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Gastrointestinal stromal tumors – Summary of mutational status of the primary/secondary KIT/PDGFRA mutations, BRAF mutations and SDH defects

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ABSTRACT

The most important findings revealing pathogenesis, molecular characteristics, genotyping and targeted therapy of gastrointestinal stromal tumors (GISTs) are activated oncogenic mutations in *KIT* and *PDGFRA* genes. Imatinib mesylate (IM), which inhibits both KIT and PDGFRA receptors, significantly improved treatment of advanced (metastatic, recurrent, and/or inoperable) GISTs. However, in a significant number of patients the treatment fails due to the primary or secondary resistance to targeted therapy. Most common cause of secondary resistance is a presence of secondary mutations. Approximately 15% of adult patients with GISTs are negative for mutations in *KIT* or *PDGFRA* genes. These so-called wild-type GISTs appear to be characterized by other oncogenetic drivers, including mutations in *BRAF*, *RAS*, *NF1* genes, and subunits of succinate dehydrogenase (SDH) complex.

In the present study we investigated 261 tumour specimens from 239 patients with GIST. Primary mutations were detected in 82 % tumor specimens. 66 of them were in *KIT*, and 16 % in *PDGFRA* genes. Remaining 18 % were *KIT/PDGFRA* wild-type. Secondary *KIT* mutations were detected in 10 from 133 (7 %) patients treated with IM. We examined secondary *KIT* mutations in exons 13 and 17 and secondary *PDGFRA* mutation in exon 18 in sixteen progressive tumors and/or metastasis (from overall 22 samples). We identified *BRAF* V600E point mutation in 4 % of *KIT/PDGFRA* wild-type GIST patients. Moreover, we analysed SDH complex mutations in 4 younger patients (15, 33, 37, and 45 years old) from 44 patients without *KIT*, *PDGFRA*, and *BRAF* mutations. Two patients (a 37-year old man, and a 33-year old woman) had defects of the SDH complex.

Our findings of mutational status of the primary and secondary *KIT/PDGFRA* mutations in patients with GIST confirm mechanisms of primary and secondary resistance, and also intralesional and interlesional heterogeneity of secondary mutations within and between progressive lesions. Moreover, detection of V600E *BRAF* mutation and defects of SDH complex in *KIT/PDGFRA* wild-type GISTs confirm their activation and allow for a selection of targeted therapy.

1. Introduction

The most important finding contributing to identification of pathogenesis, molecular characteristics, genotyping and targeted therapy of gastrointestinal stromal tumours (GISTs) was the presence of activated oncogenic mutations in the *KIT* [1] and *PDGFRA* [2] genes. Tyrosine kinase inhibitor (TKI) Imatinib mesylate (IM) (Glivec®/Gleevec®, Novartis Pharma AG, Basel, Switzerland), which inhibits both KIT and PDGFRA receptors, significantly improved treatment of advanced (metastatic, recurrent, and/or unresectable) GIST [3]. Unfortunately, most patients on IM treatment will eventually develop resistance to this agent as well as to other drugs such as sunitinib, regorafenib – used in the 2nd and 3rd line TKI treatment. Resistance can be divided into primary (early) and secondary (late). Approximately 10–15 % of patients with GIST fail to respond on their initial exposure to IM [4]. Most common cause of the primary resistance to IM rests in the tumor inherent mutations in the molecular target of the drug [4]. About 50 % of the patients who benefit initially from IM treatment

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develop eventually secondary/acquired resistance [5]. The main cause of development of the secondary resistance (50–80 %) is a presence of secondary mutations. Secondary mutations are single substitutions and affect different exons than the primary mutations but on the same allele [6–8]. Secondary mutations may arise also in a distinct receptor than that affected by primary mutations but this phenomenon is uncommon [9]. An unfavourable factor of the tumor resistance is a high intralesional and interlesional variability of the secondary mutations [6]. Approximately 15 % of adult GISTs are negative for mutations in *KIT* or *PDGFRA* genes. These so-called wild-type GISTs were identified with a number of other oncogenetic drivers, including mutations in *BRAF*, *RAS*, *NF1* genes, and subunits of the succinate dehydrogenase complex [10].

In the present study, we aimed to disclose the frequency and mutational status of the primary mutations of *KIT/PDGFRA*, and most importantly, secondary *KIT/PDGFRA* mutations in patients with GIST analysed in our department. Mutational status of the patients with secondary resistance was correlated with their clinical and pathologic data.

2. Materials and methods

2.1. Patients

The study was performed using specimens of primary GISTs diagnosed at the Department of Pathology and Molecular Medicine. We analysed 261 tumor specimens from 239 patients with GIST. From 239 investigated patients, 125 (52 %) were men and 114 (48 %) women. Median age was 68 years (range, 15–100 years). The primary tumors were predominantly in the stomach (104 tumor samples, 40 %). 46 (18 %) arose in the small intestine, 6 (2 %) in the esophagus, and 9 (3 %) in the colon and rectum. Extragastrointestinal samples formed 6.5 % (17 cases). Within the latter group the tumors were localised in the omentum, peritoneum, mesentery, epigastrium, and ovary. Further, we examined 59 (23 %) metastatic specimens (in the liver, peritoneal cavity, mesentery, and kidney). In 7.5 % of cases (20 samples) data as to the primary or metastatic site were not available. From 261 tumor samples, 188 (72 %) had spindle cell morphology, 48 (18 %) were epithelioid, and 20 (8 %) tumors displayed a mixed morphology. The results of morphological examinations in 5 cases (2 %) were not specified.

All patients enrolled in the study were processed through a standard histopatological investigation involving immunohistochemical detection of CD117, CD34, DOG-1, smooth muscle actin, desmin, H-caldesmon, S100 protein, and Ki-67 (Fig. 1). The results were considered to be a basis for a further molecular evaluation, and are not a subject reported in this article. Molecular diagnosis focused on primary and secondary mutations in *KIT* and *PDGFRA* genes with regards to sensitivity or primary/secondary resistance to IM targeted treatment. A group of *KIT/PDGFRA* wild-type GISTs is characterized by a complexity and a high heterogeneity of molecular features, resistance to IM, and it is a challenge for new molecular-targeted therapeutic strategies. Because of comprehensive molecular diagnosis of GISTs, we extended mutational analysis to *BRAF* mutations and defects of *SDH* complex.

Diagnostic tumor tissues were collected and tested for mutational status after an informed consent by the patients, and with the approval of the Ethical committee.

2.2. Sequence analysis

We performed mutational analysis of the *KIT* a *PDGFRA* genes from formalin-fixed, paraffin embedded (FFPE) (210 samples, 81 %) or fresh frozen tumor (51 samples, 19 %) specimens. Total DNA for molecular investigation from archival (FFPE) tumor tissues was extracted using QIAamp DNA Tissue FFPE Kit (QIAGEN, Hilden, Germany). In case of frozen tissues, DNA was extracted using Tri-Reagent (Invitrogen Ltd., Carlsbad, CA, US) according to the manufacturer´s instruction. Exons 9, 11, 13, and 17 of the *KIT* gene and exons 12, 14, and 18 of the *PDGFRA* gene were amplified using PCRBIO HS Taq Mix Red (PCR Biosystems Ltd., London, UK) as previously described [11]. Amplification conditions and sequence of primers of the *BRAF* gene were used as published by Hostein et al. [12]. Amplification of the exon 14 (*KIT* gene) was performed as reported by Antonescu et al. [6]. The PCR products were purified with GEL/PCR DNA Fragments Extraction Kit (GENEAID Biotech Ltd., New Taipei, Taiwan). Bidirectional DNA sequencing of the complete exons was done with BigDye® Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., Waltham, MA, US.). Cycle sequencing products were purified using NucleoSEQ® Columns (Macherey-Nagel GmbH&Co. KG, Düren, Germany) and analysed on GENETIC ANALYSER 3130 capillary electrophoresis system (Thermo Fisher Scientific Inc., Waltham, MA, US.). All sequence alterations were confirmed by an independent PCR amplification and sequencing to exclude PCR artefacts. The identity of the relevant nucleotide sequences were confirmed by database search. All investigated samples, which were analysed for presence of *KIT*, *PDGFRA* and *BRAF* mutations were morphologically reviewed. All included more than 20 % of tumor cells. Thus, we were able to use the technique of Sanger sequencing for mutational analyses. In cases with confirmation of primary *KIT* mutation and two different secondary *KIT* mutations in the same tumor nodule, two point *PDGFRA* mutations in exon 14, and *PDGFRA* mutations in exons 14 and 18, the molecular finding using the above described techniques was confirmed by Next-generation sequencing (NGS) - (TruSight® Tumor 26, Illumina® Inc. San Diego, CA, US.). NGS data were analyzed using software Illumina VariantStudio™ 3.0, Illumina® Inc. San Diego, CA, US.

3. Results

3.1. Primary KIT/PDGFRA mutations

Of the 261 specimens from 239 patients with GISTs we identified primary mutations in 214 (82 %) tumor specimens from 193 (80 %) patients. Primary mutations in the *KIT* gene were discovered in 173 (66 %) tumor samples from 154 patients, and in the gene *PDGFRA* in 41 (16 %) samples from 39 patients. The remaining 47 (18 %) specimens from 46 (20 %) patients were *KIT/PDGFRA* wild-type.

In 154 tumor samples from 142 patients we detected primary *KIT* mutations in exon 11 (59 %). Simple deletions were found most frequent (54 samples from 51 patients, 35 %). Deletions were located between codons 551–565, and a spectrum of in-frame deletions included 3–51 base pairs. Most commonly we detected deletions of two codons (557–558) (19 cases). Single substitutions were discovered in 42 specimens from 40 patients (27 %). The substitutions were localized in three codons: 557, 559 and 560, with exception of 5 cases (point mutations present in codons 558, 566, and 576). Duplications (internal tandem duplications) were third most common mutations in exon 11 (11 cases, 7 %). Simple insertions were present in 3 patients (2 %). The inserted codons were 558 and 559 (same insertions in 2 patients). Complex mutations (deletion-insertion, duplication-substitution, deletion-substitution) were found in 44 samples (29 %). These mutations consisted of one to several nucleotide deletion or duplication coexisting with small insertions or substitutions, respectively. Primary mutations in *KIT* exon 9 were found in 13 specimens from 10 patients (5 %). In all of these specimens we detected identical 2 codon duplication introducing a tandem alanine-tyrosine pair (AY502-503). Single nucleotide substitutions in *KIT* exon 13 were identified in 6 specimens of 2 patients (2 %). In all these 6 specimens there were same point mutations, which involve codon 642 (K642E). No primary *KIT* mutations were found in exon 17.

Most common primary *PDGFRA* mutations (34 samples/32 patients,

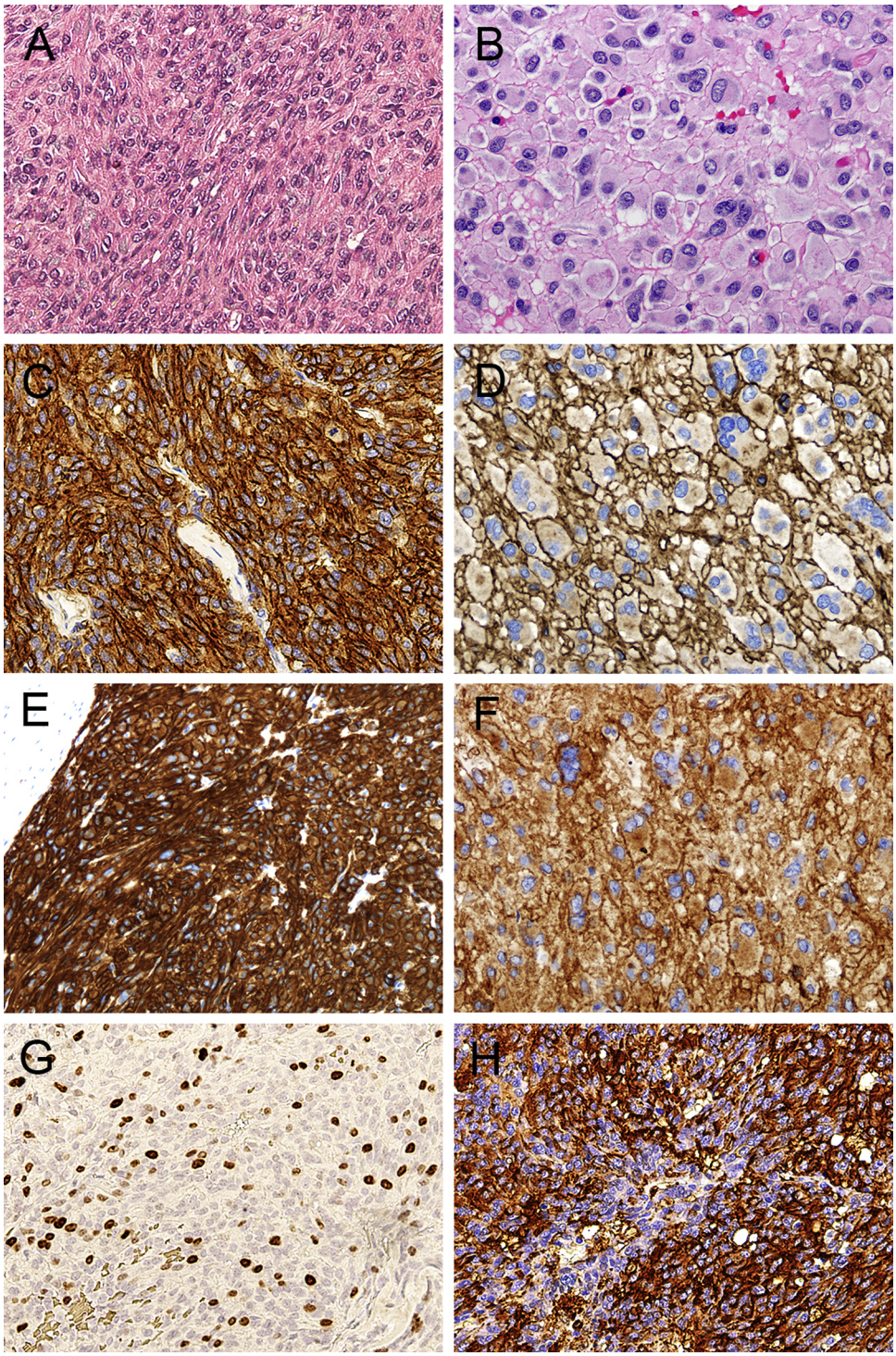

Fig. 1. GIST – histopathology findings: A.- B. Haematoxylin-eosin staining: A. Spindle cell GIST; B. Epitheloid GIST; C.- H. Immunohistochemical staining: C. CD117, spindle cell GIST, a strong membrane and a weaker cytoplasmatic positivity; D. CD34, epitheloid GIST, a strong membrane positivity; E. DOG1, spindle cell GIST, membrane and cytoplasmatic positivity; F. DOG1, epitheloid GIST, membrane and a weak cytoplasmatic positivity; G. Ki-67 antigen, spindle cell GIST, nuclear positivity in about 10% of the tumor cell population; H. Smooth muscle actin in a combined spindle and epitheloid GIST, a strong cytoplasmatic positivity (relatively an uncommon feature).
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13 %) were localized in exon 18. A majority of *PDGFRA* exon 18 mutations were single nucleotide substitutions in codon 842 (25 samples/23 patients, 75 %), leading to D842 V mutation. D842 V primary mutation is resistant to targeted IM therapy. Simple deletions and deletions-insertions in exon 18 were detected in 9 patients (25 %). Mutations in *PDGFRA* exon 12 were detected in 5 samples from 5 patients (2 %). There were missense mutations leading to D561 V substitution (3 cases). However, simple deletion (one case), and in frame deletion and insertion were also identified (one case). Mutations in *PDGFRA* exon 14 are found rarely. We detected nucleotide substitutions in two patients. One patient had two point mutations in one biopsy specimen, specifically M642 T and H659 N (see data A in Supplementary Table). Both cases with *PDGFRA* mutations in exon 14 were confirmed using NGS technique.

3.2. Secondary KIT/PDGFRAmutations

Secondary *KIT* mutations were detected in 10 from 133 (7 %) patients treated with IM. Overview of the secondary mutations and clinicopathological data are summarized in Tables 1 and 2. We investigated 16 progressive lesions (from a total 22 tumor tissue samples) and found secondary *KIT* mutations in exons 13 and 17, and secondary *PDGFRA*

Table 1
Summary of the primary and secondary KIT/PDGFRA mutations.

PATIENT No.	SEX	TUMOR LOCALIZATION	TUMOR MORPHOLOGY	TIME OF EXAMINATION	**PRIMARY MUTATION**	EXAMINETED SAMPLES No.	**SECONDARY MUTATION**
1.	F	primary tumor (peritoneal cavity)	spindle cell morphology, high mitotic activity	September 2009	***KIT* gene exon 9** (DUP AY 502/503)	1	–
		relapse of the disease specimen from retroperitoneum	spindle cells with circular nucleus, focal epithelioid cells with perinuclear vacuolization of cytoplasm and greater nucleus	April 2016	***KIT* gene exon 9** (DUP AY 502/503)	3 nodules	**D820Y exon 17 *KIT* gene** – **D820Y exon 17 *KIT* gene**
2.	M	primary tumor (stomach)	spindle cells with vacuolization of cytoplasm/ in part of tumor cells with epithelioid appearance	June 2006	***KIT* gene exon 11** (SUB/DEL)	1	–
		relapse of the disease specimen from stomach	part of tumor with spindle cells/part with greater polygonal cells tumor with necrosis and mitotic activity	April 2009	***KIT* gene exon 11** (SUB/DEL)	1	**V654A exon 13 *KIT* gene**
3.	M	metastasis into subcutaneous	spindle cell morphology, nuclear atypia and high mitotic activity	February 2007	***KIT* gene exon 11** (SUB/DEL)	1	–
		2 samples from necropsy	spindle cell morphology	June 2008	***KIT* gene exon 11** (SUB/DEL)	2 nodules	**N822 K exon 17 *KIT* gene N822 K exon 17 *KIT* gene**
4.	M	relapse of the small intestine tumor	spindle cell morphology focal tumor cell vacuolization	February 2007	***KIT* gene exon11** (ITD)	1	**V654A exon 13 *KIT* gene**
5.	M	relapse of the disease	spindle cell morphology	August 2007	***KIT* gene exon 11** (SUB/DEL)	1	**V654A exon 13 *KIT* gene**
6.	M	primary tumor (peritoneal cavity)	spindle cell morphology with focal epithelioid morphology mitotically active cells	October 2002	***KIT* gene exon 11** (SUB)	1	–
		metastasis of the liver	spindle cell morphology with focal epithelioid morphology	December 2008	***KIT* gene exon 11** (SUB)	1	**V654A exon 13 *KIT* gene**
7.	M	specimen from abdominal wall	spindle cell morphology with focal nuclear atypia high mitotic activity	October 2014	***KIT* gene exon 11** (SUB)	1	**D820E exon 17 *KIT*gene N822 K exon 17 *KIT* gene**
8.	M	relapse of the disease (colon)	polygonal cells with oval-shaped nucleus and nucleolus	March 2011	***KIT* gene exon 11** (INDEL)	1	**D823Y exon 17 *KIT* gene**
		relapse of the disease (rectum)	polyglonal cells with epithelioid morphology focal mitotically active	April 2012	***KIT* gene exon 11** (INDEL)	1	**D823Y exon 17 *KIT* gene**
9.	F	residuum after chemotherapy	mixed morphology (spindle/ epithelioid)	May 2005	***KIT*gene exon 13** (SUB) K642E	1	–
		relapse of the disease	epithelioid morphology	May 2005	***KIT* gene exon 13** (SUB) K642E	1	–
		metastasis of the abdominal wall	spindle morphology	October 2008	***KIT* gene exon 13** (SUB) K642E	1	**N822 K exon 17 *KIT*gene**
		metastasis of the peritoneum	spindle morphology	October 2008	***KIT*gene exon 13** (SUB) K642E	1	**D820 G exon 17 *KIT*gene**
		metastasis of the retroperitoneum	spindle morphology, slight nuclear atypia high mitotic activity	May 2009	***KIT* gene exon 13** (SUB) K642E	1	**D820A exon 17 *KIT* gene**
10.	M	expansion in omentum	epithelioid morphology with focal spindle cells, nuclear atypia mitotically active	September 2013	***PDGFRA* gene exon 14** (SUB) P653L	1	**D846H exon 18 *PDGFRA* gene**

mutation in exon 18, respective. We did not find secondary *KIT* mutations in exon 14. Nine patients died of the disease progression, one patient with the disease (No. 9) commited suicide. In patient No. 1 we examined three tumor tissue samples during the disease progression. Interestingly, in two tumor samples of the latter case we detected two identical secondary *KIT* mutations in codon 820 (ex 17), but a third tumor nodule was *KIT* wild-type. This was the only patient with secondary mutations whose GIST also changed morphological appearance. A thoroughly sampled tumor from September 2009 showed spindle cell morphology; but specimen from April 2016 showed mixed spindle/ epitheloid morphology. Secondary V654A mutation (exon 13) was revealed in four patients. Secondary D823Y mutation was detected in patient No. 8. We analysed two biopsy specimens of the latter patient. First specimen was examined on March 2011 (tissue of a progressive tumor – recurrent GIST in the colon) and a second on April 2012 (a tumor tissue of the rectum). Both samples showed the same primary *KIT* mutation in exon 11 (insertion and deletion) and a secondary mutation in exon 17 (D823Y). In patient No. 7 we identified two different secondary point mutations (D820E, and N822 K) in the same lesion (a needle biopsy of the neoplastic nodule from the abdominal wall). Both secondary *KIT* mutations in exon 17 were confirmed using NGS technique (see data B in Supplementary Table). Patient No. 9 had three different secondary *KIT* missense mutations in exon 17 (see Fig. 2), each from a different tumor sample (metastasis in the abdominal wall, metastasis in the peritoneum - both from October 2008, and metastasis in the retroperitoneum from May 2009). We identified two point *PDGFRA* mutations in patient No. 10. The first single substitution was detected in codon 653 (specifically P653 L, exon 14) and a second mutation in

Table 2
Summary of the clinicopathological data of patients with secondary mutations.

PATIENT No.	DIAGNOSIS	1st LINE THERAPY	PROGRESSION	2nd/3rd LINE THERAPY	FOLLOW UP
1.	09/09 tumor of the peritoneal cavity ●**PM**	10/09-10/10 adjutant IM	05/11 relapse of the disease 07/11 palliative IM 04/14 escalation of IM (800 mg/day) (partial regression) 11/15 progression	12/15 2nd line: SM (primary progression) 04/16 relapse of the disease ●●**PM and SeM** 05/16 3rd line: regorafenib 5/17 progression	DOD 2017
2.	06/06 inoperable tumor of the stomach ●**PM**	09/06 paliative IM 400 mg/day regression until 05/07	10/07 progression (PET/CT) 02/09 local relapse (radical resection) ●●**PM and SeM** 06/09 paliative IM (400 mg/day) decrease IM (200 mg/day): renal insufficiency 08/09 IM interrupted		DOD 01/2010
3.	04/01 radical resection of the tumor (jejunum and ileum)	06/04 initial dose of IM (400 mg/day) 06/04-07/06 stable disease	07/06 progression 08/06 escalation of IM (800 mg/day) 02/07 metastases into subcutaneous ●**PM** 04/07 progression of disease	04/07-06/07 2nd line: SM 07/07 progression on SM	DOD 06/2008 ●●**PM and SeM**
4.	06/01 surgical resection of the tumor (small intestine) 02/04 metastasis	05/04 initial dose of IM (partial remission)	01/07 progression 01/07 escalation of IM (800 mg/day) 02/07 relapse of the disease ●**PM and SeM**		DOD 02/2007
5.	10/02 malignant GIST 07/03 metastasis	08/03 initial dose of IM (strong partial remission) 06/04 regression of tumor			since 06/2004 loss of contact (according last information died) investigation biopsy from 08/07 ●**PM and SeM**
6.	01/01 surgical resection of tumor (peritoneal cavity) 10/02 progression in abdominal cavity ●**PM**	01/03 IM 400 mg/day 10/03 300 mg/day (hepatotoxicity) 11/03 progression of disease	01/05-11/05: 400 mg/day progression of the disease 11/05-02/06: 600 mg/day progression of the disease 02/06-08/06: 800 mg/day progression of the disease 08/06 IM interrupted	09/06 2nd line: SM 50 mg/day (4 weeks) 10/06 reduction of SM 37.5 mg/day (hepatotoxicity) SM until 3/07 progression 05/07-02/08 3rd line: nilotinib 02/08 progression 03/08-04/09 IM 800 mg/day progression of the disease 12/08 biopsy metastasis of the liver ●● **PM and SeM** 05/09 chemotherapy 08/09 progression of the disease	09/2009 loss of contact (according last information died)
7.	10/06 advanced GIST of the peritoneal cavity (with metastases in the liver)	10/06 IM 400 mg/day	03/10 local progression 04/10 escalation of IM dose 800 mg/day 10/10 local progression	08/11 2nd line: SM 50 mg/day 05/13 progression 05/13 started 3rd line: regorafenib 10/13 finish regorafenib because of progression 08/14 salvage therapy by IM 09/14 interrupted 10/14 palliative surgery ●**PM and SeM**	DOD 2014
8.	03/05 resection of the tumor (colon)	06/07 IM 400 mg/day	03/11 local progression ●**PM and SeM** 04/12 surgical resection of tumor ●●**PM and SeM** 11/12 remission (PET/CT) 02/13 subileosis status 03/13 kidney failure inoperabile GIST	03/13 2nd line: SM 50 mg/day massive progression 3rd line: regorefenib	DOD 11/2013
9.	since 1991 pain of abdomen 1996 surgery of small intestine 04/05 2nd relapse ●**PM** 04/06 3rd relapse ●●**PM**	09/06 IM 400 mg/day (complete remission) 10/08 progression ●●●/●●●● **PM and SeM/PM and SeM**	05/09 surgery of the metastases ●●●●●**PM and SeM** 06/09 escalation of the IM (800 mg/day) progression of the disease	10/09 2nd line: SM (50 mg/day) progression of the disease 5/10 salvage IM (600 mg/day)	Suicide 07/2012

(*continued on next page*)

Table 2 (*continued*)

PATIENT No.	DIAGNOSIS	1st LINE THERAPY	PROGRESSION	2nd/3rd LINE THERAPY	FOLLOW UP
10.	09/13 tumor of the omentum **●PM and SeM**	10/13-12/13 IM 400 mg/day 12/13 IM 300 mg/day IM interrupted (urinary inflammation and bronchial pneumonia)	progression of the disease before IM therapy/primary progression?	palliative therapy	DOD 03/2014

Legend: ● first molecular analysis, ●● second molecular analysis, ●●● third molecular analysis, ●●●● forth molecular analysis, ●●●●● fifth molecular analysis, DOD – died on disease, IM – imatinib mesylate, SM – sunitinib malate, PM – primary mutation, SeM- secondary mutation.
Note: In each molecular analysis primary and secondary mutations were detected and verified.

codon 846 (specifically D846H, exon 18) of the *PDGFRA* gene (see data C in Supplementary Table). Only one progressive tumor from the omentum (September 2013) was available for molecular investigation. Therefore, we were not able to distinguish whether this case had two primary *PDGFRA* mutations or whether these mutations represented one primary and one secondary *PDGFRA* mutation. We suppose that missense mutation in exon 14 (P653 L) may be a primary mutation, and point mutation in exon 18 (D846 H) a secondary mutation. All the primary mutations were always detectable along with the secondary mutations in each tumor.

3.3. BRAFmutations

We identified *BRAF* V600E point mutation in two patients (a 30-year old woman, and a 77-year old man) out of 46 patients (4 %) without *KIT* and *PDGFRA* mutations. Both patients had the tumor localized in the small bowel. There were differences in the morphology and results of immunohistochemical investigations in both tumors. In the first case, the tumor showed a spindle cell morphology, positivity of CD117, and in the second case the tumor had a mixed spindle cell/epitheloid morphology, and it was CD117 negative.

3.4. SDH complex mutations

In four younger (15, 33, 37 and 45-year old) patients out of 44 patients without *KIT*, *PDGFRA*, and *BRAF* mutations we examined DNA for the presence of SDH complex mutations (*SDHA*, *SDHB*, *SDHC* and *SDHD* genes). Two patients (a 37-year old man, and a 33-year old woman) had defects in the SDH complex. Analysis of the other two patients did not establish any defects of this complex. Defects of the SDH complex were detected using MLPA (Multiplex ligation-dependent probe amplification) technique. In a specimen from the 37-year old man we found deletion of the whole *SDHB* gene, deletion of exons 2–4 of the *SDHC* gene, and deletion of exons 2–4 of the *SDHD* gene. This patient had GIST of the small intestine which presented with a number of small tumors in a length of 100 cm in the intestine, and they all had spindle cell morphology. Analysis of DNA sample from the 33-year old woman showed deletion of *SDHAF2* exons 1–4 and deletion of *SDHD* exons 1–4. Tumor cells of this patient showed spindle cell morphology with markedly anaplastic cells. The tumor was bulky and it was localized in the pelvic peritoneum.

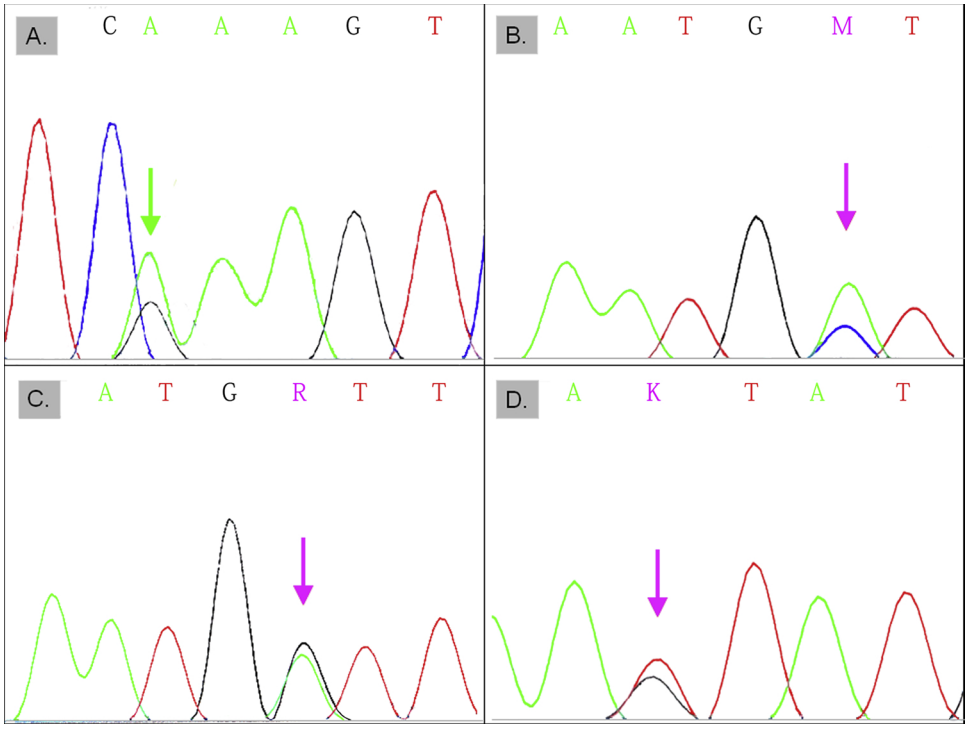

Fig. 2. Sequencing chromatogram of mutational analysis of the *KIT* gene (patient No. 8). **A.** primary *KIT* mutation in exon 13 (K642E), **B.** secondary *KIT* mutation in exon 17 (N822 K), biopsy specimen from metastasis of the abdominal wall, October 2008, **C.** secondary *KIT* mutation in exon 17 (D820 G), biopsy specimen from metastasis of the peritoneum, October 2008, **D.** secondary *KIT* mutation in exon 17 (D820A), biopsy specimen from metastasis of retroperitoneum, May 2009.

4. Discussion

GISTs constitute a majority of primary mesenchymal tumors of the gastrointestinal system [13]. The most powerful predictor of targeted therapy efficacy of the GISTs is *KIT* and *PDGFRA* gene mutational status [14,15]. In this study we detected primary *KIT/PDGFRA* mutations in 82 % of tumor tissue specimens. The vast majority of *KIT* mutations are present in exon 11 [16]. Mutations in exon 11 affect autoregulatory function of KIT protein, and promote spontaneous kinase activation [17]. *KIT* exon 9 mutations, characterized by A502-Y503 codon tandem duplications are almost exclusively localized in intestinal tract. They have been associated with a more aggressive phenotype [16] and lead to spontaneous receptor dimerization [17]. Primary mutation in exon 13 (K642E) encoding the ATP-binding region are rare and may be activating by alteration of the tree-dimensional structure of the mutant protein [18]. Mutations in exon 17 encoding the activation loop of the kinase seem to stabilize the active conformation [17]. *PDGFRA* mutations are mutually exclusive with *KIT* mutations. These mutations represent a mechanism alternative to KIT mutation for pathologic activation of tyrosine kinase receptor signalling pathways. Mutations in *PDGFRA* gene affecting the most common the activation loop (exon 18), and rarely in juxtamembrane domain (exon 12) and kinase I domain (exon 14) [19,20]. A majority of *PDGFRA* exon 18 mutations were single nucleotide substitution in codon 842 (75 %), leading to D842 V mutation. Although the most common D842 V *PDGFRA* mutation is intrinsically imatinib resistant, approximately 30 % of the *PDGFRA*-mutated GISTs other than D842 V, are known to be potentially imatinib sensitive [21] Mutations in exon 12 are associated with good response to IM [20]. In exon 14 is described one single rare mutation (N659 K) [20].

Secondary resistance to IM is a critical problem in patients with advanced GISTs [3]. Secondary *KIT* mutations associated with IM resistance are preferentially located in the first (TK1, encoded by *KIT* exons 13 and 14), or in the second (TK2, encoded by *KIT* exons 17 and 18) KIT tyrosine kinase domain [14,22,23]. In the present study we identified secondary V654A mutation (exon 13, *KIT* gene) in four patients (4/9). Furthermore, we found secondary *KIT* mutations in exon 17 in five patients (5/9). Unlike primary IM resistance, secondary resistance is most often associated with expansion of the tumor clones with secondary *KIT* or *PDGFRA* mutations. More importantly, Gramza et al. [24] reported a clinical study of patients who progressed on IM therapy. They revealed that approximately 70 % of patients with acquired resistance had tumor clones with one or more secondary kinase mutations. Intralesional and interlesional heterogeneity of the secondary mutations were detected by others authors either [7,23,25,26]. These authors described an occurrence of two to five different secondary mutations in separate metastases or a presence more than one acquired mutation in the same metastasis. Furthermore, these authors pointed out that each tumor nodule under progression developed an individual clonal evolution. The most effective clones with regard of proliferation will lead to the tumor progression, which may vary in individual metastases. Beside establishing unfavourable prognosis of patients with secondary resistance some authors focused on patients with secondary *KIT* mutations in activation loop (A-loop, exon 17). Wang et al. reported molecular mechanisms, which stabilize the open active conformation of the A-loop [4]. They identified secondary *KIT* mutations in 4/10 imatinib-resistant GIST patients (40 %). Each of these four patients developed an identical type of secondary *KIT* mutation, specifically D823Y (exon 17). More importantly, Guo et al. [27] found that secondary *KIT* mutations in the A-loop are associated with sunitinib resistance. Furthermore, Yeh et al. [28] investigated the effect of targeted therapy using regorafenib (3rd line of targeted therapy) on prolonged PFS (progression free survival) in patients with *KIT* secondary mutations in exon 17. The authors focused on intra-tumoral and inter-tumoral heterogeneity which represents always a substantial problem for therapy. GIST heterogeneity may explain different treatment efficacies of regorafenib between patients with secondary *KIT* mutations. Beside establishing new therapeutic strategies in patients with resistant or wild-type GISTs, some authors focused on alternative therapeutics targets [29,30].

In contrast to secondary *KIT* kinase mutation, secondary *PDGFRA* kinase mutation is much less common in imatinib-resistant GISTs [31]. In the present study, we found *PDGFRA* mutations in exon 14 (P653 L) and exon 18 (D846 H) in one patient (patient No. 10 with omental GIST, data C in Supplementary Table). Tumor cells in this case showed epitheloid morphology. Murayama et al. [32] focused on a patient with omental (extragastrointestinal) GIST and mutations in *PDGFRA* gene and reviewed data published in the literature. Authors reported that a majority of KIT-negative extra-gastrointestinal omental tumors is characterized by epitheloid cell morphology, and all cases of omental GISTs had mutations in *PDGFRA* gene.

Approximately 15 % of GISTs do not have detectable mutations in either *KIT* or *PDGFRA* genes. These *KIT/PDGFRA* wild-type GISTs are clinically indistinguishable from *KIT/PDGFRA* mutated GISTs. They have identical morphology, express high levels of KIT protein, and occur anywhere in the GI tract. Phosphorylated KIT is detectable in these tumors, suggesting that KIT is still activated, but tumor cells use alternative mechanisms of their activation [10]. Mutations of the *BRAF* gene are commonly detected in diverse benign and malignant tumors, including benign melanocytic nevi (81 %), melanomas (50–70 %), thyroid papillary carcinomas (35–60 %), colorectal adenomas/carcinomas (5–20 %), and other tumors [33]. V600E *BRAF* mutations were also reported in a subset of GISTs [34]. We identified primary V600E *BRAF* mutations in 2/46 of adult *KIT/PDGFRA* wild-type patients (4%). The frequency of V600E *BRAF* mutations in literature varies from 3.5 to 7% of the wild-type GISTs [33–38]. Jasek et al. concluded that mutational analysis of wild-type GISTs should include the *BRAF* gene because of their mutational status contributes to understanding of pathogenesis and might be important for therapy [37]. In contrast, Huss et al. [38] pointed out that *BRAF* mutations are rare events in wild-type GISTs. However, they underline an important role of BRAF inhibition as a therapeutic option to control the disease in advanced stages. The *BRAF* mutation causes both, primary resistance to imatinib treatment, and acquired resistance when it occurs as a secondary event in *KIT/PDGFRA*-mutated GISTs relapsing under therapy [39]. The aim of the present study was to ascertain whether secondary resistance of GIST patients without *KIT/PDGFRA* secondary mutations is generated by the presence of *BRAF* mutations. We analysed 76 patients under the treatment of IM. No *BRAF* mutations were observed. In contrast with our results, Agaram et al. [34] investigated presence of *BRAF* mutation in patients with secondary IM resistance. The resistant tumor clone of the patient harboured a primary *PDGFRA* mutation (deletion) and a secondary V600E *BRAF* mutation. Interestingly, Miranda et al. [40] reported GIST with dual *BRAF* and *KIT* activating mutations in an untreated patient. This finding challenges the concept of the *KIT/PDGFRA* and *BRAF* mutations are mutually exclusive in primary GISTs. None of the patients carrying concomitant mutations of *KIT* and *KRAS* or *BRAF* genes were treated with imatinib. These data suggest, that the concomitance of *KIT* and *BRAF* mutations might explain the resistance phenomena observed in a fraction of patients with GISTs carrying imatinib-sensitive *KIT* mutations (about 5 %) [39]. First study of treatment *BRAF* – mutated GIST was reported by Falchook 2013 et al. [41]. The authors described a patient with V600E *BRAF* mutated GIST treated with dabrafenib, a potent ATP-competitive inhibitor of BRAF kinase. After 8 months of therapy they identified tumor progression. Because of an intratumoral heterogeneity, which can be a factor in tumor adaptation and treatment failure, the authors analyzed three progressive lesions. All three lesions were clonally related by identical V600E *BRAF* mutations (and an aberration in *CDKN2A* gene). In one of the three lesions (lesion 1) they identified somatic gain-of-function *PIK3CA* mutation (H1047R). Lesions 2 and 3 were *PIK3CA* wild-type. Somatic *PIK3CA* mutation (H1047R) as well as a *CDKN2A* aberration may have contributed to the eventual resistance to treatment.

Approximately half of all wild-type GISTs show defects of SDH complex [10]. SDH deficiency characterizes subsets of several tumors (GISTs, paragangliomas, renal cell carcinomas, and pituitary adenomas) [42]. GISTs with the defect of SDH complex demonstrate specific pathological and clinical features, including the absence of activating mutations of *KIT* and *PDGFRA* genes, and primary resistance to imatinib [43]. They are always found in the stomach, show epitheloid morphology and are often multiple. Moreover, in contrast to GISTs with *KIT/PDGFRA* mutations, they metastasize to lymph nodes and show activation of insulin growth factor receptor (IGFR). They have an indolent course and even with liver metastases, the patients live long. A majority of pediatric GISTs have defects of SDH complex. SDH deficient GISTs comprise two syndromes: Carney triade and Carney-Stratakis syndrome [42,44]. We investigated defects of SDH complex in two patients. Interestingly, in one patient (33-year old woman), we ascertained deletion of exons 1–4 of the *SDHAF2* gene. *SDHAF2* (succinate dehydrogenate complex assembly factor 2) gene encodes a mitochondrial protein needed for the flavination of SDH complex subunit A (SDHA) required for activity of this complex. Mutations in this gene were described recently and they are associated with paraganglioma [45]. No paraganglioma was found in our patient so far. Celestino et al. [46] evaluated SDH mutations in a series of 25 primary sporadic wild-type *KIT/PDGFRA/BRAF* GISTs. They detected *SDHB* germline mutations in 12 % of the patients. All patients had no apparent personal or familial history of paraganglioma and/or pulmonary chondroma. Same results were reported by Janeway et al. [45]. These authors tested 34 wild-type GISTs without personal or family history of paraganglioma and found that 12 % of the patients had germline mutations in *SDHB* or *SDHC* genes.

According recent studies, GISTs lacking mutations in *KIT*, *PDGFRA*, *BRAF*, *SDH*, *NF1* and/or *RAS* signalling pathway may include other genomic alteration, e.g. point mutations or fusions (FGFR1/HOOK3, FGFR1/TACC1) in *FGFR1* gene, *NTRK* fusions (specifically ETV6/NTRK3 fusion), mutations in *TP53*, *MAX*, *MEN1*, *CHD4*, *CTDNN2*, *BCOR, ARID1A* and *APC* genes [47–50]. This small subgroup of GISTs could represent another unique group of GIST family with alternative therapeutic possibilities [51].

5. Conclusions

Targeted TKI therapy significantly improved prognosis of patients with advanced GIST. Unfortunately, a vast majority of responding patients fail due to the primary or secondary resistance to targeted therapy. Our findings of mutational status of the primary and secondary *KIT/PDGFRA* mutations in patients with GIST confirm mechanisms of primary and secondary resistance, and also confirm an intralesional and interlesional heterogeneity of the secondary mutations within and between progressive lesions. Moreover, V600E *BRAF* mutation and defects of SDH complex in *KIT/PDGFRA* wild-type GIST confirm mechanism of their activation and allow for a selection of appropriate targeted therapy. A growing knowledge of the complexity of primary and secondary resistance (multiple and intra/interlesional variability of secondary mutations), and molecular identifications of individual subgroups of *KIT/PDGFRA* wild-type GISTs may improve strategies of individual targeted therapies.

Declaration of Competing Interest

The authors declare that they have no conflict of interest in this study.

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Appendix A. Supplementary data

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