

# Correlation of treatment outcome in sanger/RT-qPCR *KIT/PDGFR*A wild-type metastatic gastrointestinal stromal tumors with next-generation sequencing results: A single-center report

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**Abstract.** In patients with gastrointestinal stromal tumors (GIST), it has become mandatory to determine the driver mutation in order to predict the response to standard treatment with tyrosine kinase inhibitors (TKI). A total of 10-15% of all GIST lack activating mutations in *KIT* proto-oncogene, receptor tyrosine kinase (*KIT*)/platelet-derived growth factor receptor alpha (*PDGFRA*) and have been classified as *KIT/PDGFR*A wild-type (WT) GIST. They are characterized by poor response to TKI. From a group of 119 metastatic GIST patients, 17 patients with *KIT/PDGFR*A/BRAF WT GIST as determined by reverse transcription-quantitative (RT-q) PCR and Sanger sequencing were profiled by a targeted next-generation sequencing (NGS) approach and their treatment outcome was assessed. In the present study, 41.2% of patients as *KIT/PDGFR*A/BRAF WT GIST examined with RT-qPCR and Sanger sequencing were confirmed to be carriers of pathogenic *KIT/PDGFR*A mutations by NGS and were responsive to TKI. The percentage of genuinely *KIT/PDGFR*A WT GIST in the present study thereby dropped from the initial 14.3% detected with the RT-qPCR and Sanger sequencing to 7.6% after NGS. Their outcome was universally poor. The reliability of RT-qPCR and direct Sanger sequencing results in this setting is therefore insufficient and it is recommended that NGS becomes a requirement for treatment decision at least in *KIT/PDGFR*A/BRAF WT GIST as determined by RT-qPCR and Sanger sequencing.

## Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors affecting the gastrointestinal tract but still represent less than 1% of all gastrointestinal tract tumors. The estimated annual incidence of clinically relevant GIST is ~1/100,000 annually (1,2). A total of ~85% of GIST harbor mutually exclusive activating mutations of the genes encoding *KIT* proto-oncogene, receptor tyrosine kinase (*KIT*) or platelet-derived growth factor receptor alpha (*PDGFRA*) tyrosine kinase receptors leading to ligand-independent activation of these receptors and making tumor cells sensitive to tyrosine kinase inhibitors (TKI) (3,4). Imatinib mesylate inhibits the *KIT/PDGFR*A receptor by directly binding to the ATP-binding site within the amino-terminal domain of the kinase and thus competitively inhibits ATP binding (3). In prospective randomized clinical phase III trials, imatinib achieved disease control in 70-85% of patients with advanced *KIT/PDGFR*A-mutant GIST, and the median progression-free survival was 20-24 months. The median survival in patients with advanced disease who are treated with front-line imatinib is up to 5 years (5-7).

A total of ~10-15% of all GIST lack mutations in either of the aforementioned genes and have been classified as *KIT/PDGFR*A wild-type (WT) GIST (4,8-10). The molecular mechanism underlying tumorigenesis in this subgroup is not fully understood. ~A total of 20-40% of *KIT/PDGFR*A WT GIST represent succinate dehydrogenase complex (SDH)-deficient GIST. SDH-deficiency is caused by the loss of function mutations in one of the genes encoding the SDH subunits (*SDHA*, *SDHB*, *SDHC* and *SDHD*) or *SDHC* epimutation resulting in transcriptional silencing of the gene (11,12). Another subgroup of *KIT/PDGFR*A WT GIST with competent SDH complex activity is defined as RAS-pathway (RAS-P)-mutant GIST. This group includes GIST with inactivating mutation of the *NFI* gene, an activating mutation of *BRAF*, or *RAS* (13,14). SDH-deficient and *RAS* mutant GIST represent half of the *KIT/PDGFR*A WT GIST. In the

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remaining *KIT/PDGFR* WT GIST, the oncogenic driver remains unknown. Of late, certain extremely rare genetic alterations have been demonstrated, such as *ETV6-NTRK3* fusion, and alterations in *FGFR1*, or *FGF4*, *MAX* and *MEN1* genes (15–17). Metastatic *KIT/PDGFR* WT GIST demonstrates a poor response to imatinib mesylate treatment, and there are no prospective data on response, disease progression and overall survival in this setting (18).

Standard methods currently used to determine activating mutations in the *KIT* and *PDGFR* genes are reverse transcription-quantitative (RT-q) PCR and direct Sanger sequencing. The disadvantages of these methods are that they detect either predetermined mutations (mostly hot spot mutations) or in the case of Sanger sequencing, mutations in a specific gene segment, but only if allele frequency of mutation is at least 20% in the sample. They also do not detect all possible mutations in the *KIT* and *PDGFR* genes. Alternatively, next-generation sequencing (NGS) enables the identification of numerous changes in a wider range of investigated genes that cannot be easily detected with standard methods.

This study was performed with the aim to identify somatic mutations using NGS in metastatic *KIT/PDGFR* WT GIST patients and analyze their treatment outcomes in a real-world setting.

## Materials and methods

**Patients.** Between January 2002 and December 2020, 119 patients with metastatic *KIT*-positive GIST started their treatment at the Institute of Oncology Ljubljana (Ljubljana, Slovenia). From this population of patients, only patients with *KIT/PDGFR/BRAF* WT tumors as assessed by standard methods were included in further analysis. The present study was approved (approval no. 0120-204/2021/3) by the local Institutional Review Board of the Institute of Oncology Ljubljana and by the National Medical Ethics Committee of the Republic of Slovenia. All procedures in the present study were performed following the ethical standards of the responsible committees on human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Individual patient consent was waived for the present study as it was a retrospective study, the research involved no risk to the subjects, and the institutional informed consent forms for treatment included consent for the use of patient's data, materials, and/or test results for research purposes.

**Immunohistochemistry (IHC).** Formalin-fixed, paraffin-embedded (FFPE) tissue samples were examined and evaluated by a dedicated pathologist from the Department of Pathology at the Institute of Oncology Ljubljana (Ljubljana, Slovenia). Diagnosis of GIST was confirmed based on microscopic findings and CD117 antigen expression as previously described by the authors (19). The determination of DOG1 antigen expression was performed after 2010 in selected cases.

In *KIT/PDGFR* WT GIST patients, SDH deficiency was assessed using IHC. Specifically, CD117 and SDHB were detected on 2–4  $\mu$ m FFPE tissue sections and dried at 56°C for 2 h. Until 2017, CD117 was detected according to a previously described protocol by the authors (19). From 2017, both CD117 and SDHB were detected using fully automated IHC

system Ventana Benchmark XT (Ventana ROCHE Inc.). Both epitopes were retrieved on board employing HIER using high pH Cell Conditioning Solution 1 (cat. no. 950-124; Ventana ROCHE Inc.) for 88 min at 100°C. CD117 was detected using a commercially available rabbit monoclonal antibody CD117 (clone YR145; cat. no. 117R; Cell Marque; Merck KGaA). Primary antibody was diluted 1:200 using DAKO REAL™ antibody diluent (cat. no. S2022; DAKO Agilent Technologies, Inc.). SDHB was detected using commercially available rabbit monoclonal antibody SDHB (clone EP288; cat. no. 466R; Cell Marque; Merck KGaA). Primary antibody was diluted 1:100 using DAKO REAL™ antibody diluent (cat. no. S2022; DAKO Agilent Technologies, Inc.). Both antibodies were incubated on board for 60 min at 37°C. Bound primary antibodies were visualized using 3-step multimer detection system OptiView DAB IHC Detection kit (cat. no. 760-700; Ventana ROCHE Inc.) according to the manufacturer's protocol.

The determination of DOG1 was introduced in 2010 using a fully automated IHC system Ventana Benchmark XT (Ventana ROCHE Inc.). The epitope was retrieved on board employing HIER using high pH Cell Conditioning Solution 1 (cat. no. 950-124; Ventana ROCHE Inc.) for 88 min at 100°C. Until 2019, DOG1 was detected using a commercially available rabbit monoclonal antibody DOG-1 (clone SP31; cat. no. M331; Spring Bioscience; Abcam). Primary antibody was diluted 1:50 using DAKO REAL™ antibody diluent (cat. no. S2022; DAKO Agilent Technologies, Inc.). Since 2019, DOG1 was detected using a commercially available rabbit monoclonal antibody DOG-1 (clone SP31; cat. no. 244R; Cell Marque; Merck KGaA). Primary antibody was diluted 1:200 using DAKO REAL™ antibody diluent (cat. no. S2022; DAKO Agilent Technologies, Inc.). Both DOG1 antibodies were incubated on board for 60 min at 37°C. Bound primary antibody was visualized using 3-step multimer detection system OptiView DAB IHC Detection kit (cat. no. 760-700; Ventana ROCHE Inc.) according to the manufacturer's protocol.

**Tumor genotyping.** Genomic DNA and RNA for tumor molecular analysis were extracted from FFPE primary tumor samples. Macro-dissection of FFPE sample slides followed by tumor genomic DNA/RNA extraction was performed as previously described by the authors (19,20).

Detection of somatic mutations in *KIT* (exons 9, 11, 13 and 17) and *PDGFR* (exons 12, 14 and 18) was performed in all GIST samples (n=119) from the present study by RT-qPCR and direct Sanger sequencing. Additionally, patients with *KIT/PDGFR* WT GIST were tested for the presence of hot spot mutations in *BRAF* gene as previously described by the authors (19).

A total of 17 samples defined as *KIT/PDGFR/BRAF* WT GIST (including the immunohistochemically SDHB deficient patients) were analyzed by NGS using TruSight Oncology 500 DNA kit (Illumina, Inc.): TSO500-DNA and 8 samples were additionally examined with TruSight Tumor 170 RNA kit (Illumina, Inc.): TST170-RNA. One sample was examined for germline alterations using TruSight Cancer Panel (Illumina, Inc.). Libraries were prepared according to the manufacturer's protocol and sequenced on NextSeq 550 Sequencing System. Read alignment to the hg19 reference genome and variant calling was performed using TruSight Tumor 170 v2

Local App software (Illumina, inc.). Bioinformatical analysis was performed as previously described by the authors (20). Targeted analysis of following genes was performed: *KIT* (LRG\_307t1), *PDGFRA* (LRG\_309t1), *BRAF* (LRG\_299t1), *SDHA* (LRG\_315t1), *SDHB* (LRG\_316t1), *SDHC* (LRG\_317t1), *SDHD* (LRG\_9t1) and *NFI* (LRG\_214t1). Variants were described according to HGVS v2019.05 nomenclature (21).

**Clinical evaluation, follow-up and outcome analysis.** Patients were followed-up every 3-4 months with clinical evaluation, laboratory testing (complete blood count, biochemistry), abdominal ultrasound, abdominal contrast-enhanced computerized tomography. RECIST criteria until 2007 and Choi criteria were used thereafter for defining response and disease progression (22,23). In case of no disease progression, treatment was continued. In case of progression on imatinib, sunitinib has been used since 2007 and for those progressing on sunitinib, regorafenib since 2014. Patients with a documented local progression were managed surgically if that was feasible.

**Statistical analysis.** Overall survival was assessed from the beginning of treatment until the date of death from any cause by Kaplan-Meier test. To test the difference in survival between patients with *KIT/PDGFRA/BRAF* WT GIST vs. mutated tumors as assessed by RT-qPCR and Sanger sequencing, the log-rank test was used (24).  $P < 0.05$  was considered to indicate a statistically significant difference. Swimmer plot was applied to present the clinical outcome of patients with *KIT/PDGFRA/BRAF* WT tumors and their NGS molecular status (25). Statistical analyses were performed using SPSS Statistics for Windows (version 25; IBM Corp.).

## Results

A total of 119 patients with metastatic GIST, all older than 18 years, were registered between 2002 and 2020. All 119 tumor samples stained positive for CD 117 staining (Fig. S1), the staining for DOG1 was performed after 2010 in 53 (44.5%) samples. The median follow-up at the time of analysis in October 2021 was 11.5 years. Clinicopathological characteristics of the whole group are presented in Table S1.

In 2 patients, tumor specimens were inadequate for molecular analysis. In the remaining 117 patients, mutational status and mutation location were assessed using standard methods RT-qPCR and Sanger sequencing. Single *KIT* or *PDGFRA* mutations were detected in 85% of all GIST patients: *KIT* exon 11 mutation in 81 patients (68.1%), *KIT* exon 9 mutation in 9 patients (7.6%), and *KIT* exon 13 in 1 patient (0.8%). A total of 2 patients had co-primary mutations: one in *KIT* exons 9 and 13 and one in *KIT* exons 11 and 13. In 3 patients, samples were taken and analyzed after the start of treatment with imatinib and revealed compound mutations: twice in *KIT* exons 11 and 17 and once in *KIT* exons 13 and 17. A total of 5 patients (4.1%) were *PDGFRA* mutated (one in *PDGFRA* exon 14, three in *PDGFRA* exon 18 and one in *PDGFRA* exon 12). None of the 17 *KIT/PDGFRA* WT patients was positive for *BRAF*. Thus, 17 patients (14.3%) were designated as *KIT/PDGFRA/BRAF* WT GIST (Table I). All 17 tumor samples stained positive for CD 117 antigen, while staining for DOG1 antigen was performed in 16 (94.1%) cases, one sample did not stain positive, probably due to an inadequate tissue sample.

The majority of 17 patients identified as *KIT/PDGFRA/BRAF* WT GIST were women (70.6%), their median age was 60 years, and 15 (88.2%) received therapy with imatinib. The majority of tumors were large, with a high mitotic rate and a high risk of progression. The most frequently affected sites were the small intestine in 47.0% and the stomach in 29.4%-similar to the whole study group. The clinicopathological characteristics of *KIT/PDGFRA/BRAF* WT GIST patients and their tumors are presented in Table II.

The treatment decision in 15 *KIT/PDGFRA/BRAF* WT GIST patients as assessed by standard methods receiving initial therapy with imatinib differed regarding the period when they were treated. Before 2016, the decision for imatinib treatment in Slovenia was based on a pathohistological diagnosis of GIST solely (13 patients). After 2016, two patients were offered imatinib treatment based on reports of clinical responses to imatinib also in *KIT/PDGFRA/BRAF* WT patients (26,27). One of the two (2/17) *KIT/PDGFRA/BRAF* WT GIST patients not treated with imatinib, had a solitary lesion in the liver and was treated with radiofrequency ablation. The disease did not recur. The second patient was treated with upfront sunitinib. Altogether, five patients were treated with sunitinib occasionally in the course of their disease and none received regorafenib.

The median overall survival of patients with *KIT/PDGFRA/BRAF* WT GIST vs. mutated tumors as assessed by standard methods was 4.02 years (95% CI 0.17-7.86) and 5.98 years (95% CI 4.92-7.05), respectively (log-rank  $P = 0.232$ ; Fig. S2).

Results of IHC assessment of SDH competence/deficiency in 16 out of 17 *KIT/PDGFRA/BRAF* WT GIST patients as assessed by standard methods RT-qPCR and Sanger sequencing are provided in Table III. Specifically, loss of IHC staining for SDHB protein was detected in 4 patients.

Molecular status of genes frequently altered in GIST was reevaluated using a targeted NGS approach in all 17 *KIT/PDGFRA/BRAF* WT GIST samples. In one case the quantity of remaining DNA was insufficient for NGS analysis. Mutational status, mutation location, variant allele frequency, and pathogenicity of detected alterations in 17 *KIT/PDGFRA/BRAF* WT GIST patients are presented in Table III. The deposition of the full sequences of *KIT* and *PDGFRA* in 17 GIST patients in the National Center for Biotechnology Information (NCBI) gene bank is in progress. A total of 7 out of 16 samples with sufficient DNA yield for NGS (43.8%) were found to carry a pathogenic alteration, either in *KIT* (4 patients) or *PDGFRA* (3 patients). They all responded to imatinib-most surprisingly, including the patient with *PDGFRA* D842V mutation. Besides pathogenic alterations in the aforementioned genes, NGS also revealed *SDH* alterations in 3 patients that had already been suspected based on the loss of IHC staining for SDHB protein (Fig. S3). One sample demonstrated a pathogenic variant in *NFI*, likely in the context of neurofibromatosis type 1. The detected *NFI* pathogenic variant in this patient was confirmed to be of germline origin. No fusions in genes included in TruSightTumor 170-RNA kit were detected in examined samples. Only 5 out of 16 cases with sufficient DNA (31%) of *KIT/PDGFRA/BRAF* WT GIST were confirmed to be *KIT/PDGFRA/BRAF/SDH/NFI* WT also by NGS, and none of them responded to TKIs. Their

Table I. Mutational status and mutation location for all 119 patients with GIST, assessed by standard methods reverse transcription-quantitative PCR and Sanger sequencing.

Mutation genotype	n (%)
<i>KIT/PDGFR A/BRAF</i> -mutant GIST	
<i>KIT</i> exon 11	81 (68.1)
<i>KIT</i> exon 9	9 (7.6)
<i>KIT</i> exon 13	1 (0.8)
<i>KIT</i> exons 9 and 13 <sup>a</sup>	1 (0.8)
<i>KIT</i> exons 11 and 17 <sup>b</sup>	2 (1.7)
<i>KIT</i> exons 13 and 17 <sup>b</sup>	1 (0.8)
<i>PDGFRA</i> exon 12	1 (0.8)
<i>PDGFRA</i> exon 14	1 (0.8)
<i>PDGFRA</i> exon 18	3 (2.5)
<i>BRAF</i>	0
<i>KIT/PDGFR A/BRAF</i> wild-type GIST	17 (14.3)
Not determined/Unknown	1 (0.8)

<sup>a</sup>two primary mutations; <sup>b</sup>sample taken and analyzed after beginning of treatment with imatinib; GIST, gastrointestinal stromal tumors.

survival was typically poor except for one patient who remains alive with metastatic disease but has been treated surgically.

A total of 7 out of 8 patients that responded to imatinib had activating mutations in *KIT* or *PDGFRA* confirmed by NGS. One patient that had clinical benefit with stable disease, had the *NF1* mutation (c.4537C>T; p. Arg1513\*; Table III). Type of treatment, duration of response, survival, and NGS molecular status of 17 *KIT/PDGFR A/BRAF* WT GIST patients as assessed by standard methods is presented in Fig. 1.

The percentage of *KIT/PDGFR A/BRAF* WT GIST patients as determined by standard methods (14.3%; 17 out of 119 patients) was reduced to 7.6% (9 out of 119 patients) after re-evaluation with NGS. However, only five out of 119 patients (4.2%) were truly WT GIST patients (excluding those without *SDH* and *NF1* variants).

## Discussion

In patients with GIST, it has become mandatory to determine the driver mutation in order to predict the response to TKI treatment. The European Society for Medical Oncology (ESMO) guidelines, however, do not specify the screening method for the detection of somatic mutations (28). RT-qPCR, direct Sanger sequencing or NGS may be used, but in the past, the first descriptions of gain of function mutations in *KIT/PDGFR A* were based on RT-qPCR and direct Sanger sequencing (4,29,30). These methods have remained the cornerstone for detecting oncogenic drivers in GIST until today. Nevertheless, it has been observed in routine clinical practice that there are patients with *KIT/PDGFR A* WT GIST by conventional RT-qPCR and direct Sanger sequencing that respond to imatinib. With these observations, the reliability of the results of RT-qPCR and direct Sanger sequencing in the identification of genuinely *KIT/PDGFR A* WT GIST are questioned.

Table II. Clinicopathological characteristics of 17 *KIT/PDGFR A/BRAF* wild-type GIST patients (as assessed by standard methods reverse transcription-quantitative PCR and Sanger sequencing) and their tumors.

Clinicopathological characteristic	n (%)
Sex	
Male	5 (29.4)
Female	12 (70.6)
Age, years	
Median	60
Range	32-76
≤60	9 (53)
>60	8 (47)
Imatinib initial therapy	
Yes	15 (88.2)
No	2 (11.8)
Primary tumor location	
Esophagus	0 (0)
Stomach	5 (29.4)
Small intestine	8 (47.0)
Rectum	2 (11.8)
Other	2 (11.8)
Mitotic rate <sup>a</sup>	
≤5	6 (35.3)
5.1-10	2 (11.8)
>10	9 (52.9)
Primary tumor size, cm	
0-5	1 (5.9)
5.1-10	5 (29.4)
10	11 (64.7)
Risk classification <sup>b</sup>	
High	11 (64.7)
Intermediate	5 (29.4)
Low	1 (5.9)
Very low	0 (0)

GIST, gastrointestinal stromal tumors; <sup>a</sup>Mitotic rate counted as the number of mitoses per 5 mm<sup>2</sup>; <sup>b</sup>Risk group for GIST adapted from Miettinen and Lasota (53).

In the present study, mutations detected by NGS as well as clinical outcomes of the *KIT/PDGFR A/BRAF* WT GIST were reported (n=17). Patients were selected from a larger series of GIST patients (n=119) analyzed for mutational status with conventional methods RT-qPCR and direct Sanger sequencing.

Among initially included 119 patients, the clinicopathological characteristics showed no difference when compared with published data. As previously reported, also in the present study, there was a slight male predominance (50.4%) (5,6,31). The age at diagnosis of our patients ranged from 32 to 89 years, with the median age being 63.5 years. The median age across most studies falls in the mid-60s (1,2,5-7). In our cohort, GIST most commonly arose in small intestine and

Table III. Mutational status, mutation location, allelic frequency, pathogenicity, and clinical benefit to imatinib in *KIT/PDGFR $\alpha$ /BRAF* WT GIST patients as assessed by standard methods reverse transcription-quantitative PCR and Sanger sequencing.

n	SDHIHC	Clinically significant gene variants in GIST (NGS)		cDNA variant description	Predicted protein	Pathogenicity ACMG/AMP <sup>f</sup>		VAF (%)	TMB (mut/MB) ns/(ns+s)	Clinical benefit to imatinib	RECISt evaluation (22)
		variants in GIST (NGS)	CDNA variant description			ACMG/AMP <sup>f</sup>	VAF (%)				
1 <sup>a</sup>	Loss	WT						6/7	NA	NT	NA
2	Comp.	WT						1/2	NA	No	PD
3 <sup>b</sup>	Comp.	WT						1/1	NA	No	PD
4	Comp.	WT						0/1	NA	No	PD
5	Comp.	WT						2/3	NA	No	PD
6 <sup>b,c</sup>	Comp.	NF1 (LRG_214t1)		c.4537C>T	p. (Arg1513*) R1513*	P		96.27	96.66	Yes	SD
7	Loss	SDHA (LRG_315t1)		c.776delinsGC	p. (Tyr259Cysfs*62) Y259Cfs*62	LP		69.66	4/4	No	PD
8	Loss	SDHB (LRG_316t1)		c.268C>T	p. (Arg90*) R90*	LP		30.4	NA	No	PD
9 <sup>b,d</sup>	Loss	SDHB (LRG_316t1)		c.688C>T	p. (Arg230Cys) R230C	LP		79.31	4/4	NT	NA
10 <sup>b,g</sup>	Comp.	PDGFRA (LRG_309t1)		c.2525A>T	p. (Asp842Val) D584V	P		41.17	0/0	Yes	CR
11 <sup>b</sup>	Comp.	PDGFRA (LRG_309t1)		c.1682T>A	p. (Val561Asp) V561D	P		84.61	2/3	Yes	CR
12 <sup>b</sup>	Comp.	PDGFRA (LRG_309t1)		c.1936A>G	p. (Lys646Glu) K646E p. (Asn659His) N659H	LP LP		40.56	2/3	Yes	CR
13 <sup>b</sup>	Comp.	PDGFRA (LRG_309t1)		c.1975A>C		P		41.27	1/1	Yes	CR
14	Comp.	KIT (LRG_307t1)		c.1679T>A	p. (Val560Asp) V560D	P		43.35	0/0	Yes	CR
15	Comp.	KIT (LRG_307t1)		c.1669_1674del	p. (Trp557_Lys558del) W557_K558del	LP		47.21	1/3	Yes	PR
16 <sup>b</sup>	Comp.	KIT (LRG_307t1)		c.1671_1676del	p. (Trp557_Val559delinsCys) W557_V559delinsC	LP		41.2	2/2	Yes	PR
17 <sup>e</sup>	NA	NA		c.1648-3_1673del	p. (Lys550_Lys558del) K550_K558del	LP		39.9	NA	Yes	PR
	NA	NA		NA	NA	NA		NA	NA	No	PD

WT, wild type; P, pathogenic; LP, likely pathogenic; VAF, variant allele frequency; TMB, tumor mutational burden; ns, nonsynonymous; s, synonymous; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; RECISt, response evaluation criteria in solid tumors; NA, not applicable; NT, not treated; comp., competent; \*upfront treated with sunitinib; <sup>b</sup>besides DNA, also RNA was sequenced by TruSight Tumor 170-RNA; <sup>c</sup>confirmed germline mutation; <sup>d</sup>radiofrequency ablation of solitary liver metastasis; <sup>e</sup>not enough material for additional testing; <sup>f</sup>Pathogenicity determined according to ACMG/AMP guidelines (58); <sup>g</sup>resistant mutation *PDGFRA* D84-2V.

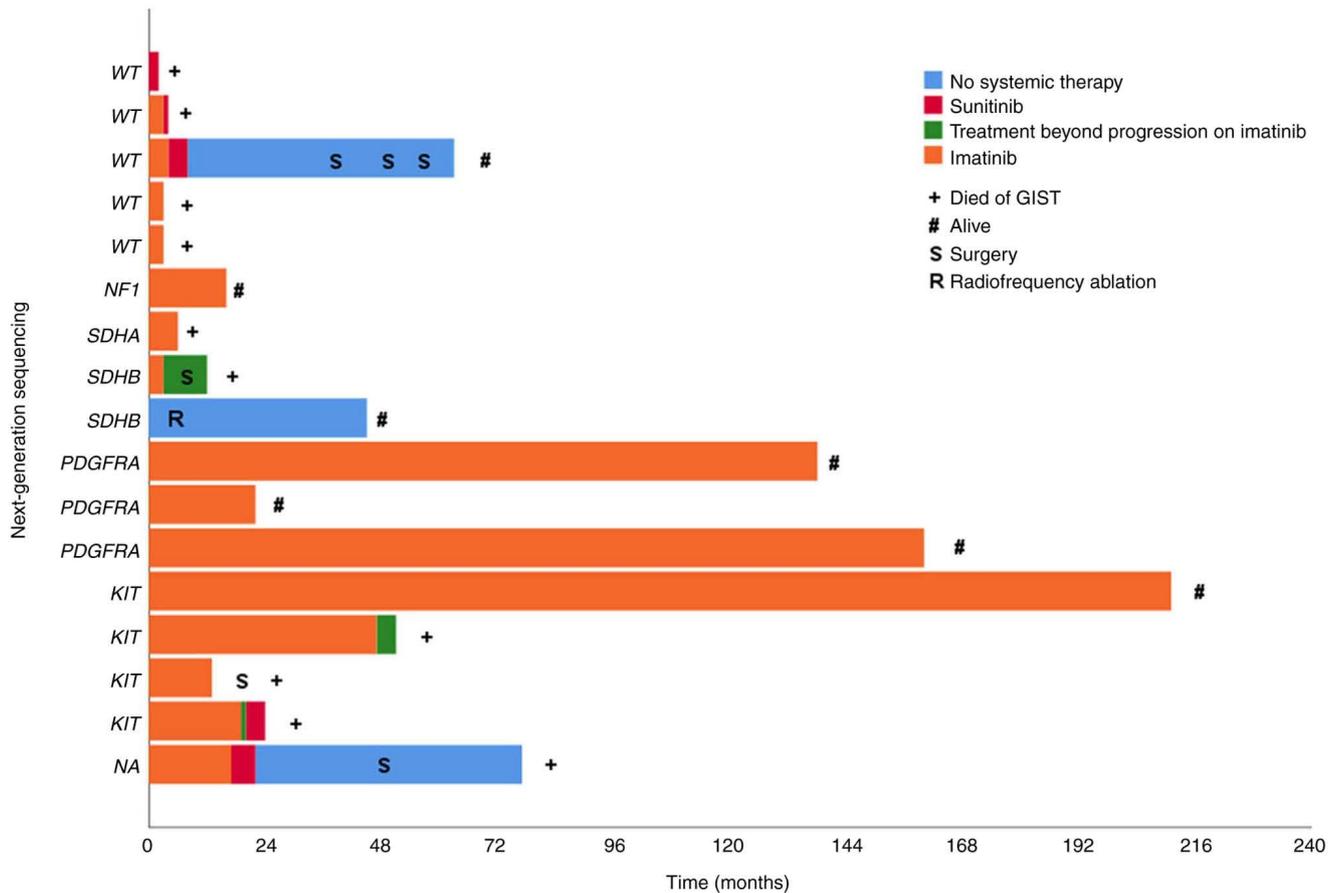


Figure 1. Clinical outcome in 17 *KIT/PDGFRΑ/BRAF* wild-type GIST patients (as assessed by standard methods reverse transcription-quantitative PCR and Sanger sequencing) and their next-generation sequencing molecular status. Patients are listed according to Table III. *KIT*, *KIT* proto-oncogene, receptor tyrosine kinase; *PDGFRA*, platelet-derived growth factor receptor alpha; GIST, gastrointestinal stromal tumors.

stomach (37.0%) while in the previous study, the majority of GIST were found in the stomach and less commonly in the small intestine (1,2,8,32). This may be an area of interest for future research. There was also no difference in the proportion of *KIT/PDGFRΑ/BRAF* WT GIST as determined with conventional RT-qPCR and direct Sanger sequencing between our study (14.3%) and data published in the literature, where they observed 7.1-15% of WT GIST (2-4).

In the present study, *KIT/PDGFRΑ/BRAF* WT GIST by conventional RT-qPCR and direct Sanger sequencing were found to arise predominantly in females (70.6%), at a younger age than the whole study population (median age 60 years), while other clinical characteristics (primary location, mitotic count, risk group) did not differ from the whole study population. *KIT/PDGFRΑ/BRAF* WT GIST by conventional RT-qPCR and direct Sanger sequencing were reported to arise predominantly in the stomach if *SDH* mutant or epimutant and predominantly in the small intestine if *SDH* competent (33). In the present analysis, they were found more often in the small intestine than in the stomach, regardless of *SDH* status. Importantly, only adult GIST patients were included in the present analysis. The natural history of *KIT/PDGFRΑ/BRAF* WT GIST by conventional RT-qPCR and direct Sanger sequencing has been suggested to be more indolent than that of *KIT/PDGFRΑ*-mutated GIST, but the present study demonstrated a shorter (yet not significantly)

overall survival compared with the whole study group (median overall survival 4.02 years vs. 5.87 years) (8,34,35). *KIT/PDGFRΑ/BRAF* WT GIST by conventional RT-qPCR and direct Sanger sequencing, that are also confirmed to be WT (for *KIT/PDGFRΑ* alterations) by NGS, can be associated with hereditary syndromes, such as type 1 neurofibromatosis (NF1), Carney triad (23), Carney Stratakis syndrome and hereditary paraganglioma/pheochromocytoma (HPGL/PCC) syndromes (8,33). In the present study, only one patient had a hereditary syndrome (NF1).

In the present study, 15 of total 17 *KIT/PDGFRΑ/BRAF* WT GIST patients by conventional RT-qPCR and direct Sanger sequencing were available for assessing response to imatinib. Patients (n=7) that responded to imatinib were, however, diagnosed to carry pathogenic alterations by NGS; either in *KIT* or *PDGFRA*. One patient with long-term, on-going complete response on imatinib was a carrier of *PDGFRA* D842V mutation. Patients with *PDGFRA* D842V mutation rarely respond to imatinib (34-37). Farag *et al* (37) reported partial response in 2 out of 16 *PDGFRA* D842V-mutated GIST patients and stable disease in 3 of totally 16 patients. The percentage of patients carrying pathogenic alterations either in *KIT* or *PDGFRA* in the present study (41.2%) was higher than the one reported by Heinrich *et al* (7) demonstrating pathogenic alterations either in *KIT* or *PDGFRA* in 3 of total 20 patients (15%) (5-7). No new mutations of *KIT* and *PDGFRA* were observed in the

present study; all mutations discussed in Table III have already been previously reported in NCBI ClinVar and/or Cosmic and discussed (30,38-46).

One patient that had clinical benefit with stable disease was a carrier of *NFI* germline pathogenic variant and the reason for benefit could only be hypothesized. Putatively, some of the lesions followed with CT scans could have been other entities (not verified) in the context of confirmed *NFI* and not GIST metastases. A possible explanation for response to TKI could also be attributed to the increased KIT activation through phosphorylation. Namely, the efficacy of TKI in patients with increased KIT activation (detected in some of *KIT/PDGFR*A/*BRAF* WT tumors), suggested that in some of these patients KIT activation may play a role in the pathophysiology (26). Moreover, similar to our observation for the patient carrying pathogenic variant in *NFI* exon 34 (c.4537C>T), Mussi *et al* (47) reported stable disease in one patient with *NFI* exon 18 mutation after therapy with imatinib.

The remaining 7 out of 15 patients available for assessing response to imatinib did not respond. Among them, two samples demonstrated the *SDH* alteration which had already been suspected by IHC and was later verified with NGS. Patients with *SDH* alterations (mutation or epigenetic silencing) are known not to respond to imatinib (7,12). A total of 5 patients remained WT also by NGS, among whom 4 were initially treated with imatinib and did not respond, and one was treated with sunitinib and did not respond. This is consistent with the published literature stating that NGS WT GIST does not respond to TKI, regardless of whether *SDH* deficient or *SDH* competent by IHC (9). In the remaining not responding patient the quantity of DNA was not sufficient for NGS analysis.

In 16 GIST patients, the tumor mutational burden (TMB) was assessed. The calculated TMB was low in all patients (less than 10 mutations/MB). These results are in accordance with the previous study by Chalmers *et al* (48) but differ from the results by Feng *et al* (49). High TMB correlates with the response to immune checkpoint inhibitors in solid tumors and pembrolizumab was approved for adults and children with TMB-high solid tumors (50). In oncogene-driven disease, it would be expected that a lower TMB correlates with a longer response to TKI. At present, to the best of our knowledge, no aware study exists on the correlation between the TMB score and the largest clinical benefit of imatinib in GIST patients.

*KIT/PDGFR*A WT GIST has been reported to comprise 10-15% of all GIST with standard diagnostic methods RT-qPCR and direct Sanger sequencing. In large randomized clinical trials that establish the recommendations for the treatment of metastatic GIST, NGS has not been performed. In the phase III BFR14 trial, mutational status was determined by PCR and Sanger sequencing and identified 11.4% WT GIST (51). In the B2222 study, mutational status was determined by PCR and high-pressure liquid chromatography (HPLC) (3). Activating mutations were identified in 92.9%. In EORTC 62005 study mutations were screened by denaturing high-performance liquid chromatography (DHPLC) and characterized by bi-directional DNA sequencing (52). Activating mutations of *KIT* or *PDGFR*A were found in 84% of GIST. In SWOG S0033/CALGB 150105 study, a combination of PCR amplification, DHPLC screening, and sequencing was used and demonstrated 16.9% *KIT/PDGFR*A WT GIST (83.1%

of samples carried *KIT/PDGFR*A mutations) (18). In the follow-up analysis of the SSG XVIII/AIO trial, conventional Sanger sequencing was used and identified 7% *KIT/PDGFR*A WT GIST (53). The results of the present study are absolutely in concordance with the results of the aforementioned studies. Namely, in the present study, 85% of samples were identified to be *KIT/PDGFR*A mutation-positive with RT-qPCR and Sanger sequencing, while 14.3% of samples were *KIT/PDGFR*A WT GIST.

However, in recent years, studies of NGS in GIST have been published, demonstrating that this technique has the potential to detect genetic alterations otherwise not detectable with conventional techniques, particularly in *KIT/PDGFR*A WT tumors (8,13,17,54,55). Indeed, ESMO guidelines recommend tumor molecular testing in GIST patients if TKI are considered as a part of the management, however, the preferential method is not specified (28). On the other hand, the latest National Comprehensive Cancer Network guidelines recommend that all GIST lacking a *KIT* or *PDGFR*A mutation should be examined for *SDH* deficiency and alternative driver mutations using NGS (56).

When comparing NGS with RT-qPCR and direct Sanger sequencing, NGS provides the highest positivity rate (43). This conclusion indicates a potential advantage of NGS for the detection of targetable mutations in real-world clinical routine setting. In the present study, using the NGS in 16 out of 17 *KIT/PDGFR*A/*BRAF* WT GIST as determined by conventional RT-qPCR and Sanger sequencing, mutated genotype was identified in 69% (11 patients). NGS identified 4 cases with *KIT* mutation, 3 cases with *PDGFR*A mutation, both missed in conventional analysis. It also identified 3 cases with *SDH* complex mutation (all suspicious on IHC staining) and 1 *NFI* germline pathogenic variant. All patients with *KIT/PDGFR*A mutated genotype by NGS responded to imatinib. Similar results have also been reported by previous studies. In the report from Heinrich *et al* (18), the presumed causative mutation was identified with a combination of PCR and Sanger sequencing in 83% of GIST (*KIT* and *PDGFR*A analysis only). Using the NGS technologies, Heinrich *et al* (7) estimated that 97.5% of GIST can be assigned a mutated genotype. NGS of 20 cases originally classified as *KIT/PDGFR*A WT GIST disclosed 17 (85%) carrying a mutation, most commonly (in 60%) in *SDH* complex. Astolfi *et al* (43) indicated that ~20% of patients, assessed by Sanger and IHC, with *KIT/PDGFR*A/*SDH*/*RAS*-P WT GIST are carriers of pathogenic *KIT* mutations, thus expected to be eligible for and responsive to the various therapeutic lines of TKI in use for *KIT/PDGFR*A mutant GIST. Vanden Bempt *et al* (57) also demonstrated that the percentage of WT GIST decreases with the use of NGS. Optimizing detection assays of genetic alterations led to the identification of *KIT* or *PDGFR*A mutations in 92.0% of GIST samples and reduced the number of WT GIST to 1.2% only.

In conclusion, the results of the present study demonstrated that ~41.2% of patients diagnosed through standard methods (RT-qPCR and Sanger sequencing) with the *KIT/PDGFR*A/*BRAF*-pathway WT GIST are actually carriers of pathogenic *KIT/PDGFR*A mutations when re-evaluated by NGS, and are thus expected to be eligible for and responsive to the various therapeutic lines of TKI in use

for *KIT/PDGFR*A-mutant GIST. The percentage of genuinely *KIT/PDGFR*A WT GIST in the present study decreased from the initial 14.3% when assessed with the RT-qPCR and Sanger sequencing to 7.6% after NGS. Therefore, it is strongly considered that NGS should become prerequisite for treatment decision at least in *KIT/PDGFR*A/*BRAF*-pathway WT GIST as determined by RT-qPCR and Sanger sequencing.

The limitation to the present study is a small number of NGS samples, as well as a long follow-up with different molecular methods used throughout the course of study, diverse methods of evaluation, and variable access to treatment.

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### Availability of data and materials

The data presented in this study are openly available in Sequence Read Archive (NCBI-SRA), accession number PRJNA824718.

### Authors' contributions

MU, SN and BBN conceptualized the present study. Formal analysis, MU, AB, SN, VS, VSD, OB, GK and BBN performed formal analysis. MU, AB, SN, VS, VSD, OB, GK and BBN conducted investigation. MU, SN and BBN provided methodology. MU and BBN performed project administration. MU, AB, SN, VS, VSD, OB, GK and BBN provided resources, SN and BBN supervised the present study. MU and SN conducted visualization. MU wrote the original draft. MU, AB, SN, VS, VSD, OB, GK and BBN wrote, reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript. SN and BBN confirm the authenticity of all the raw data.

### Ethics approval and consent to participate

The present study was approved (approval no. 0120-204/2021/3) by the local Institutional Review Board of the Institute of Oncology Ljubljana and by the National Medical Ethics Committee of the Republic of Slovenia (Ljubljana, Slovenia). All procedures in the present study were done following the ethical standards of the responsible committees on human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Individual patient consent was waived for this study as it was a retrospective study, the research involved no risk to the subjects, and the institutional informed consent forms for treatment included consent for the use of patient's data, materials, and/or test results for research purposes.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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