Genetic alterations in cell cycle regulation-associated genes may promote primary progression of gastrointestinal stromal tumors

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Abstract

Gastrointestinal stromal tumors (GISTs) are one of the most common mesenchymal tumor types and usually contain *KIT* or *PDGFRA* mutations. GISTs with concomitant low- and high-grade components are seen in clinical practice. Herein, we retrospectively analyzed the histological characteristics and immunohistochemical results of 22 GIST cases with concomitant low- and high-grade tumors. Whole-exome sequencing (WES) was performed on ten pairs of high-grade GIST specimens and matched low-grade samples. Differential oncogenes mutated only in high-grade GISTs were identified, which were confirmed by Sanger sequencing. Fluorescence in situ hybridization was employed to detect *MYC* copy number variation. High-grade GISTs were more likely to have lower CD34 expression and a higher Ki-67 proliferation index compared to the matched low-grade tumors. WES identified 30 differential cancer-associated genes mutated only in high-grade GISTs; Sanger sequencing confirmed ten relevant differential oncogenic mutations in nine genes (*MGA*, *ARID1A*, *LATS2*, *MAX*, *PIK3CA*, *RBI*, *RPS6KB2*, *SDHA*, and *SETD2*). Two patients had *MGA* mutations, whereas other gene mutations occurred in only one patient. Most of the differential cancer-associated genes are mainly involved in cell cycle control. *MYC* copy number gain was a common genetic variation. High-grade GISTs revealed more *MYC* copy number gains than matched low-grade tumors, and low-grade GISTs with coexisting high-grade components showed more *MYC* copy number gains than pure low-grade GISTs. Moreover, *MYC* copy number gain was positively correlated with the mitotic index and Ki-67 proliferation index. Alterations in cell cycle regulation-associated genes, such as genetic mutations and *MYC* copy number gain, may promote primary progression from low-grade GISTs to high-grade tumors by regulating tumor cell proliferation.

Introduction

Gastrointestinal stromal tumors (GISTs), which are the most common mesenchymal tumors in the digestive tract, span a biological behavior spectrum that ranges from benign to

malignant. Activating mutations in the *KIT* or *PDGFRA* (platelet-derived growth factor A) genes are frequently identified in most GISTs. It is estimated that these mutations result in structural changes to tyrosine kinase receptors and ligand-independent constitutive autoactivation of kinases, which function in signal transduction pathways that promote proliferation and inhibit apoptosis; this in turn results in GIST tumorigenesis [1]. In addition, most *KIT*/*PDGFRA* wild-type GISTs usually have loss of function of succinate dehydrogenase (SDH) or mutations in the *BRAF*/*KRAS* or *NF1* genes [2, 3]. With advances in comprehensive genomic profiling, many novel molecular events have been described in GISTs that lack or feature *KIT*/*PDGFRA* mutations, such as mutations in *PIK3CA*, *MEN1*, *MAX*, and *ETV6-NTRK3* fusion [4–6]. Some mutations are related to the progression of GIST toward a tumor with more aggressive biological behavior. *PIK3CA* mutations have been detected in large or metastatic *KIT*-mutant GISTs, which suggest that *PIK3CA*-mutant clones

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have a proliferative advantage during disease progression [4]. *SETD2* mutations have been identified only in high-risk/metastatic GISTs, and assessing *SETD2* genetic status has been recommended to guide risk stratification [7]. However, most of these studies of large sample sizes were based on individual cases of either low- or high-risk GIST, and thus a wide comprehensive genomic study of paired samples of different GIST grades from the same cases is lacking.

In this study, for the first time, we identified differential mutations in a series of GISTs with concomitant low- and high-grade tumors via whole-exome sequencing (WES) and Sanger sequencing and analyzed the potential driver molecular abnormalities. Nine differential cancer-associated genes that were mutated only in high-grade GISTs were identified, most of which are mainly involved in cell cycle control. Mutations in *MGA* and *MAX* were detected in three high-grade GISTs, which suggest that the *MGA/MAX/MYC* network plays a role in tumor progression. Furthermore, we determined the *MYC* gene status using fluorescence in situ hybridization (FISH). Mutations in cell cycle regulation-associated genes and *MYC* copy number gain may promote cell proliferation and result in the progression of GISTs.

Materials and methods

Patients and tumor samples

We re-reviewed the histological characteristics and immunohistochemical results of 1183 GIST cases from January 2012 to June 2018 in the archived pathology database of Xijing Hospital (Xi'an, China) and identified 22 GIST cases with concomitant low- and high-grade components. In addition, *MYC* gene status was determined in 86 cases of pure low- or high-grade GIST. By definition, the low-grade component contains a population of spindle cells without marked cellular atypia and hypercellularity and ≤ 5 mitoses per 50 high-power fields (HPF) (a total area of 5 mm²). In contrast, the high-grade component consists of spindle or epithelioid cells with obvious pleomorphism or atypia and hypercellularity and with > 5 mitoses per 50 HPF. GISTs with concomitant low- and high-grade tumors contain two morphologically different neoplastic components, which either gradually or abruptly transition to one another; these components also show dramatic differences in mitotic activity. To distinguish these two coexisting components more strictly, the mitotic index of the high-grade tumor was found to exceed that of the low-grade tumor by ≥ 5 mitoses per 5 mm². Written informed consent was obtained from each participant, and the study was approved by the Xijing Hospital institutional ethical committee. This study was performed in accordance with the principles embodied in the Declaration of Helsinki.

Whole-exome sequencing

We selected 22 clinical cases of GIST with concomitant low- and high-grade components, and of these, ten pairs of high-grade tumor specimens and matched low-grade samples had sufficiently high DNA quality for WES. Hematoxylin-eosin (HE)-stained tumor tissue sections were used as a guide for localizing the high- or low-grade tumors. The targeted neoplastic tissues containing a minimum of 85% tumor nuclei were manually microdissected from unstained, 10- μ m-thick formalin-fixed, paraffin-embedded (FFPE) sections. All 20 GIST specimens were obtained from primary tumors, and the patients from which they were derived had not undergone radiation, chemotherapy, or targeted therapy with tyrosine kinase inhibitors prior to surgery. Genomic DNA was extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's recommendations. Hybridization-captured, adapter ligation-based libraries were synthesized using an Agilent SureSelect Human All Exon Kit (Santa Clara, CA, USA), which was designed to enrich 334,378 targeted exonic regions of 20,965 genes. The WES libraries were then amplified and quality-checked, and Illumina HiSeq Platform sequencing (Novogene Bioinformatics Technology Co. Ltd, Beijing, China) was performed. The average sequencing depth of the target regions was $> 100\times$.

Genomic analysis

After raw data generation by base calling, the paired-end reads were trimmed with AdapterRemoval (<http://adapterremoval.readthedocs.io/>) to remove stretches of low-quality bases ($< Q10$) and adapters in the sequences. The paired-end reads were then aligned on the human reference genome GRCh37 (hg19) using the Burrows-Wheeler Aligner (<http://bio-bwa.sourceforge.net/>) and Samblaster (<https://github.com/GregoryFaust/samblaster>). Variant calling was performed using the Genome Analysis Toolkit (<https://software.broadinstitute.org/gatk/>) and SAMtools (<http://www.htslib.org/>). To maximize sensitivity in heterogeneous GIST specimens, the test was validated to detect base substitutions and short insertions and deletions (indels) with a threshold depth of coverage $> 5\times$ and a variant allele frequency $\geq 10\%$. Furthermore, point mutations and small indels in genes were annotated for their functional effect on their respective proteins via ANNOVAR (Version 2013Aug23) (<http://www.openbioinformatics.org/annovar/>). Nonsynonymous and nonsense single-nucleotide variations (SNVs), frameshift/non-frameshift indels, and splice region mutations were selected. Variants present in the Exome Aggregation Consortium (<http://exac.broadinstitute.org/>) or 1000 Genomes databases (<https://www.1000genomes.org/>) with a frequency $> 1\%$,

which indicated potential germline variants, were excluded from the analysis. All indels and SNVs that were predicted to be deleterious by at least one of four prediction tools (SIFT, PolyPhen-2_HDIV, MutationTaster, CADD) were included in further analyses. Potential candidate drivers were determined according to the Catalog of Somatic Mutations in Cancer (COSMIC, <http://cancer.sanger.ac.uk/census>), MDG125 [8], SMG127 [9], CDG291 [10], and published references [11]. Similar and differential mutations in cancer-associated genes were obtained by Venn diagram analyses of high- and low-grade tumors. To identify the over-represented pathways, gene set enrichment analysis was performed using web-based OmicShare KEGG (Kyoto Encyclopedia of Genes and Genomes) Enrichment Analysis (<http://www.omicshare.com/tools/>), and related references were reviewed for mutated genes not included in the data.

Sanger sequencing

To validate the relevant differential oncogenic mutations detected only in high-grade GISTs by WES, PCR (polymerase chain reaction) primers were designed according to the mutated sites using Primer Premier 5.0 software. DNA fragments flanking the putative mutations were amplified over 39 cycles in a 50 μ l reaction mixture, which contained 45 μ l Golden Star T6 Super PCR Mix (Tsingke, Xi'an, China), 4 μ l forward and reverse primers, and 1 μ l template genomic DNA per reaction. The PCR products were purified with a Tsingke PCR Purification Kit and were sequenced for low- and high-grade tumors with a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Monza, Italy) in an ABI 3730xl Genetic Analyzer (Applied Biosystems). To improve sequencing accuracy and to exclude analytical errors, all sequencing analyses were carried out on both strands and were repeated on the PCR products. The alignments of the high- and low-grade tumor sequencing data and the reference sequence were compared using SnapGene (version 2.3.2).

Fluorescence in situ hybridization

To verify the status of *MYC* copy number variation (CNV), FISH was performed on GIST samples using a *MYC* (8q24) probe (Ambiping Pharmaceutical Technology Co. Ltd., Guangzhou, China) according to the manufacturer's instructions. Copy number gain was determined above a 15% threshold with more than two red signals per nuclei.

Statistical analysis

The Wilcoxon signed rank test was used to compare the *MYC* copy number gain and immunohistochemical expression between high-grade and low-grade tumors in

cases with both components. The Mann-Whitney U test and the Spearman rank correlation test were used to analyze the relationship between *MYC* copy number gain and tumor features in pure low- or high-grade GIST.

Results

Clinicopathological features of GISTs with concomitant low- and high-grade components

Seventeen (77.3%) of the 22 patients were male. The age at the time of diagnosis ranged from 37 to 77 years, and the average age was 54.9 ± 9.4 years (median, 56 years). The age distribution was similar to that of general GISTs. The tumor was located in the stomach in five cases, in the small intestine in 16 cases, and in the colon in one case. All patients had a maximum tumor diameter ≥ 4 cm (range, 4–18 cm), and nine cases (40.91%) had multiple nodules. Single or multiple metastases were found during surgery in six patients. The proportion of high-grade components in whole tumors of each patient was 10–85%. The risk stratification of the tumors was assigned based on location, size, and mitotic index in the high-grade component; therefore, all GISTs were high-risk or very high-risk tumors (Table 1).

Microscopically, two tumor components with different cell densities and cell morphologies were present in the same nodule or in different nodules (Fig. 1a). Low-grade GISTs usually exhibited a predominantly spindle cell morphology, a lower cell density and distinct extracellular collagen fibers with lower mitotic activity (mitotic counts were 1–2 per 50 HPF) (Fig. 1b). High-grade GISTs frequently showed rhabdoid to epithelioid histology and a significantly higher cell density that revealed sarcomatoid features with significant nuclear atypia and pleomorphism, and even multinucleation (Fig. 1c, d). Small focal or massive necrosis was present in 17 cases of high-grade GIST (Fig. 1e). The mitotic index in the high-grade tumors was 6–90 per 50 HPF, which was significantly increased compared with the low-grade components ($P = 0.000$) (Table 1).

Most of low- and high-grade GISTs demonstrated diffuse and strong immunohistochemical reactivity for CD117 and DOG1, and there was no significant difference in expression between the two tumor components (Fig. S1). Compared with the matched low-grade GISTs, 14 patients had lower CD34 expression in high-grade tumors ($P = 0.000$), and of these, five cases showed diffuse strong positivity in low-grade tumors and negativity in high-grade tumors (Fig. 2a, b). Eight patients had similar CD34 expression in the low- and high-grade components, among which five cases were diffusely positive and one case was negative. The high- and low-grade tumors had significantly different Ki-67

Table 1 The clinicopathological characteristics of 22 GISTs with concomitant low-grade and high-grade tumors

No	Age	Sex	Location	Tumor focus	Metastasis	The maximum diameter (cm)	Prognostic group			High-grade tumor			Low-grade tumor					
							Morphology	Mitotic rate per 50 HPF	Ki-67 index	CD34	Morphology	Mitotic rate per 50 HPF	Ki-67 index	CD34	Morphology	Mitotic rate per 50 HPF	Ki-67 index	CD34
1	49	Male	Duodenum	Single		11	6b	Epithelioid and spindle	73	35%	–	Spindle	1	3%	+++			
2	77	Female	Colon	Single		12.3	6b	Spindle	15	35%	+++	Spindle	2	8%	+++			
3	52	Female	Stomach	Multiple	Liver	12	6b	Spindle	44	25%	++	Spindle	2	12%	+++			
4	61	Female	Duodenum	Single		16	6b	Spindle	6	20%	–	Spindle	1	3%	+			
5	39	Male	Duodenum	Multiple	Liver	10.6	6b	Spindle	8	15%	+	Spindle	1	2%	+++			
6	61	Female	Small intestine	Multiple		7.5	6a	Spindle	8	25%	++	Spindle	1	2%	++			
7	62	Male	Duodenum	Multiple		9.5	6a	Epithelioid	25	30%	–	Spindle	1	4%	++			
8	57	Male	Small intestine	Multiple	Intra-abdominal, pelvic cavity	10.7	6b	Spindle	10	30%	+++	Spindle	1	5%	+++			
9	64	Male	Small intestine	Single		5.5	6a	Epithelioid	10	20%	+	Spindle	1	5%	+++			
10	45	Male	Jejunum, sigmoid colon	Multiple	Abdominal wall	13.5	6b	Epithelioid	8	30%	–	Spindle	1	8%	++			
11	54	Male	Small intestine	Multiple	Liver	15.5	6b	Spindle and epithelioid	38	40%	–	Spindle	2	5%	–			
12	61	Male	Stomach	Single		7.5	6a	Spindle	46	35%	+++	Spindle	1	5%	+++			
13	65	Male	Small intestine	Multiple		18	6b	Spindle and epithelioid	16	25%	–	Spindle	1	3%	+++			
14	59	Male	Small intestine	Single		5	5	Spindle	17	30%	–	Spindle	2	3%	+++			
15	58	Male	Stomach	Single		6	6a	Spindle	25	20%	+++	Spindle	2	5%	+++			
16	48	Male	Small intestine	Multiple	Pelvic cavity, abdominal wall	9	6a	Spindle	22	20%	–	Spindle	1	2%	+++			
17	55	Male	Duodenum	Single		15	6b	Spindle	6	15%	–	Spindle	1	5%	++			
18	46	Female	Duodenum	Single		8	6a	Spindle	8	15%	++	Spindle	2	3%	++			
19	37	Male	Duodenum	Single		4	5	Epithelioid	6	20%	+	Spindle	2	6%	+++			
20	44	Male	Stomach	Single		4	5	Spindle	9	20%	+++	Spindle	1	4%	+++			
21	54	Male	Stomach	Single		8	6a	Spindle	90	50%	+++	Spindle	1	7%	+++			
22	60	Male	Small intestine	Single		7.5	6a	Spindle	7	15%	+	Spindle	1	3%	+++			

– negative, + weakly positive, ++ moderately positive, +++ strongly positive

Fig. 1 Pathological characteristics of GISTs with coexisting low- and high-grade components. **a** Two tumor components with different cell densities were noticeably separated by smooth muscle tissue (hematoxylin-eosin staining (HE), $\times 6$). **b** The low-grade component displayed bland spindle cell histology, a lower cell density and rare

mitotic figures (HE, $\times 200$). **c** The high-grade tumor contained short spindle cells with obvious atypia, a brisk mitotic rate and a higher cell density (HE, $\times 200$). **d** The high-grade GIST exhibited obvious pleomorphism with tumor giant cells and multinuclear cells (HE, $\times 200$). **e** Necrosis was often observed in high-grade tumors (HE, $\times 200$)

proliferation indexes ($P = 0.000$). The Ki-67 index of high-grade GISTs ranged from 15–50%, while that of low-grade tumors ranged from 2 to 8% (Fig. 2c, d, Table 1).

High-grade GISTs contain differential mutations in cell cycle-related genes

WES was performed on ten cases of high-grade GIST and on the corresponding low-grade tumors. An average 69.5 million reads per sample were obtained, which produced an average coverage depth of 71 $\times$ –160 $\times$ on target regions per

sample. We analyzed candidate oncogenes with potentially deleterious mutations in the coding sequence and splicing regions, and 802 mutations in 247 oncogenes or tumor suppressor genes were detected (Fig. 3). The number of variants per specimen was 27–52, with a median of 37. Both high-grade and low-grade GISTs shared some of the mutated genes (193/247, 78.14%), and only a few differential oncogenes were mutated only in high-grade tumors (12.15%) or low-grade tumors (9.71%). The *KIT* gene, which was mutated in all 20 samples, showed the same mutation pattern in the two components from each patient;

Fig. 2 Immunohistochemical features of GISTs with coexisting low- and high-grade tumor components. **a** The low-grade tumors showed diffuse immunohistochemical positivity for CD34 (immunohistochemical staining (IHC), $\times 400$); **b** Matched high-grade GISTs were

CD34 negative expression (IHC, $\times 400$). The Ki-67 proliferation index in the low-grade tumor was $\sim 3\%$ (**c**), while in high-grade tumors, the Ki-67 proliferation index was $\sim 30\%$ (**d**) (IHC, $\times 400$)

this finding suggested that high-grade and low-grade tumors in the same patient might arise from a common cell. No mutations were detected in the *PDGFRA*, *KRAS*, *BRAF*, *NF1*, and *TP53* genes. *SDHA* mutations were identified only in the high-grade tumor of case 4.

Thirty genes mutated only in high-grade GISTs were detected in 247 oncogenes. Mutations in five genes were detected in two cases (*MGA*, *ARID1A*, *CLTC*, *KAT6B*, and *ZNF521*), while the others were mutated in only one patient. We detected an average of 3.5 differential cancer-associated genes per patient with a range of 1–6 genes. Twenty four oncogenes mutated only in low-grade tumors were detected, and six genes were examined in no less than two cases.

The differential cancer-associated gene mutations were verified by Sanger sequencing. Ten differential mutations found only in high-grade tumors were confirmed in nine genes: *MGA*, *SDHA*, *ARID1A*, *LATS2*, *MAX*, *SETD2*, *RB1*, *RPS6KB2*, and *PIK3CA* (Table 2 and Fig. 4), among which the first seven are tumor suppressor genes, and the latter two are oncogenes. These mutations were present in seven of ten patients. The mutational profile did not show any highly recurrent differential alterations shared by the majority of cases, and only *MGA* variants were identified in two

patients, while four patients had concurrent differential mutations. Functional enrichment analysis highlighted many significantly enriched pathways related to cell proliferation (*MGA*, *MAX*, *RB1*, *ARID1A*, *PIK3CA*, *RPS6KB1*, *LATS2*), evasion of apoptosis (*PIK3CA*, *RPS6KB1*, *SDHA*, *LATS2*), and chromatin remodeling (*ARID1A* and *SETD2*).

MGA mutations were identified in high-grade tumors from two cases of small intestinal GISTs: a stop-gain mutation (c.4741C>T, p.Q1581*) and a frameshift deletion (c.1599delT, p.H533fs*12). Both mutations were predicted to generate a premature stop codon and to cause truncation of the C-terminal region of the MGA protein, including the basic helix-loop-helix leucine zipper (bHLHLZ) domain. The patient with the *MGA* stop-gain mutation also had additional differential alterations in *SDHA*, which appeared as two consecutive base substitutions (c.363G>A, p.W121* and c.364C>T, p.H122Y), and resulted in a significant reduction in SDHA protein expression (Fig. 5) (Anti-SDHA antibody [EPR9043(B)], Abcam, epitope was a synthetic peptide within Human SDHA C-terminal 550-650aa). In addition to mutations in *MGA* and *SDHA*, stop-gain mutations were identified in the *ARID1A* (c.666C>A, p.Y222*) and *RB1* (c.496G>T, p.E166*) genes, which may lead to the

Fig. 3 The mutation features of cancer-associated genes identified by WES. **a** WES in ten cases of high-grade GIST and matched low-grade GIST showed variant mutation patterns with a predominance of missense mutations. **b** Every specimen exhibited different mutation patterns, and the number of mutations in each tumor ranged from 27 to 52. **c** Venn diagram analysis of mutational genes in all high- and low-grade GISTs revealed that both components share many similar

mutations in cancer-associated genes (numbers represent the number of genes). **d** An analysis of the proportion of mutated cancer-associated genes in high- and low-grade GISTs from each patient showed that most mutated genes were detected in both components and that a small number of differential genes were mutated only in high-grade or low-grade tumors (numbers in histograms represent the number of genes)

loss of function of these tumor suppressor genes. The tumor with an *ARID1A* mutation also had a missense mutation in *SETD2* (c.6074T>C, p.I2025T). A *PIK3CA* missense SNV (c.333G>T, p.K111N) was confirmed in one case of gastric GIST. The frequency of *PIK3CA* mutations in GIST is ~1.65% (14/846), while this is the first report of the *PIK3CA* mutation c.333G>T in GIST. A *MAX* somatic frameshift insertion (c.216_217insAC, p.Q73fs) in the bHLHLZ domain was detected in a female patient. This mutation may lead to amino acid changes after codon 73, which were predicted to result in the loss of MAX protein function. For the first time, missense SNVs in *LATS2* (c.2144C>G, p.A715G) and *RPS6KB2* (c.1261C>A, p.P421T) were identified in high-grade GIST components.

MYC copy number gain may promote tumor progression of GIST

Copy-number analysis based on WES data for *MYC* genes showed that three high-grade tumors carried copy-number gains compared with their corresponding low-grade tumors. One case had *MYC* amplification validated by FISH, which

illustrated four red/two green signals in the high-grade tumor and two red/two green signals in the low-grade tumor (Fig. 6), while the remaining two cases showed chromosome 8 polysomy with copy number gain in the *MYC* gene in both high-grade and low-grade tumors.

MYC CNV was assessed in 22 pairs of GIST with coexisting low- and high-grade components by FISH. In low-grade tumors, 15 cases had chromosome 8 polysomy with copy number gain of the *MYC* gene, and 7 cases showed normal *MYC* gene copy number; in contrast, in high-grade tumors, 17 cases demonstrated chromosome 8 polysomy with copy number gain, one case had *MYC* amplification, and four cases showed normal *MYC* gene copy number (Table 3). High-grade GISTs had more *MYC* gene copy number gains than low-grade GISTs ($P = 0.012$), but no significant differences were found in the number of cases of *MYC* copy number gain between high-grade tumors and low-grade tumors ($P = 0.296$, Table 3). It was suggested that the alterations in *MYC* copy number occurred in the early stages of GIST (low-grade GIST) and that more *MYC* copy number gains contributed to GIST progression.

Table 2 Differential oncogenic mutations in high-grade GISTs confirmed by WES and Sanger sequencing

Patient	Gene and exon	Chromosome	Position	Reference	Alteration (WES)	DV/DP (WES) ^a	Alteration (Sanger)	Exonic function	Amino acid change
1	RB1 exon 4	13	48919331	G	T	38/55	T	Stop gain	p.E166*
2	LATS2 exon 5	13	21557701	G	C	22/56	C	Missense mutation	p.A715G
4	MGA exon 15	15	42040990	C	T	7/8	T	Stop gain	p.Q1581*
4	SDHA exon 4	5	225584	G	A	29/60	A	Stop gain	p.W121*
4	SDHA exon 4	5	225585	C	T	30/61	T	Missense mutation	p.H122Y
6	MAX exon 3	14	65544682	G	GGT	31/35	GGT	Frameshift insertion	p.Q73fs
8	ARID1A exon 1	1	27023560	C	A	105/163	A	Stop gain	p.Y222*
8	SETD2 exon 13	3	7108595	A	G	11/19	G	Missense mutation	p.I2025T
11	MGA exon 3	15	41988806	AT	A	12/18	A	Frameshift deletion	p.H533fs
11	RPS6KB2 exon 14	11	67202158	C	A	17/46	A	Missense mutation	p.P421T
12	PIK3CA exon 2	3	178916946	G	T	3/6	T	Missense mutation	p.K111N

WES whole-exome sequencing, Sanger Sanger sequencing

^aDV represents the sequence depth of the variant bases, DP represents the sequence depth of the bases at this site

Detection of 86 cases of pure low- or high-grade GIST by FISH showed similar results. In 60 cases of low-grade GIST, 43 had normal *MYC* copy number, and 17 had chromosome 8 polysomy with copy number gain of the *MYC* gene; in 26 cases of high-grade GIST, normal *MYC* copy number was detected in ten cases, chromosome 8 polysomy with copy number gain of the *MYC* gene was detected in 15 cases, and *MYC* amplification was detected in one case (Table 3). *MYC* CNV in high-grade GIST had more copy number gains compared with low-grade GIST ($P = 0.004$, Table 3). Moreover, the tumors with *MYC* copy number gain usually had a higher mitotic index and a higher Ki-67 proliferation index ($P = 0.010$ and 0.001 , respectively, Table 3). Compared with pure low-grade GISTs, low-grade tumors with coexisting high-grade components had a higher incidence of *MYC* copy number gain ($P = 0.001$), which suggested that low-grade GISTs with *MYC* copy number gain were more likely to transform into high-grade tumors and that *MYC* copy number gain might promote tumor progression.

Discussion

Despite harboring similar driver *KIT* mutations, most GISTs exhibit extremely different clinical behaviors, which vary widely from incidental, clinically silent small tumors to locally invasive, distant metastatic tumors with high mitotic activity [12]. Additional genetic events may influence the variable behavior of these tumors, and high-grade tumors are likely to harbor many genomic alterations associated with highly aggressive behavior and high proliferative activity [13–15]. In the present study, WES results revealed 30 and 24 oncogenes that mutated only in high-grade GISTs and low-grade tumors, respectively. These mutations might occur during tumor progression, and the mutations only in low-grade tumors did not cause GIST progression to high-grade tumor. Tumor subclones with mutations only in low-grade tumors may have different subclonal origins from high-grade tumors. Furthermore, we identified ten differential genetic alterations in nine genes in seven patients by Sanger sequencing, and seven of these genes (*MGA*, *MAX*, *RBI*, *ARID1A*, *PIK3CA*, *RPS6KB1*, *LATS2*) have been documented to be involved in cell cycle regulation. Therefore, we deduced that mutations in these genes were associated with tumor progression of low-grade to high-grade tumors and that the progression of GISTs may be the result of cell cycle control via the convergent integration of various cascades, such as the *MGA*/*MAX*/*MYC* network and the *PI3K*/*AKT* signaling pathway (Fig. S2). Notably, none of the patients received chemotherapy or receptor tyrosine kinase inhibitor therapy prior to surgery, which indicates that the

Fig. 4 Representative differential oncogenic mutations identified in high-grade GISTs by Sanger sequencing. In each image, the top panel depicts the sequencing fluorescent peak trace chromatograms of high-

grade GIST, and the bottom panel depicts the low-grade tumor counterpart (mutations are highlighted by light blue squares)

mutations identified in our study were spontaneous events unrelated to any therapy.

MGA and MAX are members of the bHLHLZ family of transcription factors. In our study, truncating mutations in *MGA* were identified only in the high-grade tumors of two cases of small bowel GIST. In addition, *MGA* mutations (c.5554C>T, p.H1852Y and c.7766delT, p.I2589fs) have been reported in two other cases of small bowel GIST with *KIT* mutations [11]. All reported *MGA* mutations in GISTs occurred in high-grade tumors or metastatic tumors, which suggest that *MGA* mutations might promote tumor progression. A possible mechanism for this is that *MGA* loss-of-function mutations may reduce the suppression of MAX–MYC heterodimerization complex formation and promote GIST progression by regulating MYC-dependent transformation [16, 17], which modulates the cell cycle by enhancing cyclin-dependent kinase (CDK) functions. Furthermore, we identified a *MAX* loss-of-function mutation in the present study. *MAX* mutations are not considered a rare genetic event in GIST, and *MAX* inactivation may foster cell cycle activity by regulating p16^{INK4A} silencing [18].

PIK3CA is a downstream effector of the KIT/PDGFRA signaling pathway that drives carcinogenesis by inducing proliferation and inhibiting apoptosis [19, 20]. In the present study, a *PIK3CA* mutation was detected only in the high-grade component in a *KIT*-mutant GIST of a patient who had not undergone targeted therapy with tyrosine kinase inhibitors, which suggests that *PIK3CA* mutations may represent primary oncogenic events related to GIST progression. Although the *PIK3CA* c.333G>T mutation was first reported in GIST, it occurs in a variety of tumors (18

reported cases in COSMIC: mutation ID COSM27505), which suggests that this mutation is a recurrent genetic event that is a vital contributor to oncogenesis and progression. GISTs with *PIK3CA* base substitution mutations are frequently classified as high-risk tumors [21], and more *PIK3CA* mutations are detected in large and metastatic *KIT*-mutant GISTs, which suggests that *PIK3CA* mutant clones have a proliferative advantage during disease progression [4, 21]. Moreover, oncogenic activation of the PI3K pathway has been implicated in different cancers and contributes to a more aggressive tumor phenotype [22, 23]. In addition, the loss of *ARID1A* has recently been linked to increased AKT phosphorylation and PI3K/AKT pathway activation [24]. In the present study, a stop-gain mutation in *ARID1A* in a high-grade GIST was validated in one patient with extensive abdominal metastasis. *PIK3CA* and *ARID1A* mutations can activate the PI3K/AKT pathway and further activate CDK which is complexed with cyclins, and leads to tumor cell proliferation and inhibition of apoptosis [25].

RB1 is one of the most important genes in cell cycle regulation. In our study, one case had an *RB1* loss-of-function mutation only in high-grade GIST. Similarly, Merten et al. identified two cases of *RB1* inactivating missense mutations in GIST, which were correlated with morphologically high-risk features and clinically malignant behavior [26]. Moreover, deletion of the *RB1* gene locus identified by fluorescence in situ hybridization is frequent in high-risk GIST and may be associated with tumor progression [27]. To our knowledge, *RPS6KB2* and *LATS2* mutations in GIST have not been previously reported. *RPS6KB2* is an important kinase regulated by the mTOR signaling

Fig. 5 *SDHA* mutations resulted in decreased expression of SDHA protein. **a** Morphological characteristics of low-grade GIST components in case 4 (HE, $\times 200$); **b** Morphological features of high-grade tumors (HE, $\times 200$). **c** Immunohistochemical results of low-grade tumors revealed moderate to strong cytoplasmic expression of SDHA

protein (IHC, $\times 400$); **d** High-grade tumors showed a marked reduction in SDHA expression (IHC, $\times 400$). **e** Sanger sequencing revealed wild-type *SDHA* in low-grade GIST; **f** *SDHA* mutations (c.363_364GC>AT) in high-grade tumors were detected by Sanger sequencing

pathway, and its activity can shorten the G1 phase of the cell cycle, enabling cells to enter S phase at an increased rate by binding several heterogeneous ribonuclear proteins in a mitogen-inducible manner [28]. Since *RPS6KB2* is an oncogene, activating mutations may fuel tumor progression by regulating cell cycle control. *LATS2* is a component of the Hippo signaling pathway, which is involved in regulating cell proliferation by inhibiting CDK2/cyclin E activity and limiting G1/S transition [29, 30]. In addition, *LATS2* can promote DREAM (DP, RB, E2F, and MuvB) repressor complex assembly by phosphorylating DYRK1A and can promote RB-induced permanent cell cycle arrest in tumor cells [31]. In the present study, a *LATS2* mutation was found

in the protein kinase-like domain, which may undermine the tumor-suppressor function of this gene.

MYC is a positive regulator of G1-specific CDKs and acts at least three distinct pathways: (1) functional inactivation of the CDK inhibitors p27^{Kip1}, p21^{Cip1}, and p57^{Kip2}, (2) induction of the CDK-activating phosphatase Cdc25A and (3) deregulation of cyclin E expression [32]. *MYC* gene alterations are common genetic variations, as 21% of solid tumors show *MYC* copy number gain [17]. In this study, 50.8% of GIST samples showed *MYC* copy number gain, especially GISTs with coexisting low- and high-grade components. Low-grade GIST with copy number gain led to a higher frequency of tumor progression, and the tumors with *MYC* copy number

Fig. 6 *MYC* CNV in low-grade GIST and high-grade GIST of case 11. **a** CNV analysis revealed an increased copy number of the chromosome 8 fragment (inside the square frame) where *MYC* resides (the upper figure: red represents an increase in copy number, blue represents a decrease in copy number, and green indicates no change in

copy number; the lower figure: orange indicates the same allele distribution, while blue indicates the preference of allele distribution); **b** Normal *MYC* copy number (two red/two green signals) was detected in the low-grade tumor; **c** *MYC* copy number amplification (four red/two green signals) was identified in the high-grade tumor

Table 3 The relationship among *MYC* copy number gain and tumor grade, mitotic index and Ki-67 proliferation index

Tumor features	Coexisting GIST			Pure GIST		
	<i>MYC</i> copy number gain	<i>MYC</i> copy number normal	<i>P</i>	<i>MYC</i> copy number gain	<i>MYC</i> copy number normal	<i>P</i>
Tumor grade			0.296			0.004
Low-grade	15	7		17	43	
High-grade	18	4		16	10	
Mitotic index (/50 HPF)			0.174			0.010
≤5	15	7		17	43	
6–10	7	4		4	7	
11–20	3	0		2	0	
>20	8	0		10	3	
Ki-67 proliferation index (%)			0.153			0.001
≤5	11	6		10	27	
6–10	3	1		8	19	
11–30	14	4		8	6	
>30	5	0		7	1	

gain usually had a higher mitotic index and a higher Ki-67 proliferation index. These findings suggest that *MYC* copy number gain may promote progression of GISTs by regulating tumor cell proliferative activity.

In summary, the present study reports 22 GIST cases with concomitant low- and high-grade components, both of which displayed different histopathological and immunohistochemical characteristics. WES was performed using ten pairs of high-grade GISTs and matched low-grade GISTs, and the data showed that both components shared most of the detected mutations in cancer-associated genes. WES and Sanger sequencing identified nine cancer-associated genes that were mutated only in high-grade GISTs; the function of these genes is primarily cell cycle control. FISH revealed that *MYC* copy number gain is a common genetic alteration in GIST and that this alteration may occur in the early stages of this disease. It was also revealed that low-grade tumors with *MYC* copy number

gain were more likely to progress to high-grade tumors. GISTs with *MYC* copy number gain had higher proliferative activity. Therefore, the primary progression of GISTs may be the result of a variety of different genes acting through different signaling pathways, and cell cycle regulation-related gene mutations and copy number gain of the *MYC* gene may play important roles in these pathways.

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Compliance with ethical standards

Conflict of interest For the remaining authors none were declared.

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