISTs, secondary *KIT* and *PDGFRA* gene mutations were observed in 210 and 14 patients, respectively. As shown in Table 2, all 210 samples with secondary *KIT* mutations were missense mutations found in either kinase domain I or II. In kinase domain I (exons 13 and 14), mutations were limited to codon 654 (V654A in 134 lesions), and codon 670 (T670I in 10 lesions). In kinase domain II (exon 17), various missense mutations were found in exon 17 with several different codons (Y823D, C809G, D816H, N822K, N822Y, A829P, and

Table 1 Clinical characteristics of the imatinib-resistant GIST patients

Characteristics	No. of patients ($N = 320$)	% of patients
Gender		
Male	152	47.50%
Female	168	52.50%
Age (years)		
Median age	55	
Range	27~69	
Tumor dimension (cm)		
≤2	81	25.31%
>2, ≤5	90	28.13%
>5, ≤10	105	32.81%
>10	44	13.75%
Mitoses(/50HPF)		
≤5	145	45.31%
>5	175	54.69%
Appearance of resistance		
Nodule within a mass	92	28.75%
Enlargement or regrowth of a pre-existing mass	104	32.50%
Appearance of a new lesion	124	38.75%

Table 2 Secondary *KIT* and *PDGFRA* mutation genotypes in imatinib-resistant GISTs

Secondary *KIT* mutation site	No. of patients	Mutations type	% of patients
KIT gene ($N = 210$)			
Exon 13	134	V654A	134 (100%)
Exon 14	10	T670I	10 (100%)
Exon 17	66	Y823D	15 (22.73%)
		C809G	3 (4.55%)
		D816H	10 (15.15%)
		N822K	29 (43.94%)
		N822Y	3 (4.55%)
		A829P	2 (3.03%)
		D820Y	4 (6.06%)
PDGFRA gene ($N = 14$)			
Exon 14	3	H687Y	3 (100%)
Exon 18	11	D842V	11 (100%)

D820Y, corresponding with 15, 3, 10, 29, 3, 2, and 4 lesions, respectively). In the 14 samples with secondary *PDGFRA* mutations, 3 had H687Y mutations in exon 14, and 11 had D842V mutations in exon 18.

We further compared the primary and secondary mutation sites in these 224 imatinib-resistant GISTs. After imatinib treatment, three types of clonal or polyclonal evolution were observed in *KIT*, including primary *KIT* mutations transforming into multiple *KIT* mutations or secondary *PDGFRA* mutations. With multiple *KIT* mutations, in addition to the primary mutations in exon 9 and exon 11, tumors harbored secondary mutations in *KIT* exon 13, exon 14, and exon 17. As shown in Table 3, the most commonly identified secondary mutation was in exon 13. Of the 14 samples with secondary *PDGFRA* mutations, 4 had secondary mutations in exon 18 (primary mutation in exon 12) and 10 had *PDGFRA* mutations. These included 2 cases of primary *KIT* mutations in exon 9, and 8 cases of primary *KIT* mutations in exon 11.

Genetic Mutations Detected by NGS in Imatinib-Resistant Cases without Secondary KIT and PDGFRA Mutations

There were 96 imatinib-resistant patients with GISTs that did not possess secondary *KIT* or *PDGFRA* mutations. Figure 2 shows the mutations detected in these cases. Overall, the most common mutations were found in retinoblastoma protein 1 (*RB1*) (18 cases), SWI/SNF-related matrix associated actin-dependent regulator of chromatin subfamily B member 1 (*SMARCB1*) (16 cases), and myc-associated factor X (*MAX*) (10 cases). The less common mutations were found in *MCL1* (6 cases), *RICTOR* (6 cases), *GNAS* (6 cases), *IL7R* (6 cases), *ARID1A* (4 cases), *CDKN* (4 cases), *IGF2* (4 cases), *VEGFA* (4 cases), *CCNE1* (4 cases), *MYC* (4 cases), *NF1* (4 cases), *PTEN* (4 cases), *ATR* (4 cases), and *DNMT3A* (4 cases). Additionally, there were occasional mutations seen in *EGFR, ATM, BAI3, DDR2,* and *ERCC4*.

Table 3 Comparison of the primary and secondary mutation sites in imatinib-resistant GIST

Primary Status → Secondary Status ($N = 224$)	No. of patients
KIT mutation	210
Exon 9→ Exon 9 + 13	8
Exon 9 → Exon 9 + 17	7
Exon 11 → Exon 11 + 13	126
Exon 11 → Exon 11 + 14	10
Exon 11 → Exon 11 + 17	59
PDGFRA mutation	14
KIT Exon 9 → PDGFRA Exon 14	1
KIT Exon 11 → PDGFRA Exon 14	2
KIT Exon 11 → PDGFRA Exon 18	6
PDGFRA Exon 12 → PDGFRA Exon 12 + 18	5

Characteristics of Imatinib-Resistant Cases where the most Common Mutations Are Not Secondary KIT or PDGFRA Mutations

Detected mutations in *RB1*, *SMARCB1*, and *MAX* were independent of *KIT* or *PDGFRA* mutations in these cases. Some cases (6/18) in the patients with *RB1* mutations (Fig. 3), and almost all cases with *SMARCB1* mutations were present with other mutations (Fig. 4). In cases with *MAX* mutations, there were no other mutations present. These patient characteristics

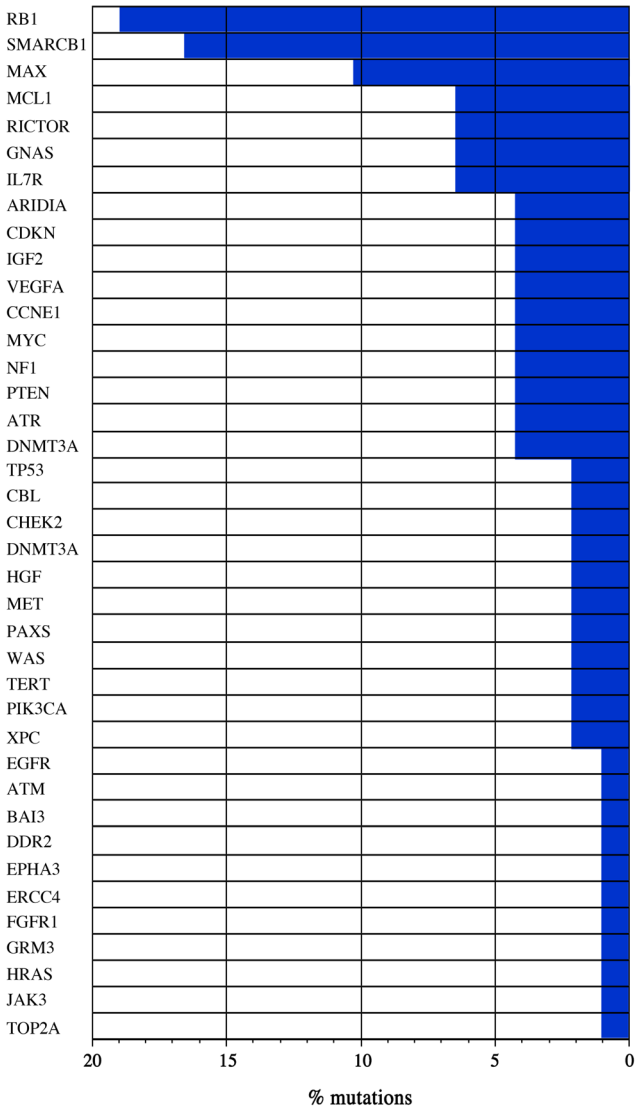

Fig. 2 Molecular findings in quadruple-negative cases by next-generation sequencing The mutation frequency of *RB1*, *SMARCB1* and *MAX* were 18.75%, 16.67% and 10.42%, respectively. Following, *MCL1* (10.42%), *RICTOR* (6.25%), *GNAS* (6.25%), *IL7R* (6.25%), *ARID1A* (4.17%), *CDKN* (4.17%), *IGF2* (4.17%), *VEGFA* (4.17%), *CCNE1* (4.17%), *MYC* (4.17%), *NF1* (4.17%), *PTEN* (4.17%), *ATR* (4.17%), and *DNMT3A* (4.17%) were found in these cases. Lower frequency in mutation events was found for TP53 (2.08%), CBL (2.08%), and others. The lowest mutations were shown in *EGFR, ATM, BAI3,* and others at 1.04% frequency

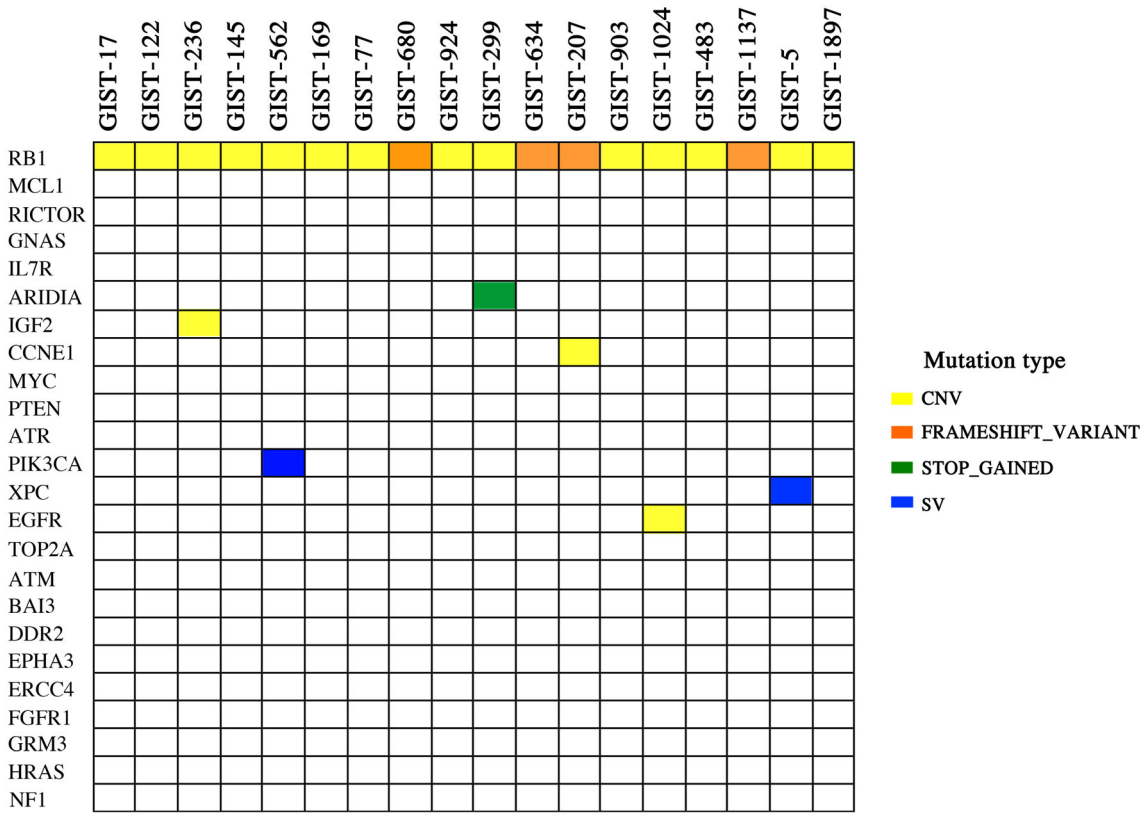

Fig. 3 There are six cases in the imatinib-resistant GISTs with *RB1* were accompanied by additional multiple mutations

are summarized in Table 4. We noted a significantly higher association of mutations in *SMARCB1* or *RB1* with increased tumor size and mitoses. We also noted that mutations in *MAX* were associated with smaller tumors that were less than 2 cm. Additionally, mutations in *SMARCB1* or *RB1* were associated with cases with enlarged lesions or regrowth of pre-existing masses (77.78% and 75.00%, respectively). *MAX* mutations were always associated with the appearance of new lesions (70.00%).

Discussion

Secondary *KIT* mutations contribute to acquired resistance in most cases of GISTs [29–31]. Our present study revealed that secondary KIT mutations were found in most cases of *KIT*-mutant GISTs with acquired resistance to imatinib treatment.

In 65.63% (210/320) of resistant patients with *KIT* secondary mutations, most acquired secondary mutations were preferentially located in tyrosine kinase domain I or II of *KIT*. Domain I is primarily composed of exons 13 and 14, which encode the drug/ATP binding pocket of the receptor. Domain II is composed of exons 17 and 18, which form the kinase activation loop [32–34]. In 210 patients, secondary kinase mutations were significantly more common in exon 13, but not in those with the primary mutation. Secondary mutations observed in the ATP-binding domain are limited to V654A and T670I, and the substitution of these residues induces substantial modifications in the conformation of the kinase domain [35–37]. In addition, V654 interacts with the diaminophenyl ring of imatinib, and changes in this amino acid to an alanine (V654A) reduces the binding affinity. T670I acts as the gate-keeper mutation, causing steric hindrance for imatinib binding [38–40]. Compared to the ATP-binding domain, mutations in the activation loop are variable, which may destabilize the inactive conformation by introducing charged side chains into the binding pocket.

In contrast to secondary *KIT* kinase mutations, secondary *PDGFRA* kinase mutations are much less common in imatinib-resistant GISTs. The most common *PDGFRA* mutation was D842V found in exon 18. These cases suggested that mutant *PDGFRA* yields oncogenic signals similar to those of mutant *KIT*.

No secondary *KIT* or *PDGFRA* mutations were found in 96 imatinib-resistant lesions, demonstrating that some resistant GISTs were independent of *KIT* and *PDGFRA*. Therefore, other recurrent causes of TKI resistance, such as acquired mutations of downstream signaling effectors, were also identified in the present study. The most common events were found in *RB1* (18 cases), *SMARCB1* (16 cases), and *MAX* (10 cases).

Mutations of the cell cycle inhibitor RB1 ranked as the most common genomic mutation events in the acquired

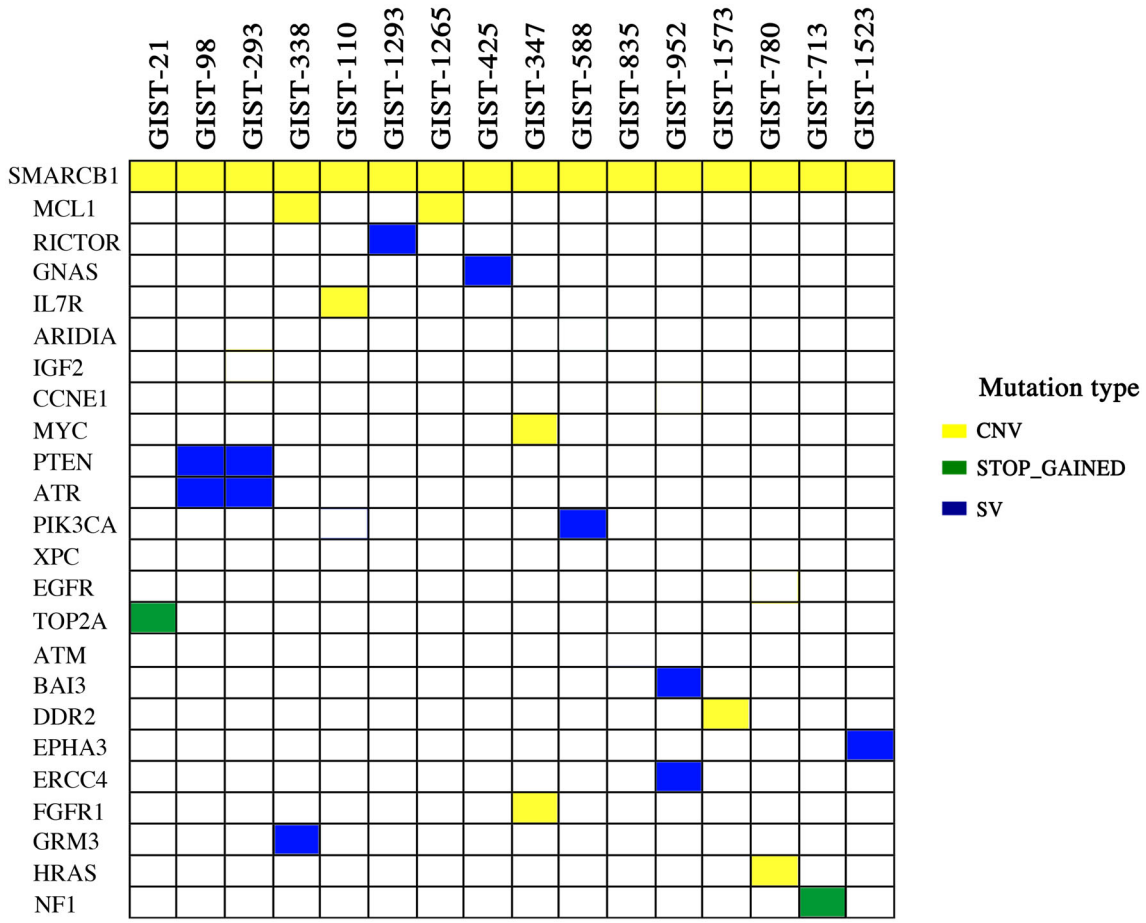

Fig. 4 Almost all cases were present with other mutations in the resistant GISTs with *SMARCB* mutation

resistance group, and few of the cases showed additional mutations. *RB1* mutations contribute to cell proliferation, and play and important role in tumor progression [41, 42]. Consistent with prior sequencing studies of cell cycle regulator genes in GISTs, we also found that *RB1* mutations were associated with increased risk of recurrence. Our study also provides evidence that *RB1* mutations contribute to the recurrence and progression of GISTs.

Notably, *SMARCB1* mutations were the second most common type to contribute to resistance in GISTs. SMARCB1, a core subunit of the SWI/SNF complex, is a known tumor suppressor, and its loss is associated with rhabdoid tumor onset [43–45]. In our study, *SMARCB1* mutations were significantly enriched in GISTs with larger tumor sizes and increased mitoses. Several pathways are regulated through SMARCB1, such as chromatin remodeling, cyclin D1/CDK4 activation, and WNT/β- catenin, possibly explaining why most of them showed an additional mutation [46–50]. In addition, SMARCB1 interacts with GLI molecules and, through its removal, the Hedgehog (Hh) pathway is activated. Activation of this pathway has been implicated in the development of various cancers, and several activity-modulating molecules have been developed and studied [51–53]. GIST-like mouse models triggered by the inactivation of patched 1 (PTCH1) suggest that Hh could also have a role in GIST biology [54]. These findings indicate that GIST progression and malignancy can be comprehensively explained by aberrant *SMARCB1*-related pathways.

We also identified mutations in *MAX* in 10 cases. No additional mutations were observed in cases with *MAX* mutations. MAX is a basic helix-loop-helix leucine zipper (bHLHZ) transcription factor and is an essential binding partner of MYC. MYC is involved in regulating cell proliferation, differentiation, and apoptosis through heterodimerization with MYC-family proteins. However, MAX homodimers can also regulate transcription in a MYC-independent manner [55–58]. Most *MAX* mutations were associated with smaller GISTs, or new lesions, suggesting that MAX is involved in the early stages of GIST development. These findings suggest that MAX mutation causes cell cycle dysregulation at an early point in GIST progression, probably enabling progression to GIST stages with greater proliferative potential. Altogether, our studies demonstrate frequent disruption of MAX in early progression of *KIT*-mutant GISTs by promoting cell cycle dysregulation, ultimately contributing to increased GIST formation and transition to more advanced cancer.

Table 4 Characteristics of imatinib-resistant cases with the most common mutation events

Characteristics	RB1 ($N = 18$) No. of patients (%)	SMARCB1 ($N = 16$) No. of patients (%)	MAX ($N = 10$) No. of patients (%)
Gender			
Male	6 (33.33%)	8 (50.00%)	6 (60.00%)
Female	12 (66.67%)	8 (50.00%)	4 (40.00%)
Age (years)			
Median age	45	58	39
Range	41~52	30~67	35~50
Tumor dimension (cm)			
≤ 2	0 (0%)	1 (6.25%)	8 (80.00%)
> 2, ≤5	2 (11.11%)	3 (18.75%)	2 (20.00%)
> 5, ≤10	11 (61.11%)	9 (56.25%)	0 (0%)
> 10	5 (27.78%)	3 (18.75%)	0 (0%)
Mitoses(/50HPF)			
≤ 5	3 (16.67%)	5 (31.25%)	9 (90.00%)
> 5	15 (83.33%)	11 (68.75%)	1 (10.00%)
Appearance of resistance			
Nodule within a mass	4 (22.22%)	3 (18.75%)	3 (30.00%)
Enlargement or regrowth of a pre-existing mass	14 (77.78%)	12 (75.00%)	0 (0%)
Appearance of a new lesion	0 (0%)	1 (6.26%)	7 (70.00%)

These mutations may be useful as prognostic biomarkers, or as predictive markers to identify patients that stand to benefit the most from adjuvant imatinib therapy. Mutations in other genes, such as *TP53*, *CBL*, *CHEK2*, *DNMT3A*, and *HGF*, are rarely involved in GIST resistance to imatinib. Our findings provide a more comprehensive molecular study of GIST resistance, demonstrating the effect of *KIT*-mutant GISTs with accompanying mutations on tumor progression. These results improve our understanding of genomic aberrations and processes that drive GIST tumorigenesis, tumor progression, and malignancy.

Patients with intermediate to high risk GISTs are clinically challenging. There are currently no reliable biomarkers to identify patients in this category that have higher risk of relapse and might benefit from adjuvant imatinib therapy. Our observations also suggest that assessment of mutation events in *RB1*, *SMARCB1*, and *MAX* may be a useful addition to existing risk assessment models to identify patients that will benefit from adjuvant treatment and intensified surveillance schedules.

Conclusions

Our study showed that secondary *KIT* and *PDGFRA* mutations were major contributors to the development of resistance to imatinib treatment. Additional genetic mutation events in *RB1*, *SMARCB1*, and *MAX* that were independent of *KIT* and *PDGFRA* mutations contributed the most to the evolution of GIST resistance. GISTs that resulted from different genetic alterations presented different clinicopathological features. Identifying these genetic mutations may be a useful addition to existing risk assessment models to identify patients that will benefit from adjuvant treatment. This study presents new potential therapeutic targets to improve disease progression and survival, beyond the established treatments with *KIT*/*PDGFRA*-inhibitors imatinib, sunitinib, and regorafenib. Future studies to identify patient subsets that benefit most from long-term adjuvant TKI therapy are expected to further improve individualized treatment options.

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Authors' Contributions Conceived and designed the experiments: Jiang Du, Yang Liu. Performed the experiments: Si Wang, Rui Wang and Si-Yao Wang. Analyzed the data: Qiang Han, Hong-Tao Xu and Peng Yang. Contributed reagents/materials/ analysis tools: Jiang Du, Hong-Tao Xu, and Yang Liu. Wrote the paper: All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

Ethics Approval and Informed Consent This study was approved by the Ethics committee of the First Affiliated Hospital of China Medical University and was performed according to the principles of the Declaration of Helsinki. Written informed consents were obtained from the patients in this study.

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