

Frequency and clinicopathologic profile of *PIK3CA* mutant GISTs: molecular genetic study of 529 cases

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Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors usually driven by the mutational activation of receptor tyrosine kinases, KIT, or PDGFRA. Oncogenic activation of phosphatidylinositide-3-kinase (PI3K), a downstream effector in the KIT signaling pathway, has been identified in different types of cancer, with the PI3K 110 α subunit encoded by *PIK3CA* being a common mutational target. In this study, the mutational hotspot in the *PIK3CA* kinase domain encoded by exon 20 was evaluated in 529 imatinib-naive GISTs using PCR amplification and Sanger sequencing. Eight mutations (two co-existing in one tumor) were identified. Subsequently, The cobas *PIK3CA* Mutation Test was employed to evaluate mutational hotspots in exons 1, 4, 7, and 9 in 119 *PIK3CA* exon 20-wild type tumors. In two cases, mutations in exons 1 and 9 were identified. In one GIST, previously undetected by Sanger sequencing, the exon 20 mutation was discovered. Altogether, eight primary and two metastatic GISTs carried *PIK3CA* mutations. The size of primary *PIK3CA*-mutant GISTs was ≥ 14 cm (mean size 17 cm), and mitotic activity varied from 0 to 72 per 50HPF (mean 5/50HPF). Follow-up data showed short survival in 6 of 7 studied cases. Detection of *PIK3CA* mutations in large or metastatic *KIT*-mutant GISTs may suggest that *PIK3CA*-mutant clones have a proliferative advantage during disease progression. Tyrosine kinase inhibitors have been successfully used in GIST treatment. However, resistance frequently develops due to secondary *KIT* mutations or activation of downstream to KIT signaling pathways, such as the PI3K/AKT/mTOR pathway. *PIK3CA* mutations similar to the ones detected in GISTs have been shown to cause such activation. Therefore, genotyping of *PIK3CA* in GISTs might help to pinpoint primary and metastatic tumors with the potential to develop resistance to tyrosine kinase inhibitors and guide therapy with PI3K inhibitors.

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Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal (GI) tract. In general, GISTs are driven by mutational, oncogenic activation of receptor tyrosine kinases, KIT, or platelet-derived growth factor receptor α (PDGFRA), although subsets of KIT/PDGFRA-wild-type tumors have been

identified and include succinate dehydrogenase (SDH)-deficient GISTs, neurofibromatosis 1 (NF1)-associated GISTs, BRAF mutant GISTs, and the so-called ‘quadruple wild-type tumors’ that lack mutations in *KIT*, *PDGFRA*, *BRAF*, and *SDH* genes.^{1,2} Phosphatidylinositol 3-kinase, catalytic, alpha (*PIK3CA*) encodes the p110 α catalytic subunit of phosphatidylinositide-3-kinase (PI3K), a lipid kinase, downstream effector in the KIT signaling pathway that promotes cellular growth, proliferation, and survival. Activation of PI3K/AKT/mTOR signaling pathway has been reported in different types of human cancer, and is often caused by *PIK3CA* mutations.³ *PIK3CA* mutations frequently coexist with mutational activation of other

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oncogenes, such as *BRAF* and *RAS*.⁴ Because the presence of *PIK3CA* mutations might negate the effect of the KIT inhibitor and indicate tumor response to PI3K/AKT/mTOR axis inhibitors, the mutation status of *PIK3CA* could be a factor in the selection of targeted therapy.^{5,6} Recently, *PIK3CA* mutations have been reported in two primary gastric GISTs: one concurrent with a *KIT* mutation and another with the *HRAS* mutation, and in treatment-resistant metastasis of a *BRAF*-mutant GIST.⁷⁻⁹ Thus, activation of the PI3K/AKT/mTOR signaling pathway may have a role in both pathogenesis and the progression of GISTs. The presence of the *PIK3CA* mutation could indicate an alternative treatment inhibiting the PI3K/AKT/mTOR signaling pathway for patients with advanced and metastatic tumors, as opposed to KIT inhibitor therapy. The aim of this study was to determine the frequency of *PIK3CA* mutations in imatinib-naive GISTs and their role in GIST biology and pathogenesis.

Materials and methods

Study Material

The analyzed cohort of 529 GISTs contained primary tumors from different locations, including the esophagus ($n=7$), stomach ($n=214$), small intestine ($n=127$), large intestine with rectum and anus ($n=32$), and intra-abdominal ($n=141$) and metastatic extra-abdominal GISTs ($n=4$). The intra-abdominal category included tumors involving omentum, retroperitoneum, and GISTs largely disseminated in the abdominal cavity at the time of diagnosis. In all cases, GIST diagnosis was confirmed by positive KIT (CD117) and/or ANO1 (DOG1) immunohistochemistry. Among gastric GISTs, 16 SDH-deficient tumors were identified following SDHB and SDHA immunohistochemistry. In the majority of cases, CD34, SMA, and S100 immunohistochemistry and *KIT*, *PDGFRA*, *BRAF*, *HRAS*, and *KRAS* genotyping data were available. Tumor size was documented in 347 primary GISTs. There were 195 tumors < 10 cm and 152 tumors > 10 cm. Furthermore, in 71 cases, intra-abdominal dissemination or extra-abdominal metastases were present at the diagnosis.

Study Design

A total of 529 GISTs were examined for *PIK3CA* exon 20 mutations by PCR amplification and Sanger sequencing. In addition, 119 cases were examined by The cobas *PIK3CA* Mutation Test (Roche Molecular Systems, Pleasanton, CA, USA) in order to detect mutations in other exons, as their detection by Sanger sequencing is hampered by homologous pseudogene sequences. Seventy-five GISTs evaluated by The cobas *PIK3CA* Mutation Test were either large (> 10 cm) or metastatic tumors. In

addition, two cases were analyzed by the Ion Torrent next generation sequencing platform (Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA) to further confirm Sanger sequencing results. In one case, a double *PIK3CA* mutation was further analyzed through cloning and sequencing.

Molecular Studies

DNA extraction. DNA was extracted from formalin-fixed paraffin-embedded tissue samples. Five to ten 5- μ -thick sections were deparaffinized with xylene, washed twice in ethanol, lyophilized, and incubated with 10 μ g/ μ l proteinase K (Roche Diagnostics, Indianapolis, IN, USA) in Hirt-Buffer at 55 °C for 24 h. Subsequently, DNA was recovered using the Maxwell 16 robotic system and DNA IQ Casework Pro Kit (Promega, Madison, WI, USA).

PCR amplification and Sanger sequencing. *PIK3CA* exon 20 was PCR amplified with AmpliTaq Gold DNA polymerase (Applied Biosystems by Life Technologies, Austin, TX, USA) following a standard three-temperature protocol with denaturing at 94 °C, annealing at 48 °C, and extension at 72 °C. Two sets of primers were used: (1) *PIK3CA1F* 5'-AGGAGATGTGTTACAAGGCT-3' and *PIK3CA1R* 5'-TTGTGTGGAAGATCCAATCC-3' and (2) *PIK3CA2F* 5'-TGCATACATTCGAAAGACC C-3' and *PIK3CA2R* 5'-TTGTGTGGAAGATCCAATC C-3'. The PCR product sizes for these two assays were 243 and 128 bp, respectively. PCR amplification products were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) following agarose gel electrophoresis and then sequenced directly with forward and reverse primers. Cases revealing sequence variations were evaluated at least twice to ensure reproducibility of the PCR amplification and sequencing results. In one case, *PIK3CA* exon 20 PCR amplification products were cloned into plasmid using the TOPO TA Cloning Kit (Invitrogen by Life Technologies) and subsequently sequenced. The sequences were analyzed following alignment with *PIK3CA*, NG_012113.2 and NM_006218.2:c reference sequences (www.ncbi.nlm.nih.gov). Known *PIK3CA* mutant colon carcinomas were used as a positive controls. The Sanger sequencing service was performed by MacroGenUSA (Rockville, MD, USA).

The cobas *PIK3CA* Mutation Test. The cobas *PIK3CA* Mutation Test (Roche Molecular Systems, Pleasanton, CA, USA), a real-time PCR assay, allows detection of mutations affecting the following codons: p.R88 in exon 1, p.N345 in exon 4, p.C420 in exon 7, p.E542, p.E545 and p.Q546 in exon 9, and p.M1043, p.H1047, and p.G1049 in exon 20. Genotyping using The cobas *PIK3CA* Mutation Test was performed following the protocol provided by the manufacturer.

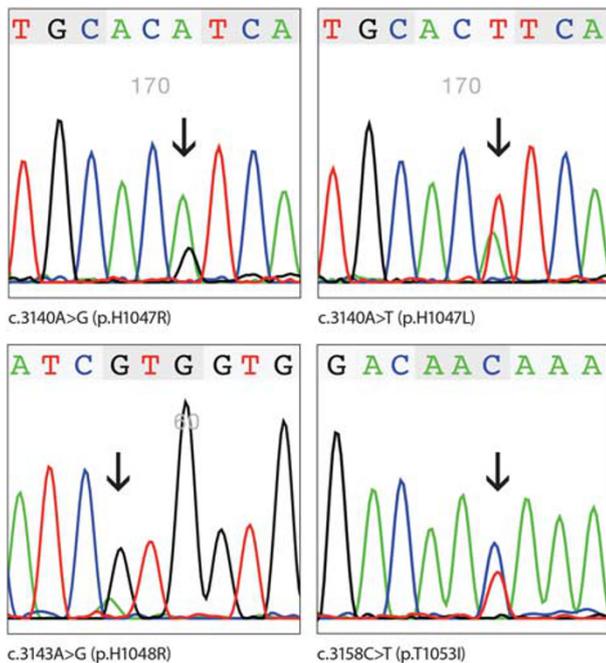


Figure 1 Examples of *PIK3CA* mutations detected by at least two independent PCRs and Sanger sequencing.

Next-generation sequencing. Next-generation sequencing was performed using the Ion Torrent NGS platform in accordance with the manufacturer's instructions (Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA). Libraries were prepared using 10 ng of tumor DNA, Ion AmpliSeq Library Kit 2.0, Ion AmpliSeq Cancer Hotspot Panel v2 Kit, and Ion Xpress Barcode Adapters 1-16 Kit. Template preparation was done using emulsion PCR with Ion OneTouch 200 Template Kit v2 DL and Ion OneTouch DL and Ion OneTouch ES devices. Sequencing was performed using a Personal Genome Machine (PGM) and the Ion PGM 200 Sequencing Kit. The data were processed by Torrent Server Suite 4.2 and sequences aligned to the human genome reference sequence HG-19 (The Genome Reference Consortium). Variant calling was performed using Variant Caller v4.2, which is compatible with the Integrative Genomics Viewer (Broad Institute, Cambridge, MA, USA), a high-performance visualization tool for interactive exploration of large, integrated data sets.

Results

Molecular Studies

Sanger sequencing. Sanger sequencing of the PCR amplified *PIK3CA* exon 20 revealed eight mutations in seven GISTs. Examples of Sanger sequencing are shown in Figure 1. All mutations consisted of single-nucleotide substitutions: c.3103G>A, c.3127A>G, c.3137C>T, c.3139C>T, c.3140A>G,

c.3140A>T, c.3143A>G, and c.3158C>T. At the protein level, these substitutions would lead to p.A1035T, p.M1043V, p.A1046V, p.H1047R, p.H1047L, p.H1047Y, p.H1048R, and p.T1053I mutations, respectively. In one tumor, two substitutions, c.3103G>A and c.3158C>T, were detected. The cloning and sequencing of PCR products confirmed the presence of these mutations on two different alleles. All mutations identified in this study were previously reported in other tumors (Catalog of somatic mutations in cancer, cancer.sanger.ac.uk/cosmic).

In three cases, initially identified substitutions (c.3117C>T, c.3152G>A, and c.3153G>A, the cause of p.F1039F and p.W1051* *PIK3CA* mutations) could not be confirmed by the sequencing of two independent PCR amplification products (Figure 2). Subsequently, two of these cases were evaluated by Ion Torrent next-generation sequencing, which did not confirm the presence of clones with c.3152G>A and c.3153G>A *PIK3CA* mutations. However, due to the poor DNA quality, the sequencing coverage of the analyzed regions was only 353 and 145 reads, respectively.

The cobas *PIK3CA* Mutation Test. Three additional *PIK3CA* mutants were identified in 120 GISTs evaluated using The cobas *PIK3CA* Mutation Test (Table 1). These mutants involved p.R88 in exon 1, p.E545 in exon 9, and p.H1047 in exon 20. The latter mutant was not detected via Sanger sequencing, while the two former ones were not tested by that assay.

Clinicopathological Profile of *PIK3CA* Mutant GISTs

The clinicopathologic data of *PIK3CA* mutants are summarized in Table 2. The 10 patients included 7 men and 3 women (mean age, 58 years). There were four gastric and one small intestinal primary GIST, one liver metastasis of a small bowel GIST, three retroperitoneal GISTs with small intestinal GIST-like morphology, and one GIST metastatic to the abdominal wall from an unknown primary tumor. The tumor size of all primary *PIK3CA* mutant GISTs was ≥ 14 cm (mean and median size, 17 cm), and mitotic activity varied from 0 to 72 per 50HPF (median, 5/50 HPFs).

Nine *PIK3CA* mutant GISTs showed spindle cell or spindle to ovoid cell morphology (Figure 3). Epithelioid cell morphology was seen in the abdominal wall metastatic GIST with unknown primary tumor. All tumors were KIT and DOG1 positive, while CD34 and SMA were expressed in seven and one cases, respectively (Figure 4). The follow-up available in the seven cases revealed death of the disease for two cases, with median survival time <9 months and overall short survival <35 months in four additional cases (in which the cause of death was not available). *PIK3CA* mutations were concurrent with *KIT* exon 11 mutations in all 9 analyzed cases.

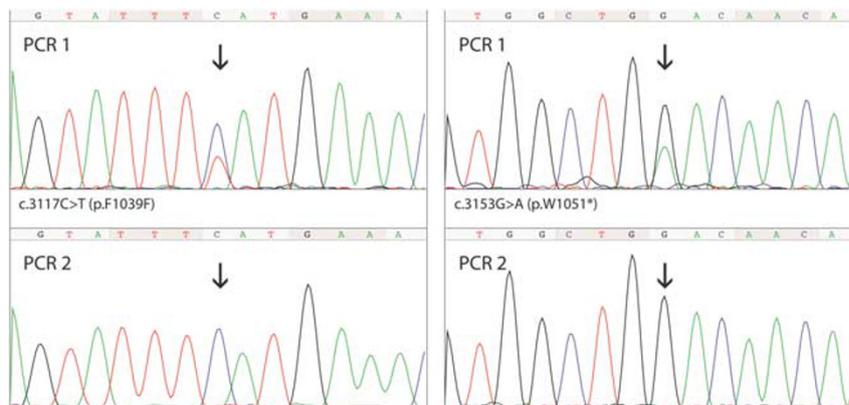


Figure 2 Two *PIK3CA* mutations initially detected by PCR amplification and Sanger sequencing that could not be confirmed by independent assays.

Table 1 Genotype of *PIK3CA* mutant GISTs reported in this study

<i>PIK3CA</i> exon	<i>PIK3CA</i> mutation	<i>PIK3CA</i> Sanger seq.	Cobas <i>PIK3CA</i> Mutation Test	<i>KIT</i> exon 11 mutation	Case no.
1	p.R88?	Not done	Mutant	p.550_555delinsI	3
9	p.E545?	Not done	Mutant	p.553_556del	10
20	p.A1035T	c.3103G>A	Not done	p.I563L	8
20	p.M1043V	c.3127A>G	Not done	p.V560_564delinsD	5
20	p.A1046V	c.3137C>T	Not detected	p.W557R	4
20	p.H1047Y	c.3139C>T	Invalid	p.557_559del	9
20	p.H1047R	c.3140A>G	Not done	No data	7
20	p.H1047L	c.3140A>T	Mutant	p.553_556del	2
20	p.H1047?	WT	Mutant	p.550_554del	1
20	p.H1048R	c.3143A>G	Not done	p.558_562del	6
20	p.T1053I	c.3158C>T	Not done	p.I563L	8

Frequency of *PIK3CA* Mutations in GIST

PIK3CA exon 20 mutations were identified by Sanger sequencing in 1.3% (7 of 529) of analyzed GISTs. However, if tumors < 10 cm were excluded, then the frequency rose up to 2.7% (6 of 223). In a similar cohort, the frequency of *PIK3CA* mutations other than those in the exon 20 *PIK3CA* hot-spot was 2.7% (2 of 75), as documented by The cobas *PIK3CA* Mutation Test. Also, this real-time PCR assay was more sensitive than Sanger sequencing and identified one additional exon 20 mutant previously undetected by the Sanger sequencing. Based on the above data, we estimated that *PIK3CA* mutations in large (> 10 cm) and advanced GISTs might reach a 5–6% frequency.

Discussion

Phosphatidylinositol 3-Kinase, or PI3K, is a lipid kinase, a downstream effector in the KIT signaling pathway that is thought to promote processes, such as cellular growth, proliferation, and survival. Oncogenic activation of the PI3K pathway has been implicated in different types of cancer. Activation of the PI3K/AKT/mTOR pathway contributes to a more aggressive tumor phenotype, which results in shorter

survival and poor treatment outcome.¹⁰ Genetic or epigenetic mutations affecting PI3K, PI3K downstream kinases, such as AKT or mammalian target of rapamycin (mTOR), and PTEN, a major PI3K-negative regulator, have been reported.^{3,5,6}

Relatively little is known about the genetic alteration of PI3K/AKT/mTOR pathway in GISTs, although one study reported frequent mono-allelic loss of *PTEN*, a negative regulator of PI3K.^{11,12} In addition, activation of the PI3K/AKT pathway was proposed to be involved in tumor cell survival in imatinib-sensitive and -resistant GISTs.¹³

PIK3CA, a gene that encodes the p110α catalytic subunit of PI3K, has been shown to be mutated in common carcinomas, malignant melanoma, and some sarcomas. The majority of *PIK3CA* mutations cluster in exon 9 and exon 20, encoding helical and kinase domains respectively, although activating mutations in other *PIK3CA* regions including exons 1, 4, and 7 have been reported.³

In this study, a large cohort of imatinib-naive GISTs was screened for *PIK3CA* mutations and 10 mutants were identified. A great majority of these mutations were located in exon 20, with four of them affecting codon 1047. This codon was mutated in all three previously reported *PIK3CA* mutant GISTs.^{7–9} Thus, similarly to other cancer, p.H1047 appears to

Table 2 Clinicopathologic profile of *PIK3CA* mutant GISTs reported in this study

Case no.	Age	Sex	Location	Tumor size (cm)	Mitosis/50HPF	Group ^a	Histology	Follow-up (months)
1	64	M	Stomach	22	21	6b	Spindle cell	DOD (11)
2	48	F	Stomach	19	72	6b	Spindle to ovoid cell	DUNK (8)
3	79	M	Stomach	14	1	3b	Spindle cell	DUC (279)
4	61	F	Stomach	N/A	0	N/A	Spindle cell	N/A
5	55	M	Small bowel	17	29	6b	Spindle cell	DOD (6)
6	51	M	Retroperitoneum	20	4	3b	Spindle to ovoid cell	DUNK (35)
7	51	M	Retroperitoneum	>15	3	3b	Spindle cell	DUNK (10)
8	45	F	Retroperitoneum	14	5	3b	Spindle cell	DUNK (27)
9	40	M	Metastasis in the liver (primary tumor, small bowel GIST)	N/A	N/A	N/A	Spindle cell	N/A
10	84	M	Metastasis in the abdominal wall (primary tumor unknown)	N/A	N/A	N/A	Epithelioid	N/A

Abbreviations: DOD, died of disease; DUC, died of unrelated causes; DUNK, died of unknown causes; N/A, not available.

^aBased on previously published classification.¹

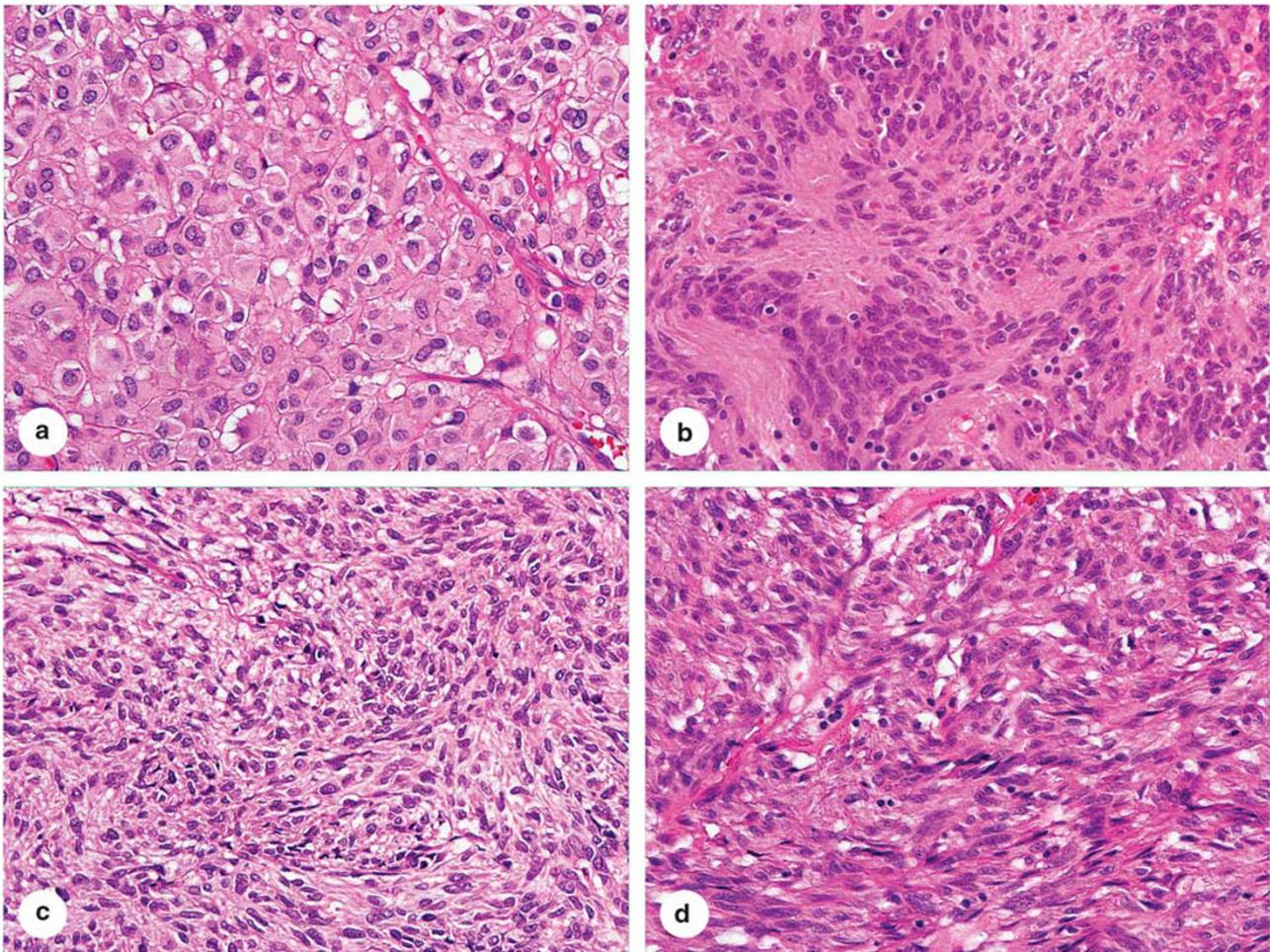


Figure 3 Four examples of *PIK3CA* mutant GISTs. (a) An epithelioid GIST of the stomach. (b) A retroperitoneal spindle cell GIST with Verocay body-like, prominent aggregates of cell processes, typically seen in GISTs of intestinal origin. (c) A GIST with spindled to ovoid cell morphology. (d) A spindle cell GIST representing the morphology often seen in intestinal GISTs.

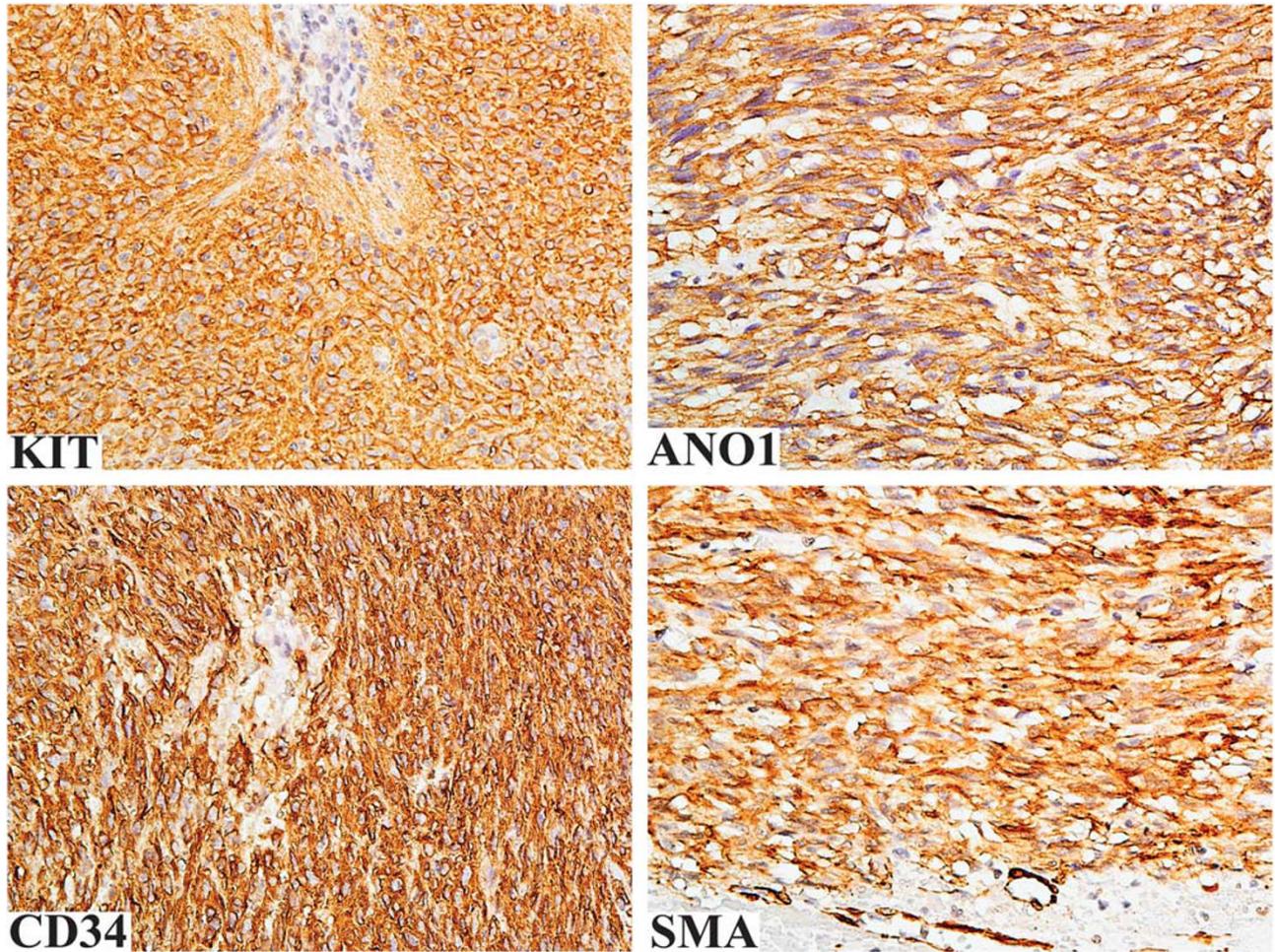


Figure 4 Immunohistochemical findings in *PIK3CA* mutant GISTs. The tumor cells are positive for KIT, DOG1, CD34, and SMA.

be the most common mutational hotspot in GISTs. One tumor revealed two co-existing *PIK3CA* exon 20 mutations. The co-existence of two different *PIK3CA* mutations has been previously reported in breast cancer and linked to more aggressive clinical outcomes.^{14,15}

A previous study based on immunohistochemistry suggested that activation of the mTOR signaling pathway is characteristic for the *PDGFRA* mutant and wild-type GISTs, rather than *KIT* mutant GISTs.¹⁶ In our study, all nine analyzed *PIK3CA* mutants turned out to be *KIT* exon 11 mutants. Similarly, one of three previously reported *PIK3CA* mutant GISTs carried *KIT* K558_E562del, typical of GISTs.⁷

One *PIK3CA* mutation was reported in *KIT*/*PDGFRA*-wild type, *BRAF* V600E mutant GIST metastasis during *BRAF*-inhibitor treatment, at the time of disease progression.⁸ Primary GISTs driven by *BRAF* mutations are exceptionally rare.^{7,17} In the current study, *PIK3CA* was evaluated in one *BRAF* mutant GIST and revealed wild-type sequences.

Nevertheless, a p.H1047R *PIK3CA* mutation co-existing with a p.G12V *HRAS* mutation was

identified by next-generation sequencing in a high-grade gastric tumor reported as 'quadruple wild type' GIST.⁹ Thus, in rare cases, *PIK3CA* mutations might have a role in wild-type GIST pathogenesis. In our study, no tumors with co-existing *PIK3CA* and *HRAS* or *KRAS* mutations were identified by Sanger sequencing.

Previous studies documented p.G12R and p.G12V *KRAS* mutations, respectively, in an imatinib-resistant lesion of disseminated GIST and in an anaplastic *KIT*-negative tumor developed following long-term imatinib therapy for CML.^{9,18} Based on previous studies, the frequency of *RAS* mutations in imatinib-naïve GISTs remains very low.¹⁹ More recently, a *KRAS* G12V mutation at 29% allele frequency has been reported in an *SDH*-deficient highly malignant gastric GIST.²⁰ No *PIK3CA*, *HRAS*, and *KRAS* mutations were identified in 16 *SDH*-deficient GISTs analyzed in this study.

In this study, all GISTs harboring *PIK3CA* mutations were large (>10 cm) tumors. Also, previously published *PIK3CA* mutants represented high-grade primary or metastatic GISTs. The presence of *PIK3CA* mutations in such tumors may

suggest that *PIK3CA* mutant clones have proliferative advantage and can become dominant in a late stage of the GIST genetic evolution. These mutations can also arise without the selection pressure of a KIT tyrosine kinase inhibitor, such as imatinib mesylate.

Imatinib mesylate, a KIT and PDGFRA tyrosine kinase inhibitor, has been used for the treatment of primary advanced and disseminated GISTs. However, both primary and secondary resistance acquired after or during initially successful treatment occurs.²¹ The resistance could be related to the mutational activation of downstream pathways, such as the PI3K/AKT/mTOR pathway. Thus, identification of *PIK3CA* mutations in primary *KIT* mutant GIST may have significant clinical implications in the choice of a kinase inhibitor therapy, as the mutational activation of PI3K may nullify the effect of KIT inhibition due to independent downstream activation of the signaling pathways. Also, the type of *PIK3CA* mutation might influence the response to PI3K/AKT/mTOR inhibitors.²²

Applicability of Sanger sequencing in the analysis of *PIK3CA* mutations has limitations. The presence of the pseudogene on chromosome 22 with high (>95%) homology to the *PIK3CA* exon 9 sequence hampers detection of mutations by Sanger sequencing. Thus, in this study, The cobas *PIK3CA* Mutation Test was employed to search for common *PIK3CA* exon 9 mutations. This real-time PCR assay, developed to detect changes in *PIK3CA* mutational hotspots, is more sensitive than Sanger sequencing.²³ In our study, the comparison of these two methods revealed a high concordance with only two variances. In one case, a rare mutation identified by Sanger sequencing was not detectable by The cobas *PIK3CA* Mutation Test and, in the second case, Sanger sequencing turned to be less sensitive in the detection of a mutant minority clone.

The reproducibility of Sanger sequencing results was generally good. However, in three cases, initially detected heterozygous G>A and C>T mutations were not seen in subsequent PCR amplifications. Although such results might be a consequence of intratumor heterogeneity,²⁴ the next-generation sequencing failed to detect subclones carrying these mutations. Because the C:G>T:A substitutions represent the most common sequencing artifacts detected in PCR of formalin-fixed paraffin-embedded DNA,²⁵ it is possible that early sequencing results from these two cases represented random PCR amplification artifacts. Studies of multiple, well-preserved DNA samples with next-generation ultra-deep sequencing could help to distinguish intratumor clonal heterogeneity from random PCR amplification artifacts.²⁶

In summary, we reported the rare occurrence of *PIK3CA* mutations in imatinib-naive GISTs. These mutations occurred predominantly in large and clinically aggressive GISTs, suggesting that the expansion of *PIK3CA* mutant clones is an additional

oncogenic event that can also develop without selection pressure caused by a kinase inhibitor. Detection of these mutations is significant for the selection of targeted therapy as their presence may preclude the use of a tyrosine kinase inhibitor and instead require inhibitors for the PI3K/AKT/mTOR pathway.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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