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MECHANISMS CONTRIBUTING TO TYROSIN KINASE INHIBITOR RESISTANCE IN GISTs: TOWARD A PERSONALIZED THERAPY

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General background

1. Gatrointestinal stromal tumor

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors in the gastrointestinal (GI) tract [1]. For decades, prior to the 1990s, these mesenchymal tumors arising in the GI tract were often classified as smooth muscle tumors or neural tumors [2]. In 1983, Mazur and Clark introduced the term "stromal tumor", but it was not broadly accepted until the early 1990s, when CD34 was discovered as a marker for stromal tumors growing in the GI tract [3-4].

In the 1990s, investigators noted similarities between GIST cells and the interstitial cells of Cajal (ICC), a group of cells located in the musculature and around the myenteric plexus throughout the GI tract, working as pacemakers for peristaltic contraction. Further studies revealed that ICC express KIT and are developmentally dependent on stem cell factor (SCF) [5-7]. In 1998, a revolutionary publication by Hirota and colleagues, showed activating mutations in the KIT receptor tyrosine kinase (RTK) gene in GISTs as well as expression of KIT protein by immunohistochemistry [6]. In 2003, Heinrich and colleagues additionally identified platelet-derived growth factor receptor alpha (PDGFRA) gene mutations, as an alternative pathogenetic event in GISTs lacking KIT gene mutations [8]. To date, approximately 85% of GISTs are reported to harbor activating mutations in KIT or the homologous RTK gene, PDGFRA [8–11].

1.1 Oncogenic KIT and PDGFRA mutations and signaling pathways in GIST

The KIT and PDGFRA genes map to chromosome 4q12. Both encode type III receptor tyrosine kinases with closely related structural features. These kinases are constituted by an extracellular (EC) ligand-binding domain containing five immunoglobulin-like repeats, a transmembrane sequence, a juxtamembrane domain (JM), and two cytoplasmic kinase domains (TK[I]: ATP-binding pocket and TK[II]: kinase activation loop, Figure. 1) [11-12].

KIT and PDGFRA are activated by binding of their ligands, SCF and PDGFA respectively, to the EC domain. Ligand binding brings to the receptor homodimerization and subsequent cross-phosphorylation of cytoplasmic tyrosines, which operate as binding sites for various signalling proteins: KIT and PDGFRA tyrosine kinase activity is regulated by phosphorylation cascades with activation of signaling substrates regulating cell proliferation, adhesion, motility, and survival [13]. On the whole, KIT and PDGFRA activation regulates important cell functions including proliferation, apoptosis, adhesion, and chemotaxis (Figure 1) [13-14]. In addition it is critical for the development and maintenance of several cell types, as well as hematopoietic cells, ICC, germ cells,

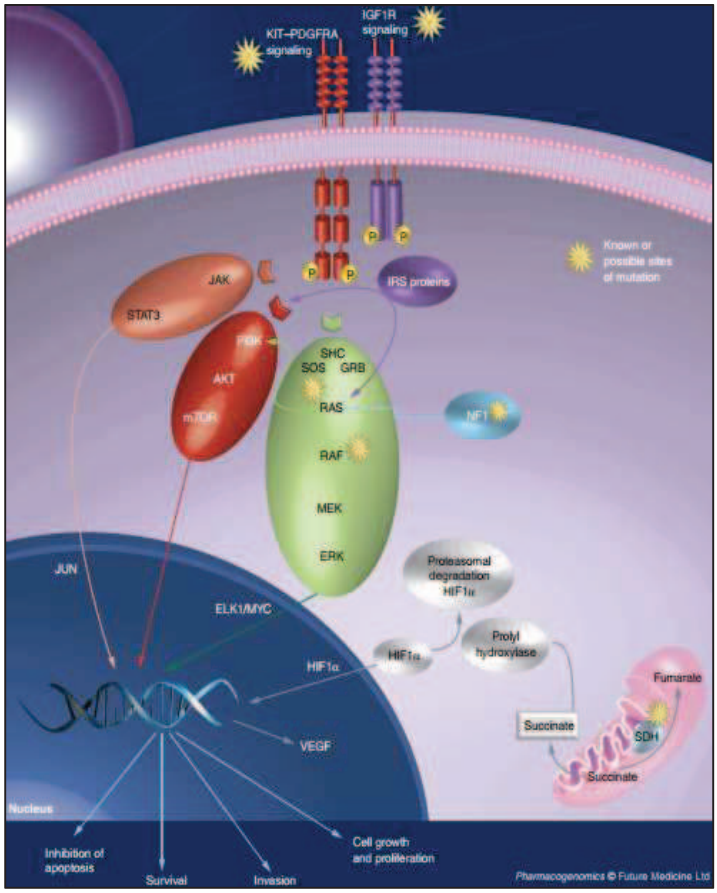


Figure 1. Oncogenic signaling in KIT and PDGFRA GISTs dimerization of KIT and PDGFRA leads to phosphorylation of tyrosine residues and the activation of different pathways, including RAS/RAF/MEK and PI3K/AKT/mTOR. RAS (green cascade) activates the MAPK cascade (RAF, MEK and ERK), and stabilizes ETV1 expression, leading to changes in gene expression. In wild-type GIST, mutation in NF1 (light blue), RAS or BRAF can all activate the signaling through the MAPK cascade. PI3K (red cascade) activates the mTOR pathway that leads to alteration in protein translation and cell survival. In wild-type GISTs, mutation in RAS may activate the PI3K cascade, as well. Phosphorylation of JAK leads to STAT3 activation (orange cascade), which promotes JUN transcription, important for cell proliferation and survival. Wild-type GISTs show high expression of IGF1R (purple cascade), which signals through both the MAPK and the PI3K pathways. Recently, defects in SDH have been identified in wild-type GIST, (gray cascade). Loss-of-function mutation in any of the SDH subunits leads to cytoplasmic accumulation of succinate, which downregulates prolyl hydroxylase. In turn, prolyl hydroxylase promotes proteasomal degradation of HIF1α. Therefore, succinate cytoplasmatic accumulation leads to increased levels of HIF1α, which enters the nucleus and activates, among others, the transcription of the VEGF gene.

mast cells, and melanocytes [13-14]. Mutations in KIT or PDGFRA gene involve two main regions, the receptor regulatory domains (dimerization region in the EC and JM domains) and the enzymatic domains (TK[I] and TK[II]). In GISTs most KIT mutations (~65%) involve the JM domain (exon 11) followed by mutations involving the EC dimerization domain (exon 9) which are seen in about 9% of cases. Primary KIT mutations can also occur in exon 13 (TK [I) and exon 17 (TK[II]), but these mutations are rare events (~2%) and data are quite limited (Figure 2).

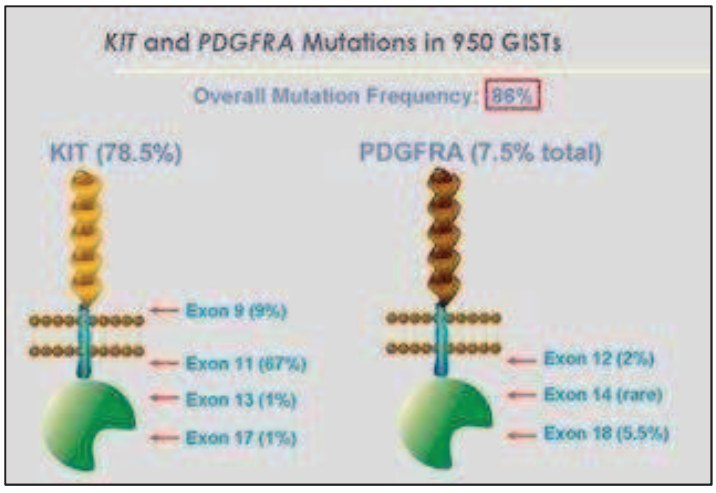


Figure 2. Kit mutations in untreated GISTs in exon 9, 11, 13 and 17, encoding part of the EC and JM domains, respectively. PDGFRA mutations, found in <10% of GISTs, involve the same domains.

1.2 Epidemiology, clinical features and prognosis of GISTs.

The exact incidence of GIST in the USA and Europe is very difficult to estimate, since GISTs have only been properly recognized and uniformly diagnosed as an entity starting only in late 1990s. Recent population-based studies performed in Sweden [14], Holland [15] and Iceland [16] found incidences of approximately 14.5, 12.7, and 11 cases/million per year, respectively. These findings would translate into an annual incidence in Europe of ~8,000-9,000 cases and in the USA of ~ 4,000-5,000 cases a year.

GIST patients range in age from the teens to the 90s, but peak age is around 60 years. The tumors are generally between 2 and 30 cm in diameter at the time of diagnosis and may cause mass-related symptoms or anemia as a result of mucosal ulceration. Not infrequently, however, GISTs are discovered incidentally during radiologic imaging for unrelated conditions, or as a secondary finding in a surgical resection or autopsy specimen [18].

GISTs occur throughout the entire GI tract and are most commonly found in the stomach (60%), jejunum and ileum (30%), duodenum (5%), colorectum (4%), and rarely in the esophagus and appendix [1,4,17]. Clinical symptoms associated with GIST include abdominal pain, fatigue, dysphagia, obstruction and satiety. Patients may suffer of with chronic GI bleeding (causing anemia) or acute GI bleeding (caused by erosion through the gastric or bowel mucosa) or rupture into the abdominal cavity causing life-threatening intra-peritoneal hemorrhage. Previously, a population-based study revealed that about 70% of GISTs were associated with clinical symptoms, 20% were not, and 10% were detected at autopsy [19].

1.3 Histopathology

Morphologically, GISTs can have three main histological subtypes: I) spindle cell type (accounting 70% of the cases), II) epithelioid type (20-25%), and III) mixed spindle cell and epithelioid type (10%) (Figure 3). In general, GISTs have a wide variation ranging from hypocellular to highly cellular with higher mitotic rates.

Spindle cell type of GIST is made up of cells in short fascicles. They have clear eosinophilic fibrillary cytoplasm, ovoid nuclei, and ill-defined cell borders. Gastric spindle cell GISTs often reveal extensive perinuclear vacuolization, a diagnostic feature formerly used for tumors of smooth muscle origin. Distinctive histological patterns among spindle cell GISTs including sclerosing type and vacuolated type [1].

Epithelioid cell GISTs are characterized by round cells arranged in nests or sheets and with eosinophilic to clear cytoplasm.

Finally, approximately 10% of GISTs show mixed morphology, being composed of both spindle and epithelioid cells [1].

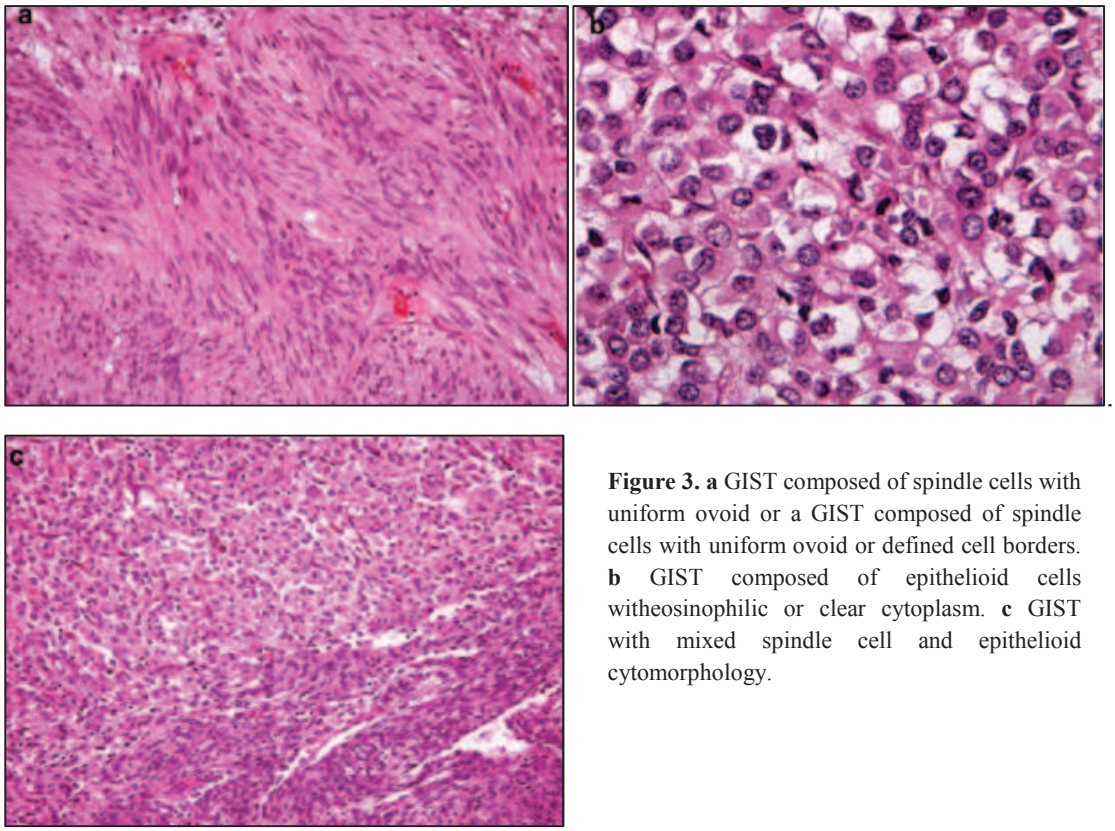


Figure 3. a GIST composed of spindle cells with uniform ovoid or a GIST composed of spindle cells with uniform ovoid or defined cell borders. **b** GIST composed of epithelioid cells witheosinophilic or clear cytoplasm. **c** GIST with mixed spindle cell and epithelioid cytomorphology.

1.4 Wild-type GIST

About 85% of paediatric GIST and about 10–15% of adult GISTs do not harbour any mutations in the KIT and PDGFRA genes and are defined as KIT/PDGFRA wild type (WT) [10].

WT GIST occur more often in women, often arise from the stomach, are multifocal, have a frequent epithelioid morphology and show an indolent course even if metastatic on diagnosis [20,21]. KIT/PDGFRA WT GISTs represent a highly heterogeneous group of patients, profoundly different from mutant tumors in their genomic background. About 50% of KIT/PDGFRA WT GISTs present an overexpression of insulin-like growth factor 1 receptor (IGF1R) at the mRNA and protein level [22,23]. Differences in the expression of genes that belong to neural tissue were also seen between tissue from mutated GIST and murine mature ICC, suggesting that KIT/PDGFRA WT GIST may have a different origin [24]. Furthermore, in KIT/PDGFRA WT GIST, several mutations have been described with uncertain pathogenic significance. In particular, BRAF exon 15 V600E substitution is present in up to 13% of GISTs [25]. In the past years interesting data on succinate dehydrogenase (SDH) complex deficiency in KIT/PDGFRA WT GIST, have also emerged, delineating a small group of GISTs with peculiar clinical and molecular features [26,27].

SDH complex, or mitochondrial complex type II, represents one of the five complexes belonging to the mitochondrial respiratory chain and is involved in the Krebs cycle and electron transport of oxidative phosphorylation. It shows a tetrameric structure composed of two hydrophilic subunits

with catalytic activity (SDHA and SDHB) and two hydrophobic subunits linked to the inner mitochondrial membrane (SDHC and SDHD). Germline or somatic inactivating SDH-inactivating mutations have been described in several tumours, such as paragangliomas / pheocromocitomas and renal-cell carcinoma [26-28].

The first evidence of germline SDHB- and SDHC-inactivating mutations in sporadic KIT/PDGFRA WT GISTs was reported in 2011 as 'type 2 GIST' [26]. Despite the low incidence of SDH-inactivating mutations, it has been reported that about 5–7.5% of sporadic KIT/PDGFRA WT GISTs show negative immunohistochemistry staining of SDHB [28]. SDH-deficient GIST comprise the great majority of gastric GISTs in children and young adults and a small proportion of gastric GISTs in older adults displaying a distinct clinical and pathological phenotype with respect to KIT/PDGFRA mutant GISTs.

1.5 Familial GIST

Several progenies with heriTable mutations in the juxtamembrane domain (exon 11) of the *KIT* gene have been identified. The first to be reported was a Japanese family in which a deletion of one of two consecutive valine residues (codon 559 or 560, GTTGTT) was traced through three generations. Affected individuals had hyperpigmentation of perineal skin and suffered the development of multiple benign and malignant GISTs [29]. A germline V559A substitution has been described in an italian family and in another one from Japan [30,31]. Affected members in both progenies had pigmented macules involving the skin of the perineum, axilla, hands, and face (with the exception of lips and buccal mucosa), as well as evidence of skin mastocytosis (urticaria pigmentosa) on biopsy. In addition, patients in both families developed multiple GISTs in the stomach and small bowel as early as age 18 years. A germline mutation in the kinase I domain of KIT was reported in a 67-year-old mother and her 40-year-old son from France. Both patients had more than a dozen of duodenal and jejunal GISTs, and both were found to have a constitutional K642E substitution in exon 13 of the *KIT* gene [32]. A mutation in the activation loop of KIT has been recently described by Hirota *et al* in a kindred with multiple gastric and small bowel GISTs [33]. The D820Y mutation found in affected family members caused diffuse ICC hyperplasia and GIST formation but was not associated with skin hyperpigmentation or mast cell disease.

2. Treatment of GIST

Important improvements have occurred in GISTs treatment in recent decades. Before 1990s, GISTs have been erroneously diagnosed as smooth-muscle tumors of the GI tract such as leiomyoblastomas and leiomyomas. Due to the wrong diagnosis GISTs erroneously were unsuccessfully treated with radiotherapy and conventional chemotherapy as mesenchymal neoplasms [34,35]. When it was clear that GISTs were a distinct tumor, the paradigm of GIST treatment has dramatically changed. Currently, GIST has been changed from an incurable disease to a manageable, chronic condition for a significant proportion of patients [36].

The two gold standards of GISTs treatment are surgery and imatinib. Complete surgical removal is the standard therapy for localized resecTable GIST; in locally advanced and metastatic GIST imatinib is the first choice drug

2.1 Imatinib

The initial pilot study of imatinib in advanced GIST occurred in the year 2000 with a Finnish patient with metastases to the peritoneum and liver which progressed despite multiple lines of chemotherapy [36]. With a 400 mg/daily dose, after 8 months of treatment, six of twenty-eight liver metastases were no longer detecTable. The successful treatment of the first patient with metastatic GIST set off a fast succession of pivotal trials.

Imatinib mesylate (GLEEVEC® formerly STI571, Novartis Pharma AG, Basel, Switzerland) is a tyrosine kinase inhibitor (TKI) with activity against ABL, BCR-ABL, KIT, PDGFRA, PDGFRB and CSF1R. Its structure mimics adenosine triphosphate (ATP) and it binds competitively to the ATP binding site of the target kinases. This prevents substrate phosphorylation and signaling, thereby inhibiting proliferation and survival [38, 39] (Figure 4).

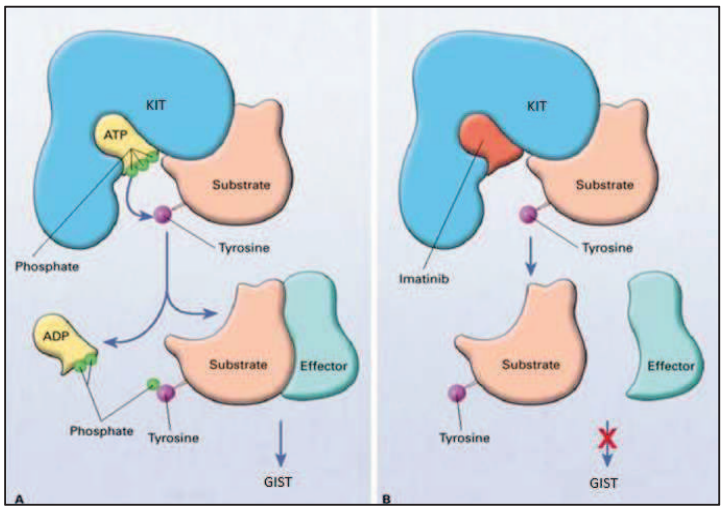


Figure 4. Mechanism of imatinib action

Two important observations made in 1999 suggested that imatinib might be effective against GISTs. The first was that imatinib could block the *in vitro* kinase activity of both wild-type KIT and a mutant KIT isoform commonly found in GISTs (point mutation in exon 11) [40]. The second observation was that imatinib

inhibited the growth of a GIST cell line containing a *KIT* gene mutation [41]. In part, on the basis of these preclinical findings, a patient with GIST with metastasis to the liver was granted compassionate use of imatinib mesylate in March 2000 [37]. The success in treating the first GIST patient with imatinib quickly led to a multicenter trial (CSTIB2222) that involved the Dana-Farber Cancer Institute, Fox-Chase Cancer Center, Oregon Health & Science University Cancer Institute, and the University of Helsinki [42]. In this trial, 147 patients with advanced, unresecTable, KIT-positive GIST were enrolled. Patients were randomly assigned to either 400 mg or 600 mg per day in a single oral dose. With a follow-up of at least 6 months, partial responses were observed in 54% of patients, and an additional 28% had sTable disease. Disease progression was seen in only 14% of patients during initial follow-up. Similar results were reported for the European Organization for Research and Treatment of Cancer Soft Tissue and Sarcoma Group phase I study of imatinib for patients with advanced soft tissue sarcomas, including GISTs [42]. On the basis of the results of the CSTIB2222 trial and the European Organization for Research and Treatment (EORTC) of Cancer trial, IM was approved by the US Food and Drug Administration, FDA, for the treatment of unresecTable and metastatic GIST on February 1st, 2002.

2.1.1 Correlation between KIT and PDGRFA mutation status and imatinib response

The presence and the type of *KIT* or *PDGFRA* mutation status are predictive of outcome to imatinib. Exon 11 mutations occur in the KIT juxtamembrane domain and are the most common mutations in GISTs. Tumors with exon 11 mutations have better response rates to imatinib, with a longer progression free survival (PFS) and overall survival (OS). Exon 9 mutations, specific for intestinal GIST, occur in the KIT extracellular domain. Exon 9 mutations are associated with a lower response to imatinib and a poorer PFS. These differences translate into significantly longer event-free and overall survival among the exon 11–mutant group versus the other two groups. Thus, even though wild-type and exon 9–mutant forms of KIT are equally sensitive to imatinib *in vitro*, tumors with these genotypes are less responsive to treatment than are exon 11–mutant tumors. [10,42]. Patients with a PDGFRA point mutations (in particular D842V) show a scarce response to imatinib therapy. This is consistent with *in vitro* data showing relative resistance of this variant to imatinib [9,44].

2.1.2 Resistance to imatinib

Non achievement of sTable disease or progression of disease within 6 months of an initial clinical response (KIT exon 9 mutation or no detecTable kinase mutation – wild-type tumors, PDGFRA exon 18) is termed as primary resistance, occurs in 10%-20% patients and relates to the mutational profile of the tumor. Most of wild-type GISTs display primary resistance [45,46]; progression after more than 6 months of clinical response is defined as secondary resistance. This has been attributed mainly to:

- genomic amplification (Figure 5),
- overexpression of KIT/PDGFRA without new point mutations,
- loss of KIT expression, accompanied by activation of an alternative tyrosine kinase or other oncogenes.
- acquisition of new kinase mutations.

Although the 2 years survival of patients with metastatic GIST treated with imatinib, approximates 70% of the patients develop disease progression by 2 year.

The most common mechanism of secondary resistance appears to be the appearance of the KIT kinase domain mutations. Therapeutic options for patients whose GISTs progress on imatinib consider dose escalation or treatment with other TKI.

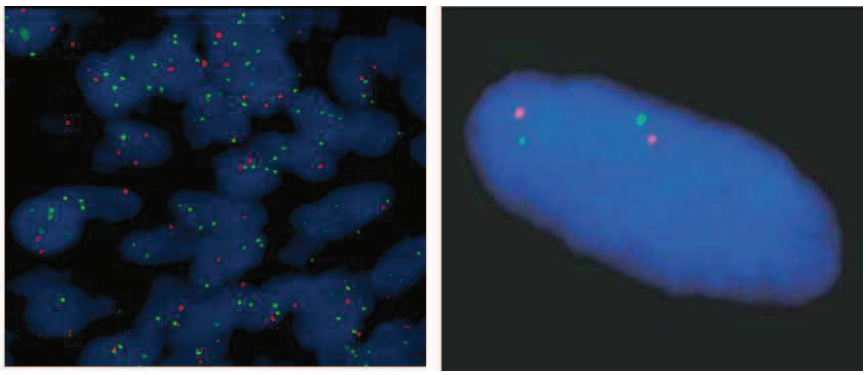


Figure 5. On the left: FISH analysis of a GIST patient with KIT amplification. Chromosome 4 centromere probe is shown in orange and the KIT probe in green. On the right analysis of GIST patient without KIT amplification (ratio KIT:centromere is 1:1

2.2 Sunitinib

For patients with GIST who develop disease progression during imatinib treatment or are intolerant to imatinib, sunitinib is the standard therapy, a second generation TKI. Sunitinib malate (SUTENT®, formerly SU11248; Pfizer, New York, USA) is an oral multitarget receptor tyrosine kinase inhibitor (KIT, PDGFR (α and β), VEGFR 1,2,3, and FLT3, CSF-1R, and RET that has shown anti-angiogenic and antitumor activities in several *in vitro* and *in vivo* tumor models. Sunitinib has been approved by the FDA on January 26th, 2006 for the treatment of patients with imatinib refractory or intolerant GIST. Currently, sunitinib is the only second line, FDA approved drug treatment for GIST.

Sunitinib, as well as imatinib, binds to the inactive conformation of the target tyrosine kinases and inhibits binding of ATP. Despite this similarity, sunitinib has the potential for activity in imatinib-resistant GIST, presumably through unique binding characteristics and broader spectrum of kinase inhibition, including the tumor-associated angiogenic VEGFR family of tyrosine kinases (Figure 6) [48].

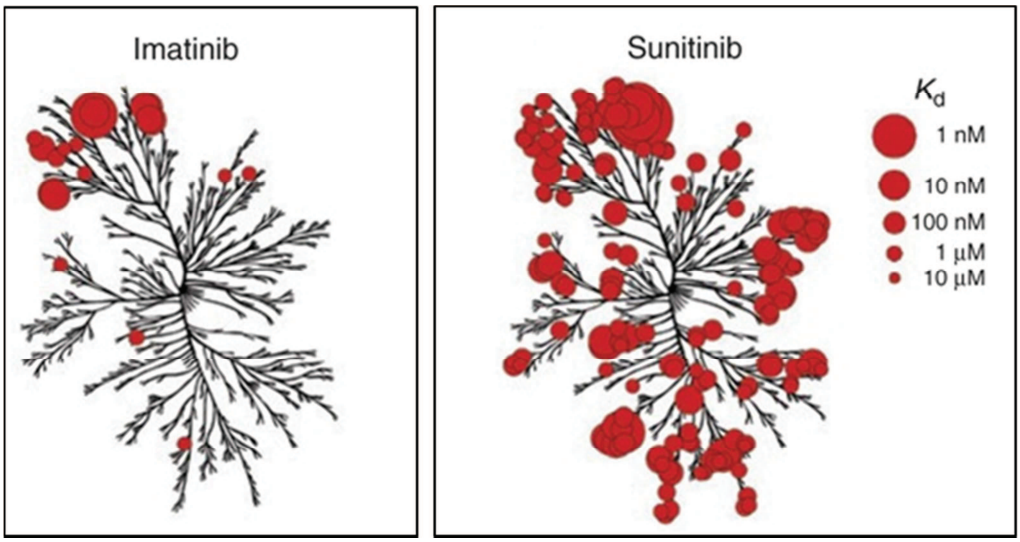


Figure 6. Kinase that bind imatinib and sunitinib are shown in red circles; larger circles indicate higher affinity binding. Interaction with Kd<10 are shown.

The clinical benefit of sunitinib is genotype-dependent on both the primary and secondary KIT mutations. It has been described it gives at least short-term clinical benefits in about 65% of GIST patients who are refractory to imatinib. Particularly, it shows superior efficacy in GIST patients hosting KIT exon 9 mutations [49]. Unfortunately, only about one-quarter of patients who are switched to sunitinib will continue to have responsive disease a year later [48].

Sunitinib has been shown to be effective with certain mutations that are resistant to imatinib; however, genotype analysis showed that patients with secondary *KIT* mutation affecting the activation-loop domain have PFS and OS. Clinically, some patients with secondary *KIT* mutation involving activation-loop domain experienced rapid disease after switching their treatment from

imatinib to sunitinib [50]. Profiling of sunitinib against imatinib-resistant kinases (i.e., KIT with primary plus secondary mutations) has shown that sunitinib potently inhibits imatinib-resistant KIT ATP/drug-binding pocket mutations, but has little activity against imatinib-resistant KIT activation loop mutations [49]. Thus, many imatinib-resistant mutations confer cross-resistance to sunitinib, thereby accounting for the relatively short PFS, approximately 6-9 months, with second-line sunitinib [51].

Despite these considerations, at the moment, sunitinib remains the standard of care for IM-refractory GISTs regardless the status of their secondary *KIT* mutation.

2.3 Regorafenib

Regorafenib (STIVARGA, formerly BAY 73-4506, BAYER, Levurkusen, Germany) is an orally available multikinase inhibitor with activity against multiple targets, including KIT, PDGFR, VEGFR1, R2, R3, TIE2, RET, FGFR 1, RAF, and p38 mitogen-activated protein kinase (MAPK) [52,53]. On February 25th, 2013 the US FDA approved regorafenib to handle patients with advanced GIST that cannot be surgically removed and no longer respond to other FDA-approved treatments for this disease. Safety and effectiveness of regorafenib were assessed in a clinical study involving 199 patients with unresecTable GIST that progressed after treatment with imatinib or sunitinib. Patients were randomly assigned to receive either regorafenib or a placebo. All patients received optimal supportive care, which includes treatments for the management of side effects and symptoms related to the tumor. Patients enrolled in the study took regorafenib or placebo until either the cancer progressed or side effects became unaccepTable. Results showed that patients treated with regorafenib had a delay in tumor growth (PFS) that was, on average, 3.9 months later than patients who were given placebo. Patients who received the placebo were given the opportunity to switch to regorafenib when their cancer progressed [52,53].

2.4 Emerging treatment for GISTs

The progress made in the management of GISTs has been possible because researchers around the world have worked together to study new drugs. Without clinical trials and the help of those patients who take part in them, we would not have the powerful, safe, and effective drugs imatinib and sunitinib. Researchers are now studying different compounds as possible new treatments for GIST:

- **Nilotinib (Tasigna®, Novartis Pharmaceuticals)** is used as first line treatment in leukemia patients (CML, Chronic myeloid leukemia), resistant or itolerant to imatinib. Nilotinib acts

by blocking the same enzyme activity as imatinib, though in a somewhat different way. Although nilotinib has shown some benefit, there is not enough information to suggest that nilotinib is more effective than imatinib in GIST. However, nilotinib may be able to control GIST in patients resistant to both imatinib and sunitinib.

- **Pazopanib (Votrient®, GlaxoSmithKline Pharnaceuticals)** is approved for patients with kidney cancer. Some early reports suggest that pazopanib might also be useful in GIST. To date, phase II trial is completed and a phase III trial is ongoing.
- **Sirolimus (Rapamune®, Pfizer Pharmaceutical), everolimus (Afinitor®, Novartis Pharmaceuticals), temsirolimus (Torisel®, Wyeth Pharmaceuticals), and ridaforolimus (AP23573, Merck and ARIAD Pharmaceuticals).** Besides Tyrosin kinase inhibitor drugs, there are many other proteins involved in mTOR pathway that could be of interest as biological target. Many of these drugs have been tested in combination with either imatinib or sunitinib in GIST and other cancers to see whether their combined use is more effective than one drug alone. Presently these agents are in phase I or II clinical trials.
- **HSP90 inhibitors, (as AT13387, Astex Pharmaceuticals)** block a key protein inside cancer cells, HSP90. HSP90 is a chaperone for which KIT is a client protein, so it contributes to GIST growth. AT13387 combined with imatinib is currently being studied in phase II clinical trials.
- **PI3K inhibitors, (as BYL719, Novartis Pharmaceuticals)** is the first oral PI3K inhibitor that strongly and selectively inhibits the PI3K alpha isoform of PI3K. Its biological activity correlates with inhibition of various downstream signaling components of the PI3K/Akt pathway and it inhibits the proliferation of breast cancer cell lines harboring *PIK3CA* mutations. A phase I clinical trial is ongoing.

Specific background

Cancer treatment is complicated by the myriad of treatment options and the lack of patient-specific information that may help clinicians select the best option therapy. There are two genomes relevant in cancer handling: the patient (germline) and the tumor (somatic). Together, these two genomes bring to treatment outcome through four processes: the germline genome modulates treatment exposure and toxicity while the somatic genome primarily determines tumor prognosis and response [54].

3. Germline genome: pharmacogenetics and pharmacogenomics

In a large patient population, a drug that is proven effective in many patients often fails to work in many others. Moreover, when it works, it could cause important serious side effects, even death, in a limited number of patients [55]. It is well documented that the large variability in efficacy and adverse reactions (ADR) occurring among different patients is the major determinant of use and limitation of drug in the clinical setting. Factors that can cause inter-variation among different patients treated with the same drug are various and complex, and include age, sex, lifestyle, environmental factors and especially genetic factors [56].

Pharmacogenetics focuses on the variants within one or more candidate genes while pharmacogenomics evaluates the entire genome for associations with pharmacological phenotypes [57]. This discipline has its origin in 1950s with the growth of human biochemical genetics. The role of genetics as a potential cause of ADR has been review for the first time by Motulsky in 1957 in "*Drug reaction, Enzymes and biochemical Genetics*". The term pharmacogenetics was created by Friedrich Vogel in 1959. In the late 1960s Vessel showed similarity of drugs disposal in identical twins, who shared 100% of their genes, unlike fraternal twins with only the 50% in common.

In the last decades, pharmacogenetic research has been hit by an explosion of interest by physician, geneticist and the pharmaceutical industry, as reflected by the rapid increase in the number of papers in the medical literature [58]. The rapid accumulation of knowledge of genome desease and genome-drug interaction has also driven the transformation of pharmacogenetics into a new entity of human genetics – pharmacogenomics – and provided the rationale for the hope of an individualized medicine [57].

The term pharmacogenomics was introduced in 1990s as a result of the knowledge gained from of the Human Genome project, and the development of genome sciences [58,59].

The main aim of pharmacogenetics and pharmacogenomics is to individualize medicine according to the specific genetic make-up of a given patient. Environment, diet, age, lifestyle and state of health can all influence the individual response to any pharmacological treatment, but understanding the influence of genetics could be the key to develop personalized medicine characterized by greater efficacy and safety. [59].

3.1. Single Nucleotide polymorphisms (SNPs)

Polymorphisms are genetic variations that occur with different frequency in different populations. These variations could be represented by insertion or deletions, but the most common variations are SNPs. A SNP is a DNA sequence variation, with a frequence > 1%, occurring when a single nucleotide in the genome differs among members of species within an individual. In the human genome the number of SNPs is around 3.2 milions and they are responsible for the 90% of human genetic variability [60]. SNPs are classified in three groups depending on where they are located in the genome: (i) c-SNP, variations located in coding region, exons, whose presence could modify or not the aminoacid sequence of the protein (non sinonimus and sinonimus, respectively) (ii) p-SNP, located in perigenic region, and (iii) r-SNP, random SNPs located in the intragenic regions, do not influence transcript or protein, but they can modify the DNA third structure, interfering with chromatin or DNA replication (Figure 7).

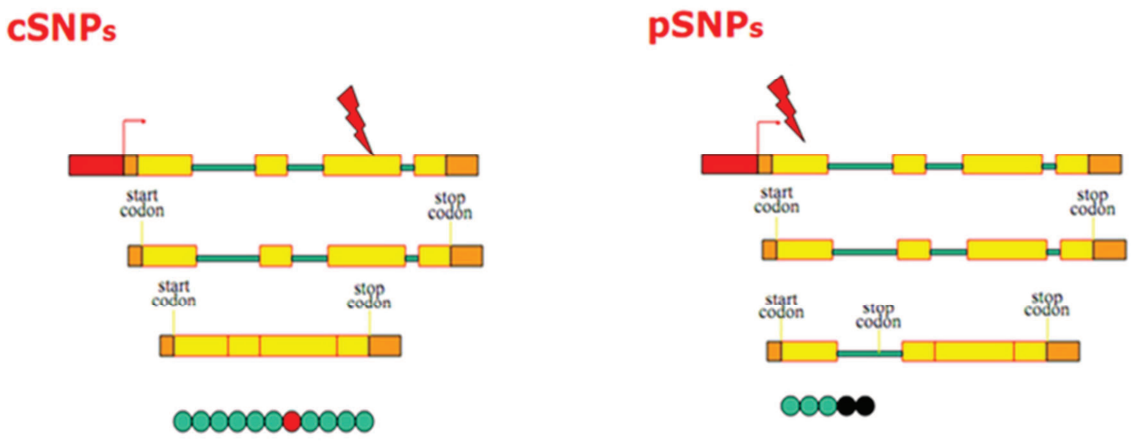


Figure 7. SNPs classification depending on their position.

Polymorphisms in key genes encoding drug transporters and metabolizing enzymes influence intracellular drug delivery. Pharmacogenetics has indeed demonstrated to be a potential source of biomarkers able to predict drug response and adverse drug reactions.

The rapid development of techniques in the area of genome analysis has eased the identification of new pharmacogenomics biomarkers. Such biomarkers mainly originated from genes encoding drug-metabolizing enzymes, drug transporters and drug targets. Some of these are now integrated by the US FDA and the European Medicines Agency (EMA) into drug label inserts [61]. In Table 1 are reported some significant examples of pharmacogenomics biomarkers in the context of cancer, describing prevalence, authority guidelines and relative importance [61].

Table 1. Pharmacogenenomic biomarkers in the context of desease, prevalence, authority guidelines and relative importance.

Area	Drug	Disease	Susceptibility	Causative genotype	Carrier frequency and type [a]	FDA and EMA advice	Importance [b]
Cancer	Imatinib (Gleevec)	Aggressive systemic Mastocytosis (ASM)	Response	c-kit mutation (D816V)	90% (non carrier)	Indicated for c-kit D816V-negative ASM (FDA)	***
	Gefitinib (Iressa)	Non-small cell lung cancer	Response	HER1 amplification or activating mutations	10–15% (carrier)	Approved for treatment of tumors with HER1 mutations (EMA)	***
	Panitumumab (Vectibix) Cetuximab (Erbitux)	Metastatic colorectal carcinoma	Response	Presence of HER1 expression Absence of KRAS mutations	HER1 expression 65–85% KRAS 70% (non-carrier)	Indicated or recommended for treatment of HER1-expressing tumors with wild-type KRAS (FDA, EMA	***
	Trastuzumab (Herceptin)	Breast cancer Metastatic gastric cancer	Response	HER2 amplification or overexpression	20–25% (carrier)	Evaluation of HER2 overexpression is necessary for treatment (FDA, EMA	***
	Azathioprine (Azasan, Imuran) 6-mercaptoropurine (Purinethol)	Leukemia Autoimmune disease Transplantation	Myelosuppression	Homozygosity for defective TPMT alleles (e.g. TPMT*2)	1% (hom)	TPMT geno- or phenotyping is recommended prior to treatment (FDA)	**
	Irinotecan (Camptosar, Campto)	Colon cancer	Neutropenia	UGT1A1*28 homozygosity	5–10% (hom)	Reduced initial dose should be considered for UGT1A1*28/*28 subjects (FDA)	**
	Tamoxifen (Nolvadex, Istubal, Valodex)	Breast cancer	Response Response	Defective CYP2D6 alleles. CYP2C19*17 (increased)	1–7% (hom) 40-50% (het) 1–8% (hom) Approx. 30% (het)	- -	** PC

[a] The frequency varies across different populations. Carrier refers to the frequency of subjects with at least one allele variant.
[b] The number of asterisks denotes the authors opinion of the raltive importance with ***denoting the highest significance.
PC Promising candidate.

Various initiatives have been suggested such as a pharmacogenetic research network that includes a series of integrate groups with expertise in pharmacology, genomic science, bioinformatics and clinical science. The group located at Standford University is responsible for the development of a public database that focuses on genotype data relevant to pharmacogenomics. This is only an example of what it is going on this panorama, although clinical translation remains the first

necessary step. Pharmacogenomics studies require a large number of subjects and multi-disciplinary teams with complementary expertise, as well as the ability to genotype a very large number of polymorphism and haplotypes [62].

The main current problems related to pharmacogenomics are: poorly defined phenotypes; significance of non-functional mutations; ethical aspects such as the use of genomic information; unclear sources for covering diagnostic costs and lack of founding for large prospective randomized studies, besides retrospective studies. Currently the most important aspect is a careful cost-benefit analysis of genetic testing closely followed by the specific need of educating the industrial and chemical research to the use of genetic testing [61].

Pharmacoepigenetics offers another level of explanation for inter-individual variations in drug response that cannot be clarified on the basis of genetic polymorphism. Many genes encoding enzymes, drug transporters, transcription factors, drug targets and nuclear receptors are under epigenetic control. Epigenetics includes covalent modifications of DNA and histones, DNA packing around nucleosomes, chromatin folding and attachment to the nuclear matrix and regulatory non coding RNAs, like small interfering RNAs (siRNAs) and microRNA (miRNAs). This emerging area is far to be fully elucidated, and represents an attractive field of investigation connecting environment and the genome [63,64].

4. Somatic genome: tumor prognosis and response

A healthy cell becomes cancerous by losing its ability to suitably regulate its replication. This initial genetic aberration can be a simple change in DNA sequence or a change that impacts an entire gene or chromosomal region. This malignant cell represents the seed from which a tumor arise, and the genome of this cell is the founder somatic genome. Through repeated cellular replication this somatic genome acquires additional abnormalities. Some of these acquired variants will further drive cancer progression, such as mutations in genes that are responsible for maintaining DNA replication accuracy or controlling metastatic spread. The initial and acquired aberrations determine the behavior of the primary tumor, in particular its ability to metastasize, and the treatment mechanisms that it will be sensitive to. Thus, prediction of tumor prognosis and response can be affectuated by understanding the somatic genetics [55].

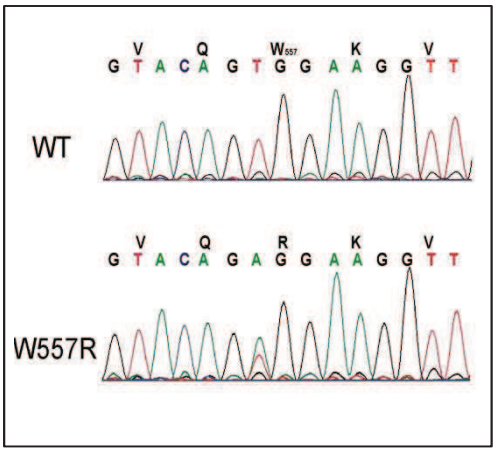


Figure 8. KIT exon sequence: tumor samples with normal DNA (upper panel) and with a W557R primary mutation (lower panel).

About 90% GISTs are characterized by one primary somatic mutation, mainly in exon 9 or 11 (Figure 8). This gain of function mutation determines a constitutive, ligand indipendent activation (Figure 9). The responsiveness of GIST to imatinib varies by primary KIT genotypes, which is now considered the most important factor in predicting outcome. In particular, patients with exon 11 mutations, despite a poor clinical prognosis prior to the imatinib era, are much less likely to experience treatment failure than patients with exon 9 mutations or without detectable mutation in KIT and PDGFRA [65].

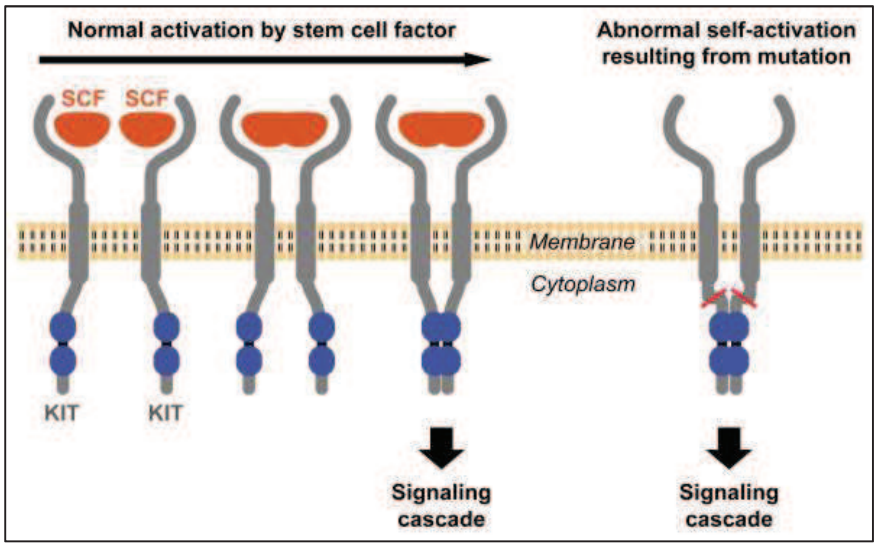


Figure 9. Two kit receptors normally dimerize in the presence of the phisiological ligand SCF to initiate downstream signaling (left). Mutations in the receptor cause abnormal constitutive signaling without SCF stimulation (right).

Mutational analysis of KIT and PDGFRA in GISTs has important implications in diagnosis and therapy decision and prediction of response to imatinib treatment.

Approximately 10% of GIST patients show primary resistance and the vast majority eventually develop secondary resistance and disease progression. Considering the heterogeneity in the mutational spectrum of GIST patients, it is interesting to thoroughly compare the treatment response, according to the mutational types, in the imatinib era. To date, according to the literatures the responsiveness of GIST to imatinib varies by primary KIT genotype [9, 66-68]. A mistake in KIT and PDGFRA mutation analysis might have dramatic consequence for patients: some will be treated with the wrong dose of imatinib (KIT exon 9 false negative), others without benefit (PDGFRA exon 18 D842V false negative) Considering these studies, on average approximately 71% of GIST exon 11 mutants reached an objective response (complete and/or partial response) compared with approximately 36% of exon 9 and 41% of WT-GISTs. Similarly, approximately 26% of wt-GISTs and approximately 16% of patients with exon 9 mutation experienced progressive disease, compared with only 5% of GISTs with exon 11 mutations. These findings are indeed corroborated by the fact that exon 11 mutant patients have a significantly better PFS and OS than patients with a tumor with exon 9 mutation or no detecTable KIT or PDGFRA mutations [65].

5. Imatinib transporter genes

It is well recognized that inter-patient variability in drug response reflects the systemic levels or intracellular concentrations of the drug, known to be associated with its pharmacokinetics (absorption, distribution, and metabolism) of the drug itself. Imatinib is metabolized by the cytochrome-P450 - mostly CYP3A4 and 3A5 isoforms. The active uptake of imatinib into cells is known to be mediated mainly by the hOCT1 transporter (encoded by the *SLC22A1* gene), whereas its efflux is mediated by the ABC transporters, in particular ABCB1 (also known as MDR1) and, to a lesser extent, ABCG2 (Figure 10) [69-73]. Besides these, other transporters may be important in the absorption, distribution, and elimination of imatinib, including the families of organic cation transporters (OCT) and organic anion transporters (OAT). In particular, a recent study by Hu *et al.* identified imatinib as a substrate of OATP1A2 (encoded by *SLCO1A2*), [72] whereas the involvement of members of the OCTN family as imatinib transporters is still uncertain.

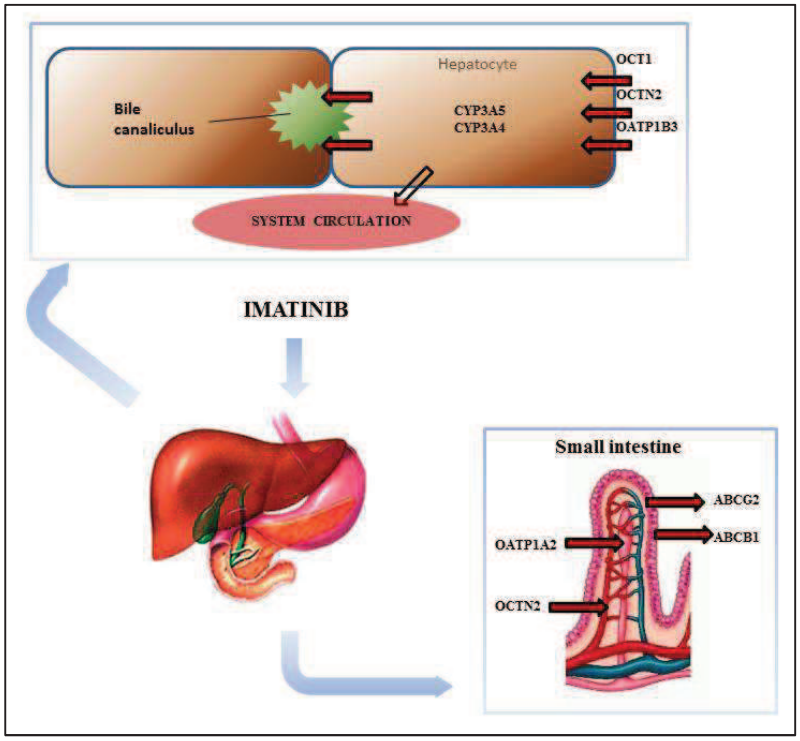


Figure 10. Main players in imatinib transport.

Inter-patient variability in imatinib metabolism/transport is substantial and thus far unexplained. A possibility is that genetic polymorphisms in genes encoding imatinib-metabolizing enzymes and transporters may influence the imatinib uptake into target cells. Consequently, genetic polymorphisms on the candidate genes *CYP3A4/3A5*, M*DR1*, *ABCG2*, *OATP1A2*, *OCTN1* (encoded by*SLC22A4*), *OCTN2* (encoded by*SLC22A5*) and *hOCT1* could affect expression of the corresponding proteins and thus may predict differences in responses to imatinib.

6. Folate metabolism pathway

Folate metabolism supports numerous critical intracellular reactions, including DNA synthesis, repair, and methylation (Figure 11). DNA methylation status is essential for healthy development and maintenance of cellular homeostasis and functions in adult organisms, including silencing of repetitive DNA elements and proper expression of genetic information [74]. The accurate maintenance of DNA synthesis, repair, and methylation patterns is fundamental, and its balance in mature cells is maintained by the concerted action of more than thirty enzymes [75]. Figure 10 represents a simplified diagram of the key enzymes that take part in the folate metabolic pathways. They include methylenetetrahydrofolate reductase (MTHFR), thymidylate synthase (TS), methionine synthase (MTR), methionine synthase reductase (MTRR), serine hydroxymethyltransferase (SHMT), dihydrofolate reductase (DHFR), reduced folate carrier (RFC), and folate receptor 1 (FOLR). These enzymes are highly polymorphic, and several functional genetic polymorphisms have been attracting research interest. In particular, several of these polymorphisms have been associated with decreased or increased enzymatic activities, with possible alteration in DNA synthesis, repair, and methylation processes. Aberrant methylation patterns, have been associated with various diseases including cancer and neurodegenerative disease. Additionally, polymorphisms in genes involved in the folate metabolisms have been associated with cancer risk, including colorectal and gastric cancer, vascular disease, depression and Down's syndrome. Currently, there are evidences of an association between DNA methylation level and polymorphisms in folate metabolism genes. The alteration of one or all of these processes could explain the association that have been found between some of these polymorphisms and risk of various malignancies, including breast, colorectal, and prostate cancer, and acute lymphoblastic leukemia [76].

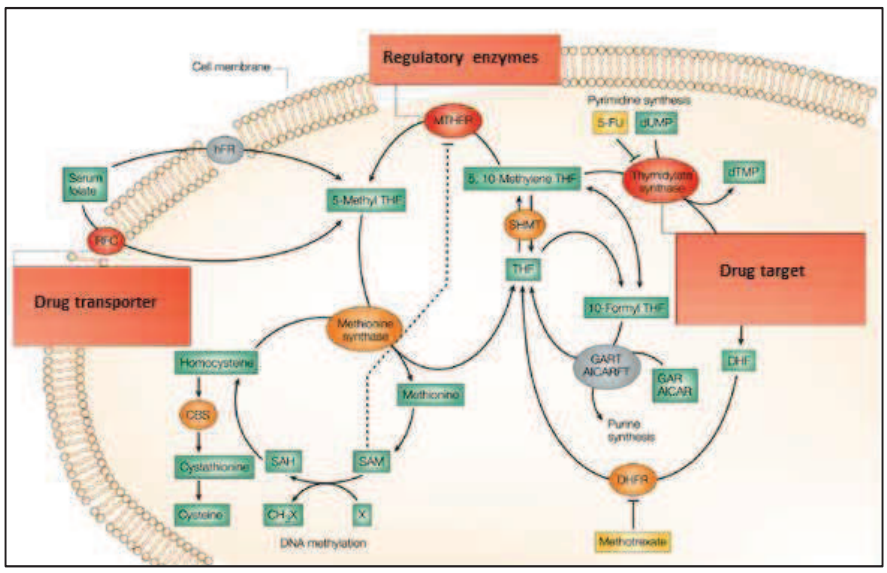


Figure 11. Key enzymes in the folate metabolic pathways. Image edited from Nat Rev Cancer. 2003 Dec;3(12):912-20. Cancer pharmacogenetics: polymorphisms, pathways and beyond. Ulrich CM, Robien K, McLeod HL.

Aim

Imatinib was the first inhibitor of c-KIT tirosin kinase to be licensed by health authorities and currently represents the first line treatment for GIST [37-39]. Despite the enormous success, resistance against imatinib emerges in a significant proportion of patients. In most GISTs initially responding to imatinib, development of resistance over time is common, and the occurrence of secondary resistance represents the main cause of disease progression. Different mechanisms leading to imatinib resistance have been identified and extensively investigated, and the most common is related to the alteration of the receptor signaling, in particular the acquisition of secondary mutations [69]. In addition, up-regulation of liver drug metabolizing enzymes [69], causing increased clearance of imatinib and up-regulation of drug transporters may be potential mechanisms [71].

An attractive alternative is represented by inter-individual differences in imatinib pharmacokinetics. In this process, the genes that control drug absorption, distribution, metabolism and excretion play a key role. Indeed, all of the drug metabolizers and transporters contain many genetic polymorphism, which might cause large inter-individual variability in imatinib plasma concentration and disposition.

For the reason mentioned above, the general aim of my three-years research period was to investigate the relationship between biomarkers and drug response – and resistance of course – on the basis of both an *in vivo* and *in vitro* approach.

Following the hypothesis that polymorphisms in genes encoding for imatinib transporters and metabolism enzymes may influence imatinib concentration delivered to target cells [70-73], the **first objective** was to conduct a retrospective study in a subset of 60 patients enrolled in a multicentric randomized phase III study, and to investigate a panel of SNPs in genes involved in imatinib transporters (I) and in the folate metabolism pathway (II) [74-76].

Based on the recognition that clinical progression of GIST during TKI therapy is often multifocal, TKI resistance mutations have been assessed in only single, or few, progressing metastases per patient and the heterogeneity of these mutations, in a given patient, remains unclear [77,78], the

second objective was to characterize in-depth the heterogeneity of KIT mutations and the drug-resistance mechanisms using a sensitive next generation sequencing approach.

First objective

The first objective is made up by two parts:

I. **Association between imatinib transporters genotype and response in GIST patients receiving imatinib therapy.**

II. **Folate-related polymorphisms in GIST: susceptibility and correlation with tumor characteristic and clinical outcome.**

I. Association between imatinib transporters genotype and response in GIST patients receiving imatinib therapy

Table 2. Patient and disease characteristics of the study population (*n* = 54).

Gender, *n* (%)	
Female	19 (35.2)
Male	35 (64.8)
Age at diagnosis, years	
median (range)	58 (18-83)
Tumour site, *n* (%)	
Stomach	30 (55.6)
Small Intestine	21 (38.9)
Other	3 (5.5)
Tumour size [a], *n* (%)	
2 - 5 cm	5 (9.2)
5 - 10 cm	17 (31.5)
≥ 10 cm	19 (35.2)
missing	13 (24.1)
Mitotic index [b], *n* (%)	
< 5	11 (20.4)
6-10	4 (7.4)
≥ 10	15 (27.8)
missing	24 (44.4)
Mutational status, *n* (%)	
KIT exon 11	31 (57.4)
Other than *PDGFRα* D842V and *PDGFRα*/*KIT* WT	4 (7.4)
missing	19 (35.2)

[a] None of the analysed subjects had tumour size < 2 cm; [b] 50 x High power filed.

Materials and methods

Study population – A total of 54 unresecTable/metastatic GIST patients receiving standard first-line imatinib 400 mg daily were retrospectively enrolled in this pharmacogenetic study. Twenty-nine patients were enrolled at *Sant'Orsola-Malpighi* Hospital, Bologna, and 25 at *Istituto Nazionale dei Tumori*, Milan, Italy. A written informed consent was required and the study was approved by the Ethics Committees of the two institutions. Patients with available peripheral blood were eligible. Patients characteristics and clinical features of their tumors are summarized in Table 2.

Evaluation of imatinib response - Time to progression (TTP) was calculated from the start of imatinib therapy to the date of disease progression documented by the CT scan performed approximately every 3-4 months. In one case, the disease re-evaluation by CT scan was done earlier due to clinical progression.

Genotyping analysis - DNA was isolated from fresh or frozen whole blood using a DNA isolation kit from QIagen (QIAamp® DNA Mini Kit, Qiagen, Hilden, Germany). Characteristics of the studied polymorphisms - three insertion/deletion and 28 single nucleotide polymorphisms, from now on all referred as SNPs in the text - are reported in Table 3a. Genotypes were determined by polymerase chain reaction (PCR) - based assays [restriction fragment length polymorphism (RFLP) and/or real-time] according to published methods [79] or by Taqman® assay PCR. Positive and negative controls were included in each reaction as quality control. In addition, accuracy of genotyping was confirmed by repetition of 100% of the samples.

PCR RFLP – the main components to perform a PCR are primers, nucleotide sequences complementary to the target region, DNA polymerase, necessary to enzymatically assemble a new strand of DNA, deoxynucleotide triphosphates (dNTPs), the building blocks from which the DNA polymerase synthesize a new DNA strand, buffer solution and usually divalent cations that help the reaction (i.e. $MgCl_2$) 10ng DNA was added at this mixture and the thermal cycling program were performed (Table 3). The DNA is replicated in every cycle and the amount increased exponentially. The amplification of the sequence of interest is followed by the incubation with an appropriate restriction enzyme that recognize, if present, the restriction site, creating DNA fragments. The fragments obtained were separated through electrophoresis at 200 V for 30 minutes, on pre-cast polyacrylamide gels 10% TBE (Bio-rad, Hercules, CA, USA). The DNA was then analyzed using Ethidium Bromide, an intercalating agent commonly used as a fluorescent tag. When exposed to UV ray its fluorescence is 20-fold higher after binding DNA. The image of the gel was acquired using a digital photo camera connected to VERADOC-4000 (Bio-rad) and visualized with QUANTITY ONE software. Three were the possible situations that can emerge from the analysis: homozygosis for the wild-type (wt) allele, homozygosis for the SNP or heterozygosis, in which one allele is wt and the other one is SNP.

Real Time PCR – Real-time PCR (RT-PCR or qRT-PCR) is a variation of the standard PCR technique used to quantify DNA o mRNA in a sample. Using sequence specific primers, the relative numbers of copies of a particular DNA or RNA sequence can be determined. Quantification of amplified product is obtained using fluorescent probes and specialized machines that measure fluorescence while performing temperature changes needed for the PCR cycles (Figure 12). More specifically for our use, an allelic discrimination assay was used to detect variants of a single nucleic acid sequence. One fluorescent dye detector is a perfect match to the wt (allele 1) and the other one is a perfect match with the SNP allele (allele 2). The allelic discrimination assay classifies unknow samples as i) homozygotes (samples having only allele 1 or 2) and ii) heterozygotes (samples having both allele 1 and 2). In particular, the allelic discrimination assay measures the change in fluorescence of the dyes associated with the Taqman probes VIC® and FAM® (Applied Biosystems, Foster City, CA), that selectively bind wt or SNP allele (Figure 12). The reaction was prepared using 10ng DNA, Taqman genotyping assay(20X or 40X), Taqman Universal Master Mix (2X) and water RNAse free, for a total volume of 25ul. The analysis was performed using 7900 HT Fast Real Time PCR system (Applied Biosystem).

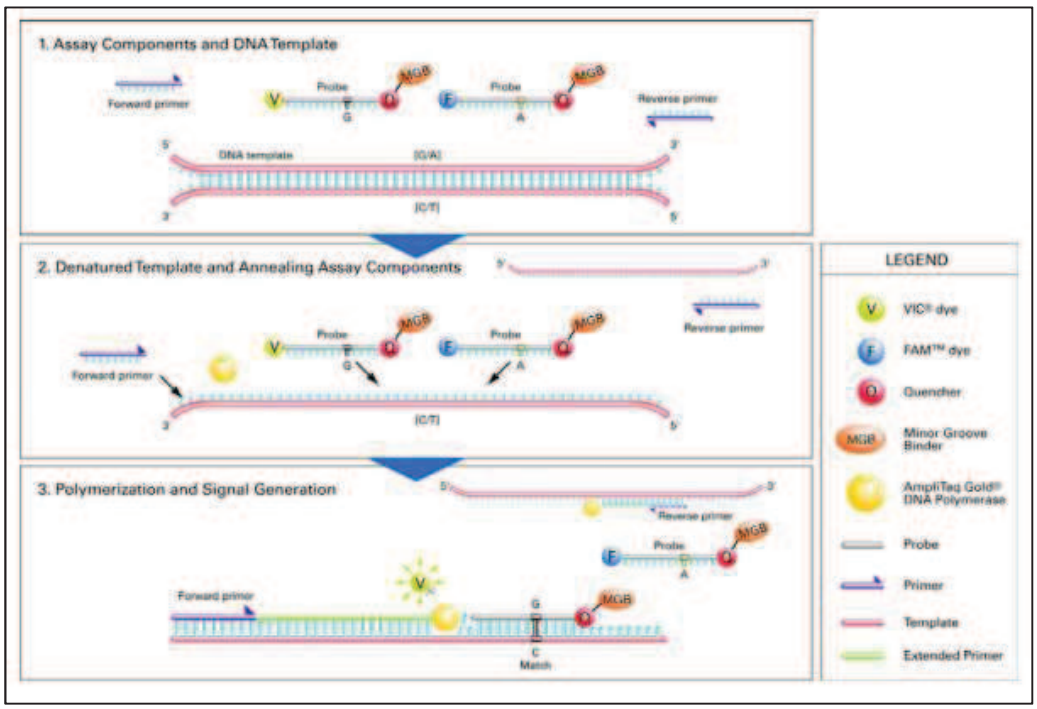


Figure 12. Each TaqMan® MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites. When the oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye quenches the reporter signal. AmpliTaq Gold® DNA polymerase extends the primers bound to the genomic DNA template. AmpliTaq Gold® DNA polymerase (with its 5´ nuclease activity) cleaves probes that are hybridized to the target sequence. Cleavage of the probes hybridized to the target sequence separates the quencher dye from the reporter dye, resulting in increased fluorescence by the reporter. The fluorescence generated by PCR amplification indicates which alleles are present in the sample.

Table 3. SNPs description.
[A] RT = Real-Time PCR with TaqMan allelic discrimination assay [Applera, Foster City, USA];
RFLP = PCR-RFLP and M = Multiplex PCR, analysis carried out according to published methods [reference parenthetically] or as standardized in our laboratory (primer set, T (°C) of annealing and restriction enzymes (Fermentas, Vilnius Lithuania) described].

Table 3. SNPs description.

Gene [full name; Protein name]	**SNP ID**	**METHOD** [A]
SLC22A1 [Solute carrier family 22 (organic cation transporter - OCT1) family 22, member 1]		
	rs12208357 [$R^{61}C$]	RT TaqMan assay C_30634096_10
	rs683369 [$L^{160}P$]	RT TaqMan assay C_928536_30
	rs4646277 [$P^{283}L$]	RT TaqMan assay C_30634088_10
	rs4646278 [$R^{287}G$]	RFLP f_GCGATGGCTCCCTTTTG r_TTAGACCCCGACCCAAGACCAC
	rs2282143 [$P^{341}L$]	RT TaqMan assay C_15877554_40
	rs72552763 [$MI^{420}I$]	RT TaqMan assay C_34211613_10
SLC22A4 [solute carrier family 22 (organic cation transporter - OCTN1), member 4]		
	rs1050152 [$L^{503}F$]	RT TaqMan assay C_3170459_30
SLC22A5 [Solute carrier family 22 (organic cation transporter - OCTN2), member 5]		
	rs2631367 [5' UTR]	RFLP [Török *et al*., 2005] [79]
	rs2631370 [5'- near gene]	RT TaqMan assay C_2843383_10
	rs2631372 [5'- near gene]	RT TaqMan assay C_26479165_10
SLCO1A2 [Solute carrier organic anion transporter family (OATP) member 1A2]		
	rs11568563 [$E^{172}D$]	RT TaqMan assay C_25605897_10
SLCO1B3 [Solute carrier carrier organic anion transporter family (OATP) member 1B3]		
	rs4149157 [5' UTR]	RFLP [Tsujimoto *et al*., 2008] [80]
	rs4149158 [5' UTR]	RFLP [Tsujimoto *et al*., 2008] [80]
	rs4149117 [$S^{112}A$]	RT TaqMan assay C_25639181_40
	rs7311358 [$M^{233}I$]	RT TaqMan assay C_25765587_40
ABCA3 [ATP-binding cassette sub-family A, (ABC1) member 3]		
	rs323040 [Intronic]	RT TaqMan assay C_11292220_10
	rs4146825 [5' UTR]	RT TaqMan assay C_32374235_10
ABCB1 [ATP-binding cassette sub-family B, (MDR/TAP) member 1; P-gp (P-glycoprotein)]		
	rs10245483 [Promoter region]	RT TaqMan assay C_2573447_20
	rs3213619 [Promoter region]	RT TaqMan assay C_27487486_10
	rs1128501 [$G^{185}V$]	RT TaqMan assay C_7586664_10
	rs1128503 [$G^{412}G$ also C1236T]	RT TaqMan assay C_7586662_10 RFLP [Goreva *et al*., 2004][81]
	rs60023214 [$I^{1145}I$ also C3435T]	RFLP [Jamroziak *et al*., 2004] [82]
	rs2032582 [$A^{893}S$/T also G2677T/A]	M [Kurzawski *et al*., 2006] [83]
ABCC4 [ATP-binding cassette sub-family C, (CFTR/MRP) member 4]		
	rs3765534 [$E^{757}K$]	RT TaqMan assay C_27478235_20
	rs9561765 [Intronic]	RT TaqMan assay C_31356298_10
ABCG2 [ATP-binding cassette sub-family G, (WHITE) member 2]		
	rs2231137 [$M^{12}V$]	RFLP [Hu *et al*, 2007] [84]
	rs2231142 [$Q^{141}K$]	RT TaqMan assay C_15854163_70
CYP3A4 [cytochrome P450, family3, subfamily A, polypeptide 4; CYP3]		
	rs2740574 [5' Near Gene]	RFLP [Radriguez-Antona *et al*., 2005] [85]
	rs28371759 [$L^{293}P$]	RT TaqMan assay C_27859823_20
CYP3A5 [cytochrome P450, family3, subfamily A, polypeptide 5; CYP3]		
	rs776746 [Splicing site]	RFLP [Hu *et al*., 2005] [86]
	rs28365083 [$T^{398}N$]	RFLP [van Schaik *et al*., 2002] [87]

Statistical analysis - The distribution of genotypes was tested for Hardy-Weinberg (HW) equilibrium using the online HW test tool offered by the Institute for Human Genetics, Technical University Munich (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Survival analysis methods were used to examine the relationship between genotypes [homozygous wild-type, heterozygous and homozygous for the variant allele (SNP)] and GIST time to progression. In univariate analysis, the survival curves were estimated and plotted with the Kaplan-Meier method. The curves were compared with log-rank test of equality of survivor functions (statistical significance defined as $p < 0.05$). In multivariate analysis, hazard ratios (HR) and 95% confidence interval (95% CI) were estimated with Cox proportional hazards models, using gender, age, tumour site and size, mutational status and status at diagnosis, as covariates in addition to the genotype. The proportional hazards assumption was tested ($p > 0.05$) using Schoenfeld residuals. Multiple logistic regression was used to assess the relation between individual SNPs and primary resistance. Statistical analysis was conducted using Stata Intercooled version 11.0.

Results

Characteristics of the study population - The cohort of 54 Caucasian GIST patients was made up of 65% men and 35% women (median age at diagnosis 58 years). The most common primary sites were the stomach (55.6%) and the small intestine (38.9%). Nearly three-quarters of patients had metastatic disease, with liver and peritoneum as the most common sites of metastases. Thirty-one patients (57.4%) harboured *KIT* exon 11 mutations, while only one patient had a *KIT* exon 9 mutation. Three patients (5.6%) had a *PDGFRα* mutation, excluding the D842V, since GISTs harbouring this mutation, as well the *PDGFRα*/*KIT* WT GISTs, were not included in the study being assumed to be resistant to imatinib. In nineteen GISTs, mutational status was unknown, due to insufficient or unavailable biological material for the analysis.

Genotype distribution. Genotype distribution of the 31 candidate SNPs are summarised in Table 4.

Table 4. Genotype frequency of the 31 candidate SNPs.

Gene SNP ID	Major/Minor allele	MAF	HWE	*P* value
SLC22A1 **– OCT1**				
rs12208357	**C/T**	0.09	0.37	
rs683369	**C/G**	0.17	0.62	
rs4646277	**C/T**	0.00	---	
rs4646278	**C/G**	0.00	---	
rs2282143	**C/T**	0.00	---	
rs72552763	**GAT/-**	0.18	0.34	
SLC22A4 **– OCTN1**				
rs1050152	**C/T**	0.43	0.78	
SLC22A5 **– OCTN2**				
rs2631367	**C/G**	0.51	1.00	
rs2631370	**A/G**	0.32	0.37	
rs2631372	**C/G**	0.40	0.25	
SLCO1A2 **– OATP1A2**				
rs11568563	**A/C**	0.04	1.00	
SLCO1B3				
rs4149157	**ATATTCACTTGGTATCTG/-**	0.31	0.75	
rs4149158	**TTTA/-**	0.31	0.75	
rs4149117	**G/T**	0.10	1.00	
rs7311358	**A/G**	0.10	1.00	
ABCA3				
rs323040	**G/A**	0.17	1.00	
rs4146825	**C/T**	0.00	---	
ABCB1				
rs10245483	**G/T**	0.48	0.79	

rs3213619	**A/G**	0.00	---
rs1128501	**C/A**	0.00	---
rs1128503	**C/T**	0.47	0.01
rs60023214	**C/T**	0.54	0.58
rs2032582	**G/T or A**	0.46	0.10
ABCC4			
rs3765534	**C/T**	0.00	---
rs9561765	**G/A**	0.06	1.00
ABCG2			
rs2231137	**G/A**	0.08	1.00
rs2231142	**G/T**	0.08	0.30
CYP3A4			
rs2740574	**A/G**	0.02	1.00
rs28371759	**A/G**	0.00	---
CYP3A5			
rs776746	**G/A**	0.04	1.00
rs28365083	**C/A**	0.00	---

Nine SNPs (*SLC22A1* rs4646277, rs4646278, rs2282143, *ABCA3* rs4146825, *ABCB1* rs3213619, rs1128501, *ABCC4* rs3765534, *CYP3A4* rs28371759, and *CYP3A5* rs28365083) were homozygous for the major allele in all patients and were excluded from further analyses. As expected from the literature, in the *SLCO1B3* gene we found the rs4149157 in complete linkage *disequilibrium* with the rs4149158, and the rs4149117 completely consistent with the rs7311358. Deviation from the HW equilibrium was observed for a single SNP (*ABCB1* rs1128503); departure from HW equilibrium was not observed for any other SNPs. The distribution of genotypes was similar to those previously reported by us in CML Caucasian patients [79] or in the publicly available database NCBI (dbSNP) for Caucasians.

Treatment outcome of imatinib therapy and genotypes. With a median duration of imatinib administration of 36,9 months, the best results during therapy were partial response and sTable disease in 90.7% of patients. With a median follow-up of 36.9 months, progressiove disease was observed in 26 cases (48.1%), with a median TTP of 21.8 months (range 1.6-58.2).

Presence of the C allele in *SLC22A4* (OCTN1 rs1050152) had a significantly favourable impact on TTP [HR 2.94, 95% CI 1.20-7.17, P = 0.018; (Table 5)]. The two minor alleles (G) in *SLC22A5* (OCTN2 rs2631367 and rs2631372) were also significantly associated with a prolonged TTP (P = 0.049 and P = 0.050 respectively; Table 5). None of the other analysed SNPs correlated with the TTP.

Table 5. Most relevant association between TTP and candidate genotypes.

Gene SNP ID	Referent/adverse Genotype	Hazard ratio [95% CI]	*P* value
SLC22A4 **– OCTN1**			
rs1050152	**CC or CT/TT**	2.94 [1.20-7.17]	0.018
SLC22A5 **– OCTN2**			
rs2631367	**CG or GG/CC**	0.43 [0.18-1.00]	0.049
rs2631372	**CG or GG/CC**	0.46 [0.21-1.00]	0.050

Alleles were correlated with TTP based on the Kaplan-Meier method. Presence of the minor allele in *SLC22A4* (TT rs1050152), was associated with reduced TTP ($P = 0.013$; Figure 13).

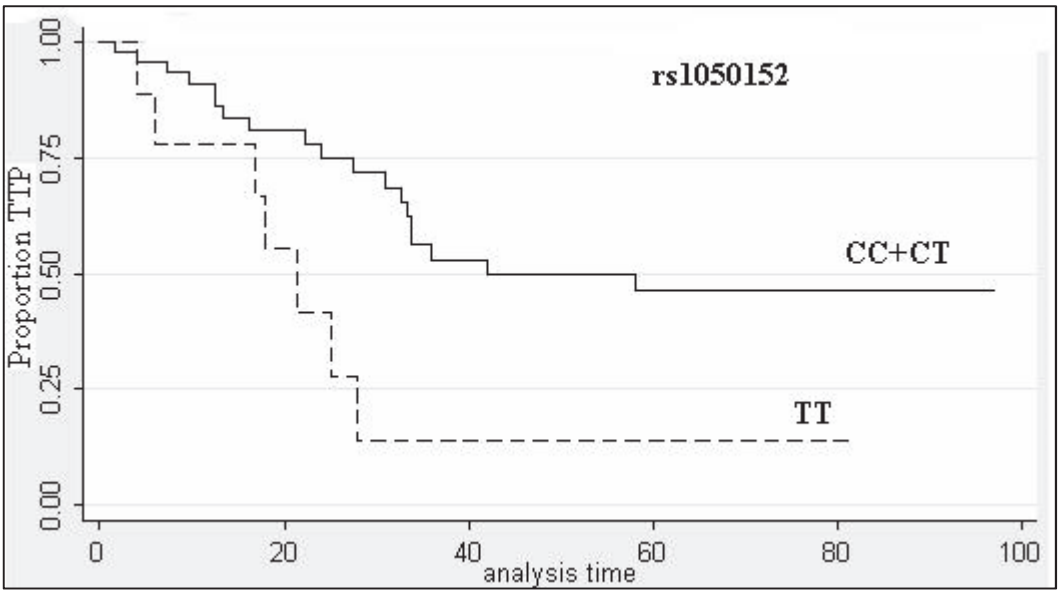
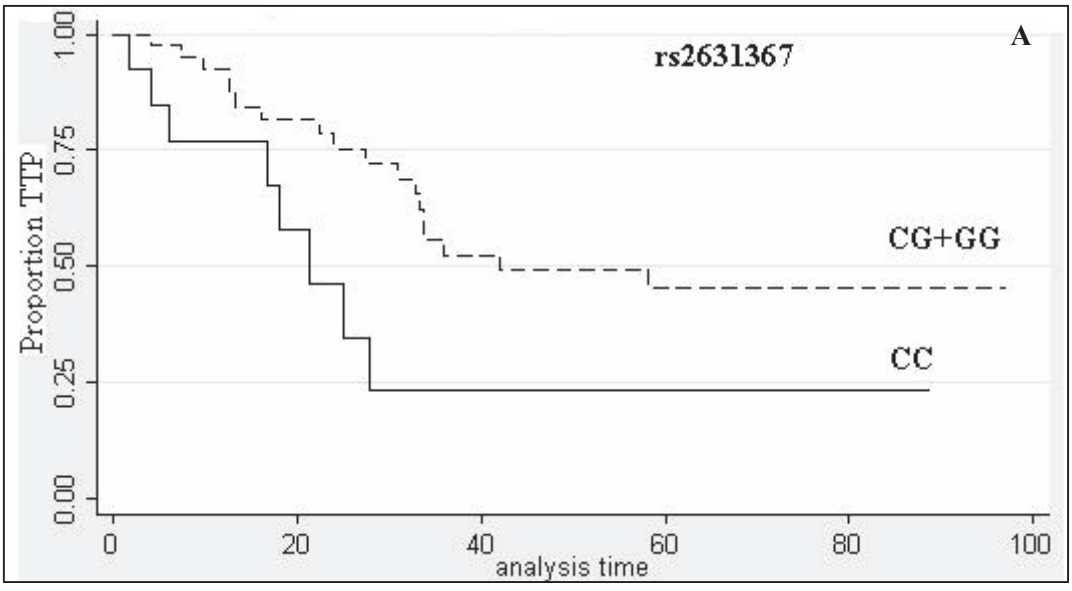


Figure 13. . Kaplan–Meier TTP estimates for SLC22A4 (OCTN1) genotypes – rs1050152.

Similarly, presence of the *SLC22A5* CC (rs2631367) and CC (rs2631372) genotype was associated with reduced TTP in both cases ($P = 0.042$ and $P = 0.045$ respectively; Figure 14).



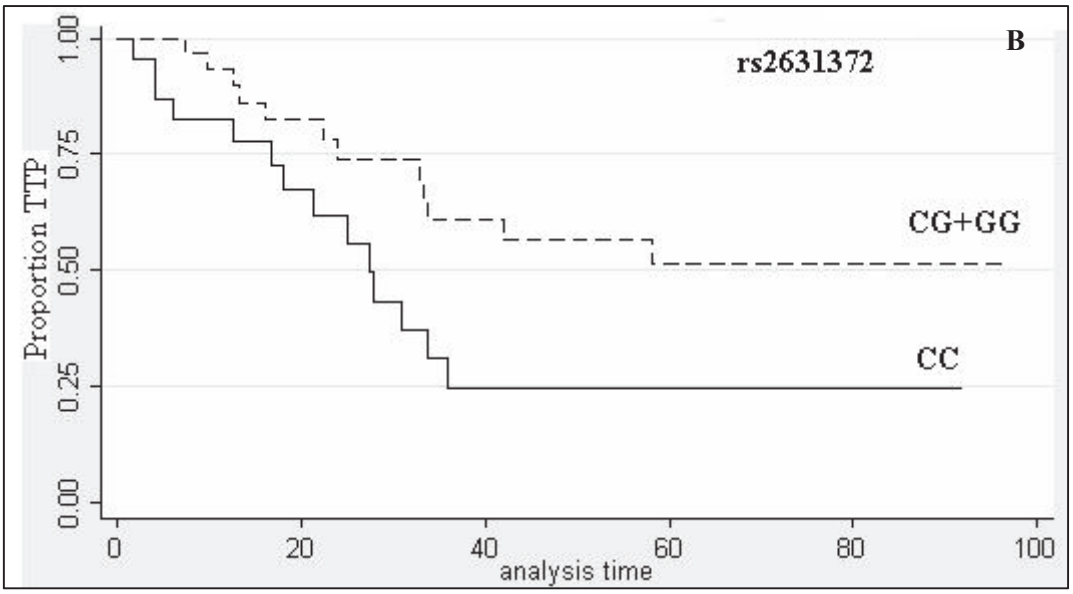


Figure 14. A. Kaplan–Meier TTP estimates for SLC22A5 (OCTN2) genotypes – rs1050152. **B**. Kaplan–Meier TTP estimates for SLC22A5 (OCTN2) genotypes – rs261372.

In addition *ABCC4* – GA genotype (rs9561765) showed a trend for a possible association with prolonged TTP ($P = 0.084$). Considering the low statistical power because of the sample size, and the low frequency ($q = 0.06$), this SNP may be worthy of further consideration. Based on the findings of the present study we stratified the population according to the number of favourable genotypes. In particular, we assigned a score of 3 in the presence of all three favourable genotypes – *SLC22A4* – CC+CT (rs1050152), *SLC22A5* – CG+GG (rs2631367) and CG+GG (rs2631372) – and a score of 0 to 2 according to the presence of zero to two favourable genotypes. A score of three was significantly associated with improved TTP ($P = 0.040$; Figure 15).

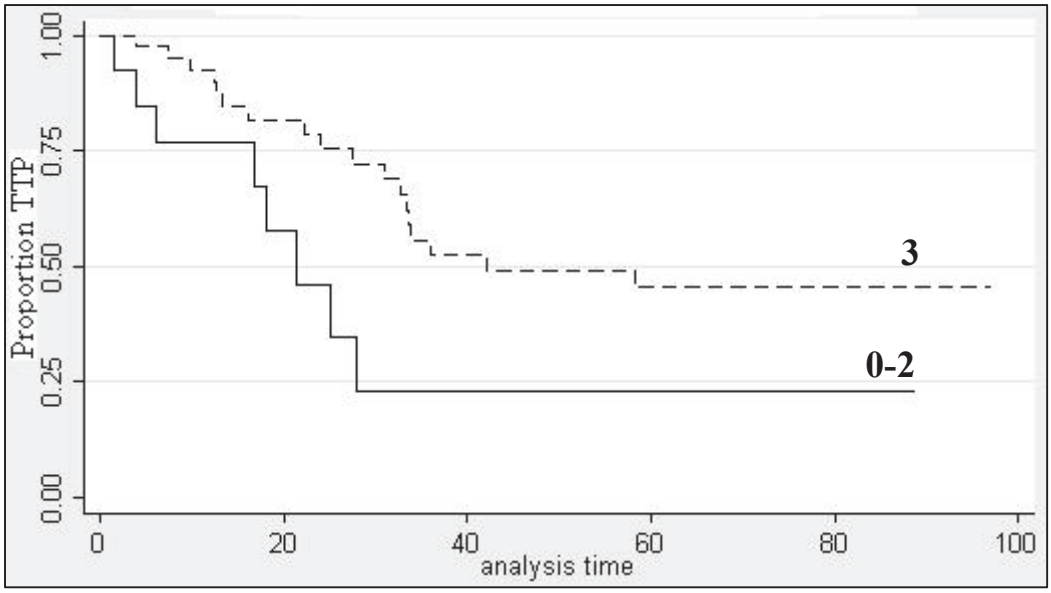


Figure 15. Kaplan–Meier TTP estimates for SLC22A4 (rs1050152) and SLC22A5 rs2631367 and rs2631372) genotypes combination. The numbers of 0–3 indicate the number of favourable genotype associates with improved TTP. (— 0 to 2 favourable genotypes; ---- 3 favourable genotypes).

Cox proportional hazards models examining the association between genotypes and TTP, were adjusted for known prognostic factors, including age, sex, site of localization, tumour size, and mutational status (Table 6). As expected, size ≥10 cm was always significantly associated with a reduced TTP ($P \leq 0.05$). In these models *SLC22A4* (rs1050152) and *SLC22A5* (rs2631367 and rs2631372) genotypes, as well as the combination of favourable - *SLC22A4* (rs1050152) and *SLC22A5* (rs2631367 and rs2631372) - genotypes maintained independent predictive value (Table 6). In addition to these, the *ABCC4* AA genotype (rs9561765) was also associated with reduced TTP ($P = 0.032$; Table 6).

Logistic analyses were performed for association with primary resistance to imatinib. However, the primary resistance rate was very low (only 5 patients, the 9.3%, had a primary resistance), and no correlation could be detected.

Table 6. Multivariate Cox regression models for TTP.

SNP	HR (95% CI)	*P* value*	SNP	HR (95% CI)	*P* value*
***SLC22A4* - rs1050152**			***SLC22A5* rs2631367**		
TT *vs* TC+CC	5.74 (2.01-16.40)	0.001	CG + GG *vs* CC	0.15 (0.05-0.44)	< 0.001
Tumour size			**Tumour size**		
≥10 cm *vs* < 10 cm	3.01 (1.13-8.06)	0.028	≥10 cm *vs* < 10 cm	3.11 (1.20-8.11)	0.020
***SLC22A5* rs2631372**			***ABCC4* rs9561765**		
CG + GG *vs* CC	0.46 (0.22-0.97)	0.043	GA *vs* AA	0.12 (0.02-0.83)	0.032
Tumour size			**Tumour size**		
≥10 cm *vs* < 10 cm	2.87 (1.07-7.70)	0.036	≥10 cm *vs* < 10 cm	2.71 (0.96-7.64)	0.060
SLC22_A4-A5 Haplotype **					
3 *vs* 1+0	0.15 (0.05-0.44)	< 0.001			
Tumour size					
≥10 cm *vs* < 10 cm	3.11 (0.02-0.44)	0.002			

* Gender, age, and mutational status adjusted.

** A score of 3 corresponds to the presence of the three favourable genotypes – *SLC22A4* – CC+CT (rs1050152), *SLC22A5* – CG+GG (rs2631367) and CG+GG (rs2631372) – and a score of 0 to 2 to the presence of zero to two favourable genotypes.

Discussion

To the best of our knowledge, this is the first pharmacogenetic study in GIST patients undergoing imatinib therapy. Since the introduction of imatinib as standard treatment in GIST patients with metastatic and/or unresecTable disease, significant efforts have been made to elucidate the mechanisms that could affect its efficacy. As is well known, *KIT* and *PDGFRα* mutational status has a significant impact on response to imatinib treatment. In particular Heinrich *et al*. reported that 83.5% of patients with exon 11 *KIT* mutations achieved an objective response after imatinib therapy, compared to 48% of patients with exon 9 mutations [9]. Patients harbouring the *PDGFRα* exon 18 - D842V - mutation are usually non responsive to imatinib [8,64]. Finally, *KIT* and *PDGFRα* GISTs are also poorly responsive to imatinib, though the percentage of patients achieving an objective response or sTable disease is variable, according to different studies [8,9,64]. Conversely, patients with *KIT* exon 9 mutations show better outcomes in response to higher dose of imatinib (600/800 mg daily vs 400 mg day) as well as to sunitinib [49]. Certainly, KIT and *PDGFRα* mutational analysis represents a good predictive marker of responsiveness to tyrosine kinase inhibitors. However, GISTs characterized by the same mutational spectrum often show different responses in the clinical practice. In addition, the inability to find secondary mutations in some progressive GISTs has suggested the possibility that, besides *KIT* and *PDGFRα* secondary mutations, additional mechanisms may be involved in imatinib resistance [88]. The theory that the mutational status cannot explain all the cases of primary/secondary resistance observed, is corroborated by the finding that GISTs with secondary *KIT* activation-loop mutations, expected to be insensitive to both imatinib and sunitinib, may still be susceptible to sunitinib [49]. It thus appears indispensable to better elucidate the different mechanisms of resistance, which will help to define subpopulation of GIST patients who will truly benefit from second-generation tyrosine kinase inhibitors, or maybe from imatinib dose escalation or discontinuation.

Retrospective data suggested that imatinib plasma levels are associated with progression-free survival in advanced GISTs [90-93]. On the contrary, finding have been reported in a recently published prospective pharmacokinetic study, the first in GIST patients [94]. The putative role of pharmacokinetic in imatinib resistance is still largely unexplored, and today imatinib plasma level assessment do not represent a standard procedure in the clinic. However, we cannot exclude that imatinib pharmacokinetic properties may impact efficacy of treatment, e.g. time to progression. In this perspective, considering the possible influence of polymorphisms in key genes encoding drug transporters and metabolizing enzymes on intracellular drug delivery, pharmacogenetics might represent a potential source of biomarkers of imatinib effectiveness as highlighted in pharmacogenetic studies in CML [79]. However, to our knowledge, the identification of different

polymorphisms in imatinib transporters and metabolizing genes has led to the conduction of several pharmacogenetic studies in CML patients but not in GISTs [79,95].

In this retrospective study, using a multiple candidate gene approach, we found that polymorphisms in imatinib transporters genes are associated with TTP. In particular we found that the *SLC22A4* variant allele (rs1050152) was significantly associated with a reduced TTP. To us, this finding is intriguing, as we found the same polymorphism correlating with the major molecular response rate in our set of CML patients [79]. None of the other pharmacogenetic studies has investigated this gene, as *SLCO1A2*, *SLC22A5*, and *ABCC4* genes only have been assumed to be good candidates for imatinib absorption [96]. We also analysed polymorphisms in these three genes with interesting results. In particular, the minor alleles in *SLC22A5* rs2631367 and rs2631372 were associated with prolonged TTP. As far as we know, none of these two polymorphisms has ever been investigated in any pharmacogenetic study related to imatinib, making it mandatory to validate this observation in independent data sets (GIST as CML as well). With regard to the *ABCC4* gene - also not investigated in previous pharmacogenetics studies – of the two investigated polymorphisms, the rs9561765 variant resulted marginally associated with a prolonged TTP. Unfortunately, due to the sample size, we had limited power to detect modest effects, as well as concrete possibility of detecting apparent correlations by chance. However, given the function assigned to the *SLC22A5* and *ABCC4* genes in imatinib absorption, this finding is biologically plausible, and further *in vitro* studies assessing the functional significance of this polymorphisms are warranted.

Interestingly, none of the *ABCB1* investigated polymorphisms have been associated to TTP. Three variants have been extensively studied in the literature - rs2032582, rs60023214 and rs1128503 - both individually and as a haplotype in CML patients and the results have been inconsistent. Kim and colleagues [94] found no association between *ABCB1* genotypes/haplotypes and imatinib efficacy. On the other hand we found a weak association between *ABCB1* CC carriers (rs60023214) only in a subset of Caucasian CML patients, which is consistent with the finding of Angelini and colleagues [79]. These results, although controversial, suggest a role of the *ABCB1* gene in determining response to imatinib in CML patients. It remains to elucidate which is the causal variant, and the importance in GIST patients.

The correlation between polymorphisms in members of the OCTN family transporters and imatinib efficacy might be very important in relation to the use of imatinib in the adjuvant setting. Currently, the optimal adjuvant treatment duration and the optimal patients' selection remain the subjects of extensive researches

[97]. Recently, Joensuu reported that three years of adjuvant imatinib substantially improves the disease-free survival and overall survival of patients with a high risk of recurrence, compared with one year [98]. However, among these patients the observed rate of recurrence is quite low (20-30%), which makes the identification of pharmacokinetic markers useful for a further sub-classification of the patients. This may be very important to identify patients that might benefit from intervals longer than three years.

Conclusion (I)

Imatinib has offered unprecedented improvements in GIST treatments. *KIT/PDGFR*α mutational status or acquisition of secondary mutation is a common mechanism, however not the unique, of imatinib resistance. The heterogeneity in mechanisms of secondary resistance unquestionably highlights the need of biomarkers of efficacy. Thus, collecting data on the role of polymorphisms in imatinib response, at the moment scarce in CML patients and unavailable in GIST, represents a clinical priority. Our study identified polymorphisms in imatinib transporters genes SLC22A4 and SLC22A5 that improved time to progression, suggesting that genotyping should be taken into account in an attempt to individualize GISTs treatment, representing an attractive opportunity for new clinical trials.

II. Folate-related polymorphisms in GIST: susceptibility and correlation with tumor characteristic and clinical outcome

A recent finding suggests that the DNA methylation profile may be associated with aggressive clinical behavior and unfavorable prognosis in gastrointestinal stromal tumor (GIST) [99]. However, as far as we are aware, there have been no investigations exploring the influence of genetic polymorphisms in enzymes that take part in the folate metabolic pathways GIST.

Materials and methods

Study population - A total of 60 unresecTable/metastatic GIST patients were retrospectively enrolled in this study. Thirty-two patients were enrolled at Sant'Orsola-Malpighi Hospital, Bologna, and 28 at *Istituto Nazionale dei Tumori*, Milan, Italy. Clinical information was collected retrospectively from the patients' medical records. Overall survival (OS) was defined as the time from the first day of treatment to death from disease. Dates of death were obtained and cross-checked using the inpatient medical records. If a patient was alive, OS were censored at the time of the last follow-up. For the 54 patients on standard first-line imatinib therapy time to progression (TTP) was calculated from the start of imatinib therapy to the date of disease progression documented by the CT scan performed approximately every 3-4 months. Data for patients who did not progressed at the last follow-up TTP evaluation were censored at that time. In order to exclude disease susceptibility we also genotyped 153 controls, anonymous blood donors from the *Centro Trasfusionale*, Sant'Orsola-Malpighi Hospital, Bologna. The study was approved by the Ethics Committees of the two institutions. The analysis was done after written informed consent for study participation and anonymous data publication in accordance with national legislation. Any subjects could cancel participation at any time during the study, according to Helsinki Declaration and later Amendments.

Genotyping analysis - We selected thirteen common [minor allele frequency (MAF) > 0.05 in Caucasian], well-studied functional variants - located in regulatory region, cause non-synonymous amino acid changes and/or have been repeatedly associated with cancer risk, survival or treatment response. Patients with available peripheral blood were eligible for this retrospective study. DNA was isolated as previously described. Characteristics of the studied polymorphisms - two insertion/deletion, one tandem repeat and ten single nucleotide polymorphisms - are reported in Table 7. Genotypes were determined by PCR RFLP and RT-PCR as previously described. Positive and negative controls were included in each reaction as quality control. In addition, for internal

quality control (accuracy of genotyping) 90% of samples were repeated. The concordance between the original and the duplicate samples for all the analysed polymorphisms was 100%.

Statistical analysis - The distribution of genotypes was tested for departures from the Hardy-Weinberg equilibrium using the χ^2 test. Survival analysis methods were used to examine the relationship between genotypes [homozygous wild-type, heterozygous and homozygous for the variant allele] and GIST time to progression. In univariate analysis, the survival curves were estimated and plotted with the Kaplan-Meier method. The curves were compared with log-rank test of equality of survivor functions (statistical significance defined as $p < 0.05$). In multivariate analysis, hazard ratios (HR) and 95% confidence interval (95% CI) were estimated with Cox proportional hazards models, using gender, age, and status (localised/metastatic) at diagnosis, as covariates in addition to the genotype. The proportional hazards assumption was tested ($P > 0.05$) using Schoenfeld residuals. Multiple logistic regression was used to assess the relation between individual polymorphisms and primary resistance. Statistical analysis was conducted using Stata Intercooled version 12.0.

Table 7. SNPs description

Gene [full name; Protein name]	**SNP ID**	**METHOD** [A]
RFC [Reduced folate carrier 1]		
	rs1051266 [Arg^{27}His]	RFLP[Shimasaki et al.,2006][98]
FOLR [Folate receptor 1]		
	rs2071010 [5' UTR]	RT TaqMan assay C_15861044_10
DHFR [Dihydrofolate reductase]		
	rs70991108 [19bp ins/del;Intronic]	RFLP [Johnson *et al*., 2004] [99]
MTHFR [Methylenetetrahydrofolate reductase]		
	rs1801133 [Ala^{222}Val]]	RFLP [Sanyal et al., 2004] [100]
	rs1801131 [Glu^{429}Ala]	RFLP [Sanyal et al., 2004] [100]
MTR [Methionine synthase]		
	rs1805087 [Asp^{919}Gly]	RT TaqMan assay C_12005959_10
MTRR [Methionine synthase reductase]		
	rs1801394 [Ile^{49}Met]	RT TaqMan assay C_3068176_10
	rs1532268 [Ser^{175}Leu]	RT TaqMan assay C_3068164_10
	rs162036 [Lys^{350}Arg]	RT TaqMan assay C_3068152_10
	rs10380 [His^{595}Tyr]	RT TaqMan assay C_7580070_1

SHMT [Serine hydroxymethyltransferase]	
rs1979277 [Leu^{474}Phe]	RT TaqMan assay C_3063127_10
TYMS [Thymidylate synthase]	
rs45445694 [6bp ins/del; enhancer region]	RFLP [Skibola et al, 2002] [101]
rs34489327 [28bp tandem repetition; 3'- near gene]	RFLP [Skibola et al, 2002] [101]

[A] RT = Real-Time PCR with TaqMan allelic discrimination assay [Applera, Foster City, USA];

RFLP = PCR-RFLP and M = Multiplex PCR, analysis carried out according to published methods [reference parenthetically] or as standardized in our laboratory (primer set, T (°C) of annealing and restriction enzymes (Fermentas, Vilnius Lithuania) described].

Results

Characteristics of the study population. The cohort of 60 Caucasian GIST patients (Table 8) was made up of 65% men and 35% women (median age at diagnosis 58.0 years; range 18-83 years). The most common primary sites were the stomach (55.0%) and the small intestine (40.0%); the remaining 5%, the sites of onset were oesophagus (1 case) and rectum (2 cases).

Table 8. Patient and disease characteristics of the studied populations.

	Cases (*n* = 60)	**Controls (*n* = 153)**
Gender, *n* (%)		
Female	21 (35.0%)	61 (39.9%)
Male	39 (65.0%)	92 (60.1%)
Age at diagnosis or selection, years		
median (range)	58.0 (18-83)	47 (21-79)
Tumour site, *n* (%)		
Stomach	33 (55.0%)	
Small Intestine	24 (40.0%)	
Other	3 (5.0%)	
Tumour size, *n* (%)		
≤ 2 cm	1 (1.6%)	
2 - 5 cm	6 (10.0%)	
5 - 10 cm	18 (30.0%)	
≥ 10 cm	22 (36.7%)	
Missing	13 (21.7%)	
Mitotic index [t], *n* (%)		
< 5	13 (21.7%)	
6-10	5 (8.3%)	
≥ 10	17 (28.3%)	
Missing	25 (41.7%)	
Mutational status, *n* (%)		
KIT exon 11	35 (58.3%)	
PDGFRα/*KIT* WT	9 (15.0%)	
Other than *PDGFRα* D842V and *PDGFRα*/*KIT* WT	4 (6.7%)	
Missing	12 (20.0)	

[t] 50 x High power filed.

[§] Numbers for certain variables may not add up to the total number because of missing information.

Twenty-three patients (38.3%) had metastatic disease, with liver and peritoneum as the most common sites of metastases. Thirty-five patients (58.3%) harboured *KIT* exon 11 mutations, while only one patient had a *KIT* exon 9 mutation. Three patients (5.6%) had a *PDGFRα* mutation, excluding the D842V, and nine (15.0%) were *KIT/PDGFRα* WT GISTs. In twelve GISTs, mutational status was unknown, due to insufficient or unavailable biological material for the analysis. Control group (n = 153) was made up of 60% men and 40% women (median age 47 years, range 21-79 years).

Genotypes distribution in the two studied populations. Genotype frequencies of the thirteen polymorphisms were found to be in Hardy-Weinberg equilibrium ($P > 0.05$) in both patients and controls, with the exception of rs1801133 in the MTHFR genes in controls only ($P = 0.016$). MAF and Hardy-Weinberg equilibrium *P* value are presented in Table 9.

Table 9. Allele frequency

	Genotypes distribution *n*				
	wt	HE	SNP	Frequency variant allele	P_{HWE}
RFC – rs1051266					
Cases	21	31	7	$q = 0.381$	0.383
Controls	59	64	29	$q = 0.401$	0.127
FOLR – rs2071010					
Cases	51	6	0	$q = 0.053$	0.675
Controls	131	20	1	$q = 0.072$	0.805
DHFR – rs70991108					
Cases	22	31	6	$q = 0.364$	0.302
Controls	60	69	24	$q = 0.382$	0.576
TS – rs45445694					
Cases	11	33	16	$q = 0.542$	0.404
Controls	38	66	49	$q = 0.536$	0.100
TS – rs34489327					
Cases	25	24	4	$q = 0.301$	0.588
Controls	54	65	34	$q = 0.435$	0.094
SHMT – rs1979277					
Cases	27	30	3	$q = 0.300$	0.140
Controls	85	55	10	$q = 0.250$	0.785
MTHFR - rs1801133					
Cases	22	28	10	$q = 0.400$	0.830
Controls	36	91	26	$q = 0.467$	**0.016**
MTHFR - rs1801131					
Cases	24	28	8	$q = 0.367$	0.976
Controls	84	57	12	$q = 0.264$	0.595
MTR - rs1805087					
Cases	40	18	2	$q = 0.183$	0.988
Controls	101	48	4	$q = 0.183$	0.543
MTRR - rs10380					
Cases	51	9	0	$q = 0.075$	0.530
Controls	121	30	1	$q = 0.105$	0.556
MTRR – rs162036					
Cases	47	11	1	$q = 0.110$	0.706
Controls	112	38	2	$q = 0.138$	0.539
MTRR – rs1801394					
Cases	21	30	9	$q = 0.400$	0.747
Controls	56	64	33	$q = 0.423$	0.075
MTRR - rs1532268					
Cases	26	25	9	$q = 0.358$	0.467
Controls	66	66	21	$q = 0.353$	0.492

Details of the results of association tests for each genetic polymorphism chosen for analysis in our case-control study population are presented in Table 9 and 10. The most significant result was found for the 6bp ins/del in TS gene (rs34489327). In particular the allele with the 6bp deletion was significantly less common in cases compared to controls (30.2% *vs* 43.5%; OR 1.84, 95% CI 1.29-3.01; $P = 0.014$ Tab 9). Genotype analysis also showed a similar significant association ($P = 0.019$)

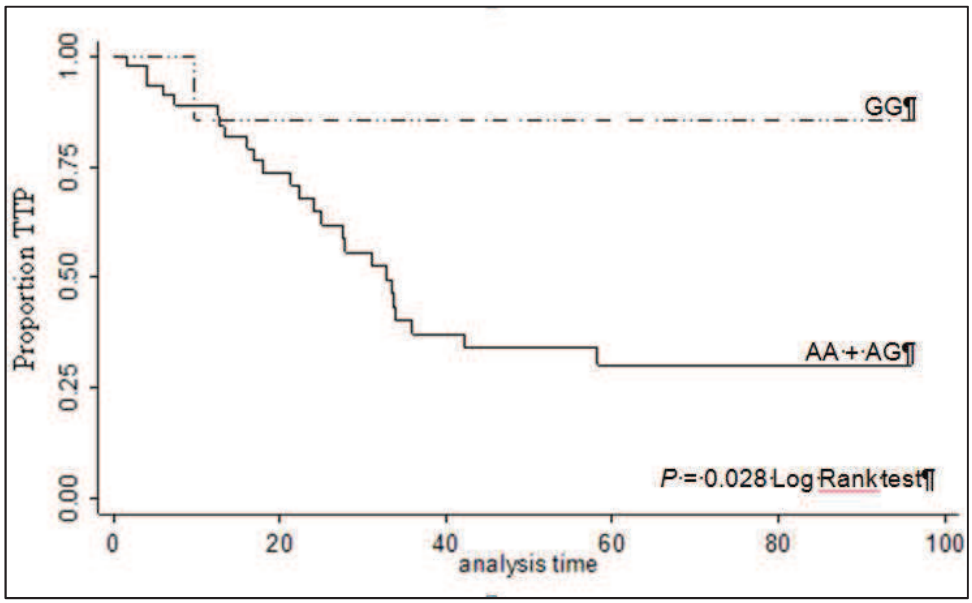
with the 6bp del/del genotype less frequent in cases compared to controls (7.5% *vs* 22.2%). There was no significant difference in genotype distribution or allele frequencies between cases and controls for any of the other polymorphisms tested across the key enzymes that take part in the folate metabolic pathways.

Association between genetic polymorphisms and clinical features at diagnosis. Results of the associations of each genotype and clinical variables are presented in Tables 11 (RFC, FOLR, DHFR, TS, SHMT and MTHFR). Univariate analysis showed an excess of wild-type MTR - rs185087 - genotype in patients aged less than 60 years when compared with those over 60 (84.6% *vs* 51.5; $P = 0.008$), and in patients with mitotic index greater than 10, compared to patients with lower mitotic index (88.2 *vs* 55.6; $P = 0.032$). In addition an excess of wild-type RFC, FOLR and MTHFR (rs1801130) genotypes was observed in patients with tumor size greater than 10 cm, compared to patients with smaller tumor.

With regard to mutational status, we divided the patients in three groups – KIT or PDGFRA mutated and wt GIST. Results of the associations of each genotype and mutational status are included in Tables 11 (RFC, FOLR, DHFR, TS, SHMT and MTHFR). According to this stratification, we observed an excess of wild-type RFC genotype in patients with PDGFRA mutations compared to KIT mutated and wild-type GIST (100% *vs* 37.1% and 11.1% respectively; *P* 0.021). We also observed that the TS 2R2R genotype (rs45445694) was more represented in PDGFRA mutated patients compared to KIT mutated and wild-type GIST (66.7% *vs* 16.7% and 0% respectively; $P = 0.027$). Another finding was an excess of the presence of at least a variant MTRR rs10380 allele in wild-type GIST compared to KIT/PDGFRA mutated patients (33.3% *vs* 2.8% and 0% respectively; $P = 0.011$). The same finding was seen for MTRR rs162036 (44% *vs* 8.6% and 0% respectively; *P* 0.020). A borderline association was also seen for MTHFR (rs1801133) genotype. In particular we observed an excess of the wild-type genotype in PDGFRA-mutated and wild-type GIST compared to KIT-mutated patients (66.7% both *vs* 27.8%; $P = 0.055$).

Association between time to progression and genetic polymorphisms. Fifty-four patients received standard first-line imatinib 400 mg daily, with a median duration of imatinib administration of 36,9 months. Demographic and disease characteristic of the subgroup of GIST has been extensively previously described. Regarding imatinib response, the best results during therapy were partial response and sTable disease in 90.7%. With a median follow-up of 36.9 months, progression of disease was observed in 26 cases (48.1%), with a median TTP of 21.8 (range: 1.6-58.2).

In the univariate analysis, we observed that the presence of the variant allele RFC rs1051266 - in homozigosity is associated, at the limit of statistical significance, with a reduced risk of disease progression (HR 0.144, 95% CI 0.019 – 1.07; $P = 0.059$). The relationship resulted significant after type allele in RFC (AA/AG), was associated with reduced TTP ($P = 0.028$; Figure 16). None of the other analysed SNPs correlated with the progression. Noteworthy, none of the five patients with correction for gender, age, and status at diagnosis (HR 0.107, 95% CI 0.014-0.82; $P = 0.032$). Alleles were correlated with TTP based on the Kaplan-Meier method. Presence of at least one wild-heterozygote FOLR genotype showed progression.



. **Figure 16**. Kaplan-Meier estimated. Presence of at least one wild-type allele in RFC (AA/AG), was associated with reduced TTP.

Table 10. RFC, FOLR, DHFR, TS, SHMT and MTHFR genotype frequencies according to clinical features.

2071010	DHFR rs70991108 19bp Ins/del		TS rs45445694 28bp repeat		TS rs34489327 6bp Ins/del		SHMT rs1979277		MTHFR rs1801130		MTHFR rs1801133	
AG/GG	+/+	+/-_-/-	2R2R	2R3R/3R3R	6 +/+	6 +/-_-/-	CC	CT/TT				
3 (9.7)	10 (31.3)	22 (68.7)	6 (18.2)	27 (81.8)	15 (48.4)	16 (51.6)	16 (48.5)	17 (51.5)	13 (39.4)	20 (60.6)	15 (45.5)	18 (54.5)
3 (11.5	11 (42.3)	15 57.7)	5 (19.2)	21 (80.8)	10 (45.5)	12 (54.5)	11 (40.7)	16 (59.3)	8 (30.8)	18 (69.2)	9 (34.6)	17 (65.4)
82	0.384		0.918		0.695		0.441		0.492		0.400	
1 (4.8)	7 (33.3)	14 (66.7)	3 (14.3)	18 (85.7)	9 (47.4)	10 (52.6)	11 (52.4)	10 (47.6)	5 (23.8)	17 (43.6)	8 (38.1)	13 (61.9)
5 (13.9)	15 (39.5)	23 (60.5)	8 (20.5)	31 (79.5)	16 (47.1)	18 (52.9)	16 (41.0)	23 (59.0)	16 (76.2)	22 (56.4)	16 (41.0)	23 (59.0)
79	0.641		0.552		0.983		0.399		0.129		0.825	
5 (15.6)	15 (45.4)	18 (54.5)	6 (18.2)	27 (81.8)	13 (46.4)	15 (53.6)	18 (54.5)	15 (45.4)	12 (36.4)	21 (63.6)	12 (36.4)	21 (63.6)
0	5 (21.7)	18 (78.3)	5 (20.8)	19 (79.2)	11 (50.0)	11 (50.0)	8 (33.3)	16 (66.7)	9 (37.5)	15 (62.5)	9 (37.5)	15 (62.5)
1 (33.3)	2 (66.6)	1 (33.3)	0	3 (100)	1 (33.3)	2 (66.7)	1 (33.3)	2 (66.7)	1 (33.3)	2 (66.7)	3 (100)	0
77	0.109		0.679		0.858		0.259		0.989		0.093	
5 (21.7)	7 (29.2)	17 (78.8)	3 (12.0)	22 (88.0)	9 (39.1)	14 (60.9)	14 (56.0)	11 (44.0)	5 (20.0)	20 (80.0)	13 (52.0)	12 (48.0)
0	9 (40.9)	13 (59.1)	5 (22.7)	17 (77.3)	10 (58.8)	7 (41.2)	9 (40.9)	13 (59.1)	12 (54.5)	10 (45.5)	6 (27.3)	16 (72.7)
23	0.404		0.329		0.218		0.302		**0.014**		0.085	
4 (23.5)	5 (29.4)	12 (70.6)	2 (11.1)	16 (88.9)	5 (31.2)	11 (68.8)	11 (61.1)	7 (38.9)	4 (22.2)	14 (77.8)	6 (33.3)	12 (66.7)
1 (5.9)	8 (47.1)	9 (52.9)	3 (17.6)	14 (82.3)	7 (50.0)	7 (50.0)	9 (52.9)	8 (47.1)	8 (47.1)	9 (52.9)	6 (35.3)	11 (64.7)
46	0.290		0.581		0.296		0.625		0.122		0.903	

3 (9.1)	17 (48.6)	18 (51.4)	6 (16.7)	30 (83.3)	14 (46.7)	16 (53.3)	16 (44.4)	20 (55.6)	16 (44.4)	20 (55.6)	10 (27.8)	26 (72.2)
0	1 (33.3)	2 (66.7)	2 (66.7)	1 (33.3)	2 (100)	0	2 (66.7)	1 (33.3)	1 (33.3)	2 (66.7)	2 (66.7)	1 (33.3)
0	1 (11.1)	8 (88.9)	0	9 (100)	4 (44.4)	5 (55.6)	3 (33.3)	6 (66.7)	1 (11.1)	8 (88.9)	6 (66.7)	3 (33.3)
57	0.120		**0.027**		0.320		0.593		0.179		0.055	

Table 11. Most relevant results correlation between mutational status and candidate genotypes.

	n (%)									
	RFC rs1051266		**TS rs45445694 28bp repeat**		**MTHFR rs1801133**		**MTRR rs10380**		**MTRR rs162036**	
Mutational status										
KIT	13 (37.1)	22 (62.9)	6 (16.7)	30 (83.3)	10 (27.8)	26 (72.2)	35 (97.2)	1 (2.8)	32 (91.4)	3 (8.6)
PDGFRA	3 (100)	0	2 (66.7)	1 (33.3)	2 (66.7)	1 (33.3)	3 (100)	0	3 (100)	0
wt	1 (11.1)	8 (88.9)	0	9 (100)	6 (66.7)	3 (33.3)	6 (66.7)	3 (33.3)	5 (55.6)	4 (44.4)
P- value	**0.021**		**0.027**		0.055		**0.011**		**0.020**	

Association between overall survival and genetic polymorphisms. The genotype distribution of the thirteen polymorphisms in genes coding for the key enzymes of the folate metabolic pathways and the association with OS are summarized in Table 12. Compared with the homozygote SHMT1 CC genotype, the TT genotype was significantly associated with a hazard of early death in the univariate analysis (HR = 6.53, 95% CI 1.17 – 36.36; $P = 0.032$). After a multivariate adjustment for gender, age, and status at diagnosis only a tendency for statistical significance of early death was obtained (HR = 4.53, 95% CI 0.77 – 26.58; $P = 0.095$). Interestingly, of the five patients with heterozygote FOLR genotype none experienced mortality. None of the other analyzed polymorphisms showed an association with the OS.

Table 12. Univariate and multivariate analyses of the different genotypes and OS in GIST patients.

Genotypes	**Patient** *N*	**Event** *n*	**Crude HR (95% CI)**	***P*** [§]	**Adjusted HR (95% CI)**	***P*** [#§]
RFC rs1051266						
AA	21	7	1.		1.	
AG	28	6	0.52 (0.17 – 1.55)	0.239	0.70 (0.22 – 2.22)	0.548
GG	7	1	0.22 (0.03 – 1.78)	0.155	0.35 (0.04 – 3.24)	0.353
FOLR rs2071010						
AA	48	12	-	-	-	-
AG/GG	5	0	-	-	-	-
DHFR rs70991108						
19 +/+	21	5	1.		1.	
19 +/-	30	6	0.99 (0.30 – 3.26)	0.983	0.94 (0.30 – 3.19)	0.925
19 -/-	5	2	2.01 (0.40 – 10.83)	0.389	1.40 (0.26 – 8.74)	0.718
TS – rs45445694						
2R2R	11	1	1.		1.	
2R3R	30	6	1.48 (0.18 – 12.36)	0.717	1.57 (0.19 – 13.23)	0.677
3R3R	16	7	3.93 (0.48 – 32.01)	0.201	3.63 (0.44 – 30.09)	0.232
TS rs34489327						
6 +/+	24	5	1.		1.	
6 +/-	22	3	0.39 (0.091 – 1.67)	0.205	0.30 (0.065 – 1.40)	
6 -/-	4	2	1.94 (0.94 – 10.25)	0.436	1.38 (0.24 – 8.04)	
SHMT rs1979277						

CC	25	4	1.		1.	
CT	29	8	1.73 (0.52 – 5.75)	0.374	2.01 (0.59 – 6.83)	0.266
TT	3	2	6.53 (1.17 – 36.36)	0.032	4.53 (0.77 – 26.58)	0.095
MTHFR rs1801133						
CC	20	5	1.		1.	
CT	28	8	1.00 (0.33 – 3.08)	0.995	0.99 (0.31 – 3.18)	0.988
TT	9	1	0.41 (0.048 – 3.50)	0.414	0.46 (0.053 – 3.99)	0.479
MTHFR rs1801131						
AA	22	6	1.		1.	
AC	28	7	0.97 (0.33 – 2.90)	0.957	0.93 (0.27 – 3.22)	0.911
CC	7	1	0.56 (0.066 – 4.57)	0.579	0.40 (0.048 – 3.35)	0.398
MTR rs1805087						
AA	37	11	1		1.	
AG	18	3	0.55 (0.15 – 1.97)	0.356	0.55 (0.15 – 2.01)	0.363
AG/GG	20	3	0.53 (0.15 – 1.90)	0.331	0.54 (0.15 – 1.99)	0.355
MTRR rs10380						
CC	49	13	1.		1.	
CT/TT	8	1	0.36 (0.047 – 2.77)	0.328	0.43 (0.056 – 3.34)	0.421
MTRR rs162036						
AA	44	10	1.		1.	
AG/GG	12	3	0.89(0.24 – 3.24)	0.856	1.04 (0.29 – 3.80)	0.953
MTRR rs1801394						
AA	20	6	1.		1.	
AG	29	7	0.83 (0.28 – 2.47)	0.737	0.88 (0.29 – 2.65)	0.814
GG	8	1	0.29 (0.035 – 2.42)	0.252	0.33 (0.037 – 2.90)	0.315
MTRR rs1532268						
AA	20	6	1.		1.	
AG	28	7	0.83 (0.28 – 2.47)	0.737	0.49 (0.13 – 1.86)	0.291
GG	8	1	0.29 (0.035 – 2.42)	0.252	1.45 (0.36 – 5.79)	0.600

Discussion

To the best of our knowledge this is the first study to investigate the association between tumors characteristics and clinical outcomes and potentially functional polymorphisms in genes of the folate pathway in GIST patients. Genetic approaches to define mechanisms of GIST development, with the recognition of KIT/PDGFRA mutation as key player, have delivered meaningful insights in the development of treatment strategies. Nevertheless, imatinib remains the only first-line treatment approved, even in patients for whom we might anticipate a lack or a subsequent failure of efficacy [64]. In addition, the mechanisms of refractoriness or resistance, in addition to the acquisition of secondary mutations in known *KIT* and *PDGFRA* exons, remains still unknown in most patients [68]. We now recognize the enormous scope of genetic variation among humans, which can be used to probe the genetics of treatment response and disease susceptibility. Basically all genes are subject to genetic variability, which can be associated with the altered efficiency of a biological pathway. These genetic variations can be associated with a person's risk for developing cancer as a result of environmental exposures, as well as variability in drug response. Genetic polymorphisms in xenobiotic/drug metabolizer, transporters and targets loci were a natural starting point to study their relevance in susceptibility and treatment efficacy. Besides these, the biological components that may also influence susceptibility and therapies outcome include enzymes that repair DNA damage, factors that regulate cell cycle control, cell division and cell death, and

enzyme involved in the immune response [104]. Many findings support the idea that different mechanisms may be involved in the GIST tumorigenesis processes, as emphasized by the widely different clinical behavior of each GIST patient, regardless of the *KIT/PDGFRA* mutational status. In this regard we thought that polymorphisms in candidate genes related to the folate metabolism may be a good choice for investigating association with clinic-pathological features, response to treatment and survival. Due to the role of these genes in DNA synthesis, DNA repair and DNA methylation, it is plausible that genetic variants of these genes may influence their function, resulting in aberrant methylation or DNA synthesis inhibition. These modifications may be thus involved in the tumor progression and consequently may play a relevant role in the long term outcome of patients. In the present study we investigated the frequencies of selected polymorphisms in patients stratified by age, gender and other main molecular and clinical characteristics, and found that few genotypes may show a likely correlation. However, the most remarkable results are the association with disease risk, TTP and OS. In particular, we found evidence for an association between a polymorphism in the TS gene (rs34489327) and GIST susceptibility. The TS gene product, a folate dependent enzyme, works converting dUMP to dTMP, which is responsible for thymine incorporation during DNA-synthesis [105]. The rs34489327 is a 6bp ins/del in the 3'- UTR region, and together with rs34743033, located in the 5'-UTR region, is the most extensively reported TS variant. These two variants are associated with the occurrence of various tumors, such as colorectal cancer, lymphoma, and acute lymphocytic leukemia [105-107]. The 6bp deletion polymorphism has been associated with a decreased protein expression due to an effect on secondary mRNA structure or mRNA stability [108,109]. Since the polymorphism has been linked to a reduced enzymatic activity, and considering its critical function we would hypothesized that the TS 6bp del may be associated with a higher cancer risk. TS inhibition results in depletion of deoxythymidine triphosphate (dTTP) pools followed by thymine-less death or, in some instances, high levels of uracil mis-incorporation in DNA followed by extensive repair and subsequent double-strand breaks in DNA, that promote chromosomal instability, translocations, and aberrations [110]. Interestingly, the TS 6bp deletion was found more frequently in the healthy controls, indicating a lower risk associated with the variant allele. Given the small population size, yet similar to other studies reported in the literature, due to the rarity of the tumor, we cannot exclude that the association we found is chance. Nevertheless, we should consider that the findings of other studies on cancer risk are controversial, with both positive, negative or null results [110-114]. Different hypothesis has been proposed to explain the observed discrepancies. It might reflect differences in the gene-disease association (i.e. different types of cancer, different association) or may reflect different intakes of micronutrients, folate and B vitamins in particular, in the different

populations [115-117]. Another alternative explanation is that TS requires 5,10-methylenetetrahydrofolate (5,10-MTHF) as a cofactor, and competes with MTHFR for available 5,10-MTHF. The TS-mediated reaction is closely linked to the reaction catalyzed by MTHFR, this implies a narrow interplay between these two enzymes. Therefore, addressing only polymorphisms in one gene may be an oversimplification of the reality and this prompted us to also include genetic polymorphisms in the key enzymes that take part in the folate metabolic pathways. However, none of the other investigated variants were associated with a higher risk of GIST. It should be noted that a better and meaningful approach would have been the gene–gene interactions analysis. Though, this approach is not practicable in our study, as it will necessarily suffer of insufficient statistical power due to the small sample size.

Through our multiple candidate gene approach, we found that polymorphisms in RFC and FOLR genes may be associated with TTP, although only RFC reached statistical significance. To us this finding is intriguing, as both the gene products are involved in the folic acid and reduced folates, as the 5'-Methyltetrahydrofolate (5'-MTHF) cellular uptake and distribution. In particular, RFC has a higher affinity for reduced folates, vice versa FOLR has higher affinity for folic acid [118,119]. With regard to RFC, previous studies have shown that individuals with the A allele, which is found significantly associated with reduced TTP in our study, had reduced plasma levels of folates and homocysteinemia than individuals carrying the G allele [120,121]. Dietary intake of folate has been associated with reduced risk for a number of cancers [122-124]. The proposed mechanisms are suboptimal DNA methylation and DNA repair capacity. Among them, altered cytosine methylation in DNA, referable to folate deficiency, may lead to inappropriate activation of proto-oncogenes and induction of malignant transformation [125]. Therefore, it is plausible that the RFC genotype may be involved in GIST tumorigenesis, by affecting plasma folate and homocysteine levels.

Advances in molecular biology have highlighted that epigenetic modifications may play important roles in tumorigenesis and tumor progression. The different methylation status of several genes has been associated with different tumor phenothypes and clinical behaviors in quite a few cancer [126]. Recently few studies have reported aberrant methylation status in GIST patients, particularly those KIT/PDGFRA wild-type [127,128]. Since methylation status of various genes greatly influences the diagnosis and prognosis of several tumors, it is reasonable to think that genetic polymorphisms in key enzymes of the folate metabolism, may perturb this pathway and have the potential of becoming biomarkers of prognosis. To explore this hypothesis, we investigated the association of the selected genetic polymorphism with OS. Among all, only the SHMT variant resulted associated with OS. SHMT is a vitamin B6-dependent SHMT1 enzyme that catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and 5,10-MTHF in the cytoplasm for

the synthesis of methionine, thymidylate, and purines. The C variant, associated to reduced OS in our population, has been associated to reduced folate levels [129,130], thus the polymorphism could mimic a situation of folate deficiency by limiting the availability of one-carbon units for both remethylation of homocysteine, important for DNA methylation, and DNA synthesis. The importance of folate status is supported by the evidence that none of the patients with heterozygous FOLR genotypes showed progression or experienced mortality. However, it must be stressed that overall survival may be affected by multiple variables, and the potential prognostic role of the folate-genotype should be verified in a larger sample size stratified according to the molecular and clinical features and the medical treatment received.

Conclusion (II)

To conclude, we report significant association between genetic polymorphisms in key enzymes of the folate metabolic pathways and GIST tumorigenesis, clinical features and outcome. Our finding should be considered in the context of both the strengths – the investigation of a large number of polymorphisms across genes with well-defined roles in the folate pathway and the robust genotyping protocols - and limitations – the small sample size - and should be viewed as exploratory. On the other hand, the rarity of GIST requires that promising genetic polymorphisms, such as those reported in the present study are subjected to further investigation. In particular, these results need to be further confirmed in larger independent studies, which will allow genome-wide association studies - only feasible when large cohort of patients is available – opening up the opportunity to identify new loci associated with GIST susceptibility and/or clinical outcome, or to definitively confirm the role of candidate genetic variations. Furthermore, the enrichment of the study by having available serum folate levels or dietary intake, associated with the opportunity of gene-gene and gene-environment interaction analysis, may lead to a better understanding of GIST pathogenesis, clinical manifestation and disease course.

Second Objective

Tyrosin-kinase Inhibitor resistance mechanisms in GISTs

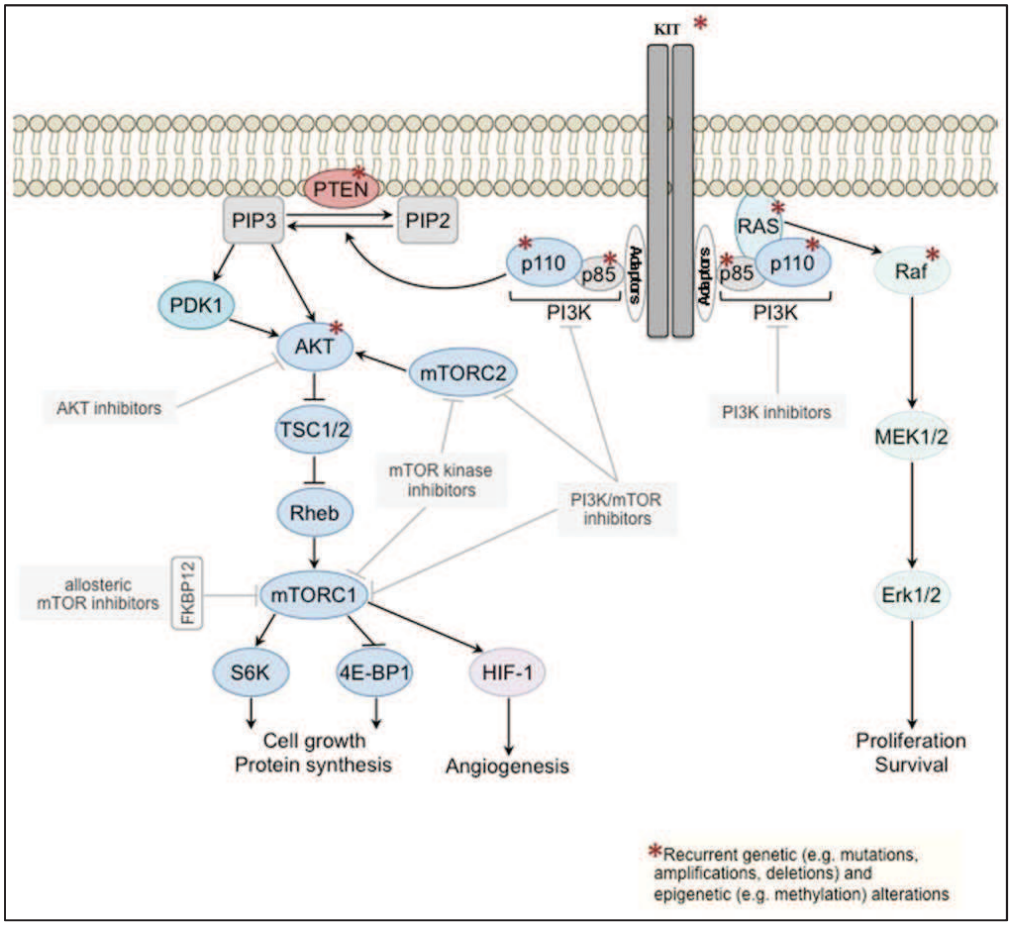


Figure 17. Main key players in KIT downstream signaling pathway.

Figure 17 highlights the main players in the KIT downstream players that will be widely discuss below.

Clinical progression of GIST, during TKI therapy, is often multifocal. However, TKI resistance mutations have been assessed in only single, or few, progressing metastases per patient, so that the heterogeneity of these mutations, in a given patient, remains unclear [131].

We screened up to 46 progressing GIST metastases per patient to evaluate TKI resistance mechanisms, part of a more extensive project aimed to characterize the secondary resistance by means of high-throughput screens.

Materials and methods

Patients - We analyzed a total of 14 GISTs patients (9 males, 5 females, age range 32-73, median age 55). All patients progressed clinically on imatinib or sunitinib, according to the conventional Southwest Oncology Group/Response Evaluation Criteria in Solid Tumours. All the patients underwent resection during 2002–20012 at the Brigham and Women's Hospital, Boston, MA, USA. Imatinib or sunitinib was discontinued within 1 week prior to debulking surgeries. This study was approved by the Institutional Review Board of Brigham and Women's Hospital.

Haematoxylin and eosin-stained sections from paraffin blocks were reviewed by pathologists to confirm the diagnoses prior to inclusion in the study. Tumour regions from different metastases or different areas within metastases were selected from each patient, with an emphasis on variation in tumour cytology, KIT expression (KIT-positive or KIT-negative) and mitotic activity. all frozen tumor specimens were analyzed histologically, and shown to be composed of > 90% neoplastic cells.

The morphological appearance (spindle cell, epithelioid cell, mixed cell type, unusual morphology), tumour size, location, treatment effects (necrosis, hyalinosis, pseudo-chondroid changes, haemorrhage) were evaluated, as well as mitotic rate [expressed as the number of mitotic Figures per 50 high power fields (HPFs) in the most mitotic area, using a × 40 objective and a × 10 ocular, field size 0.25 mm^2].

For every patient we had from a minimum of 3 different metastases samples to up to 52 (Table 14).

DNA samples. Genomic DNA was isolated, from frozen specimens, using QIAmp kit from QIagen (TurnberryLane Valencia CA, USA). All known precautions were observed during the DNA isolations to prevent cross-contamination between different samples. The extracted DNA was quantified using NanoDrop spectrophotometer (Thermo Scientific, Fisher Scientific, Pittsburgh, PA, USA). mutational analysis was performed on the extracted genomic DNA, using polymerase chain reaction with specific primer sets for *KIT* exons 9, 11, 13, 14, 15, 16, 17, 18 (Table 13). DNA were amplified in 20-µl PCR reactions for 2 minutes at 94°C, followed by 30 seconds at 94°C, 30 seconds minute at 60°C, and 1 minute at 72°C for 35 cycles, with a final step of 7 minutes at 72°C. PCR products were evaluated by ethidium bromide staining on a 1% agarose gel alongside 1 Kb Plus DNA Ladder (Invitrogen, Grand Island, NY, USA). The PCR products were purified using QIAquick PCR Purification Kit (QIagen), and Sanger sequenced.

Table 13. Primer sequences used for *KIT* mutational analysis.

Exon	Forward	Reverse
9	ATTTATTTTCCTAGAGTAAGCCAGGG	ATCATGACTGATATGGTAGACAGAGC
11	CCAGAGTGCTCTAATGACTG	CACAGAAAACTCATTGTTTCAGGTGG
13	ATTTTGAAACTGCACAAATGGTCCTT	GCAAGAGAGAACAACAGTCTGGGTAA
14	GTAGCTCAGCTGGACTGATA	AATCCTCACTCCAGGTCAGT
16	GATCTGCCTGCAAGTTCACA	GGCTCTAAAATGCTCTGTTCTCA
17	GCGTACTTTTGATTTTTATTTTTGGTG	AAATGTGTGATATCCCTAGACAGGATTT
18	CATTTCAGCAACAGCAGCAT	CAAGGAAGCAGGACACCAAT

Novel KIT mutations constructs. To evaluate if the secondary mutations were involved in the resistance we made KIT mutation constructs. Oligonucleotides representing various mutations were designed by the Stratagene online program and synthesized by Invitrogen. Wild type *KIT* or exon 9 mutant *KIT* (A502_Y503dup) or exon 11 mutant KIT (V560D) was used as the template DNA for site-directed mutagenesis reactions utilizing the mutant oligonucleotides and the QuikChange mutagenesis kit (Stratagene, Agilent Technology, Santa Clara, CA, USA, Figure 18). Successful mutagenesis was confirmed by sequence analysis of the whole coding sequence of *KIT*.

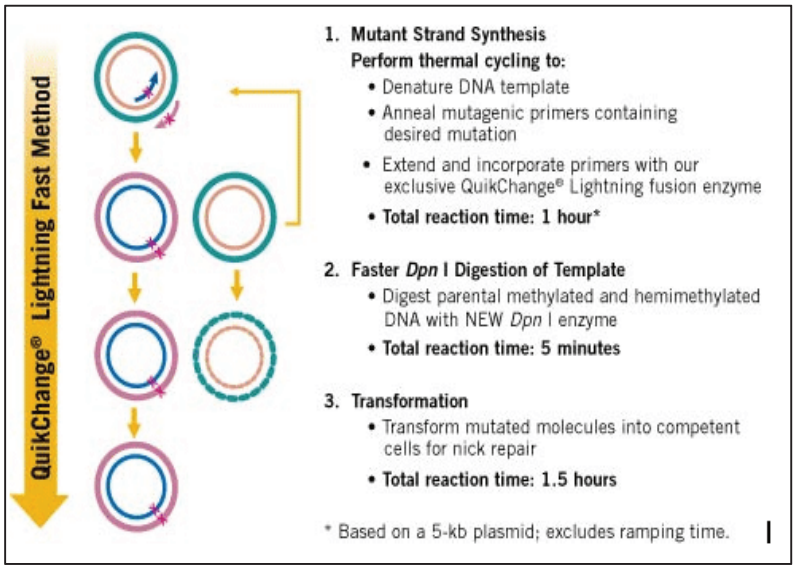


Figure 18. Overview of the site-directed mutagenesis method.

The plamid chosen for the experiments was pCDNA 3.1 (Figure 19)

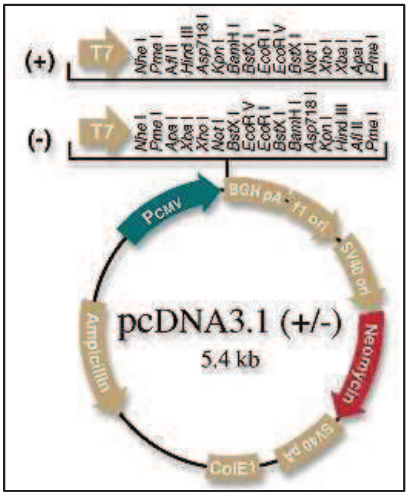


Figure 19. pcDNA plasmid.

*Functional experiments***.** Novel *KIT* mutations identified by sequencing were biochemically profiled using constructs prepared by site-directed mutagenesis. KIT autophosphorylation status was assessed by Western immunoblotting as described below. GIST48B cells, a cell line that has KIT gene but does not express KIT protein, were transiently transfected with mutated KIT constructs using the Lipofectamine Plus kit from Invitrogen. 24 hours after transfection, cells were exposed to different concentrations of imatinib, sunitinib (0.1, 0.5, 1 and 5 μmol\L). Whole-cell lysates were immunostained for p-KIT(Y721) and total KIT; p-KIT expression is inversely proportion to the drug cells response: the cells are responding to the treatment, have a lower expression of p-KIT.

Western blotting. Frozen tumor samples were diced in ice-cold lysis buffer containing protease inhibitors (10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1mM phenylmethylsulfonyl fluoride), homogenized using a Tissue Tearor (Biospec Products, USA), and immunoblotted. Uniform protein transfer was demonstrated by Ponceau S staining (Sigma Chemical), and immunostains were performed for total KIT (Dako, #A4502, 1:500, rabbit) and phospoKIT (Cell Signaling, # 3391, 1:500, rabbit); in the second part of the study, immunostains were performed, besides for KIT and phospo KIT, also for total MAPK (Cell Signaling, # 9102 1:1000, rabbit) for phospo-MAPK (Cell Signaling, # 9101, 1:1000, rabbit) for AKT (Cell Signaling, # 9272, 1:500, rabbit,) for phospo-AKT (Cell Signaling, # 9271, 1:500, rabbit); beta-actin (Sigma, #A4700, 1:500, mouse) was used as positive control. Detection was by chemiluminescence (ECL, Amersham Pharmacia Biotechnology), captured using a FUJI LAS1000-plus chemiluminescence imaging system.

Clinically progressing *KIT*-mutant GISTs were from pts formerly responding to imatinib and/or sunitinib. *KIT* exons 8 through 18 were sequenced at 2000-fold coverage (454 pyrosequencing). As a part of this more extensive study, my work was focused on the confirmation and extension of these analyses, through Sanger sequencing, to additional metastases from the same patient. Drug-response studies were performed by expressing mutant constructs in a KIT-negative GIST.

tastases #	Location	Morphology	Mitoses /10HPF	Primary mutation	Secondary mutation
1	abd wall	Spindle	11:10	A502_Y503dup	Q575_P577delinsH
2a	periumbilicus	Spindle	9:10	A502_Y503dup	S840N
2b	periumbilicus	Spindle	12:10	A502_Y503dup	S840N
3a	periabd wall	Spindle	7:10	A502_Y503dup	None
3b	periabd wall	Spindle	12:10	A502_Y503dup	None
4	periabd wall	Spindle	12:10	A502_Y503dup	S840N
5	small bowel	Mixed	4:10	A502_Y503dup	I571_D572delinsT
6a	omentum	Spindle cells	8:10	A502_Y503dup	S840N
6b	omentum	Spindle cells	6:10	A502_Y503dup	S840N
7a	omentum	Spindle cells	12:10	A502_Y503dup	S840N
7b	omentum	Spindle	4:10	A502_Y503dup	S840N
7c	omentum	Epithelioid	14:10	A502_Y503dup	I571_D572delinsT
7d	omentum	Epithelioid	31:10	A502_Y503dup	N822K
7e	omentum	Short spindle	16:10	A502_Y503dup	S840N
7f	omentum	Mixed	17:10	A502_Y503dup	N822K
7g	omentum	Spindle	19:10	A502_Y503dup	S840N
7h	omentum	Mixed	28:10	A502_Y503dup	N822K
8	abd wall	Spindle	15:10	A502_Y503dup	N822K
9a	colon, spleen, upper quadrant	Spindle	10:10	A502_Y503dup	N822K
9b	colon, spleen, upper quadrant	Spindle	20:10	A502_Y503dup	N822K
9c	colon, spleen, upper quadrant	Spindle	4:10	A502_Y503dup	S840N
9d	colon, spleen, upper quadrant	Mixed	25:10	A502_Y503dup	N822K
9e	colon, spleen, upper quadrant	Spindle	10:10	A502_Y503dup	S840N
9f	colon, spleen, upper quadrant	Spindle	28:10	A502_Y503dup	N822K
9g	colon, spleen, upper quadrant	Spindle	8:10	A502_Y503dup	S840N
9h	colon, spleen, upper quadrant	Spindle	14:10	A502_Y503dup	S840N
9i	colon, spleen, upper quadrant	Mixed	25:10	A502_Y503dup	N822K
9l	colon, spleen, upper quadrant	Spindle	16:10	A502_Y503dup	N822K
9m	colon, spleen, upper quadrant	Spindle	18:10	A502_Y503dup	S840N
9n	colon, spleen, upper quadrant	Spindle	18:10	A502_Y503dup	N822K
10	LUQ	N/A	N/A	A502_Y503dup	N822K
11a	colon	Mixed	10:10	A502_Y503dup	N655S
11b	colon	Spindle	10:10	A502_Y503dup	N822K
11c	colon	Mixed	20:10	A502_Y503dup	N822K

12	lateral segment liver	Spindle	2:10	A502_Y503dup	N822K
12	peritoneum	Spindle	10:10	A502_Y503dup	F681L
14	small bowel	Short spindle	54:10	A502_Y503dup	None
15a	Bladder	Spindle	1:10	A502_Y503dup	None
15b	Bladder	Mixed	8:10	A502_Y503dup	None
15c	Bladder	Spindle	14:10	A502_Y503dup	None
16a	Bladder	Spindle	5:10	A502_Y503dup	N680K
16b	Bladder	Spindle	8:10	A502_Y503dup	N680K
16c	Bladder	Spindle	22:10	A502_Y503dup	None
17	pelvic	Spindle	16:10	A502_Y503dup	None
18	pelvic	Short spindle	1:10	A502_Y503dup	None
19	pelvic	Short spindle	5:10	A502_Y503dup	None
20	small bowel	Spindle	11:10	A502_Y503dup	S840N
21a	Abd wall	Epithelioid	1:10	A502_Y503dup	I571_D572delinsT
21b	Abd wall	N/A	N/A	A502_Y503dup	N822K
21c	Abd wall	Epithelioid	14:10	A502_Y503dup	N822K
21d	Abd wall	N/A	N/A	A502_Y503dup	N822K
21e	Abd wall	N/A	N/A	A502_Y503dup	N822K
(NL)	Spleen			wt	
1a	lower quad	Mixed	13:10	A502_Y503dup	D816H
1b	lower quad	Mixed	15:10	A502_Y503dup	None
2	left lower quad preperitoneal fat	Mixed	20:10	A502_Y503dup	None
3a	left lower quad preperitoneal fat	Mixed	05:10	A502_Y503dup	None
3b	left lower quad preperitoneal far	N/A	N/A	A502_Y503dup	None
4a	omentum	Mixed	00:05	A502_Y503dup	None
4b	omentum	Mixed	16:10	A502_Y503dup	N680K
4c	omentum	Mixed	14:10	A502_Y503dup	None
4d	omentum	Mixed	00:02	A502_Y503dup	None
4e	omentum	Mixed	03:05	A502_Y503dup	None
4f	omentum	Spindle	01:10	A502_Y503dup	None
4g	Omentum	Mixed	17:10	A502_Y503dup	Y646S
4h	omentum	N/A	N/A	A502_Y503dup	
4i	omentum	Mixed	00:03	A502_Y503dup	
5a	omentum	Mixed	03:05	A502_Y503dup	
5b	omentum	Mixed	07:10	A502_Y503dup	
6a	transverse colon nodule	Mixed	16:10	A502_Y503dup	None

6b	transverse colon nodule	Mixed	15:10	A502_Y503dup	None
7a	proximal small bowel mesnetery	Mixed	26:10	A502_Y503dup	None
7b	proximal small bowel mesnetery	Mixed	10:10	A502_Y503dup	D820E
8	small bowel mesentery	Mixed	16:10	A502_Y503dup	None
9	small bowel mesentery	Mixed	07:10	A502_Y503dup	None
10	right colic gutter	Mixed	26:10	A502_Y503dup	N655S
11a	right colic gutter	N/A	N/A	A502_Y503dup	
11b	right colic gutter	Mixed	05:10	A502_Y503dup	N822H
12a	ileum	Mixed	03:10	A502_Y503dup	D820G
12b	ileum	Mixed	09:10	A502_Y503dup	
12c	ileum	Mixed	10:10	A502_Y503dup	None
13	small bowel mesntery nodule #4	Mixed	10:10	A502_Y503dup	N822H
14	right colon mesenteric nodule	Mixed	02:04	A502_Y503dup	D820A
15	left colic gutter	Mixed	11:10	A502_Y503dup	D820H
16a	pelvic deposits	Mixed	08:10	A502_Y503dup	
16b	pelvic deposits	Mixed	09:10	A502_Y503dup	
17	rectosigmoid nodule	Mixed	09:10	A502_Y503dup	N822K
(NL)	ileum				
1	left pelvis sigmoid epiploca	Spindle	01:10	Y553_Q556del	V654A
2	right pelvic sidewall lesion	mixed	17:10	Y553_Q556del	L783V
3a	right pelvic side wall peritoneum	mixed	01:10		V654A
3b	right pelvic side wall peritoneum	Spindle	02:10		
4a	right pelvic side wall nodules	Spindle	00:10		
4b	right pelvic side wall nodules	Spindle	00:10		
4c	right pelvic side wall nodules	mixed	00:10	Y553_Q556del	none
4d	right pelvic side wall nodules	mixed	00:10		
5a	colon serosal nodules	Spindle	01:10		
5b	colon serosal nodules	mixed	05:10	Y553_Q556del	
6a	left pelvic side wall nodules	Spindle	00:10		
6b	left pelvic side wall nodules	mixed	00:10		
6c	left pelvic side wall nodules	mixed	00:10	Y553_Q556del	None
6d	left pelvic side wall nodules	Spindle	00:10		
6e	left pelvic side wall nodules	mixed	00:10		
7a	small bowel mesenteric nodule	Spindle	00:10	Y553_Q556del	None
7b	small bowel mesenteric nodule	mixed	00:10		
7c	small bowel mesenteric nodule	N/A	00:10		
8	appendix	mixed	N/A		

9	cecal nodule	mixed	02:10		
10	mesial colon nodules	Spindle	02:10		
11	peritoneal nodules	mixed	00:10		
12a	perihepatic nodules	Spindle	00:10	Y553_Q556del	V654A
12b	perihepatic nodules	mixed	00:10		
12c	perihepatic nodules	Spindle	00:10	Y553_Q556del	None
12d	perihepatic nodules	N/A	00:10		
3 (NL)	small bowel			wt	
1a	Omentum	mixed	09:10		
1b	Omentum	mixed	16:10		
1c	Omentum	mixed	09:10	V559_E561del	D820Y
1d	Omentum	mixed	21:10	V559_E561del	D820Y
1e	Omentum	mixed	35:10	V559_E561del	D820Y
1f	Omentum	mixed	7:10		
1g	Omentum	N/A	0:10		
1h	Omentum	mixed	14:10		D820Y
1i	Omentum	mixed	8:10		N822Y
2	perivesicle fat	N/A	0:10		
3°	sigmoid mass	mixed	21:10		A829P
3b	sigmoid mass	mixed	17:10	V559_E561del	A829P
4	Omentum	N/A	N/A		
5	Omentum	N/A	0:10		
6	transverse colon	mixed	0:10		
7	prox small bowel mesentery	Epithelioid	0:10		None
8	prox small bowel serosa	mixed	0:10		
9a	mid small bowel mesenter	mixed	0:10		
9b	mid small bowel mesenter	N/A	0:10		
9c	mid small bowel mesenter	mixed	0:10		
9d	mid small bowel mesenter	N/A	0:10		
9e	mid small bowel mesenter	N/A	0:10		
9f	mid small bowel mesenter	mixed	0:10		
9g	mid small bowel mesenter	mixed	0:10		
9h	mid small bowel mesenter	N/A	0:10		
9i	mid small bowel mesenter	mixed	0:10	wt	
9l	mid small bowel mesenter	N/A	0:10		
9m	mid small bowel mesenter	mixed	4:10		D820Y
9n	mid small bowel mesenter	N/A	0:10		

9o	mid small bowel mesenter	N/A	0:10		
10	distal small bowel	mixed	0:10	V559_E561del	None
11	upper rectal mass	mixed	5:10		D820G
12	lower rectal mass	mixed	6:10	V559_E561del	N822K
13	addition/al sigmoid nodule	N/A	0:10		
14	left colic gutter nodule	N/A	0:10		
15a	sigmoid mesentery	N/A	0:10		
15b	sigmoid mesentery	N/A	0:10		
15c	sigmoid mesentery	N/A	0:10		
15d	sigmoid mesentery	mixed	0:10		
15e	sigmoid mesentery	mixed	0:10		
15f	sigmoid mesentery	mixed	0:10		
15g	spleen	N/A	0:10	wt	
6(NL)					
1	Adjacent to gallbladder	mixed	05:10	A502_Y503dup	
2	Deep pelvis, near bladder	mixed	01:10	A502_Y503dup	
3	Small bowel serosa	mixed	00:10	A502_Y503dup	
4	Small bowel mesentery	mixed	00:10	A502_Y503dup	
5	Small bowel mesentery	mixed	00:10	A502_Y503dup	
6a	Small bowel mesentery	mixed	03:10	A502_Y503dup	D820H
6b	Small bowel mesentery	mixed	00:10	A502_Y503dup	
7	Small bowel mesentery	mixed	00:10	A502_Y503dup	
8	Small bowel mesentery	mixed	00:10	A502_Y503dup	
9	Small bowel mesentery	Epithelioid	00:10	A502_Y503dup	
10	Small bowel mesentery	Epithelioid	00:10	A502_Y503dup	
11	Omentum	Epithelioid	00:10	A502_Y503dup	
2(NL)	Appendix			wt	
1	Anterior peritoneal	Spindle	03:10	Delition in exon 11	None
2a	Left diaphragmatic mass	mixed	06:10	Delition in exon 11	Y823D
2b	Left diaphragmatic mass	Spindle	04:10	Delition in exon 11	Y823D
3	LUQ post (splenic flexure)	Spindle	01:10	Delition in exon 11	V654A
1	left lower quadrant, abd wall	Epithelioid	03:10	K642E	V654A
2a	small bowel	mixed	07:10	K642E	V654A
2b	small bowel	mixed	05:10	K642E	V654A
3a	midline abdomin/al wound	N/A	N/A		
3b	midline abdomin/al wound	N/A	N/A		

4 (NL)	left upper quadrant small bowel	N/A	N/A		
1	right lower quadrant	Epithelioid	00:10		
2a	right tumor nodule	Spindled	00:10		
2b	right tumor nodule	Spindled	00:10		
3a	mesentery	mixed	05:10	Delition in exon 11	V654A
3b	mesentery	mixed	06:10		
3c	mesentery	mixed	05:10		
4	mesenteric nodule	Spindled	00:10		
5	mesenteric nodule	Spindled	00:10		
6	sigmoid nodules	Spindled	00:10		
6b	sigmoid nodules	Spindled	00:10		
6c	sigmoid nodules	Spindled	00:10		
7	right uterine nodule	leiomyoma	n/a		
8 9NL	left pelvic side wall omentum	Spindled	00:10		
1a	mesentery	spindled	11:10		None
1b	mesentery	N/A	N/A	W557R	None
1c	mesentery	N/A	N/A	W557R	None
1d	mesentery	N/A	N/A	W557R	None
2	mesentery	spindled	00:10		
3a	omentum	spindled	00:10		
3b	omentum	spindled	00:10		
3c	omentum	spindled	00:10		
3d	omentum	spindled	00:10		
4	mesentery	adipe	n/a		
5a	Serosa	spindled	04:10		None
5b	Serosa	spindled	00:10		None
6	mesentery	spindled	00:10		
7	uterus	spindled	00:10		
8a	Mesentery	spindled	00:10		None
8b	Mesentery	spindled	00:10		
(NL)	skeletal muscle	N/A	N/A		
b(NL)	omentum	N/A	N/A		
c(NL)	small bowel	N/A	N/A	wt	
1	Pelvic side wall	spindled	00:10	V559_Q575del	None
2	Liver	N/A	N/A	V559_Q575del	V654A

3 (NL)	Liver dome Liver	spindled	00:10	V559_Q575del None	
1 2 (NL)	Retroperitoneal spleen hilum spleen	spindled spindled	20:10 00:10	W557R W557R wt	V654A
1a	stomach/LUQ	spindled	30:10		A829P
1b	stomach/LUQ	spindled	48:10		
1c	stomach/LUQ	spindled	30:10		
1d	stomach/LUQ	spindled	26:10		
2a	Ligament of trietz	spindled	<1:10	V559_E561del	None
2b	Ligament of trietz	spindled	<1:10		
3	left mesocolon	spindled	00:10		
4a	right lower quadrant	spindled	50:10		A829P
4b	right lower quadrant	spindled	40:10		
5a	left rectus pelvis	spindled	42:10	V559_E561del	A829P
5b	left rectus pelvis	spindled	40:10		
6a	perivesical mass #1	spindled	32:10		
6b	perivesical mass #1	spindled	57:10		A829P
7a	mesorectal mass #1	spindled	30:10		A829P
7b	mesorectal mass #1	spindled	27:10		
8a	pelvic mass	spindled	40:10		A829P
8b	pelvic mass	spindled	13:10		A829P
9	sigmoid nodule	spindled	0:10	V559_E561del	None
10	sigmoid mesentery nodule	spindled	14:10		
11a	right perirectal tumor	spindled	0:10	V559_E561del	None
11b	right perirectal tumor	spindled	40:10		A829P
12a	left pelvic sidewall nodule	spindled	0:10		
12b	left pelvic sidewall nodule	spindled	12:10		
13	rectosigmoid serosal nodule	spindled	56:10		
14a	right external iliac nodule	spindled	14:10		V654A
14b	right external iliac nodule	spindled	2:10		V654
15a	deep rectal nodule	spindled	20:10		
15b	deep rectal nodule	spindled	0:10		
16	right perivesical fat mass	spindled	0:10		
17	right perivesical fat mass	spindled	20:10		
18	left perivesical mass	spindled	12:10		A829P

19 0 (NL)	ileocecal nodule gastric mucosa	spindled	0:10	 wt	
1a 1b 1c 1d (NL)	mesentery mesentery mesentery mesentery small intestine	spindled spindled spindled spindled	57:10 68:10 65:10 50:10	N564_Y578del wt	None
1a 1b 1c 2 3 (NL)	1a midline pelvic 1b midline pelvic 1c midline pelvic 2 small bowel mesenteric 3 (NL) Gallbladder	spindled spindled spindled spindled	0:10 4:10 0:10 1:10	A502_Y503dup A502_Y503dup A502_Y503dup A502_Y503dup wt	None None None None

Heterogeneity of kinase inhibitor resistance mechanisms within and between different GIST metastases in a given patient

Primary *KIT* mutations were found in all the samples analyzed but not in the normal tissues from the same patients. In all the tumor samples corresponding to single patient, the same primary mutation was detected.

These secondary mutations were clustered mainly in three regions of the KIT oncoprotein: the juxtamembrane domain (encoded by exon 11), the TKD 1 (tyrosin kinase domain 1, encoded by exons 13 and 14) and the TKD 2 (tyrosin kinase domain 2, encoded by exons 17 and 18). Nucleotide changes impacted three residues, D816, D820 and N822, and led to 2-4 alternative amino acid substitutions each - D816H/G, D820E/G/Y/A and N822K/Y/H. 2 small del/ins outside the kinase domain were detected: a 6-nucleotide deletion in exon 11 resulting in Q575_P57delinsH, and a 3-nucleotide deletion combined with a nucleotide substitution resulting in I571_D572delinsT. In total, 6 of the secondary *KIT* mutations detected were novel mutations (I571_D572delinsT, Q575_P577delinsH, N655S, N680K, F681L and S840N).

Up to 7 TKI resistance mutations in different progressing metastases from a typical multifocal progressing patient

In patient # 1, 46 metastases (52 samples) had an insertion of 6-bp in *KIT* exon 9 that resulted in a tandem repeat of AY502_503 in the extracellular juxtamembrane region, the most common exon 9 mutation described in GIST. 7 different predominant secondary *KIT* mutations - I571_D572delinsT, Q575_P577delinsH, N655S, N680K, F681L, N822K and S840N - were identified at the genomic level. In GIST exon 11 and an exon 9 mutations appear to be mutually exclusive.

Among the 52 GISTs from this patient, the cellular morphology ranged from typical spindle cell to epithelioid; spindle cell were characterized by eosinophilic fibrillary cytoplasm. GISTs with epithelioid morphology were composed of round cells with eosinophilic to clear cytoplasm, arranged in sheets and nests. Express and activation of KIT was evaluated in frozen metastatic GIST lesions from the same patient, showing consistent expression level of KIT. These results illustrate the remarkable heterogeneity of progressing GISTs lesion from a single, multifocal progressing patient.

Functional studies

To better understand whether these novel mutations could be involved in the mechanism of acquired resistance, secondary mutations of interest were engineered into *KIT* constructs, on their

own and in combination with the primary mutation. Transient transfection experiments in HEK293T cells and in GIST48B (that usually do not express KIT , except with a construct) with the variant *KIT* constructs were performed. The inhibition of KIT phosphorylation was examined using different concentrations of imatinib or sunitinib. Phosphorylated KIT corresponds to activated KIT

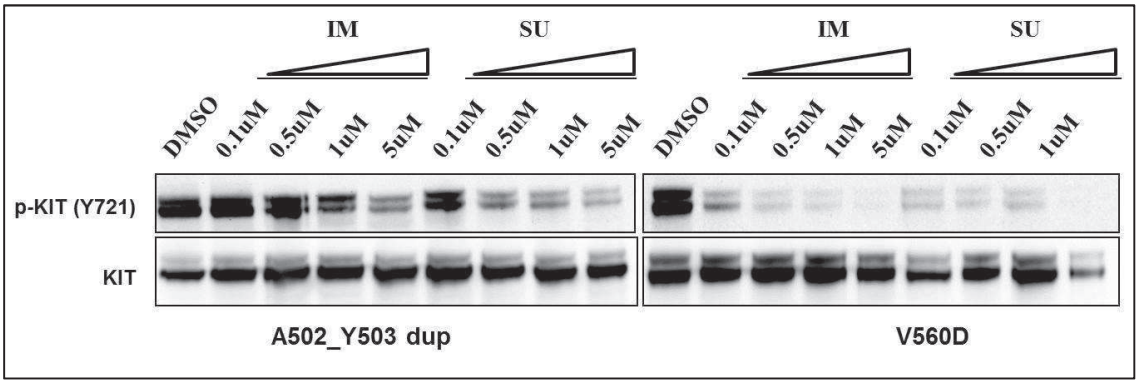


Figure 20. Immunoblot analysis of HEK293 cells transfected with mutant *KIT* constructs with primary mutation (A502_Y503duplication or V560D) and exposed to various doses of imatinib and sunitinib.

tyrosine kinase. In our experimental system, a representative *KIT* exon 11 mutation (V560D) construct, sensitive to both imatinib and sunitinib, served as negative control (Figure 20) while the N822K mutation, known to confer resistance to imatinib and sunitinib, was used as a positive control.

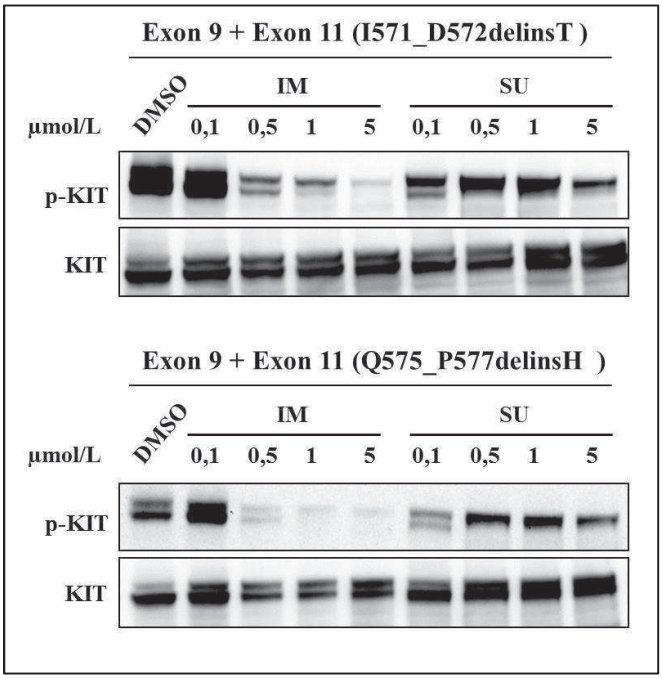


Figure 21. I571_D572delinsT and Q575_P577delinsH

The primary *KIT* A502_Y503dup mutation alone was resistant to imatinib and sensitive to sunitinib (Figure 20). I571_D572delinsT, Q575_P577delinsH, N655S, N680K, F681L and S840N resulted in sunitinib resistance, either in isolation or when co-expressed with *KIT* A502_Y503dup (Figure 21). The secondary *KIT* mutants tested showed resistance to sunitinib *in vitro,* providing strong evidence that each of these mutations plays a causal role in the clinical resistance to sunitinib.

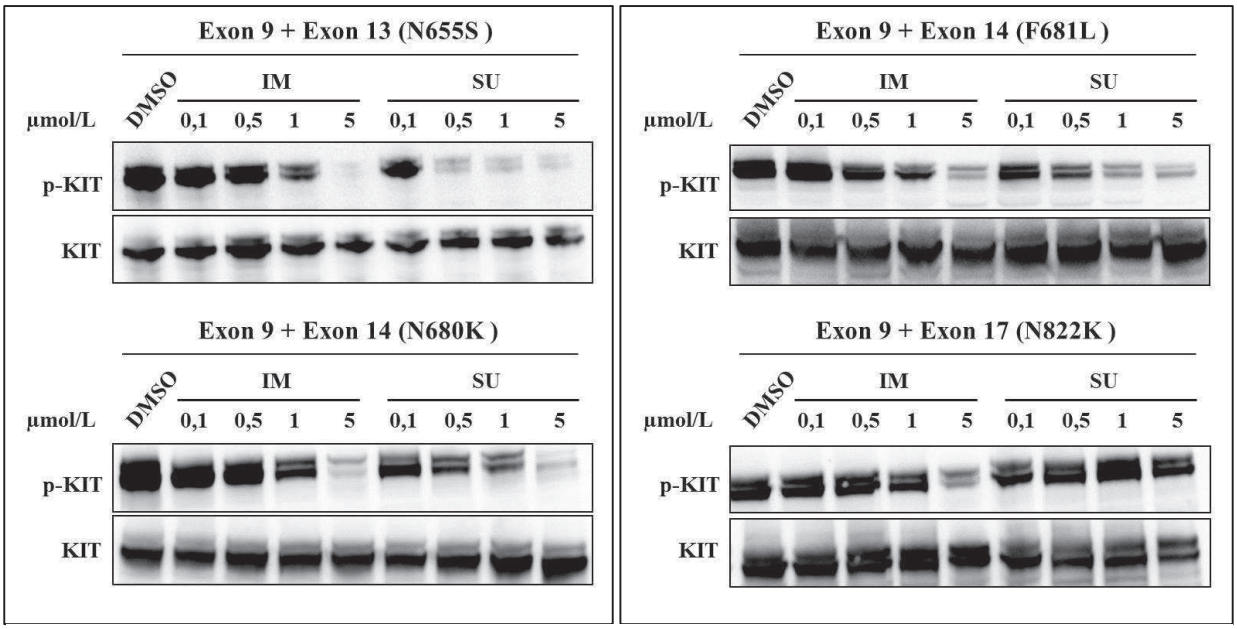


Figure 22. Immunoblot analysis of HEK293 cells transfected with mutant *KIT* constructs and exposed to various doses of imatinib and sunitinib.

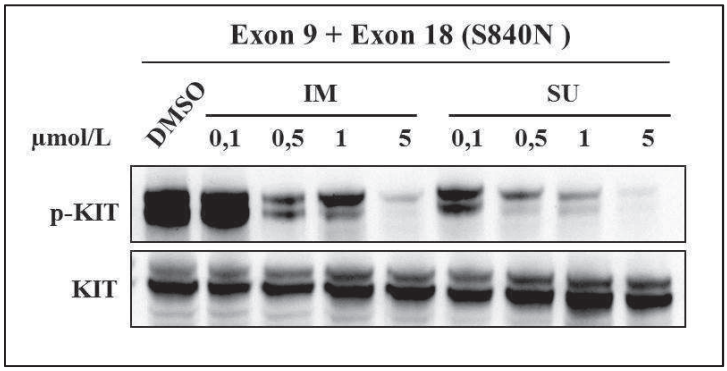


Figure 23 Immunoblot analysis of HEK293 cells transfected with mutant *KIT* constructs with S840N and exposed to various doses of imatinib, sunitinib.

The isolated *KIT* A502_Y503dup mutation was resistant to imatinib and sensitive to sunitinib (Figure 20), in keeping with the role of *KIT* exon 9 mutations in imatinib resistance. Interestingly, secondary sunitinib-resistant *KIT* mutations - I571_D572delinsT and Q575_P577delinsH - result in imatinib response when co-expressed with *KIT* A502_Y503dup (Figure 21).

These studies provide a rigorous evaluation of known and novel TKI genomic resistance mechanisms in GIST. We show that the molecular drug-resistance mechanisms can be defined in 94% of progressing GISTs after imatinib and/or sunitinib therapies. Novel TKI resistance mutations in the juxtamembrane region, TKD 1, and TKD 2 of KIT have been identified. We demonstrates the heterogeneity of molecular drug-resistant mechanisms, between different clinically-progressing metastases, in each patient with clinically progressing GIST after initial response to imatinib and/or sunitinib.

The juxtamembrane region *KIT* mutation (exon 11) is the most common mutation type in GIST, and mutation of codon 559 and/or codon 560 is often observed. These types of mutations are sensitive to both imatinib and sunitinib. The two juxtamembrane region *KIT* mutations (I571_D572delinsT, Q575_P577delinsH) reported here are unique. A502_Y503dup mutation was partially resistant to imatinib and sensitive to sunitinib. I571_D572delinsT and Q575_P577delinsH resulted in strong sunitinib resistance. These results show that different mutations, even within the same juxtamembrane domain of KIT show different inhibitory effects on TKIs.

S840N has been previously published as a presumptive non-oncogenic mutation, in a single case report (132). This S840N mutation was reported as a germline finding in a 2-year-old child with cutaneous mastocytosis. The child's father carried the same germline S840N, therefore the authors concluded the S840N was irrelevant. Interestingly, somatic S840N substitution was found in 29% of GIST metastases from patient 1. The S840N causes definite shift in sunitinib-sensitivity in our functional assay.

Discussion

The aim of this study was to understand better the TKI genomic resistance mechanisms in GIST and the relationship between kind of mutation and drug resistance.

We showed that the molecular drug-resistance mechanisms can be identified in more than 90% of GISTs progressing under imatinib and/or sunitinib irrespective of whether the primary mutation was in exon 9 or 11; the most common primary mutation in GIST is located in the justmembrane region codified by exon 11 and mutation at the codon 559 and 560 and the duplication A502_Y503 is often observed. the mutation at codons 559 and 560 are sensitive to both imatinib and sunitinib; A502_Y503dup is partially resistant to imatinib and sensitive to sunitinib.

The secondary mutations were clustered in ATP binding pocket and in the activation loop of the kinase domain.

The results showed that two or more (to up to 7) TKI-resistance mutation can be present in the same patient; in particular, patient 1 had up to seven different mutations.

We identified 6 novel mutations (2 deletion/insertions, I571_D572delinsT and Q575_P577delinsH, and 4 substitutions, N655S, N680K, F681L and S840N). To evaluate if these mutation were involved in secondary resistance acquisition, we used we engineered these into KIT construct, alone and in combination with a primary mutation. N822K, known to confer resistance to imatinib and sunitinib, was used as positive control.

I571_D572delinsT and Q575_P577delinsH are unique; these two mutations resulted in strong sunitinib resistance but resulted in imatinib response when co-expressed with A502_Y503dup. These results show that different mutations, even within the same juxtmemembrane domain of KIT display different effect of TKIs.

N655S, N680K, F681L and S840N were tested. All the mutations resulted involved in resistance acquisition.

KRAS and _KIT_ gatekeeper mutations and primary imatinib resistance in GIST: relevance of concomitant PI3K/AKT dysregulation

Approximately 10% of GIST patients have primary imatinib resistance, defined by clinical progression within three to six months after initiating therapy. Such GISTs typically lack KIT and PDGFRA mutations, or contain particular mutations, e.g. PDGFRA D842V, that are intrinsically imatinib resistant. Our study found for the first time polyclonal heterogeneity as a mechanism of primary imatinib resistance in a GIST patient.

A 61-year-old man presented to an outpatient clinic in October 2003 with an 8-week-history of progressive left shoulder pain, nausea and fatigue. Abdominal CT-scan revealed a 19.7 x 13.1 cm mass arising from the anterior wall of the stomach, accompanied by five liver metastases, all less than 1 cm in maximal diameter. Endoscopic biopsy demonstrated a spindle cell GIST with 20 mitoses per 50 hpf.

The patient received imatinib 400 mg per day and got improvement within one month (resolution of shoulder pain, softening of the palpable mass and normalization of the blood counts). CT-scan after six weeks of imatinib showed that the gastric mass had typical post-therapy changes, hypodensity as well as decrease in wall thickness. The liver metastases were not changed. CT-scan after 16 weeks from imatinib treatment beginning displayed reduction of the hypodense gastric residual mass however, a new hyperdense 2.7 x 2.0 cm nodule appeared at the caudal aspect of the mass. The patient remained under imatinib, and a follow-up CT-scan two months later showed progression of the hyperdense nodule to 4.9 x 5.6 cm, now accompanied by new progressing nodules. An upper gastrointestinal bleed caused the resection of the gastric mass, performed 24 hours after the last imatinib dose. Histologically, the gastric mass was spindle cell-type GIST. Genomic analyses by Sanger sequencing, Ion Torrent and Sequenom. were performed in clinically responding (region 1) *vs* clinically progressing (regions 2 and 3) aspects of the mass: region 1 was non-mitotic, and consistent with sTable/responding disease, whereas regions 2 and 3 had 60 and 55 mitoses per 50 hpf, respectively, and were therefore consistent with progressing, imatinib-resistant, disease. Each of these three regions expressed KIT strongly, with a homozygous *KIT* exon 11 E554_V559del

mutation (Figure 27A) and a homozygous *PTEN* missense mutation, C124S (Figure 27B). The imatinib-responsive region 1 had no additional mutations, whereas imatinib resistant region 2 had a KRAS G12R

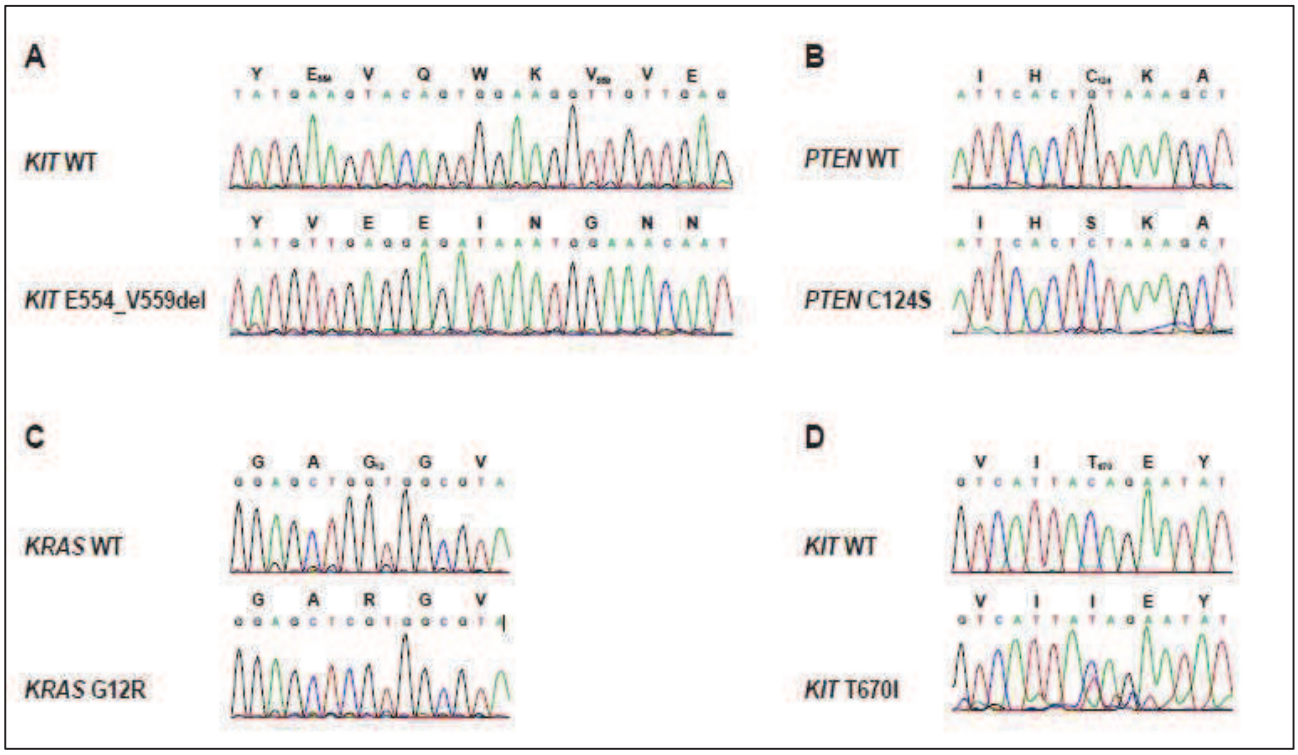


Figure 27. The three regions showed a KIT E554_V559del (27A), a PTEN missense mutation(27B). The region 2 (imatinib-resistant) had a KRAS G12R mutation (27C) and the region 3 (imatinib-resistant) had a KIT T670I mutation (27D).

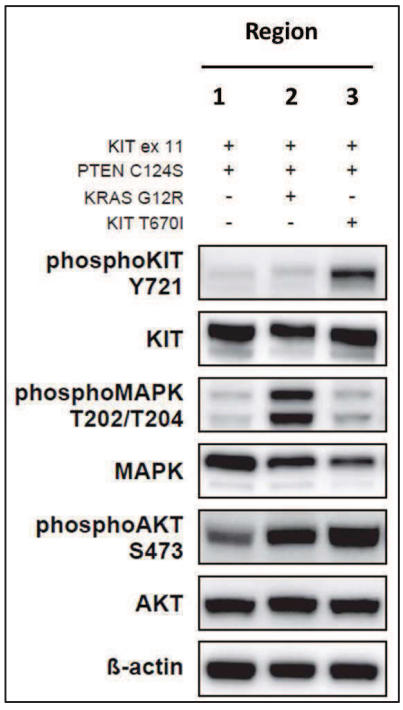


Figure 28. Results for western blotting showed KIT was activated only in region 3 with the KIT T670I mutation, MAPK was hyperactivated only in the region 2 with KRAS G12R, and AKT and S6 were hyperactivated in both of these regions.

mutation by Sequenom analysis, which was subsequently confirmed through genomic sequencing (Figure 27C). Imatinib-resistant region 3 had a KIT "gatekeeper" T670I mutation (Figure 27D), which is known to confer imatinib resistance.

Western-blotting experiments confirmed strong KIT expression in both imatinib-responsive and resistant regions (Figure 28); however, KIT was activated, as disclosed by phosphoKIT Y721 expression, only in region 3 with the KIT T670I mutation, MAPK was hyperactivated only in the region 2 with KRAS G12R, whereas AKT and S6 were hyperactivated in both of regions (Figure 28).

Imatinib was resumed, but the patient experienced progression of intra-abdominal disease, and died five months later, while receiving high-dose imatinib (800 mg/day). He did not receive sunitinib, as it was not yet approved by FDA for imatinib resistant

GIST. ISTs lacking *KIT* and *PDGFRA* mutations frequently have primary imatinib-resistance, and often they do not have known mutations. To test the hypothesis that such GISTs might contain *RAS* mutations or other KIT-downstream mutations, we used a Sequenom panel to screen for *RAS, BRAF* and *PI3KCA* mutations in *KIT/*PDGFRA-wildtype GISTs from 27 patients. Only one of 27 GISTs contained demonstrable mutations: this was a high-risk GIST (62 mitoses per 50 hpf) that contained both HRAS G12V and PIK3CA H1047R mutations. PIK3CA H1047R is a gain-of-function mutation that accounts for ~20% of PIK3CA mutations in advanced human cancers and is associated with response to PI3K/AKT/mTOR pathway inhibitors [133].

Discussion

This brief report represents the first evidence of primary imatinib resistance resulting from intratumoral genomic heterogeneity. This resistance, already at 16 weeks of treatment, was related (in separate lesions) to KRAS mutation and the KIT gatekeeper mutation T670I. Recently, *in vitro* evidence that KRAS mutations might confer imatinib resistance in GIST has been reported [134] and this case seems to confirm that KRAS gain-of-function mutation contributes to clinical imatinib resistance, in spite of therapeutic KIT oncoprotein inhibition. It is thinkable that KRAS mutations are present as minor subclones in more untreated GISTs than previously appreciated, and are then enriched for by KIT/PDGFRA-inhibitor therapies.

Conclusion

In conclusion, these novel findings demonstrate KRAS mutation and polyclonal heterogeneity as mechanisms of primary imatinib resistance in GIST, show that both KRAS and HRAS isoforms can contribute to GIST oncogenesis, and highlight the conjoined nature of the PI3K/AKT and RAS/RAF signaling pathways in GIST tumorigenesis. These findings validate the PI3K/AKT/mTOR and RAS/RAF/MEK pathways as concurrently relevant in GIST oncogenic signaling.

Discussion and Conclusions

Clinical diseases represent complex biological phenotypes reflecting the interaction of a myriad of genetic and environmental contributions.

To the best of our knowledge, this is the first work that consider the pharmacological response in GIST patients treated with imatinib by two different angles: the genetic and somatic point of view.

We first analyzed the influence of polymorphisms on treatment outcome, keeping in consideration SNPs in genes involved in the drug transport, metabolism and folate pathway.
Using a multiple candidate gene approach, we found that SLC22A4 rs1050152 was significantly associated with a reduced TTP. To us this finding seems very promising, as we found the same polymorphism correlating with the major molecular response rate in a set of CML patients [79], indicating that imatinib delivery could be really affected by this allelic variant. We also analyzed polymorphisms in three genes – SLCO1A2, SLC22A5 and ABCC4 for the first time and we identified the minor allele for SLC22A5 rs2631367 and rs2631372 associated with a prolonged TTP. The correlation between SNPs in members of OCTN family transporters and imatinib efficacy could be very important in relation to the use of imatinib in the adjuvant setting.
On the other hand, we found that polymorphism in RFC and FOLR genes may be associated with TTP; to us this finding is intriguing, as both the genes products are involved in the folic acid and reduced folates uptake.
Naturally, all these intriguing results cannot be considered as the only main mechanism in imatinib response. GIST mainly depends by oncogenic gain of function mutations in tyrosin kinase receptor genes, KIT or PDGFRA, and the mutational status of these two genes or acquisition of secondary mutation is considered the main player in GIST development and progression. To this purpose we analyzed the secondary mutations to better understand how these are involved in imatinib resistance. In our analysis we considered both imatinib and the second line treatment, sunitinib, in a subset of progressive patients.
We identified in particular 6 novel mutations and on the basis of functional studies we certainly concluded that all these variations were involved in resistance acquisition.
In general, in GIST there is a main primary mutation in exon 9 or 11, mutually exclusive; the secondary mutation does not depend by the first one and it is possible that more than 2 secondary mutations (from 2 to 7) occur in the same patient.

For the first time we demonstrated that primary imatinib resistance could result from genomic heterogeneity. In the case here described the resistance was related to KIT and KRAS mutations, validating the hypothesis that PI3K/AKT/mTOR and RAS/RAF/MEK pathways are concurrently relevant in GIST oncogenic signaling and responsible for GIST progression.

KIT/PDGFRA mutation analysis is an important tool for physicians, as specific mutations may guide therapeutic choices. Currently, the only adaptations in treatment strategy include imatinib starting dose of 800 mg/daily in KIT exon-9-mutated GISTs. In addition, the primary resistance of PDGFRA D842V GISTs which leads to the lack of rationale for the use of imatinib in these patients.

In the attempt to individualize treatment, genetic polymorphisms represent a novelty in the definition of biomarkers of imatinib response in addition to the use of tumor genotype. Accumulating data indicate a contributing role of pharmacokinetics in imatinib efficacy, as well as initial response, time to progression and acquired resistance. At the same time it is becoming evident that genetic host factors may contribute to the observed pharmacokinetic inter-patient variability. Genetic polymorphisms in transporters – some of which are already known as causes of drug resistance in cancer chemotherapy – and in metabolizing genes may affect the activity or stability of the encoded enzymes. Thus, integrating pharmacogenetic data of imatinib transporters and metabolizing genes, whose interplay has yet to be fully unraveled, has the potential to provide further insight into imatinib response/resistance mechanisms. In view of these considerations, collecting data on the role of polymorphisms in imatinib response in GISTs represents a clinical priority.

Future perspectives: ongoing studies

I. *DNA repair polymorphisms in GIST: susceptibility and correlation with tumor characteristic and clinical outcome.*
II. *VEGF pathway genes polymorphisms in GIST patients in treatment with sunitinib after imatinib failure.*

I. DNA repair polymorphisms in GIST: susceptibility and correlation with tumor characteristic and clinical outcome.

DNA repair pathway - During life, DNA accumulates changes that activate proto-oncogenes and inactivate tumor soppresor genes. The genetic instability drives the tumorigenesis and is fuelled by DNA damage and errors made by DNA repair machinery. Figure 29 summarizes some of the most common types of DNA damages and their causes [135].

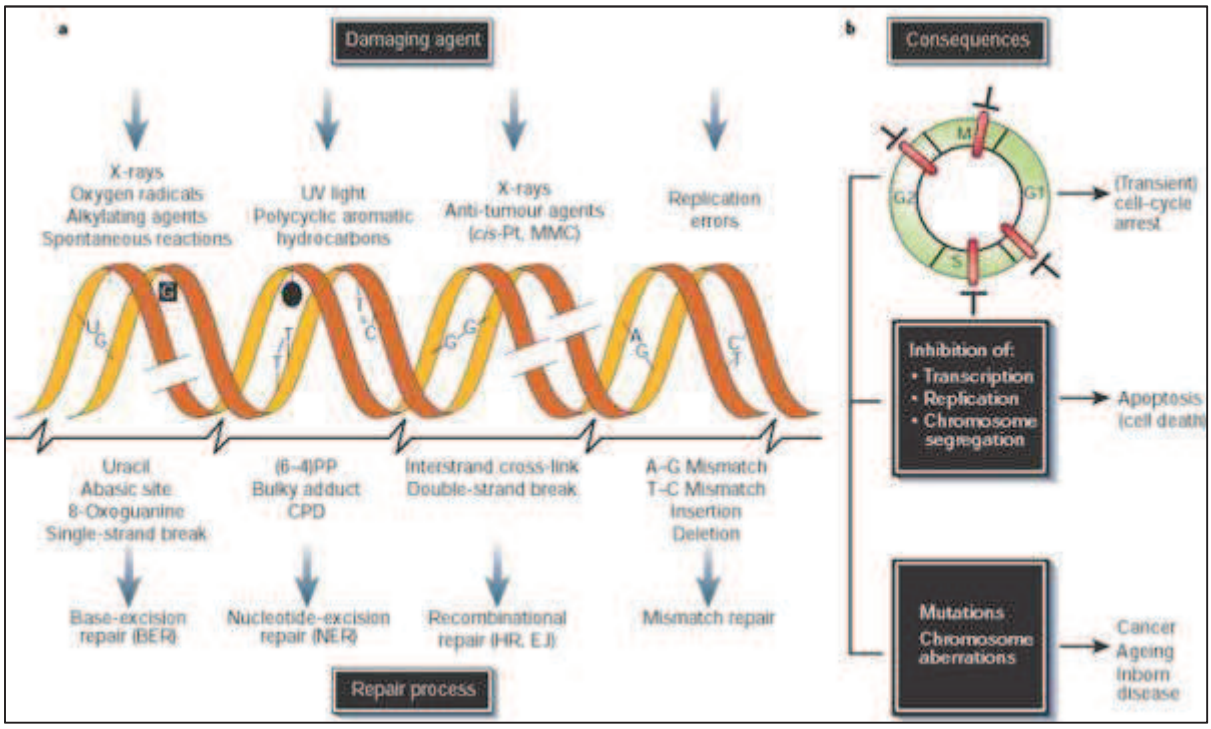


Figure 29. DNA damage, repair mechanisms (A) and consequences (B).

The main DNA repair pathways in humans are direct reversal, base and nucleotide excision, mismatch, homologous recombination repair, and non-homologous end joining [136]. When proteins/enzymes products of these pathways fail to repair damage because of a functional deficiency, the cell accumulates DNA damage and induces apoptosis. Alternatively, unrepaired damage may enhance mutation, including chromosomal aberrations that can in turn alter apoptotic signals, dysregulate cell growth, and induce carcinogenesis. Therefore, it has been hypothesized that germline or somatic variations, such as polymorphisms of DNA repair-related genes play an important role in the risk of cancer development [137,138].

On the basis of these evidences, we decided to analyze a panel of SNPs in the main genes involved in the different DNA repair pathways.

Study population - A total of 60 unresectable/metastatic GIST patients were retrospectively enrolled in this study. These patients are the same described in the "folate" study.

Genotyping analysis - We selected twenty common [minor allele frequency (MAF) > 0.05 in Caucasian], well-studied functional variants - located in regulatory region, cause non-synonymous amino acid changes and/or have been repeatedly associated with cancer risk, survival or treatment response. Characteristics of the studied polymorphisms - all single nucleotide polymorphisms - are reported in Table 15. Genotypes were determined by PCR based assays (i.e. RFLP and RT). Positive and negative controls were included in each reaction as quality control. In addition, for internal quality control (accuracy of genotyping) 90% of samples were repeated. The concordance between the original and the duplicate samples for all the analysed polymorphisms was 100%.

Table 15. SNPs description

Gene [full name; Protein name]	**SNP ID**	**METHOD** [A]
APEX [apurinic/apyrimidinic endonuclease 1]		
	rs1130409 [Asp^{148}Glu]	RT TaqMan assay
XPD [Xeroderma pigmentosus gruup D] / ERCC2[Excision repair cross-complementation group 2]		
	rs1799793 [Asp^{312}Asn]	RT TaqMan assay
	rs13181[Lys^{751}Gln]	RFLP [
hOGG1[Human 8-oxoguanine DNA glycosylase 1]		
	rs1052133 [Ser^{326}Cys]	RT TaqMan assay
XRCC1 [X-ray repair cross-complementing group 1]		
	rs1799782 [Arg^{194}Trp]]	RT TaqMan assay c__11463404_10
	rs25487 [Arg^{399}Gln]	RFLP [] [100]
XRCC3 [*X-ray repair cross-complementing group 3*]		
	rs861539 [Thr^{241}Met]	RT TaqMan assay C_12005959_10
NBS1 [Nijmegen breakage syndrome]		
	rs1801394 [Ile^{49}Met]	RT TaqMan assay C_3068176_10
XPA [Xeroderma pigmentosus gruup A]		
	rs1800975 [−4G>A]	RT TaqMan assay C___482935_1_
	rs2808668 [Intronic]	RT TaqMan assay C__9312100_10
XPC [Xeroderma pigmentosus gruup C]		
	rs2228000 [Ala^{499}Val]	RT TaqMan assay C__16018061_10
	rs2228001 [Gln^{939}Val]	RT TaqMan assay C___234281_1_
XPF [Xeroderma pigmentosus gruup F]		
	rs18000267 [Gln^{415}Arg]	RT TaqMan assay C___3285104_10
	rs3136155 [Intronic]	RT TaqMan assay C__26942939_10
XPG [Xeroderma pigmentosus gruup G]		
	rs17655 [Asp^{1104}His]	RT TaqMan assay C__1891743_10
	rs2094258 [5'UTR]	RT TaqMan assay C___1891783_10

[A] RT = Real-Time PCR with TaqMan allelic discrimination assay [Applera, Foster City, USA];

RFLP = PCR-RFLP, analysis carried out according to published methods [reference parenthetically].

Statistical analysis - The distribution of genotypes was tested for departures from the Hardy-Weinberg equilibrium using the χ^2 test. Survival analysis methods were used to examine the relationship between genotypes [homozygous wild-type, heterozygous and homozygous for the variant allele] and GIST time to progression. In univariate analysis, the survival curves were estimated and plotted with the Kaplan-Meier method. The curves were compared with log-rank test

of equality of survivor functions (statistical significance defined as $P < 0.05$). In multivariate analysis, hazard ratios (HR) and 95% confidence interval (95% CI) were estimated with Cox proportional hazards models, using gender, age, and status (localised/metastatic) at diagnosis, as covariates in addition to the genotype. The proportional hazards assumption was tested ($P > 0.05$) using Schoenfeld residuals. Multiple logistic regression was used to assess the relation between individual polymorphisms and primary resistance. Statistical analysis was conducted using Stata Intercooled version 12.0.

The analysis is ongoing, partially completed.

II. VEGF pathway genes polymorphisms in GIST patients in treatment with sunitinib after imatinib failure.

VEGF pathway - The VEGF pathway play a central role in angiogenesis throughout the tumor development. The production of VEGF is stimulated by upstream activators, including environmental cues, growth factors, oncogenes, cytokines, and hormones. The binding of VEGF to its receptors on the surface of endothelial cells activates intracellular tyrosine kinase, triggering multiple downstream signal that promote angiogenesis. Although there are multiple variants of VEGF and its receptors, the angiogenic effects of this pathway are primarily mediated through the VEGFA (the most common variant) with VEGFR-2 (Figure 30) [139-141].

As previously described, currently, the only approved second-line drug is sunitinib malate - a multitargeted agent, an inhibitor of tyrosine kinase, of KIT and PDGFRA/B and of the VEGFRs -1, -2 and 3, FMS-like tyrosine kinase-3, colony stimulating factor 1 receptor, and glial cell-line derived neurotrophic factor receptor (REarranged during Transfection; RET) [48].

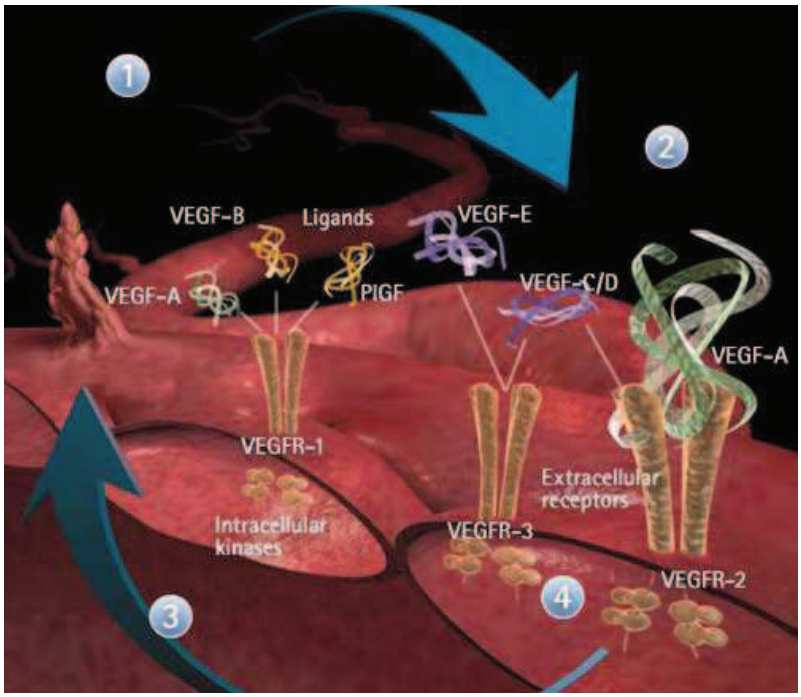


Figure 30. Main players in VEGF pathway: 1) Upstream activators stimulate the production of VEGF; 2) VEGF bind to receptors of endothelial cells; 3) angiogenesis is mediated primarily through the interaction of VEGF and VEGFR-2; 3) other variants of VEGF and its receptors play a secondary role in this process.

The aim of this analysis is to evaluate factors predicting results of sunitinib second-line therapy in inoperable/metastatic GISTs. We want to investigate the impact of the selected SNPs in *VEGFA* and *VEGFR*-1, 2, 3 genes on sunitinib-response in a group of GIST patients.

Study population - A total of 43 unresectable/metastatic GIST patients were retrospectively enrolled in this study in 2013 and 2014 at the Sant'Orsola-Malpighi Hospital, Bologna. All patients were under sunitinib as second line treatment, after imatinib failure. Overall survival (OS) was defined as the time from the first day of treatment to death from disease. Dates of death were obtained and cross-checked using the inpatient medical records. If a patient was alive, OS were censored at the time of the last follow-up. Data for patients who did not progressed at the last follow-up TTP evaluation were censored at that time.

In order to exclude disease susceptibility we also genotyped 184 controls, anonymous blood donors from the *Centro Trasfusionale*, Sant'Orsola-Malpighi Hospital, Bologna. The study was approved by the Ethics Committees of the institution. The analysis was done after written informed consent for study participation and anonymous data publication in accordance with national legislation. Any subjects could cancel participation at any time during the study, according to Helsinki Declaration and later Amendments.

Genotyping analysis - We selected nineteen common [minor allele frequency (MAF) > 0.05 in Caucasian] well-studied functional variants - located in regulatory region of four genes involved in VEGF pathway. Patients with available peripheral blood were eligible for this retrospective study. DNA was isolated as previously described. Characteristics of the studied polymorphisms - all single

nucleotide polymorphisms - are reported in Table 16. Genotypes were determined by RT-PCR as previously described. Positive and negative controls were included in each reaction as quality control. In addition, for internal quality control (accuracy of genotyping) 90% of samples were repeated. The concordance between the original and the duplicate samples for all the analysed polymorphisms was 100%.

Table 16. SNPs description

Gene [full name; Protein name]	**SNP ID**	**METHOD** [A]
VEGFA [Vascular endothelial growth factor A]		
	rs699947 [Intronic]	RT TaqMan assay C___8311602_10
	rs833061 [Intronic]	RT TaqMan assay C___1647381_10
	rs2010963 [5'UTR]	RT TaqMan assay C___8311614_10
	rs3025039 [3'UTR]	RT TaqMan assay C___16198794_10
VGFR1[Vascular Endothelial Growth Factor Receptor 1]		
	rs9513070 [Intronic]	RT TaqMan assay C__30362252_10
	rs9554320 [Intronic]	RT TaqMan assay C__32231227_10
	rs9554319 [Intronic]	RT TaqMan assay C__1910659_10
	rs9554316 [Intronic]	RT TaqMan assay C__32231224_10
	rs9582036 [Intronic]	RT TaqMan assay C__1910658_10
VGFR2[Vascular Endothelial Growth Factor Receptor 2]		
	rs1531289 [Intronic]	RT TaqMan assay C__7439188_20
	rs1870377 [$Gln^{472}His$]	RT TaqMan assay C__11895315_20
	rs2305948 [$Ile^{297}Val$]	RT TaqMan assay C__22271999_20
	rs11133360 [Intronic]	RT TaqMan assay C__26111278_10
	rs6828477 [Intronic]	RT TaqMan assay C__1673866_10
	rs6837735 [Intronic]	RT TaqMan assay C__30784758_10
VGFR3[Vascular Endothelial Growth Factor Receptor 3]		
	rs307805 [[Intronic]	RT TaqMan assay C__918880_10
	rs307822 [3'UTR]	RT TaqMan assay C___988831_1
	rs6877011 [3'UTR]	RT TaqMan assay C__29057584_10
	rs7709359 [Intronic]	RT TaqMan assay C__30240676_10

[A] RT = Real-Time PCR with TaqMan allelic discrimination assay [Applera, Foster City, USA];

RFLP = PCR-RFLP, analysis carried out according to published methods [reference parenthetically]

Statistical analysis – as previously described, the distribution of genotypes was tested for departures from the Hardy-Weinberg equilibrium using the χ^2 test. Survival analysis methods were used to examine the relationship between genotypes [homozygous wild-type, heterozygous and homozygous for the variant allele] and GIST time to progression. In univariate analysis, the survival

curves were estimated and plotted with the Kaplan-Meier method. The curves were compared with log-rank test of equality of survivor functions (statistical significance defined as $p < 0.05$). In multivariate analysis, hazard ratios (HR) and 95% confidence interval (95% CI) were estimated with Cox proportional hazards models, using gender, age, and status (localised/metastatic) at diagnosis, as covariates in addition to the genotype. The proportional hazards assumption was tested ($P > 0.05$) using Schoenfeld residuals. Multiple logistic regression was used to assess the relation between individual polymorphisms and primary resistance. Statistical analysis was conducted using Stata Intercooled version 12.0.

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