

**Clinical relevance of oncogenic point mutations and
chromosome copy number changes in
gastrointestinal stromal tumors**

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SUMMARY

Gastrointestinal Stromal Tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract. It has been shown that mutually exclusive oncogenic mutations in *KIT* or *PDGFRA*, leading to the constitutive activation of these receptor kinases, are the primary events responsible for the pathogenesis of most GIST. Additional genomic alterations, such as deletions of chromosomal regions 14q, 1p and 22q, have been proposed as secondary events required for the clinical progression of these tumors. However, the relative contribution of such primary and secondary alterations for the biology and aggressive behaviour of GIST remains elusive. In addition, the specific mutation patterns of *KIT* and *PDGFRA* seem to markedly influence patient response to currently available targeted therapies, although the predictive value of most mutations requires further validation.

In the current work we assessed the genetic background of a consecutive series of 78 patients diagnosed with GIST and treated at our institution. Primary molecular events (mutations in *KIT* or *PDGFRA*) were assessed in all samples using direct sequencing analysis. For a subset of 27 patients, comparative genomic hybridization was applied to identify secondary genetic aberrations. Genotype and genomic findings were cross-tabulated and compared with existing clinical variables and therapy response data, to determine the possible prognostic and/or predictive value of the most frequent alterations.

Our overall mutation frequency was 87.2%, with 59 cases showing alterations in *KIT* (75.7%), and 9 samples harboring *PDGFRA* mutations (11.5%). The prognostic value of several genotypic subgroups previously suggested to show prognostic value could be confirmed. In particular, patients with mutations in *KIT* exon 9 or deletions/deletions in *KIT* exon 11 showed significantly more metastatic events than those without these alterations, whereas patients with *PDGFRA* mutations have shown no disease progression. Two secondary mutations known to confer resistance to Imatinib were identified in therapy-resistant metastatic lesions from two patients with primary *KIT* mutations.

Chromosomal imbalances were detected in 23 out of 27 samples (85%), with abnormal samples displaying a median of 3 aberrations per tumor. Losses were 1.5 times more frequent than gains, and in particular 21 samples displayed complete or partial loss of chromosome 14q. Other recurrent aberrations included losses at 22q (43.5%), 1p (43.5%) and 15q (34.8%), and gains at 1q (17.4%) and 12q (17.4%). Interestingly, cases with mutations previously associated with a more aggressive clinical behaviour showed significantly more copy number changes than those without such mutations. In addition,

genomic complexity, the presence of genomic gains, deletions at 1p, or deletions at 22q was significantly associated with a shorter time to disease progression.

We conclude that identification of both primary and secondary genetic events in GIST provides relevant clinical information for the diagnosis and therapeutic management of these patients. The majority of cases in our series showed mutations in either *KIT* or *PDGFRA*, several of which with relevant prognostic and/or predictive value. In addition, secondary genomic aberrations could be seen in most cases analyzed by CGH, several of which clearly associated with a shorter disease-free survival for the patients. A multidisciplinary approach that combines clinical, pathological and genetic features thus seems mandatory to achieve a good standard of care for GIST patients.

RESUMO

Os GIST (*Gastrointestinal Stromal Tumors*) são os tumores das partes moles mais comuns no tracto gastrointestinal. Mutações pontuais nos genes *KIT* ou *PDGFRA*, promovendo a activação constitutiva destes receptores com actividade de cinase, são consideradas as alterações genéticas responsáveis pela patogénese da maioria destes tumores. Além destes eventos primários mutuamente exclusivos, várias alterações genómicas secundárias, tais como deleções cromossómicas do 14q, 1p e 22q, parecem ser requeridas para a progressão clínica destes tumores. No entanto, a contribuição relativa das alterações primárias e secundárias para a biologia e comportamento agressivo dos GIST ainda não foi determinada. Adicionalmente, os padrões específicos de mutações dos genes *KIT* e *PDGFRA* parecem influenciar marcadamente a resposta destes pacientes às actuais terapias existentes, apesar do valor predictivo das mesmas não estar bem determinado.

Neste trabalho procurámos estabelecer o perfil genético de uma série consecutiva de 78 doentes diagnosticados com GIST e tratados na IPO-Porto, realizando a pesquisa de alterações primárias nos oncogenes *KIT* ou *PDGFRA* através de sequenciação directa, e determinando eventos genómicos secundários num subgrupo de doentes recorrendo à metodologia de hibridação comparativa do genoma (CGH). Os resultados obtidos pelas duas técnicas de análise genética foram cruzados e comparados com as variáveis clínicas e dados de resposta terapêutica dos pacientes, por forma a determinar o possível valor prognóstico e/ou preditivo das alterações recorrentes.

A frequência total de mutações detectada na nossa série foi de 87.2%, tendo sido observados 59 casos com alterações no *KIT* (75.7%) e 9 amostras com mutações no *PDGFRA* (11.5%). O valor prognóstico sugerido para alguns dos grupos genotípicos observados pode ser confirmado na nossa série de amostras. Em particular, tumores com mutações no exão 9 ou deleções/delins no exão 14 do gene *KIT* apresentaram uma taxa de recorrência significativamente mais elevada que aqueles sem estas alterações, enquanto que no grupo de pacientes com mutações no gene *PDGFRA* não houve eventos de recorrência. A mesma mutação secundária de resistência a terapia foi identificada em lesões metastáticas pós-tratamento de dois pacientes com mutações primárias no gene *KIT*.

Das 27 amostras analisadas por CGH, 23 (85%) apresentaram alterações cromossómicas. Foi detectada perda total ou parcial do cromossoma 14q em 93% destas amostras, sendo ainda particularmente frequentes as perdas do 22q (43.5%), 1p (43.5%) e 15q (34.8%). Foi possível verificar que o número de alterações cromossómicas

detectado nos tumores com mutações previamente associadas a pior prognóstico era significativamente maior do que em amostras contendo outras mutações primárias. A complexidade genómica, assim como a presença de ganhos genómicos, de deleções no 1p ou de perdas do 22q também foram significativamente associadas com um menor intervalo de progressão da doença.

Foi assim possível concluir que a identificação de eventos genéticos primários e secundários em GIST proporciona informação clínica relevante para o diagnóstico e decisão terapêutica nestes doentes. A maioria dos tumores estudados apresentaram mutações nos oncogenes *KIT* ou *PDGFRA*, várias das quais associadas com uma pior resposta à actual terapia dirigida a estes receptores. Adicionalmente, aberrações genómicas secundárias foram observadas na maioria dos casos analisados por CGH, algumas das quais claramente associadas com um menor intervalo livre de doença para os doentes. Uma abordagem multidisciplinar combinando características clínicas, patológicas e genéticas parece deste modo crucial para o diagnóstico e seguimento de doentes com GIST.

RESUMÉ

Les tumeurs du stroma gastro-intestinale (GIST) sont les plus fréquents des tumeurs mésenchymateuses du tube digestif. Il a été démontré que des mutations oncogéniques mutuellement exclusifs en *KIT* ou *PDGFRA*, conduisant à l'activation constitutive de ces récepteurs kinases, sont les événements génétiques responsables de la pathogenèse de la plupart des GIST. Autres altérations génomiques, tels que les suppressions de régions chromosomiques 14q, 1p et 22q, ont été proposées comme des événements secondaires nécessaires pour la progression clinique de ces tumeurs. Toutefois, la contribution relative des modifications primaires et secondaires pour la biologie et comportement agressif des GIST reste insaisissable. En outre, les différentes mutations du *KIT* et *PDGFRA* semblent influencer la réponse des patients aux thérapies actuellement disponibles, bien que la valeur prédictive de la plupart de ces mutations nécessite plus validation.

Dans ce travail nous avons évalué le contenu génétique d'une série consécutive de 78 patients diagnostiqués de GIST et traités dans notre institution. Événements primaires (mutations du *KIT* ou *PDGFRA*) ont été évalués dans tous les lésions par séquençage direct. Dans un sous-groupe de 27 patients, hybridation génomique comparative a été appliquée pour identifier anomalies génétiques secondaires. Les résultats génétiques ont été croisés et comparés avec les variables cliniques et de réponse à la thérapie, afin de déterminer la possible valeur pronostique et/ou prédictif des modifications les plus fréquentes.

Notre fréquence de mutation a été de 87,2%, avec 59 cas présentant des altérations dans *KIT* (75,7%), et 9 dans *PDGFRA* (11,5%). Déséquilibres chromosomiques ont été détectés dans 23 tumeurs (85%). Les pertes ont été 1,5 fois plus fréquentes que les gains, en particulier dans le chromosome 14q (91%). Autres aberrations récurrents incluent pertes à 22q (43,5%), 1p (43,5%) et 15q (34,8%), et gains à 1q (17,4%) et 12q (17,4%). Fait intéressant, les cas avec mutations déjà associés à un comportement clinique plus agressif ont montré beaucoup plus de modifications du nombre de copies que ceux sans ces mutations. En outre, la présence de gains génomiques, des suppressions 1p, ou suppressions à 22q ont été significativement associés à la progression de cette maladie.

Nous concluons que l'identification des événements génétiques primaires et secondaires du GIST fournit importants renseignements cliniques pour le diagnostic et la thérapeutique de ces patients. La majorité des cas dans notre série a montré mutations dans *KIT* ou *PDGFRA*, dont quelques sont associés à la pire réponse à des

thérapeutiques ciblées. En outre, des aberrations génomiques secondaires ont été vues dans la plupart des cas analysés par CGH, dont certaines clairement associées à une courte survie sans maladie. Des approches pluridisciplinaires qui combinent des caractéristiques cliniques, pathologiques et génétiques semblent donc impératives de parvenir à un bon niveau de soins pour les patients atteints de GIST.

LIST OF ABBREVIATIONS

ABL	Abelson murine leukemia viral oncogene homolog
AKE	Hypotonic Amino-(K) Potassium-EDTA Solution
Akt	Intracellular signalling pathway Akt
AML	Acute myeloid leukaemia
ANED	Alive with no evidence of disease
ATP	Adenosine-5'-triphosphate
AWD	Alive with disease
BCR	Breakpoint cluster region
CGH	Comparative genomic hybridization
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CD34	Hematopoietic progenitor cell antigen
CML	Chronic myelogenous leukemia
DAPI	4',6-diamidino-2-phenylindole
Delins	Deletion Insertion
DFD	Dead from disease
DFS	Disease-free survival
DNED	Dead with no evidence of disease
DWD	Dead with disease
DNA	Deoxyribonucleic acid
DOG1	2-deoxyglucose-6-phosphate phosphatase 1
EDTA	Etilenodiaminotetraacetic acid
FDA	Food and Drugs Administration
FISH	Fluorescent <i>in situ</i> hybridization
GI	Gastrointestinal
GIST	Gastrointestinal stromal tumor(s)
H&E	Haematoxylin and eosin

HCL	Hydrochloric acid
HPF	High-power field
HSP90	Heat shock protein 90
ICC	Interstitial cells of Cajal
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LOH	Loss of heterozygosity
MAP	Mitogenic-activated-protein
MMR	Mismatch repair system
mTOR	mammalian target of Rapamycin
NF1	Neurofibromatosis type I
PCR	Polymerase chain reaction
PDGFRA	Platelet derived growth factor receptor alpha
PKC θ	Protein kinase C theta
PS100	S100 protein
TP53	Tumor-suppressor gene <i>p53</i>
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SMA	Smooth muscle actin
SPSS	Statistical Package for Social Sciences
SSCP	Single Strand Conformational Polymorphism
STAT	Signal transducer and activator of transcription
TK	Tyrosine kinase
TSG	Tumor-suppressor genes
VEGF	Vascular endothelial growth factor

INTRODUCTION

Cancer is one of the worldwide main causes of death. Whereas 5 to 10 % of the cases represent inherited conditions, most tumors arise from an altered somatic cell that proliferates and originates a neoplastic clone through the acquisition and accumulation of epigenetic and/or genetic alterations. These alterations usually target protooncogenes (*gain-of-function mutations*), tumor-suppressor genes (TSG) (*loss-of-function mutations*) or genes of the mismatch repair system (MMR) (*loss-of-function mutations*), leading to the accumulation of replication errors in multiple other genes and metabolic pathways. Normal cellular processes such as proliferation, differentiation, apoptosis, and adhesion become compromised, eventually resulting in self-sufficiency of growth signals, insensitivity to anti-growth stimuli, reduced apoptosis, sustained angiogenesis, limitless replicative potential and finally tissue invasion and metastasis (Hanahan D and Weinberg RA, 2000).

Background

GIST (Gastrointestinal Stromal Tumors) represent the most common mesenchymal tumors of the gastrointestinal tract (Yang J *et al*, 2008). For many years these lesions were incorrectly classified as smooth muscle tumors and grouped with leiomyomas, leiomyoblastomas or leiomyosarcomas due to their morphological similarities (Corless CL *et al*, 2004; Rubin BP, 2006). This classification persisted until electron microscopy studies showed that GIST lacked smooth muscle differentiation, leading Mazur and Clark to propose the term “stromal tumor” to identify this distinct clinicopathologic entity (Mazur MT and Clark HB, 1983). Later, in 1998, immunohistochemistry studies in GIST revealed the absence of desmin expression (characteristic of smooth muscle tumors) and the presence of KIT protein (CD117, absent in leiomyomas and leiomyosarcomas) in approximately 95% of the samples (reviewed in Corless CL *et al*, 2004 and Rubin BP, 2006). Most GIST were also shown to express the hematopoietic blast antigen CD34 (Corless CL and Heinrich MC, 2008).

Epidemiology

Most gastrointestinal tumors have an epithelial origin, and as such GIST represent only a small fraction (<1%) of the total spectrum of gastrointestinal cancers (Gupta P *et al*, 2008). Due to the misclassification problems in the early days, epidemiologic data for GIST is incomplete and likely underestimates the true incidence of these tumors. Recent studies estimate an annual incidence of 10 to 20 cases per million people (Du CY *et al*, 2008), with 3300 to 6000 new cases per year in the US (Corless CL and Heinrich MC,

2008). Incidence is identical in both sexes, with peak susceptibility between 40 to 60 years of age. There is no epidemiologic data for these tumors in Portugal. Little information is also available concerning risk factors associated with this neoplasia, even if NF1 (Neurofibromatosis type I), Carney Triad, and familial gastrointestinal stromal tumor syndromes seem to confer an increased risk to develop GIST (Rubin BP *et al*, 2007).

Survival rates

Patients with primary, non-metastatic GIST in accessible anatomic locations are routinely submitted to complete surgical resection. The 5-year survival for these patients ranges from 42 to 65% (Rossi CR *et al*, 2003; DeMatteo RP *et al*, 2008). GIST often recur locally, diffusing through the serosal surfaces. Advanced disease is characterized by a distinct metastatic pattern, with the liver and the peritoneum as primary targets. GIST rarely metastasize to the lungs, pleura, bones, brains, or lymph nodes (Rubin BP *et al*, 2007; Cichoż-Lach H *et al*, 2008).

Clinical features

A diagnosis of GIST involves a multidisciplinary approach that combines clinical, pathological, and genetic features. GIST usually occur within the entire length of the gastrointestinal tract (GI), predominantly in the stomach (~50%), followed by small intestine (~25%), rectum and colon (~10%), and esophagus (~5%). These tumors can also develop outside the GI, in locations such as the omentum, mesentery, pelvis and retroperitoneum (~10% taken together), abdomen, uterus or vagina (Miettinen M and Lasota J, 2001; Corless CL *et al*, 2004; Rubin BP, 2006, Gupta P *et al*, 2008). The clinical presentation depends on the location and size of the tumor, but approximately 10 to 30% of cases are asymptomatic and discovered incidentally in routine exams or non-related surgeries. Symptomatic tumors may cause abdominal pain, early satiety, flatulence, prolonged gastrointestinal bleeding, anemia of unknown origin, weight loss and vomiting, among others (Cichoż-Lach H *et al*, 2008). Sporadic GIST may consist of solitary primary lesions or multiple synchronous tumors. Hereditary tumors represent a minority of cases, with usually several family members being affected, some presenting multiple primary or metastatic lesions in different anatomic locations throughout their lifetime (Gupta P *et al*, 2008).

Histological features

GIST have a median diameter of approximately 50 mm, ranging in size from small 10 mm lesions, usually discovered incidentally, to large tumors with up to 35 cm. They commonly present as fleshy solid lesions with central foci of hemorrhage and/or necrosis. These tumors are frequently solitary, but may arise as multiple nodules. GIST are divided in three cytomorphological categories, namely spindle cell (70%), epithelioid (20%), or mixed (Fletcher CD *et al*, 2002; Rubin BP *et al*, 2007). GIST are thought to arise from interstitial cells of Cajal (ICC) or its precursors (Hirano K *et al*, 2008). ICC express CD34 and are known as the “pacemaker” cells of the gastrointestinal tract, as they autonomously coordinate the peristalsis in these tissues (Rumessen JJ and Thuneberg L, 1996; Rubin BP, 2006). Both GIST cells and ICC have the dual characteristics of muscle and neural cells (Du CY *et al*, 2008), also sharing several other morphologic, immunohistochemical and molecular features (Miettinen M and Lasota J, 2006).

Immunohistochemical features

In 1995, Isozaki and collaborators showed that ICC expressed CD117 and that its development was dependent on stem cell factor (SCF) signaling, through the kinase receptor coded by the gene *KIT* (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) (Isozaki K *et al*, 1995). Hirota and collaborators quickly followed up on these results and reported that 85 to 90% of GIST harbored mutations in *KIT*, and that approximately 95% stained positive for the corresponding protein CD117 (Hirota S *et al*, 1998). Nowadays, CD117 staining remains the most specific and sensitive marker used for the diagnosis of GIST (Miettinen M and Lasota J 2005a; Corless CL and Heinrich MC, 2008). KIT staining is usually strong and diffuse, with a cytoplasmic, membranous or paranuclear “dotlike” distribution (Rubin BP, 2006). Whereas expression of CD117 is a well defined characteristic of GIST, other exams are required to reliably confirm a suspected diagnosis due to the facts that: 5% of GIST are negative for this marker; other tumors also express this protein, namely malignant gliomas and small-cell lung tumors (Orosz Z *et al*, 2005); CD117 staining can differ within the same tumor and with the therapy applied; an incorrect titrating of the antibody can mimic false positive results; KIT is a transmembrane receptor (Figure 1) but in some tumors staining is predominantly cytoplasmic (Corless CL and Heinrich MC, 2008).

Additional immunohistochemical markers are therefore useful for the correct identification of GIST. Around 60 to 70% of these tumors are positive for CD34, depending on tumor location: 80-85% of gastric lesions, 50% of those arising in the small intestine

and 95-100% of oesophagus or rectum GIST show positivity for this marker. GIST may also show positivity for smooth muscle actin (SMA; 30-40%), S100 protein (5%), desmin (1-2%) and keratin (1-2%) (Rubin BP *et al*, 2007). Platelet derived growth factor receptor α (PDGFRA), a transmembrane protein (Figure 1) that belongs to the same receptor tyrosine kinase (RTK) family as KIT (and which shares several of its molecular features), should also serve as an excellent marker to identify KIT negative GIST. However, this protein is expressed in other mesenchymal tumors and currently available antibodies fail to show the necessary specificity and sensibility (Corless CL and Heinrich MC, 2008).

Much more recently, gene expression profiling studies in GIST reported that DOG1, a membrane surface protein of unknown function, was expressed in 98% of the samples, including KIT negative GIST (West RB *et al*, 2004). As this protein was rarely expressed in other tumors, it represents a very promising marker for differential diagnosis of GIST, in particular those with unclear or negative KIT staining (Miettinen M and Lasota J, 2006; Corless CL and Heinrich MC, 2008). Protein kinase C theta (PKC θ) is an additional signaling molecule selectively expressed in ICC lineages and strongly and specifically expressed in GIST. It plays an important role in the survival of lymphocytes T, positively regulating the activation of T-cell receptor signaling pathways. It is still unclear if PKC activity is dependent on KIT or PDGFRA activation or other independent mechanisms, but it represents another promising immunomarker for the identification of these tumors (Duesing *et al*, 2004).

Signal transduction pathways

The KIT protein, encoded by the *KIT* protooncogene located at chromosomal band 4q12, is a type III transmembrane receptor that belongs to the RTK family (Figure 2). It is involved in several biological mechanisms including cell differentiation, proliferation, adhesion and apoptosis (Rönnstrand L, 2004; Du CY *et al*, 2008), and is known to play an important role in the development and maintenance of melanocytes, erythroblasts, mast cells and ICC (Antonescu CR *et al*, 2003; Vu HA *et al*, 2005; Hirano K *et al*, 2008). The protein structure of KIT consists in an extracellular (receptor) domain with five immunoglobulin-like repeats, a transmembrane segment, and an intracellular (effector) domain with multiple autophosphorylation sites that mediate tyrosine kinase (TK) activity (Figure 1) (Hirota *et al*, 2003; Liu H *et al*, 2007; Corless CL and Heinrich MC, 2008). Upon binding of the ligand SCF, the receptor homodimerizes and the TK domains are activated, thus triggering the phosphorylation of downstream effectors within the MAP kinase, STAT and/or phosphatidylinositol 3 (PI3)-kinase/AKT pathways (Figure 2).

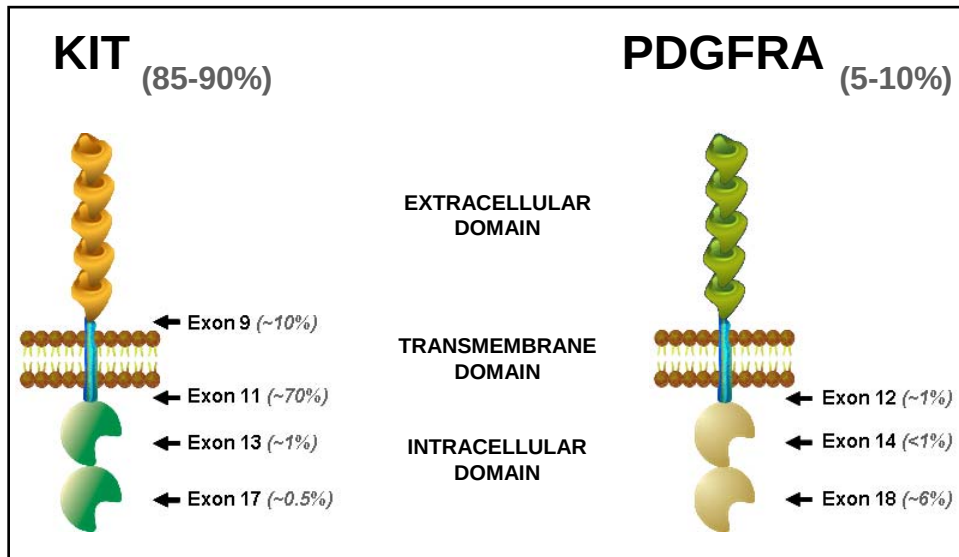


Figure 1. Structure of KIT and PDGFRA receptors with codifying exons and corresponding mutational frequencies found in GIST.

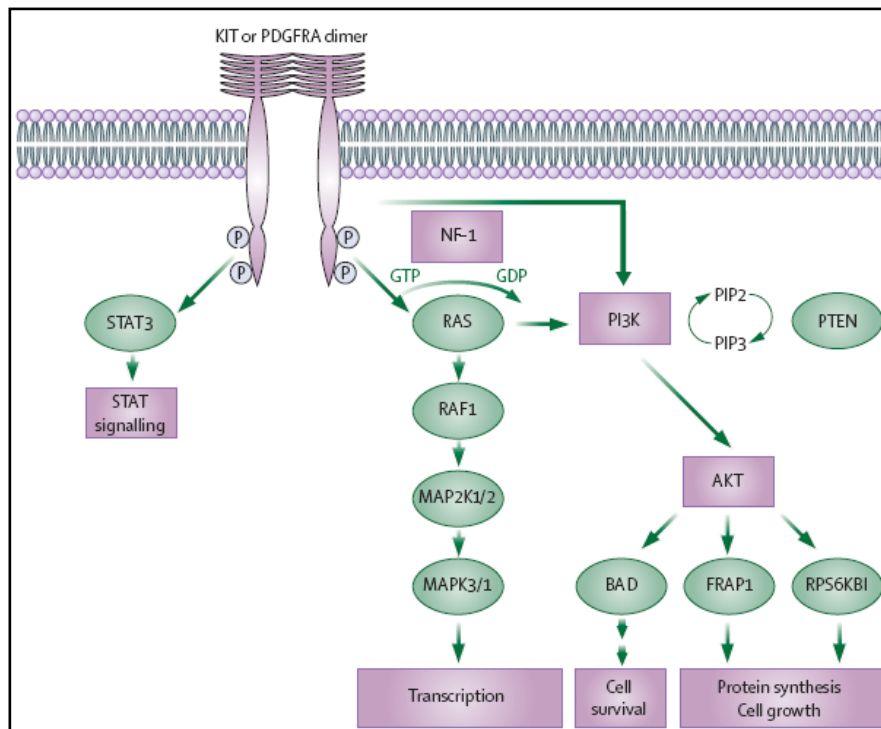


Figure 2. Signaling transduction pathways activated by KIT and PDGFRA receptors. Adapted from Rubin PR *et al*, 2007.

The PDGFRA protein, coded by the *PDGFRA* oncogene (also located at chromosomal band 4q12), shares several structural homologies with KIT and is an alternative activating receptor of its signaling cascade (Rubin BP *et al*, 2007). When connected to its respective ligand (PDGF-A), it activates similar downstream signalling molecules that promote, among other processes, cell proliferation and survival (Figure 2) (Taylor ML and Metcalfe DD, 2000; Savage DG and Antman KH, 2002; Xiang Z *et al*, 2007).

Molecular sub-classifications

In 1998, Hirota and collaborators reported that 85 to 90% of GIST harbored mutations in *KIT* (Hirota S *et al*, 1998). Later, it was shown that 5 to 10% of cases presented *PDGFRA* mutations (Heinrich MC *et al*, 2003). Overall, up to 96% of GIST will show mutations in one of these oncogenes (Heinrich MC *et al*, 2002; Hirota S *et al*, 2003; Corless *et al*, 2005). These events are mutually exclusive, emerge in the early stages of the carcinogenic process, and seem to determine the pathogenesis of GIST (Taylor ML and Metcalfe DD, 2000; Savage DG and Antman KH, 2002).

Functional studies in cell lines transfected with *KIT* or *PDGFRA* showed that mutant isoforms had constitutive kinase activity (SCF or PDGF-A independent dimerization, respectively), which resulted in continuous activation of the downstream signaling cascades (Hirota S *et al*, 1998; Tuveson *et al*, 2001; Heinrich MC *et al*, 2003; Heinrich MC *et al*, 2006). It has also been shown that a negative control of KIT receptor signaling is exerted through ubiquitination and fast proteosome degradation of the receptor upon SCF ligation. Mutant isoforms, however, have much longer half-lives due to the stabilization by the heat shock protein 90 (Corless CL and Heinrich MC, 2008). This chaperone protein is responsible for the breakdown of many RTK as well as other signaling molecules, and currently represents a promising target for tailored therapeutics. The pathogenic role of *KIT* mutations has further been demonstrated as mutant isoforms support the growth of stably transfected BA/F3 cells in nude mice (Hirota S *et al*, 1998), mice engineered to express mutant KIT develop GIST and ICC hyperplasia (Sommer *et al*, 2003; Rubin BP *et al*, 2005), and inhibition of *KIT* blocks the growth of GIST cell lines (Tuveson *et al*, 2001; Nakatani *et al*, 2005; Heinrich MC *et al*, 2006; Tarn *et al*, 2006). It is noteworthy that familial GIST syndrome has recently been found to be caused by heritable *KIT* or *PDGFRA* mutations (Isosaki K *et al*, 2000; Corless CL and Heinrich MC, 2008).

KIT mutations can be broadly assigned to one of two groups: 1) those that involve the 'regulatory' regions responsible for modulating *KIT* enzymatic activity, and 2) those that involve the enzymatic region itself (Yang J *et al*, 2008). Different mutations have distinct biological and clinical implications (Corless CL and Heinrich MC, 2008). Four different regions of *KIT* (composed of 21 exons) have been found to harbor mutations in sporadic GIST (Figure 1). Exon 11 codes for the juxtamembrane intracellular domain that is responsible for the inhibition of *KIT* dimerization in the absence of ligand. When mutated (70% of cases), the receptor dimerizes and becomes constitutively activated. Deletions and insertions predominate in the 5' and duplications are found within the 3' of the exon. Tumors with exon 11 mutations, including in-frame deletions or insertions, missense mutations or combinations thereof, occur throughout the entire GI tract (Tornillo L and Terracciano LM, 2006; Corless CL and Heinrich MC, 2008).

Exon 9 (coding for the extracellular domain) is mutated in 10% of the cases. Although the pathogenic process is less clear, it is suggested that these mutations mimic the conformational changes that occur upon ligation of the SCF (Liu H *et al*, 2007). Tumors with exon 9 mutations are often characterized as high-risk or overtly malignant, suggesting an inherently aggressive biology (Rubin BP *et al*, 2007), and approximately 95% localize in the small intestine. The mutation p.Ala502_Tyr503dup, described by Lux and collaborators (2000), is particularly recurrent (10% of these cases).

Exon 13 codes the first portion of the tyrosine kinase domain and is mutated in ~1% of cases. The mechanism by which the receptor becomes activated in these cases is yet to be discovered. Exon 17 codes for the kinase activation domain and when mutated (0.5% of cases) promotes spontaneous kinase activity (Mol CD *et al*, 2003; Foster R *et al*, 2004). Xiang and collaborators reported that a *KIT* exon 17 mutant isoform (Asp816Val) lacking the extracellular and transmembrane receptor domains still kept its kinase activity (Xiang Z *et al*, 2007). All *KIT* exon 13 or 17 mutations identified to date are missense changes (Rubin BP *et al*, 2007).

Interestingly, missense mutations in residue 816 (aspartic acid) are common in other *KIT* positive neoplasias, such as acute myeloid leukemia (AML), but were never found in GIST. In opposition, exon 11 mutations, the most frequent in GIST, occur rarely in other neoplasias. These observations suggest that *KIT* oncogene mutations are lineage specific (Rubin BP, 2006; Tornillo L and Terracciano LM, 2006).

PDGFRA mutations have been described in three different regions (Figure 1), namely exon 12 (~1%), exon 14 (<1%), and exon 18 (6%), which are homologue to exons 11, 13 and 17 of *KIT*, respectively (Rubin BP, 2006; Tornillo L and Terracciano LM, 2006). GIST harboring *PDGFRA* mutations share many clinical features with *KIT* mutated lesions, but the former are mainly gastric and present epithelioid morphology, myxoid stroma and weak or negative CD117 staining (Debiec-Rychter M *et al*, 2004; Wardelmann E *et al*, 2004; Tzen CY and Mau BL, 2005). The last feature highlights the need to combine pathological, molecular and genetic analysis in order to eliminate the risk of misdiagnosing *KIT*-negative GIST. Interestingly, evidence suggests that *PDGFRA* mutated tumors might be less aggressive (Rubin BP *et al*, 2007). It is also noteworthy that cases with the most frequent exon 18 mutation (Asp842Val, 62.6% of exon 18 mutated cases) are limited to the stomach, mesentery and omentum (Rubin BP *et al*, 2007; Corless CL and Heinrich MC, 2008). The non-random anatomic distribution of mutations seen in GIST may indicate that there is more than one population of ICC stem cells from which GIST may arise (Corless CL and Heinrich MC, 2008).

A subset of approximately 5 to 10% of GIST is negative for both *KIT* and *PDGFRA* mutations. In these cases, GIST could be originated by activating mutations either in a RTK analogous to *KIT* or *PDGFRA*, or in other downstream effectors of the signaling cascade (Heinrich MC *et al*, 2002; Rubin BP, 2006). These tumors may occur in the entire length of the GI tract (Rubin BP *et al*, 2007). It is curious that non-mutated GIST extracts have phosphorylated *KIT*, suggesting that *KIT* is still activated (Corless CL and Heinrich MC, 2008). It is also important to note that nearly 5% of GIST do not express CD117 or are equivocally positive, yet they may display *KIT* (>50%) or *PDGFRA* (~30%) mutations (Corless CL *et al*, 2004). The former occur mainly in exon 11 and have significant therapeutic implications. It is thus likely that immunohistochemistry lacks the sensitivity to detect the small amounts of mutant isoforms driving this subset of tumors.

Tumor progression and chromosome alterations

Oncogenic *KIT* or *PDGFRA* mutations are considered the primary genetic events in most sporadic and familial GIST, and the basis of its development. Whereas they seem vital to promote the neoplastic transformation, additional somatic alterations (secondary events) are presumably necessary for the biological and clinical progression of these tumors. Indeed, many studies have underlined the importance of cytogenetic alterations in GIST (El-Rifai W *et al*, 2000a; Kim NG *et al*, 2000; Debiec-Rychter M *et al*, 2001; Gunawan B *et al*, 2002; Sandberg AA and Bridge JA, 2002; Gunawan B *et al*, 2007). Genome-screening methodologies such as conventional cytogenetics and comparative

genomic hybridization (CGH), complemented by more sensitive and targeted approaches such as fluorescent *in situ* hybridization (FISH), have been applied in order to identify these changes.

Conventional cytogenetic analysis is a well-established source of diagnostic and prognostic information for hematological malignancies and sarcomas, but the requirement of fresh tissue for analysis and the difficulty in growing cancer cells *in vitro* limit its usefulness in many solid tumors. Other methodologies that could assess the genomic content of tumor samples needed to be implemented, and CGH was developed to meet this challenge (Kallioniemi A *et al*, 1992). This robust screening technique is based on competitive hybridization of tumor and normal DNA, labeled with different fluorochromes, onto normal chromosome spreads. CGH will detect genomic imbalances (specific gains and/or losses of DNA material) based on the fluorescence ratios measured after hybridization. This methodology does not require cell culture and will provide information on all chromosome pairs in one test, with the most relevant downside being the fact that it will miss balanced translocations. When specific genetic alterations are known or suspected, FISH analysis with probes targeting the regions of interest can be performed instead. The resolution and sensitivity of FISH is much higher, but the information it provides is limited to the regions under testing.

Early cytogenetic studies in tumors of the GI tract are scarce, and given the frequent misdiagnosis of GIST, mostly uninformative. It is interesting to note that some studies on GI lesions classified as leiomyosarcomas revealed a division of a particular group that did not show complex karyotypes, as expected, but instead displayed noncomplex karyotypes with deletions of chromosomes 14, 22, and 1p (Boghossian L *et al*, 1989; Bardi G *et al*, 1992; Sreekantaiah C *et al*, 1993; Saunders AL *et al*, 1996). More recent studies in sporadic GIST confirmed three major cytogenetic progression pathways, involving loss of the chromosome arm 1p, chromosome 14, or chromosome 22 (El-Rifai W *et al*, 2000a; Sandberg AA and Bridge JA, 2002; Gunawan B *et al*, 2007). It is striking that no chromosomal rearrangements targeting the 4q11-12 region (the location of both *KIT* and *PDGFRA* loci) have been described, indicating that activation of these genes occurs through oncogenic mutations (Heinrich MC *et al*, 2002).

Around two thirds of GIST samples show monosomy 14 (Kim NG *et al*, 2000; Miettinen M *et al*, 2001; Heinrich MC *et al*, 2002; Corless CL *et al*, 2004). At least two regions of this chromosome, 14q11.1-12 and 14q22-24, seem to be hotspots of deletions and likely harbor candidate TSG (El-Rifai W *et al*, 2000a; El-Rifai W *et al*, 2000b; Debiec-Rychter M *et al*, 2001). Deletions at 1p and 22q have been observed in up to 50% of the analyzed tumors, most frequently in advanced cases, suggesting the existence of TSGs in

this region that could be important in tumor progression (El-Rifai W *et al*, 2000a; Corless CL *et al*, 2004; Lasota J *et al*, 2005; Tornillo L and Terracciano LM, 2006).

Interestingly, the number and type of chromosomal changes seems to correlate with the clinical aggressiveness of the tumors (Heinrich MC *et al*, 2002). El-Rifai and cooperators analyzed a subset of 95 GIST and concluded that the mean number of changes increased from 2.6 in benign GIST to 7.5 in malignant primary tumors and up to 9 in metastatic lesions. These authors also showed that 9p deletions, 8q gains/amplifications, and 17q gains/amplifications, were found almost exclusively in high-grade or metastatic GIST (El-Rifai W *et al*, 2000a). Indeed, specific losses at 1p, 9p and 11p are clearly associated with malignancy (Bergmann F *et al*, 1998; Kim NG *et al*, 2000; Heinrich MC *et al*, 2003). A key gene in chromosome 9p is *CDKN2A* (p16INK4A), coding for an important inhibitor of the cell cycle frequently inactivated in GIST (Rubin BP *et al*, 2007). Based on these findings, a putative genetic pathway leading to the development and progression of GIST was proposed by Heinrich (2002): *KIT* or *PDGFRA* mutation → 14q deletion → 22q deletion → 1p deletion → 8q gain → 11p deletion → 9p deletion → 17q gain.

Although such a genetic fingerprint for GIST can be roughly defined, the target genes involved in these regions remain undiscovered (Heinrich MC *et al*, 2002). Also, assuming that mutant *KIT* isoforms are capable of constitutive activation and enhancement of target signaling cascades, the biological role (and mode of action) of these secondary chromosomal events remains unclear. Strikingly, only a few very recent reports have assessed both primary and secondary changes in the same samples, with limited success (Anderson J *et al*, 2002; Assämäki R *et al*, 2007; Wozniak A *et al*, 2007). Additional combined studies using molecular and cytogenetic methodologies are thus needed to clarify the relative contribution of mutations and gross chromosomal changes to the biological and clinical features of these tumors.

Prognostic factors

Considerable efforts have been placed in finding clinical, pathological or molecular markers of prognosis that could distinguish indolent GIST from the more clinically aggressive tumors, or possibly identify lesions with high metastatic potential (Liu XH *et al*, 2005; Martin J *et al*, 2005; Andersson J *et al*, 2006). This has proved a difficult task, however, and few variables currently show predictive or prognostic value. Tumor size and mitotic index remain the most relevant prognostic indicators in GIST, and the only consensus criteria were established in the GIST Workshop convened at the United States National Institute of Health in 2001 (Fletcher CD *et al*, 2002; Joensuu H, 2008).

Whereas these parameters have proved clinically useful in the classification of GIST, they are not fail-proof and additional markers are required. Indeed, it is not guaranteed that a lesion with less than 2 cm and a small mitotic index will have a benign or favorable course, as literature data shows that even these small tumors can occasionally metastasize (Fletcher CD *et al*, 2002; Rubin BP, 2006; Yang J *et al*, 2008). In view of these predicaments, many groups have shown reluctance in classifying GIST as “benign”, since any GIST presenting clinical symptoms has the potential to behave in a malignant fashion (Fletcher CD *et al*, 2002).

Tumor stage may also provide important information, as patients with resected primary GIST that recur within the peritoneum or that metastasize to the liver have poor prognosis (Fletcher CD *et al*, 2002), as do those with intra-abdominal dissemination (DeMatteo RP *et al*, 2000). Rubin and collaborators (2006) have demonstrated that anatomic location also shows prognostic significance, since gastric tumors and small-bowel lesions with the same size have distinct clinical outcomes (the former presenting better prognosis). Anatomic location also determines the risk of recurrence and progression, a fact acknowledged in the 2007 National Comprehensive Cancer Network risk stratification criteria (Demetri GD *et al*, 2007; Yang J *et al*, 2008). However, a study comprising 1765 GIST does not support this approach (Miettinen M *et al*, 2005b). Nevertheless, anatomic site was already considered as a prognostic factor in recent studies (Corless CL and Heinrich MC, 2008). Other variables such as mucosal invasion, tumor necrosis, and high cellularity have been statistically associated with an aggressive phenotype, but these have been poorly reproducible and do not show consistent results that can be used on an individual basis (Fletcher CD *et al*, 2002; Goh BK *et al*, 2008).

Interestingly, several studies indicate that the presence of *KIT* mutations confers a malignant and more aggressive behavior to GIST (Ernst SI *et al*, 1998; Taniguchi M *et al*, 1999; Kim TW *et al*, 2004; Liu XH *et al*, 2005; Martin J *et al*, 2005; Cho S *et al*, 2006). However, the presence of these alterations is not by itself able to differentiate the degree of aggressiveness of these neoplasias or predict the likelihood of recurrence after resection of a primary GIST (Rubin BP *et al*, 2001; Corless CL *et al*, 2002; Heinrich MC *et al*, 2002; Heinrich MC *et al*, 2003). Moreover, small GIST (even tumors <10 mm) have been shown to display *KIT* mutations, raising the question whether *KIT* mutations actually influence outcome (DeMatteo RP *et al*, 2008).

At the molecular level, *KIT* exon 11 point-mutations or insertions have been shown to confer a favorable prognosis, and the former are even associated with longer recurrence-free survival after surgical resection (Singer S *et al*, 2002). Some studies report that deletions in exon 11 (Andersson J *et al*, 2006; Cho S *et al*, 2006), and more

specifically in codons 557-558 (Wardelmann E *et al*, 2003; Martin J *et al*, 2005; Miettinen M and Lasota J, 2006; DeMatteo RP *et al*, 2008) are associated with poor prognosis in patients with completely resected GIST. Exon 9 mutations and exon 13 deletions have also been associated with a poor prognosis (Lasota J *et al*, 2000; Sakurai S *et al*, 2001; Antonescu CR *et al*, 2003; Lasota J *et al*, 2003). Tumors with *PDGFRA* mutations tend to be less aggressive than those with *KIT* mutations, but they may also progress and lead to patient death (Lasota J *et al*, 2004; Lasota J *et al*, 2006; Corless CL and Heinrich MC, 2008). In disagreement with these assumptions, a recent Taiwanese study of 134 GIST (99% with *KIT* mutations, 1% with *PDGFRA* mutations) found no association between the type or location of *KIT* mutations and progression-free survival rates (Yang J *et al*, 2008). This study also raises the question of the prognostic significance of different mutations according to racial differences, which requires further investigation.

It has been suggested that once GIST become metastatic, the specific kinase genotype does not influence overall survival (Gold JS *et al*, 2007), and that the prognosis at the time of clinical presentation is clearly influenced by additional genetic events (Corless CL *et al*, 2004; Rubin BP, 2006). Additional studies are therefore required to determine the possible association of molecular and/or cytogenetic alterations to the prognosis of GIST, and their possible contribution for treatment recommendations (Raut CP and DeMatteo RP, 2008).

Therapeutic options in GIST

Surgery

Surgical resection is the standard therapy for non-metastatic, operable GIST. In primary tumors, the aim is complete resection without rupture of the tumor capsule, on condition that the risk of dysfunction of the affected organ is low. However, 40 to 80% of cases are inoperable or recur. After surgery, five year disease-free survival rates for patients with primary GIST range from 43 to 65% (Di Matteo G *et al*, 2002; Rossi CR *et al*, 2003). Due to the specific metastatic pattern of GIST, lymphadenectomy is not advised, with the exception of rare cases in which ganglia involvement is clear. Surgery is not curative for recurrent or metastatic lesions (Corless CL and Heinrich MC, 2008).

Chemotherapy and/or radiotherapy

Traditional chemotherapy and/or radiotherapy are not efficient in inoperable or metastatic lesions. In general, only ~5% of these patients respond to treatment, and the median survival is ~18 months (Corless CL and Heinrich MC, 2008; DeMatteo RP *et al*, 2008).

Imatinib treatment (first line therapy)

The inefficiency of the traditional therapeutic options for metastatic or unresectable GIST and the knowledge that activating *KIT* mutations play a pivotal role in the carcinogenesis of these tumors led to the application of targeted therapy for the protein product of this oncogene. The selected drug, Imatinib mesylate (STI-571, commercial name Gleevec or Glivec) is an orally bioavailable 2-phenylpyrimidine derivative, developed originally in the 1990s to treat chronic myelogenous leukemia (CML). Indeed, Imatinib was developed to inhibit the ABL kinase (BCR-ABL protein fusion) in CML cells by occupying the ATP binding pocket of the ABL kinase domain (Antonescu CR, 2008; Corless CL and Heinrich MC, 2008). It was later demonstrated that Imatinib had the capacity to inhibit several other tyrosine kinases, namely KIT, PDGFRA and PDGFRB, ARG, and ABL (Buchdunger E *et al*, 1996; Heinrich MC *et al*, 2002; Mol CD *et al*, 2004; Dewar AL *et al*, 2005). ABL shares considerable homology with the type III receptor tyrosine kinase family and, in the particular case of the KIT receptor, Imatinib was shown to inhibit *in vitro* a mutant form of KIT commonly found in GIST, and also the growth of cultured GIST cells harboring *KIT* mutations (Tuveson DA *et al*, 2001; Heinrich MC *et al*, 2006).

As for the ABL kinase, Imatinib inhibits KIT tyrosine kinase activity, preventing phosphorylation of downstream effectors and thus suppressing the proliferation of GIST (Savage DG and Antman KH, 2002; Scheijen B and Griffin JD 2002; Tamborini E *et al*, 2004). In 2000, Imatinib was administered to a patient with metastatic GIST, unresponsive to multiple conventional therapies, and a reduction of 75% was verified in tumor size and number of metastases (Joensuu H *et al*, 2001). In 2002, after multiple clinical trials, this drug was approved by the Food and Drug Administration as a standard therapy for patients with inoperable tumors or with metastatic disease, serving as a paradigmatic example of a targeted therapy for a specific molecule in solid tumors (Rubin BP, 2006).

However, several studies quickly demonstrated an association between *KIT* and *PDGFRA* mutations and different responses to Imatinib treatment (Heinrich MC *et al*, 2003; Corless CL *et al*, 2004; Gupta P *et al*, 2008; Heinrich MC *et al*, 2006). Indeed,

patients with *KIT* exon 11 mutations have shown a more favorable response (83,5%) relatively to the ones with *KIT* exon 9 mutations (48.7%) or the ones negative for both genes (weak response). Interestingly, these results are not in accordance with previous *in vitro* trials, in which it was possible to inhibit the growth of cell lineages of GIST with *KIT* exon 9 mutations (Heinrich MC *et al*, 2002). Due to the small number of cases with *KIT* exon 13 or 17 primary mutations, only limited correlations could be inferred (Lasota J *et al*, 2008). Interestingly, the missense mutation found in *KIT* exon 13 (Lys642Glu) is thought to originate Imatinib resistance (Tornillo L and Terracciano LM, 2006), although *in vitro* studies suggested inhibition of this isoform by Imatinib (Tuveson DA *et al*, 2001).

In agreement with *in vitro* assays, patients with the Asp842Val mutation in *PDGFRA* respond weakly to Imatinib (Antonescu CR *et al*, 2003; Corless CL *et al*, 2004; Corless CL *et al*, 2005). Mutations in exon 12, however, show good response to Imatinib and the single rare mutation described in exon 14 showed *in vitro* sensitivity to Imatinib (Tornillo L and Terracciano LM, 2006). More clinical studies are clearly needed to assess the predictive value of these uncommon alterations, as well as to clarify the differences between *in vivo* and *in vitro* assays.

During treatment with Imatinib, several patients eventually develop secondary resistance that can be translated in growth of a nodule within preexisting, clinically quiescent lesions, or widespread expansion of metastatic lesions throughout the liver or abdominal cavity (Desai *et al*, 2007). Preliminary studies revealed the existence of, at least, four mechanisms that can dramatically inhibit the action of this drug: 1) acquisition of a secondary mutation in the TK domain of *KIT* or *PDGFRA*; 2) gene amplification leading to *KIT* or *PDGFRA* over-expression; 3) functional resistance in GIST that present kinase activity and that are sensitive to Imatinib *in vitro*, but not *in vivo*; 4) activation of alternative kinases in the same signaling pathways (Corless CL *et al*, 2004). Tumors showing evidence of *KIT* gene amplification have been occasionally described (Antonescu CR *et al*, 2005; Debiec-Rytcher M *et al*, 2005). More rarely, there is downregulation of *KIT* expression, suggesting the emergence of a *KIT*-independent phenotype. In their series of resected GIST from Imatinib-treated patients, Agaram and colleagues identified several mitotically active lesions that were p53 immunopositive and two tumors that had *TP53* mutations (Agaram NP *et al*, 2007). It is likely that deregulation of the cell cycle through such mutations contributes to Imatinib resistance. In the majority of primary resistance cases, however, the mechanism for drug escape remains unknown. Acquisition of secondary mutations that affect the site of Imatinib binding seems more frequent (Corless CL and Heinrich MC, 2008). Several secondary point mutations were already identified

and it was observed that generally they are present in the same allele affected by primary mutations (Miettinen M and Lasota J, 2006).

Imatinib treatment (400-800 mg/day) is generally well tolerated and, in most cases, patients achieve complete or partial remission. This treatment increased the 2-year survival of patients with advanced or metastatic disease from 20% to 70% (Glabbeke MV *et al*, 2006). Debiec-Rychter and colleagues found that the progression-free survival of GIST patients with *KIT* exon 9 mutations was significantly better when they were treated with 800 mg per day as compared with 400 mg per day (Debiec-Rytcher M *et al*, 2006). However, long-term disease control is limited by the acquisition of resistance to this drug. Due to the fact that such resistance is becoming a significant clinical problem in the treatment of this neoplasia, several trials with new targeted drugs are underway, underlining the importance of genetic studies aiming at identifying novel alterations with prognostic or predictive value or that may work as therapeutic targets in GIST.

Sunitinib treatment (second line therapy)

Patients with inoperable GIST showing Imatinib resistance or intolerance had no efficient alternative therapeutics until the introduction of SU11248 (Sunitinib Malate or SUTENT, Pfizer, New York, USA), recently approved by the FDA as a second line treatment in GIST. Sunitinib is an inhibitor of several TKs that shows anti-angiogenic and anti-tumoral activity in several tumor models both *in vitro* and *in vivo* (Demetri GD *et al*, 2006). These effects have been associated to signaling block of *KIT*, PDGFRs, VEGFRs, FLT3 and RET receptors (Corless CL and Heinrich MC, 2008). Despite the fact that both Sunitinib and Imatinib target the ATP binding domain of the TKs, they belong to different chemical classes and presumably present dissimilar characteristics and affinities of binding (Demetri GD *et al*, 2006).

Nevertheless, the inhibition of several signaling pathways by Sunitinib might be more efficient, given the fact that Imatinib has exclusive affinity to a limited number of TK domains. Patients treated with Sunitinib show good results, namely an increase in disease-free survival and overall survival rates. Future studies with Sunitinib in GIST are however needed to investigate the molecular mechanisms through which the drug exerts disease control after Imatinib failure (Demetri GD *et al*, 2006). Preliminary analysis demonstrated that treatment with Sunitinib results in better anti-tumoral response rates and clinical improvement in patients with *KIT* exon 9 primary mutations or *KIT*-negative tumors compared with exon 11 mutated lesions (Demetri GD *et al*, 2006). This data demonstrates that Sunitinib is an effective therapeutic option for patients with metastatic,

Imatinib resistant GIST. As for Imatinib, however, mutations have already been described (particularly in exon 17) that confer cross-resistance to Sunitinib. It is therefore predictable that secondary resistance may develop in patients that initially respond to this second line drug (Corless CL and Heinrich MC, 2008). The role of recurrent chromosome changes in therapy resistance to Imatinib or Sunitinib is currently unknown.

AIMS

A diagnosis of GIST requires a multidisciplinary approach that combines clinical, pathological, and genetic features. Our main goal was to characterize primary and secondary genetic events in a consecutive series of GIST, in order to contribute to their correct diagnosis and to identify genetic aberrations with prognostic and/or predictive value for these patients.

The specific aims of this project were:

- o To identify primary genetic alterations in GIST by direct sequencing of the oncogenes *KIT* and *PDGFRA*.
- o To characterize the spectrum of chromosomal alterations (secondary genetic events) in GIST using comparative genomic hybridization, a powerful whole-genome screening methodology.
- o To assess possible correlations between primary and secondary genetic events and between these and the clinico-pathological features of GIST.
- o To assess the prognostic value of genetic events (irrespective of therapy) and the possible correlations between genetic events and response to therapy, namely mutations associated with Imatinib or Sunitinib resistance.

MATERIAL AND METHODS

Patient selection

A series of 78 patients diagnosed with GIST and submitted to surgery with curative intent were included in this study. The majority of patients was diagnosed and treated at the Portuguese Oncology Institute – Porto, with the exception of six cases that were provided by other institutions. Patients had received no treatment prior to surgery. All samples were obtained after informed consent. Fresh-frozen tumor samples of 27 patients (conserved in RNA later) could be obtained. For all the remaining cases, genetic analyses were performed in formalin-fixed, paraffin-embedded tissue sections. In all cases, H&E sections from representative tissue blocks were reviewed by expert pathologists to confirm a diagnosis of GIST and to evaluate relevant histopathological parameters. Other clinical and demographic variables, such as age at diagnosis, gender, tumor size, and tumor location (divided into stomach, small intestine, rectum and colon, and outside the GI), were obtained (Table 1). Patients with tumors that eventually recurred or developed metastatic lesions were treated with Imatinib in accordance with the guidelines followed at the IPO-Porto. Second-line therapy for patients that progressed or were intolerant to Imatinib was Sunitinib.

DNA extraction from paraffin-embedded histological sections

H&E stained sections for each formalin-fixed, paraffin-embedded tumor sample were reviewed by pathologists and relevant areas with >80% neoplastic cells were delimited. Additional 10µm unstained sections were sequentially obtained from the same tissue blocks, placed in poli-L-lisine coated slides and dried at 37°C overnight. Relevant tumor areas were identified by overlapping these slides with the respective H&E sections, and after humidification with buffer, tumor fragments were macrodissected using a sterile blade and collected to 1.5 mL tubes. DNA isolation was performed using an adaptation of the technique described by Lungu and colleagues (Lungu O *et al*, 1992).

Briefly, lysis buffer was prepared with 50 µl Tris-HCL 1M (pH 8.5), 10 µl of EDTA, 5µl of Tween 20 and 935 µl of bi-distillate water. A buffer volume proportional to the tumor area and 4 µl of proteinase K (10mg/mL) were added, and samples were incubated 2 hours at 55°C. The enzyme was inactivated by temperature shock (10 minutes at 95°C followed by 15 minutes on ice), and tubes were centrifuged 5 minutes at 14000 rpm. The supernatant (containing the DNA) was transferred to a new 1.5 mL tube. Double-stranded DNA concentration and purity were quantified using a NanoDrop ND-1000

spectrophotometer. DNA quality was evaluated by electrophoresis in ethidium-bromide stained agarose gel 0.8% (p/v).

DNA extraction from fresh-frozen tumor samples

After physical disaggregation of the fresh-frozen tissue fragments using a sterile blade, DNA was extracted following the salting-out-chloroform mixed methodology described above.

DNA extraction from peripheral blood

In order to include a negative control in every set of amplifications, and to have control electrophorograms to compare with the tumor sequences, DNA from a healthy donor was extracted. A peripheral blood sample was collected inside sterilized tubes containing EDTA and placed at 4°C for immediate use. Lyses of erythrocytes was achieved by adding hypotonic solution (AKE: NH₄Cl 155mM; KHCO₃ 10mM; EDTA 0,1mM; pH 7,4) in a proportion of 10 times the available blood volume (3-5 mL). After 30 minutes incubation at 4°C, tubes were centrifuged 10 minutes at 2000 rpm. The supernatant was discarded and the process was repeated until the sediment was free of haemoglobin. The resulting nuclear sediment was transferred to 1.5 mL tubes and maintained at 80°C until DNA extraction. Genomic DNA was isolated using the salting-out-chloroform mixed technique (Müllenbach R *et al*, 1989). This protocol combines the classic phenol/chloroform methodology with the salting-out technique, eliminating the disadvantages of using phenol (classic technique), as well as reducing the DNA losses that occur when DNA is precipitated with proteins (salting-out technique).

KIT and PDGFRA mutation screening

Using the DNA extracted from each sample, *KIT* (exons 9, 11, 13, and 17) and *PDGFRA* (exons 12, 14, and 18) target sequences were amplified by polymerase chain reaction (PCR) on a standard thermocycler. Primers and conditions were as described in the literature (Penzel R *et al*, 2005; Daum O *et al*, 2007). Negative controls were included in every set of amplifications. PCR products were purified using the “GFX PCR DNA and Gel Band Purification Kit” [GE Healthcare], according to the manufacturer’s recommendations, to remove non incorporated salts, enzymes, nucleotides and primers. Samples were eluted in bi-distillate water and subsequently evaluated by electrophoresis in ethidium-bromide stained agarose gel 2% (p/v).

For the sequencing reactions, 30 to 90 ng of purified DNA were used. Primers and conditions were as described in the literature (Penzel R *et al*, 2005; Daum O *et al*, 2007). The resulting products were purified to remove excess dNTPs, labeled ddNTPs and non incorporated primers using standard methods. Briefly, each sequencing product was mixed with 2 μ L of sodium acetate 3M and 50 μ L of ethanol 96% (v/v). Tubes were vortexed and kept on ice for 30 minutes. After that, samples were centrifuged 30 minutes at 14000 rpm/4°C. The supernatant was discarded and the sediment was washed with 250 μ L of ethanol 70% (v/v), dried and eluted in deionized formamide. After denaturation (5 minutes at 95°C), the purified sequencing products were submitted to electrophoresis in a capillary column. Direct sequencing was performed on an ABI PRISM 310 automatic sequencer using the Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA), according to the manufacturer's recommendations. All results were confirmed with a second independent analysis.

Comparative genomic hybridization analysis

Fresh-frozen tumor samples from 27 patients were analyzed by CGH, following the procedure of Kallioniemi *et al* (1992), with modifications previously described (Ribeiro FR *et al*, 2006). Briefly, test (tumor tissue) and reference (peripheral blood lymphocytes from healthy donors) DNA was labeled in nick-translation reactions using SpectrumGreen and SpectrumRed-conjugated nucleotides (Vysis, Downers Grove IL), respectively. Labeled tumor and reference DNA were mixed with unlabeled Cot-1 DNA (Life Technologies, Rockville, MD) and the probe mixture was denaturated and hybridized to commercially available, normal metaphase slides (Vysis). After washing off excess probe, samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in an antifade solution (Vector Laboratories, Burlingame, CA). Samples were analyzed with a Cohu 4900 CCD camera using an automated filter wheel coupled to a Zeiss Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) and a CitoVysion system version 3.9 (Applied Imaging, Santa Clara, CA). Data from ten cells was combined to generate average ratio profiles with 99% confidence intervals for each sample. Aberrations were scored whenever the sample profile and the standard reference profile at 99% did not overlap (Kirchhoff M *et al*, 1998). Description of the CGH copy number changes followed the guidelines suggested by the International System for Human Cytogenetic Nomenclature 2005.

Statistical analysis

Relevant clinical (gender, age, tumor size, tumor location and patient risk groups), and genetic variables (mutation status and chromosomal imbalances) were cross-tabulated in order to assess possible relationships, using the chi-square or Fisher's exact test. The number of chromosomal aberrations was compared within groups of samples with different mutation genotypes using the non-parametric Mann-Whitney U test. Kaplan-Meier survival curves using log-rank test were computed for relevant clinical and genetic events. A p-value lower than 0.05 was considered statistically significant. All statistical analysis were performed using the Statistical Package for Social Sciences (SPSS) software, version 15.

RESULTS

Clinicopathologic characteristics of the patients

A total of 78 patients diagnosed with GIST were enrolled in this study. Clinical and demographic variables are summarized in Table 1. Out of the 78 patients, 32 were male (41%) and 46 female (59%), with a median age at diagnosis of 61 years (ranging from 24 to 84). Tumor location was obtainable in 76 cases, from which 64 corresponded to primary lesions. Nine metastatic lesions were analyzed due to lack of the primary sample. For the three remaining patients, the type of tumor sample collected was unclear. A total of 36 tumors were located in the stomach (47.4%), 20 in the small intestine (26.3%), four in the colon and one in the rectum (6.6%), and 15 outside the GI (19.7%). For four of the patients, a second sample collected after disease progression could additionally be assessed (Table 1), increasing the number of lesions submitted to sequencing analysis to 82. Tumor size was recorded in 70 cases and varied from 1.2 to 45 cm (average 8.8 cm). Concerning cellular morphology, the series included 52 spindle cell tumors (73.2%), six epithelioid lesions (8.5%), and 13 mixed tumors (18.3%). Based on the consensus criteria proposed by Fletcher (2002), tumors in this series could be classified as low/very low risk (n=16), intermediate risk (n=19) and high risk (n=37) (Table 2).

Immunohistochemistry

Expression of the KIT protein (CD117) was assessed in 74 cases. A total of 70 lesions (94.6%) showed a positive staining pattern, whereas two cases were negative and two cases presented unclear results. Concerning other protein markers, 57 out of 70 cases (81.4%) showed positivity for CD34, 35 in 68 (51.5%) were positive for SMA, 6 out of 66 (9.1%) for S100 protein, 4 out of 57 (7%) for desmin, and 41 out of 46 (89.1%) for vimentin.

KIT and PDGFRA mutation screening

Samples from all 78 patients were screened for mutations within exons 9, 11, 13, and 17 of the oncogene *KIT*. Mutations were detected in 59 tumors (75.7%), namely in exon 11 (n=52, 66.7%) and exon 9 (n=7, 9%). No primary mutations were found in exons 13 or 17. All *KIT* negative cases (n=19) were then analyzed for mutations in exons 12, 14, and 18 of *PDGFRA*. A total of nine samples (11.5%) showed mutations in this gene, namely in exon 18 (n=6, 7.7%), exon 12 (n=2, 2.6%) and exon 14 (n=1, 1.3%).

Interestingly, CD117 staining was seen in six out of eight *PDGFRA* positive cases. The overall mutation frequency for both genes in this series was 87.2% (68 out of 78 tumors). A comprehensive list of the detected mutations, together with relevant clinical parameters, is detailed in Table 3.

All *KIT* exon 9 positive cases harbored the same hotspot duplication p.Ala502_Tyr503dup (Figure 3). Tumor location was available for all samples and varied markedly, with three lesions arising in the small intestine and the remaining in the stomach (n=2) or peritoneum (n=2). *KIT* exon 11 mutations were all in-frame, namely deletions (n=16), insertions (n=1), delins (n=10), duplications (n=7), and missense (n=18). Whereas missense mutations affected exclusively codons 556, 557, 560 and 576, duplications clustered in the end of the exon and deletions were found mostly in the beginning of the exon (Figure 4). Tumor location was available for 50 of these patients, with most lesions appearing in the stomach (n=22, 44%), followed by those in the small intestine (n=14, 28%), outside GI [peritoneum (n=8, 16%) or liver (n=2, 4%)], and colon or rectum (n=4, 8%). Of note, two tumors with *KIT* exon 11 primary mutations and with an initial positive response to Imatinib, acquired resistance and developed peritoneal or hepatic metastases. Samples of a metastatic lesion of each patient could be analyzed, and the same secondary mutation (p.Val654Glu) was found in *KIT* exon 13 in both cases (Figure 5).

Regarding *PDGFRA* mutations, four positive cases for exon 18 displayed the hotspot missense change p.Asp842Val (Figure 6), associated with primary resistance to Imatinib, whereas two showed an in-frame deletion in this exon. The two mutations in exon 12 were delins and missense, respectively, whereas the sole mutation in exon 14 was missense. All *PDGFRA* positive tumors were located in the stomach, except for one in the small intestine and one in the peritoneum. The 10 tumors (12.8%) negative for both *KIT* and *PDGFRA* mutations were located in the stomach (n=6), colon, small intestine, and peritoneum (n=2).

Comparative genomic hybridization findings

Out of the 27 GIST submitted to whole-genome screening, 23 (85%) displayed copy number changes (Table 4, Figure 7). Most abnormal samples displayed non-complex profiles, with a median of three aberrations per tumor (ranging from one to 28 changes). Losses were 1.5 times more frequent than gains, and it is noteworthy that complete or partial loss of chromosome 14q was seen in 21 samples (91.3%). In four patients, loss of 14q was the sole copy number change detected. Other frequent changes

included losses at chromosomal regions 22q (43.5%), 1p (43.5%), and 15q (34.8%) and gains at 1q (17.4%) and 12q (17.4%). All 23 cytogenetically abnormal GIST presented at least one of the losses 1p, 14q, and 22q (Figures 8-9).

KIT/PDGFR α genotype and correlations with cytogenetic changes

Based on previous literature findings, samples submitted to CGH analysis were divided according to mutation genotypes to test for possible correlations. Genomic results were thus compared between samples with *KIT* exon 9 mutations (n=3), *KIT* exon 11 deletions/delins (n=8) or samples with no detectable mutations (n=1), totaling 12 cases associated in the literature with bad prognosis, versus samples with *KIT* or *PDGFR α* mutations not previously associated with a worse prognosis (n=11). Strikingly, the former showed significantly more copy number changes (median of 6.5 versus 2 aberrations per tumor, $p=0.025$, Mann-Whitney U test). The three cases with *KIT* exon 9 mutations showed the most complex CGH profiles (median of 9 aberrations per tumor), followed by those with exon 11 deletions/delins (median of 4 aberrations per tumor). It is noteworthy that three of the four cases without detectable CGH alterations showed no mutations in either *KIT* or *PDGFR α* . No significant associations were observed between specific copy number changes and different mutation subgroups (Table 3). Indeed, tumors with *PDGFR α* mutations showed the same pattern of alterations seen in those with *KIT* mutations, even if genomic complexity was much reduced (median of 2 vs. 6 alterations per tumor, respectively).

Therapeutic correlations and survival data

Follow-up data (median of 30 months, ranging from 8 to 123 months) was available in 74 cases. During this period, 26 patients (35%) showed disease progression and were subsequently treated with Imatinib. According with available clinical records, 19 responded partially to this therapy, but most tumors eventually progressed. The latest data indicates that six of the 26 patients died from their cancer, whereas one died from non-related causes. Fifteen are alive with disease and only three remain free of disease (after secondary surgery for metastatic events). Most of these samples showed *KIT* mutations, namely in exon 11 (n=21) and exon 9 (n=3), with two patients showing no mutations in either gene. The 48 patients that received no adjuvant therapy are currently alive without evidence of disease, with the exception of three non disease-related deaths (also without evidence of disease). Within this group, 41 tumors harbored mutations, namely in *KIT* exon 11 (n=28), *KIT* exon 9 (n=4), *PDGFR α* exon 12 (n=2), *PDGFR α* exon

14 (n=1), and *PDGFRA* exon 18 (n=6). All *PDGFRA* positive patients are thus alive with no evidence of disease.

Disease-specific survival curves could not be computed due to the reduced number of valid death-from-disease events (n=4). Given the significant worse prognosis seen in patients presenting metastatic disease at diagnosis (p=0.002, Figure 10a), five-year disease-free survival curves were computed only for the subgroup of patients with primary disease (n=60 valid cases, 12 progression events). Stratification according to tumor location showed that lesions in the small intestine or colon progressed much more frequently than those located in the stomach or outside the GI (p<0.001, Figure 10b). Most progression events were also seen in lesions categorized as high risk, with those in the low or intermediate groups showing significantly less recurrences (p=0.026, Figure 10c).

When patients were categorized based on genetic variables, namely the presence of mutations in either *KIT* or *PDGFRA*, a trend was observed towards a more aggressive outcome in patients with *KIT* mutations compared to those with *PDGFRA* mutations (p=0.081, Figure 11a). Based on previous literature reports, patients were additionally categorized according to specific mutations associated with worse prognosis. Interestingly, patients with *KIT* exon 9 or *KIT* exon 11 deletions/delins (n=22 valid cases) showed significantly worse progression-free intervals (p=0.031, Figure 11b) than those showing mutations in *PDGFRA* or other mutations in *KIT* (n=31 cases). Within the subgroup of patients with *KIT* exon 11 mutations (n=52), the number of progression events in lesions with deletions/delins was significantly higher than those with other mutations (p=0.003, Fisher's test). In multivariate analysis including mutation status, tumor location and risk groups, tumor location was selected in the Cox-regression model as the best predictor of disease relapse (p=0.016, 95% confidence interval 1.6-100.7).

In the smaller subgroup of patients with CGH data and valid follow-up (n=26, metastatic lesions included), genomic complexity, coded as more than 3 aberrations per tumor, was very strongly associated with a worse outcome for the patients (p=0.003, Figure 11c). The presence of genomic gains (p=0.020, Figure 11d), 1p deletions (p=0.036, Figure 11e) or 22q deletions (p=0.031, Figure 11f) was also significantly associated with a shorter progression-free period. Multivariate survival analysis in this subset (using tumor location, risk groups, genomic gains and losses at 1p and 22q) showed that the best predictor of progression was the presence of genomic gains (p=0.05, 95% CI 1.0-69.5).

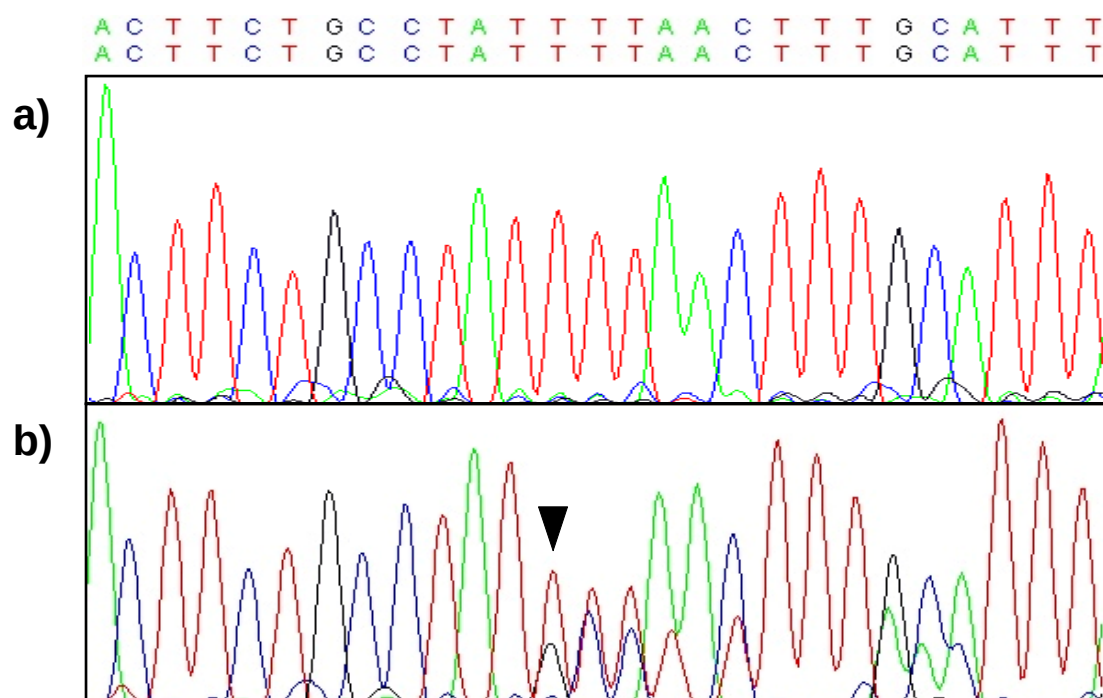


Figure 3. Electropherogram illustrating a partial sequence of *KIT* exon 9. **a)** normal control; **b)** mutation p.Ala502_Tyr503dup (arrow).

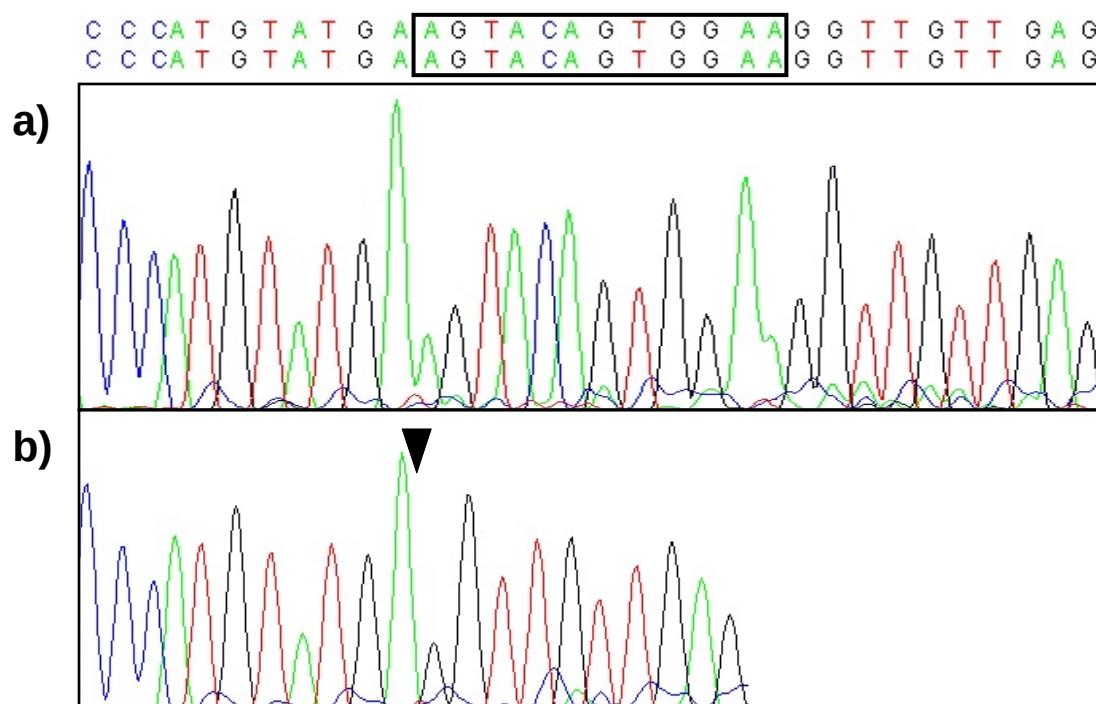


Figure 4. Electropherogram illustrating a partial sequence of *KIT* exon 11. **a)** normal control; **b)** mutation p.Trp557_Lys558del (box and arrow).

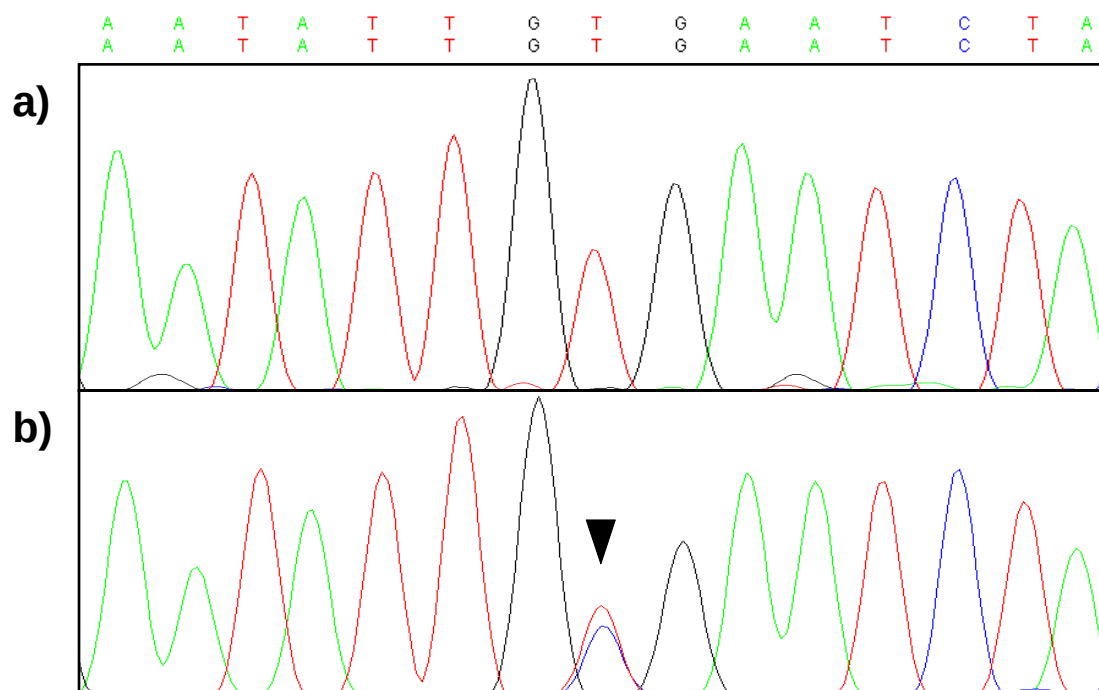


Figure 5. Electropherogram illustrating a partial sequence of *KIT* exon 13. **a)** normal control; **b)** mutation p.Val654Glu (arrow).

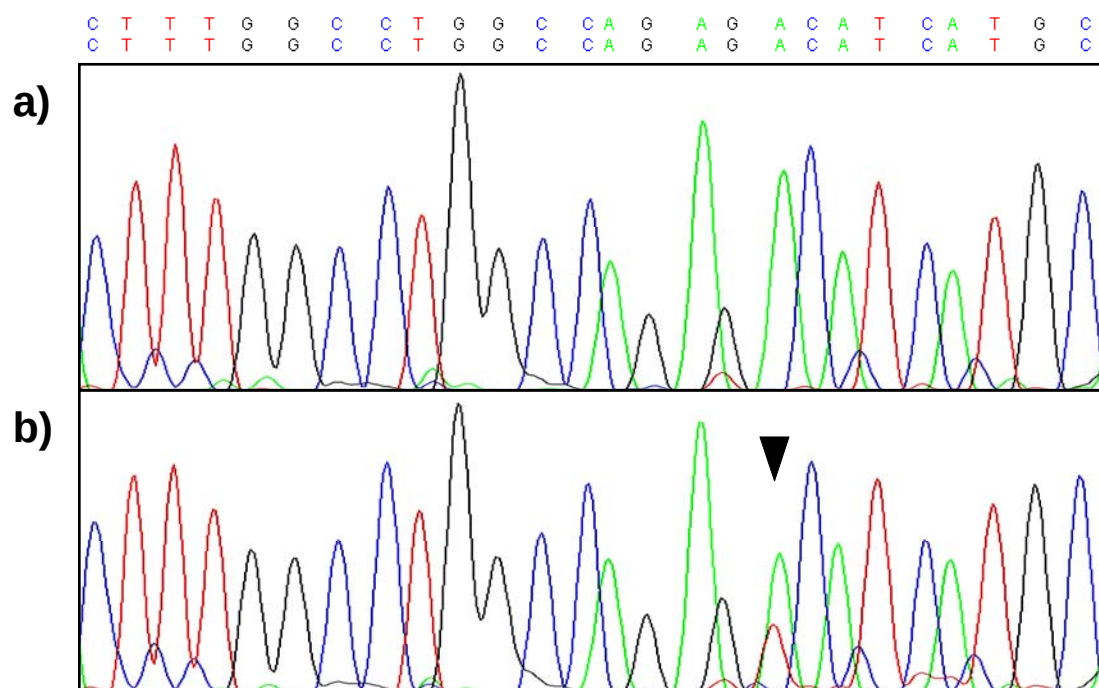


Figure 6. Electropherogram illustrating a partial sequence of *PDGFRA* exon 18. **a)** normal control; **b)** mutation p.Asp842Val (arrow).

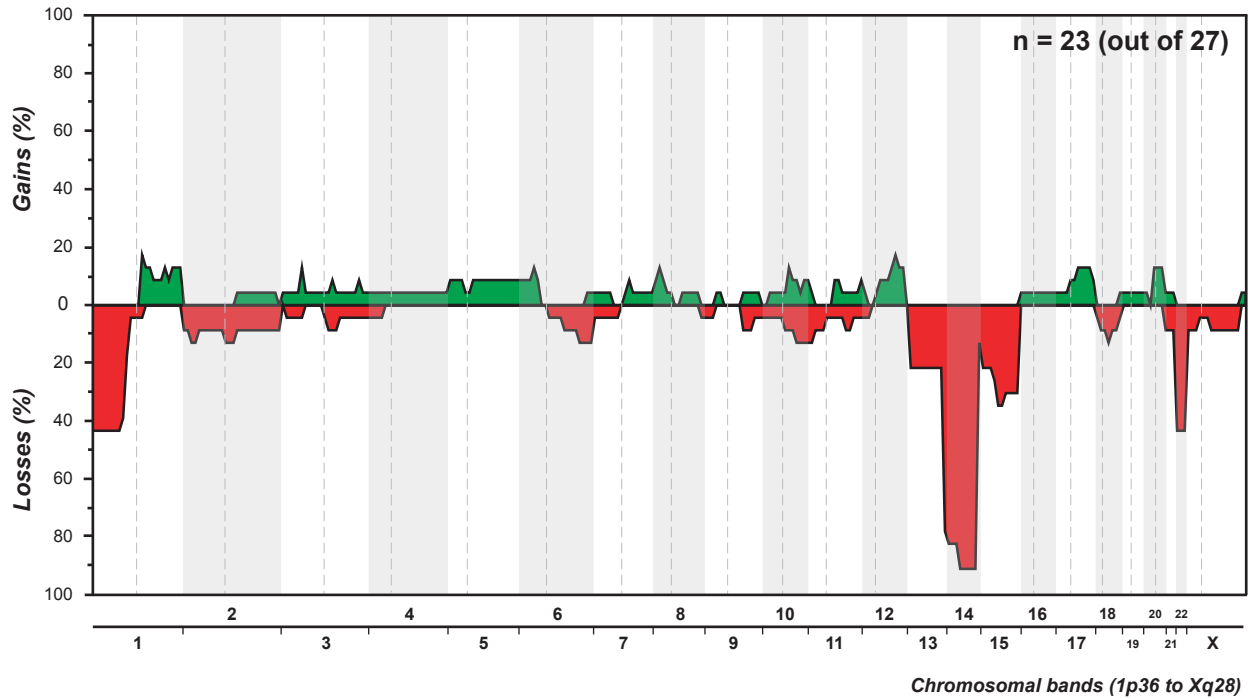


Figure 7. Copy number profile of patients diagnosed with GIST (n=23 abnormal samples). Gains and losses of genetic material are depicted along all chromosomes (X axis).

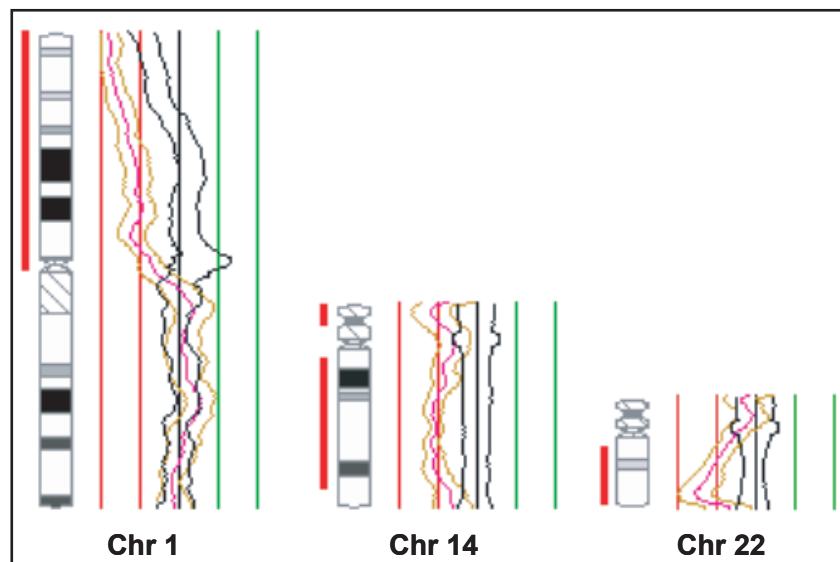
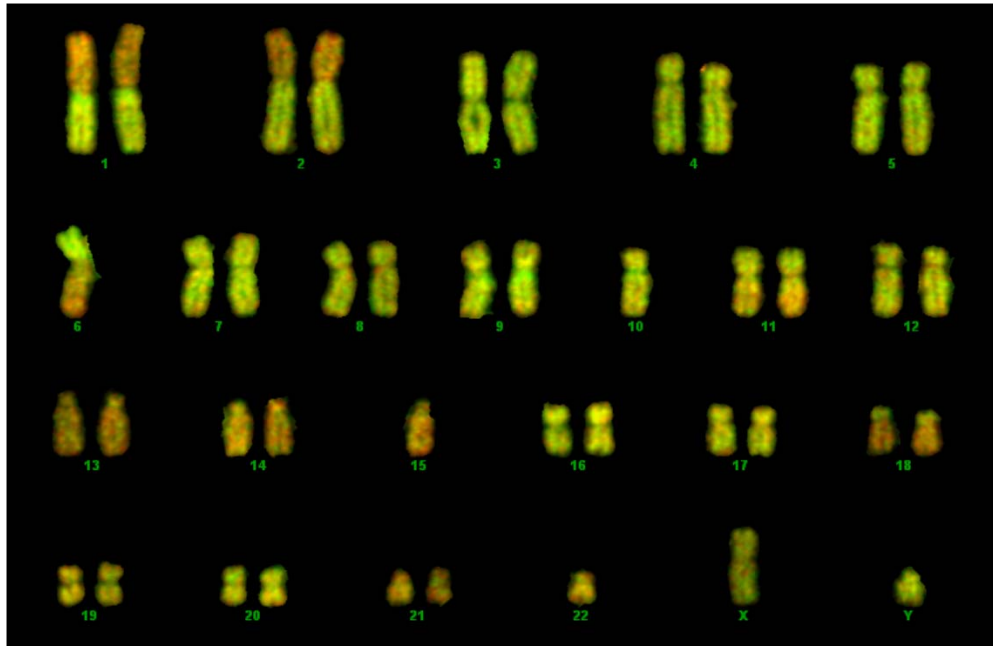


Figure 8. Partial ideograms of the chromosomal imbalances most frequently found in GIST, namely losses at 1p, 14q and 22q

a)



b)

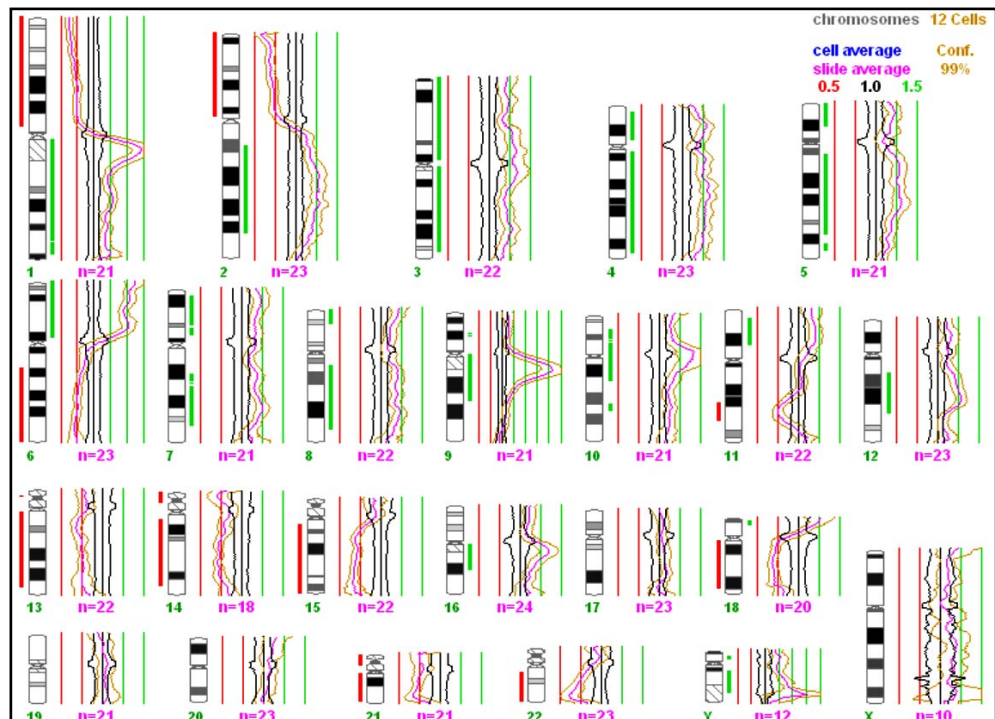


Figure 9. Genomic imbalances detected in patient 33 using comparative genomic hybridization. **a)** representative karyogram; **b)** Ideogram showing score results. Gains are shown in green and losses are shown in red. Note the coexistence of deletions at 1p, 14q and 22q.

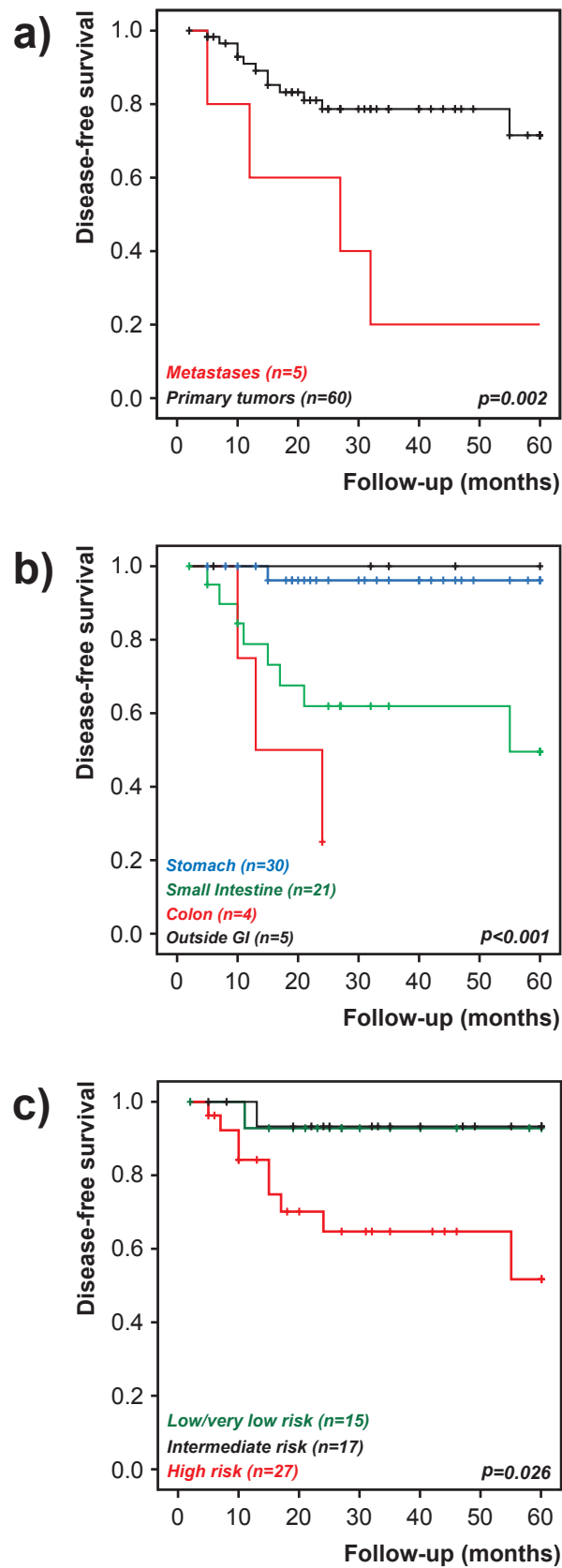


Figure 10. Five-year disease-free survival curves based on clinical variables:
a) type of sample at diagnosis; **b)** tumor location; **c)** risk groups.

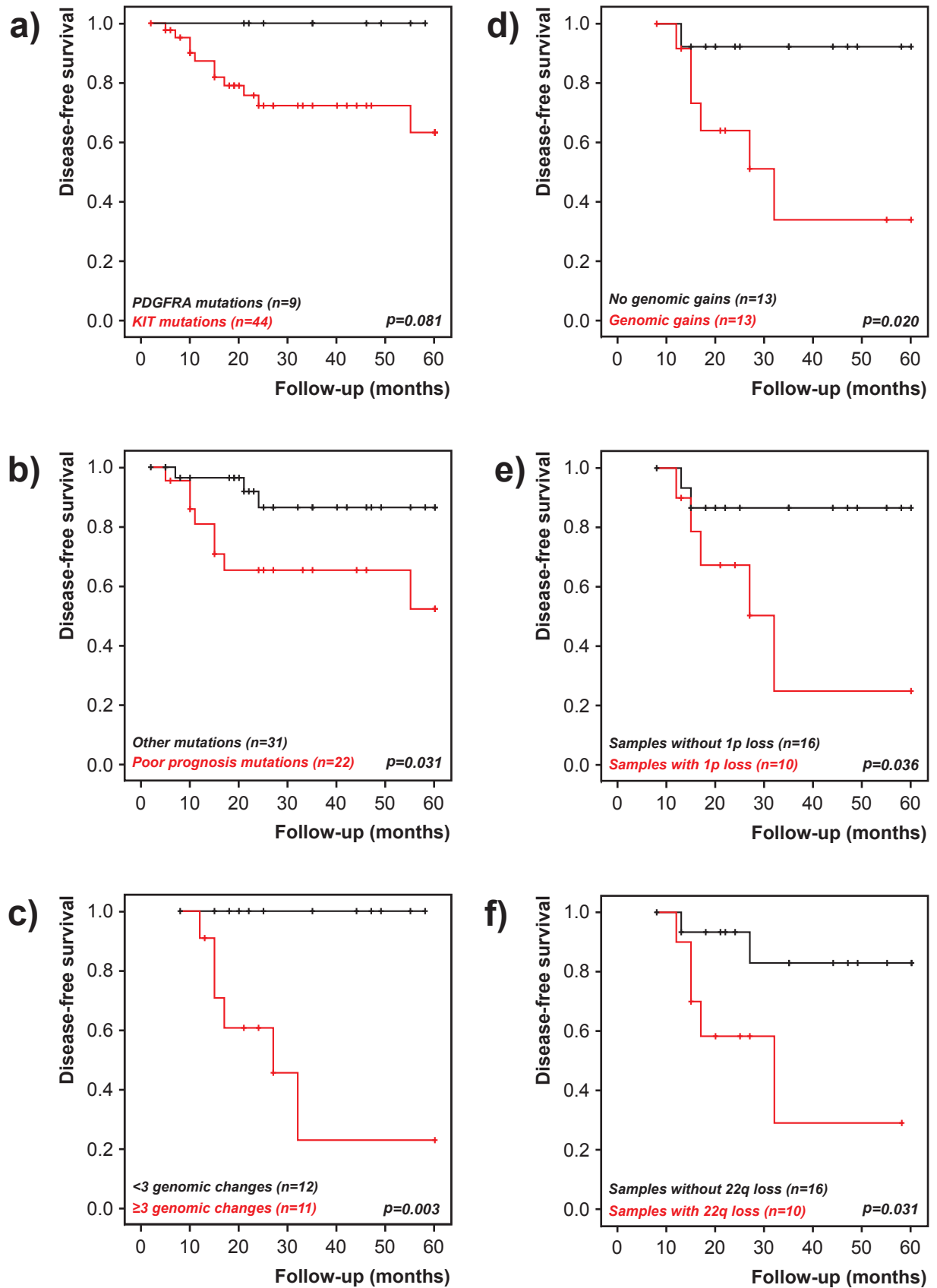


Figure 11. Five-year disease-free survival based on genetic variables: **a)** gene group; **b)** mutation type (*KIT* exon 9 or *KIT* exon 11 deletions/delins vs mutations in *PDGFRA* or other mutations in *KIT*); **c)** genomic complexity; **d)** presence of genomic gains; **e)** presence of 1p deletions; **f)** presence of 22q deletions.

DISCUSSION

Recent years have seen important breakthroughs that resulted in better diagnostic, prognostic and therapeutic tools for patients with gastrointestinal stromal tumors. Less than 10 years ago, most GIST were misclassified as muscle neoplasias, with which they shared morphological features, and it was not until the early 90's that this independent entity was properly acknowledged. More importantly, a distinctive genetic fingerprint was uncovered that allowed targeted therapies for these aggressive tumors, bringing some hope to patients whose live expectancy was otherwise much reduced. The current project aimed to characterize the mutation profile of *KIT* and *PDGFRA* in a series of GIST diagnosed and followed at our institution, and to determine the possible prognostic and/or predictive value of these alterations. Secondary genetic changes were additionally assessed using a whole-genome screening methodology to determine the relative contribution of primary and secondary genetic events in GIST establishment and progression.

Until 2007, tumor size and mitotic index remained the most important prognostic indicators in GIST (Fletcher CD *et al*, 2002; Joensuu H, 2008). In our series, tumors averaged 9 cm, and risk stratification based on these criteria grouped most lesions into the high risk category (Table 2). However, it has been shown that even low risk GIST can behave aggressively, progress and develop metastases. In accordance, two of the patients in our low risk group developed metastases, with one of them dying from this cause. Within the group of intermediate risk, most patients are currently alive with no evidence of disease, whereas those in the group of high risk showed significantly more progression events (Figure 10c). Indeed, only two of these patients are currently disease-free (following additional surgical treatment). In 2007, anatomic location was also considered of relevance and included in the determination of the risk of recurrence and progression (Demetri GD *et al*, 2007; Yang J *et al*, 2008). In our series, lesions located in the stomach (51.4%) or outside the GI (15.3%) showed no progression events, whereas the majority of those in the small intestine (26.4%) or colon (6.9%) developed metastasis in a significantly shorter period ($p < 0.001$, Figure 10b).

The main contribution to this field, however, was the identification of the causative role of KIT (and PDGFRA) receptors in these tumors (Hirota S *et al*, 1998), which not only helped establish a correct diagnosis but also revealed important prognostic and predictive value. Detection of KIT protein antigen (CD117) by immunohistochemistry is fundamental for the diagnosis of GIST, as 95% of cases show positivity for this marker (Corless CL *et al*, 2004). Interestingly, CD117 staining may show cytoplasmic, membranous or

paranuclear “dotlike” distribution (Rubin BP, 2006). One possible reason for these heterogeneous patterns is that different *KIT* mutations may express mutant isoforms with preferred cellular distributions. Furthermore, many groups quickly suggested that the type and molecular location of *KIT* or *PDGFRA* alterations is related with the risk of recurrence or progression, and also therapy response in GIST (Ernst SI *et al*, 1998; Liu XH *et al*, 2005; Martin J *et al*, 2005; Andersson J *et al*, 2006; Cho S *et al*, 2006; Lasota J *et al*, 2006; DeMatteo RP *et al*, 2008; Corless CL and Heinrich MC, 2008).

In our series, expression of CD117 was assessed in 75 cases. A total of 71 lesions showed a positive staining pattern (with considerable heterogeneity between different lesions). Even if the positive rate is in accordance with the literature, further studies are needed to clarify the dissimilar cellular distribution of KIT also observed in our study, as several reports strangely refer a predominance of cytoplasmic staining (Corless CL and Heinrich MC, 2008). Interestingly, it has been shown that within the 5% of GIST that do not express CD117, oncogenic mutations of *KIT* (>50%) or *PDGFRA* (~30%) may be found (Corless CL *et al*, 2004). Among our four CD117 expression-negative cases, one showed indeed a *PDGFRA* mutation, but no mutations were found in the remaining three. Our results for CD34, desmin, SMA, and S100 protein expression were also in accordance with previous literature reports.

The overall frequency of *KIT* and *PDGFRA* mutations in GIST varies in different studies, but is usually very high (Rubin BP, 2006). These mutually exclusive primary events are critical in the pathogenesis of most GIST, as they promote the constitutive activation of the receptors and initiate the downstream signaling pathways that translate into aberrant cell proliferation, apoptosis, chemotaxis, and adhesion. Mutation screening of both oncogenes was performed in 78 samples, and an overall mutation frequency of 87.2% (n=68) was obtained, with 59 cases harboring *KIT* mutations (75.7%) and 9 cases showing *PDGFRA* mutations (11.5%). In particular, *KIT* mutations were detected in exons 11 (n=52, 66.7%) and 9 (n=7, 9%), whereas *PDGFRA* mutations were observed in exons 18 (n=6, 7.7%), 12 (n=2, 2.6%) and 14 (n=1, 1.3%) (Table 3).

A Portuguese study with a comparable series of cases (n=78) has been recently published (Gomes AL *et al*, 2007), with the authors reporting an overall incidence of 63% mutations, with 44 cases involving *KIT* (56%), namely in exons 11 (n=40, 51%) and 9 (n=4, 5%), and 5 cases involving *PDGFRA* (6%), specifically exons 12 (n=2, 3%) and 18 (n=3, 4%). The authors state that these results are in accordance with published ranges, in particular those of the Iberian Peninsula (Martin J *et al*, 2005), even if the overall incidence in other studies (and our own) is considerably higher (Rubin BP, 2006; Miettinen M and Lasota J, 2006). Two main reasons likely account for this variability. The first refers

to the material used, as the previous Portuguese and Spanish studies screened formalin-fixed, paraffin-embedded GIST (n=78 and n=162 cases, respectively), whereas 27 of our lesions corresponded to fresh frozen tumors. It is known that DNA extracted from paraffin-embedded tissue is more degraded, enhancing the probability of obtaining false negative results. The second reason concerns the methodologies used for *KIT* mutational screening. While we used PCR followed by direct sequencing in all cases, Gomes and collaborators (2007) used a Single Strand Conformational Polymorphism (SSCP) approach (a less sensitive method) for an initial screening, and only those samples showing abnormal migration patterns were subjected to direct sequencing.

It has been suggested that the type and molecular location of different mutational events in GIST carry distinct biological and clinical implications (Corless CL and Heinrich MC, 2008; Lasota J *et al*, 2008). Indeed, different regulatory or effector domains of *KIT* and *PDGFRA* receptors may be targeted, and thus oncogenic activation occurs through distinct mechanisms. Mutations in the *KIT* extracellular regulatory domain, coded by exon 9, seem to mimic the conformational changes that follow SCF ligation. The most common mutation found within this location (p.Ala502_Tyr503dup) corresponds to an insertion of six nucleotides (Lux ML *et al*, 2000). All our samples with exon 9 mutations displayed this hot-spot alteration (Figure 3). According to literature reports, tumors with *KIT* exon 9 mutations are mainly characterized as high-risk or overtly malignant, and approximately 95% are localized in the small intestine (Rubin BP *et al*, 2007). In our series, only two out of the seven cases had origin in the small intestine and were classified as high risk. Both patients are still alive (with disease persistence).

The major mutational hotspot in *KIT* is exon 11, which encodes the juxtamembrane intracellular domain responsible for modulating *KIT* enzymatic activity (Yang J *et al*, 2008). Mutations within this exon include in-frame deletions or insertions, missense mutations or combinations thereof. These lesions may occur throughout the entire GI tract. *KIT* exon 11 deletions, and more specifically those affecting codons 557-558, have been linked to an aggressive behavior comparing with missense and insertion mutations, independently of Imatinib therapy (Wardelmann E *et al*, 2003; Martin J *et al*, 2005; Cho S *et al*, 2006; Lasota J *et al*, 2008). In our series, 26 out of 52 mutations in this domain corresponded to deletions or delins. Interestingly, 15 of these 26 patients showed disease progression, (five died from their tumors), whereas only four patients in the group with insertions, duplications or missense mutations showed disease progression (p=0.003). Most of the deceased patients had tumors classified as high risk, but at least one was considered low risk at diagnosis. It is also noteworthy that in two patients with primary *KIT* exon 11 mutations treated with Imatinib, and in which additional metastases developed, the same

secondary mutation in *KIT* exon 13 (p.Val654Glu), known to confer drug resistance (Debiec-Rychter M *et al*, 2005), was detected (Figure 5).

GIST harboring *PDGFRA* mutations share many clinical features with *KIT* mutated lesions, but are mainly gastric and present weak or negative CD117 staining (Wardelmann E, 2004). Evidence suggests that *PDGFRA* mutated tumors might be less aggressive (Rubin BP *et al*, 2007). In our series, three important observations should be highlighted: from the nine cases with *PDGFRA* mutations, 3 showed weak or only focal CD117 staining; most tumors (n=7) were located in the stomach; and all patients are currently alive with no evidence of disease. Interestingly, the hotspot p.Asp842Val mutation was detected in four of the cases (Figure 6). This mutation has been associated with primary resistance to Imatinib (Heinrich MC *et al*, 2003), but these patients were classified in the low and intermediate risk groups, showed no signs of progression, and were not treated with Imatinib thus far.

KIT and *PDGFRA* negative GIST account for 5 to 10% of all cases and may occur in the entire length of GI tract (Rubin BP *et al*, 2007). It is likely that analogous receptor tyrosine kinases or other downstream effectors of their signaling cascades harbor activating mutations in these lesions (Heinrich MC *et al*, 2002; Rubin BP, 2006). The ten tumors in our series negative for both *KIT* and *PDGFRA* mutations were mostly located in the stomach (n=6) and were classified as very low, low (n=2), intermediate, or high risk (n=5). Only one high risk patient died as a consequence of the disease, whereas two others died of unrelated causes.

In addition to the early mutation events activating *KIT* or *PDGFRA*, cytogenetic studies have shown several secondary genetic changes associated with GIST progression (El-Rifai W *et al*, 2000a; Sandberg AA and Bridge JA, 2002; Gunawan B *et al*, 2007). Few studies so far have performed genotype and genome analysis in the same samples, preventing a reliable assessment of correlations between primary and secondary genetic events, or their combined prognostic/predictive value (Anderson J *et al*, 2002; Assämäki R *et al*, 2007; Wozniak A *et al*, 2007). In our work, 85% of the analyzed GIST displayed copy number changes (Figure 7). Complete or partial deletions of chromosome 14 were seen in 91% of the abnormal cases, and in four patients this was the sole change detected. Additional recurrent aberrations included losses at 22q (43.5%), 1p (43.5%) and 15q (34.8%), as well as gains at 1q (17.4%) and 12q (17.4%) (Table 4, Figure 7). Based on previous literature findings, samples submitted to CGH analysis were divided according to mutation genotypes. Interestingly, lesions harboring mutations associated with a bad prognosis (n=12) showed significantly more copy number changes than those without such mutations (median of 6.5 versus 2 aberrations per tumor,

$p=0.025$). The presence of gains, deletions at 1p, and deletions at 22q could also be associated with a shorter disease-free survival for these patients. In accordance with other groups, genomic complexity (more than 3 aberrations per tumor) was also significantly associated with a worse outcome for the patients. It is also noteworthy that tumors with *PDGFRA* mutations showed the same pattern of alterations seen in those with *KIT* mutations (namely losses of 14q, 22q, and 1p, and gains at 10q), even if the complexity was much reduced (median of 2 vs. 6 alterations per tumor, respectively).

Taken together, these findings suggest that secondary chromosome changes play a more relevant role than it was recognized until now, and that cytogenetic screening might be required in the future to fully characterize the malignant potential of GIST. It is also noteworthy that of the four cases without detectable copy number changes, three samples were negative for *KIT* or *PDGFRA* mutations (Table 4). As CD117 staining for these three samples was focal or absent, one must consider the possibility that these lesions have been misclassified as GIST. Interestingly, histological evaluation of a recent metastatic lesion of one of these cases resulted in a diagnosis of leiomyosarcoma (patient number 62).

The introduction of targeted therapy against the tyrosine kinase domain of *KIT* (and related receptors) remarkably increased the overall survival of patients with metastatic or inoperable GIST. The molecular location and nature of *KIT* and *PDGFRA* activating mutations is known to influence the likelihood of clinical response to this drug. In our series, 26 patients (35%) showed disease progression after initial surgery and were subsequently treated with Imatinib. All but two of these tumors showed *KIT* exon 11 mutations, in particular deletions/deletions. Strikingly, this group was also the most complex regarding genomic alterations, together with lesions harboring *KIT* exon 9 mutations. Patients with inoperable GIST and showing intolerance or resistance to Imatinib now have the possibility to be treated with the second line drug Sunitinib. Preliminary data suggest this drug might even be more efficient, given the fact that Imatinib has exclusive affinity to a limited number of TK domains (Demetri GD *et al*, 2006). Unfortunately, tumors treated with this second line therapy also tend to progress, namely by acquiring *KIT* exon 17 mutations (Corless CL and Heinrich MC, 2008). Due to the fact that such resistance is becoming a significant clinical problem in the treatment of this neoplasia, several trials with new targeted drugs are underway, underlining the importance of genetic studies aimed at identifying novel alterations with prognostic or predictive value or that may work as therapeutic targets in GIST.

CONCLUSIONS

During the past decade, GIST have emerged from historic anonymity to center stage in the field of solid tumor management. Progresses concerning their biology and genetic background, namely the discovery of a defining molecular and immunohistochemical profile, laid the groundwork for the first largely successful targeted therapy protocols in solid tumors. In this study we report the genetic characterization of a consecutive series of 78 patients diagnosed with GIST, in which primary mutation events in *KIT* or *PDGFRA*, as well as secondary chromosomal changes (for a subset of 27 patients), were assessed and correlated with available clinico-pathological and therapy response data.

Using a direct sequencing approach, we conclude that most GIST show activating mutations in either *KIT* (75.7%) or *PDGFRA* (11.5%). The importance of a molecular-based approach in the diagnosis of GIST is well illustrated in the fact that within the group of cases with negative or doubtful positivity for CD117, one harboured a *PDGFRA* mutation. We further show that the majority of GIST display chromosomal alterations, namely deletions at chromosomal regions 14q, 1p and 22q, and that a significantly higher number of these secondary genetic aberrations can be seen in tumors with mutation genotypes associated with aggressiveness, such as those harboring *KIT* exon 9 mutations and *KIT* exon 11 deletions/delins.

The worse outcome previously associated with *KIT* exon 11 deletions/delins was additionally demonstrated by the fact that most patients with disease progression showed these mutations. Genomic complexity, the presence of gains, losses at 1p, or losses at 22q, could also significantly distinguish patients at a higher risk of disease progression. In opposition, all patients with *PDGFRA* mutations, associated in the literature with less aggressive phenotypes, are currently alive with no evidence of disease. These also displayed significantly less aberrations as detected by CGH.

We deem to have successfully demonstrated the relevant contribution of genetic analysis in the diagnosis and risk stratification of patients with GIST. Our findings further suggest that cytogenetic screening is a valuable tool that should be included in future studies, as secondary chromosomal changes were strongly associated with the clinical aggressiveness of these tumors. Additional follow-up is required to fully assess the prognostic and predictive value of several other recurrent genetic features.

FUTURE PERSPECTIVES

The present work focused on the genetic characterization of a series of patients diagnosed with GIST, assessing both primary mutation events as well as secondary genomic aberrations. Given the relatively small number of patients analyzed by CGH, the correlations between genotype and genomic data were limited. The reduced follow-up period for patients under Imatinib therapy further prevented a reliable determination of the predictive value of several genetic alterations. To clarify these issues and achieve a more complete characterization of our series, several steps are being implemented and prepared, namely:

- o To update and monitor patient status and response to first line and second line therapies, in order to reliably determine the prognostic and/or predictive value of genetic changes in this series of GIST.
- o To monitor Imatinib or Sunitinib resistance in treated patients and assess if new molecular events leading to this resistance have occurred in the respective tumors.
- o To apply the FISH methodology to assess the most common secondary alterations in all samples for which whole-genome screening (CGH) could not be applied.

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ATTACHMENTS

Table 1. Clinicopathologic characteristics of 78 patients diagnosed with GIST

Patient ID	Age at Diagnosis	Tumor Location	Tumor Type	Tumor pattern	Risk Group	Current status
1	80	Stomach	Primary	Spindle cell	Very low	ANED
2	59	Small intestine	Primary	Mixed	High	ANED
3	42	Peritoneum	Metastasis	Spindle cell	High	DFD
4	65	Stomach	Primary	Spindle cell	High	ANED
5	84	Peritoneum	Metastasis	Mixed	High	DFD
6	64	Stomach	Primary	Spindle cell	Low	ANED
7	58	Stomach	Primary	Mixed	Intermediate	ANED
8	77	Colon	Primary	Spindle cell	Intermediate	ANED
9	62	Stomach	Primary	Spindle cell	Intermediate	ANED
10	52	Stomach	Primary	Epithelioid	Intermediate	ANED
11	70	Peritoneum	Metastasis	Mixed	High	ANED
12	53	Peritoneum	Primary	Spindle cell	High	AWD
13	34	Stomach	Primary	Spindle cell	High	ANED
14	73	Small intestine	Primary	Epithelioid	High	AWD
15	74	Liver	Metastasis	Spindle cell	nd	DFD
16	44	Peritoneum	Primary	Epithelioid	High	ANED
17	61	Peritoneum	Metastasis	Spindle cell	Low	AWD
18	73	Colon	Primary	Spindle cell	High	DFD
19	61	Small intestine	Primary	Spindle cell	Low	DFD
20	53	Stomach	Primary	Mixed	Intermediate	ANED
21	42	Small intestine	Primary	Spindle cell	Low	ANED
22	76	Stomach	Primary	Spindle cell	Low	ANED
23	65	Peritoneum	Primary	Spindle cell	High	AWD
24	46	Small intestine	Primary	Spindle cell	High	DFD
25	42	Small intestine	Primary	Spindle cell	High	ANED
26	26	Stomach	Primary	Spindle cell	Intermediate	ANED
27	73	Small intestine	Primary	nd	Intermediate	ANED
28	73	Stomach	Primary	Mixed	Intermediate	ANED
29	77	Stomach	Primary	Spindle cell	High	ANED
30	69	Small intestine	Primary	Spindle cell	High	ANED
31	72	Stomach	Primary	Spindle cell	Low	DNED
32	59	Small intestine	Primary	Mixed	High	AWD
33	59	Small intestine	Primary	Spindle cell	High	AWD
34	77	Stomach	Primary	Mixed	Intermediate	ANED
35	46	Peritoneum	Primary	Spindle cell	High	ANED
36	46	Small intestine	Primary	Mixed	Intermediate	ANED
37	67	Stomach	Primary	Spindle cell	High	ANED
38	30	Peritoneum	Primary	Spindle cell	High	ANED
39	69	Stomach	Primary	Mixed	Low	ANED
40	66	Stomach	Primary	Mixed	Low	ANED
41	49	Stomach	Primary	Epithelioid	High	AWD
42	52	Stomach	Primary	Spindle cell	Intermediate	ANED
43	58	Rectum	Primary	Spindle cell	Intermediate	ANED

Patient ID	Age at Diagnosis	Tumor Location	Tumor Type	Tumor pattern	Risk Group	Current status
44	72	Stomach	Primary	Spindle cell	Low	ANED
45	61	Peritoneum	Primary	Spindle cell	High	DWD
46	37	Peritoneum	Metastasis	Spindle cell	High	AWD
47	38	Small intestine	Primary	Spindle cell	High	AWD
48	61	Stomach	Primary	Spindle cell	Intermediate	ANED
49	74	nd	nd	nd	nd	nd
50	57	Peritoneum	Metastasis	Spindle cell	High	AWD
51	68	Stomach	Primary	Spindle cell	Low	ANED
52	66	Stomach	Primary	Spindle cell	High	ANED
53	63	Small intestine	Primary	Epithelioid	High	AWD
54	nd	Stomach	Primary	nd	nd	nd
55	57	Stomach	Primary	Spindle cell	Intermediate	ANED
56	61	Liver	Metastasis	Spindle cell	nd	AWD
57	75	Stomach	Primary	Spindle cell	Low	ANED
58	71	Stomach	Primary	Spindle cell	High	ANED
59	50	Peritoneum	Metastasis	Spindle cell	Intermediate	AWD
60	62	Stomach	Primary	Spindle cell	Low	ANED
61	49	Stomach	Primary	Spindle cell	High	ANED
62	68	Peritoneum	nd	Spindle cell	High	DWD
63	68	Colon	Primary	Mixed	High	AWD
64	83	Stomach	Primary	Spindle cell	Low	ANED
65	55	Stomach	Primary	Spindle cell	Low	ANED
66	24	Stomach	Primary	Epithelioid	Intermediate	ANED
67	60	Small intestine	Primary	Mixed	High	ANED
68	41	Stomach	Primary	Spindle cell	High	ANED
69	51	Small intestine	Primary	Spindle cell	High	AWD
70	76	Stomach	Primary	Spindle cell	Intermediate	ANED
71	43	nd	nd	nd	nd	nd
72	73	Small intestine	Primary	Spindle cell	High	ANED
73	76	Stomach	Primary	Spindle cell	High	ANED
74	45	Stomach	Primary	nd	nd	nd
75	70	Small intestine	Primary	Spindle cell	Very low	ANED
76	72	Small intestine	Primary	Spindle cell	Intermediate	ANED
77	56	Small intestine	Primary	nd	High	AWD
78	71	Small intestine	Primary	nd	Intermediate	AWD

Abbreviations: ANED, alive, no evidence of disease; AWD, alive with disease; DNED, dead, no evidence of disease; DWD, dead with disease; DFD, dead from disease; nd - not determined.

Table 2. Risk stratification groups in 72 patients as suggested by Fletcher CD *et al*, 2002.

Risk group	Tumor Size	Mitotic Count	No. of patients (%)
Very low risk	<2 cm	<5/50 HPF	2 (2.8%)
Low risk	2–5 cm	<5/50 HPF	14 (19.4%)
Intermediate risk	<5 cm	6–10/50 HPF	19 (26.4%)
	5–10 cm	<5/50 HPF	
High risk	>5 cm	>5/50 HPF	37 (51.4%)
	>10 cm	Any mitotic rate	
	Any size	>10/50 HPF	

Abbreviations: HPF, high power (microscope) field.

Table 3. Genotype status of *KIT* and *PDGFRA* in 84 samples from 78 patients diagnosed with GIST

Patient ID	Risk Group	Gene	Exon	Mutation*	Type
2	High	<i>KIT</i>	9	p.Ala502_Tyr503dup	duplication
12	High	<i>KIT</i>	9	p.Ala502_Tyr503dup	duplication
17	Low	<i>KIT</i>	9	p.Ala502_Tyr503dup	duplication
17 (met)	Low	<i>KIT</i>	9	p.Ala502_Tyr503dup	duplication
22	Low	<i>KIT</i>	9	p.Ala502_Tyr503dup	duplication
53	High	<i>KIT</i>	9	p.Ala502_Tyr503dup	duplication
66	Intermediate	<i>KIT</i>	9	p.Ala502_Tyr503dup	duplication
72	High	<i>KIT</i>	9	p.Ala502_Tyr503dup	duplication
3	High	<i>KIT</i>	11	p.Trp557_Lys558del	deletion
4	High	<i>KIT</i>	11	p.Lys550_Gln556del	deletion
18	High	<i>KIT</i>	11	p.Tyr570_Leu576del	deletion
23	High	<i>KIT</i>	11	p.Asp579del	deletion
27	Intermediate	<i>KIT</i>	11	p.Trp557_Glu561del	deletion
34	Intermediate	<i>KIT</i>	11	p.Asp579del	deletion
35	High	<i>KIT</i>	11	p.Val560del	deletion
42	Intermediate	<i>KIT</i>	11	p.Val559_Glu561del	deletion
45	High	<i>KIT</i>	11	p.Glu554_Val559del	deletion
46	High	<i>KIT</i>	11	p.Val559_Asp572del	deletion
49	nd	<i>KIT</i>	11	p.Glu554_Val561del	deletion
50	High	<i>KIT</i>	11	p.Pro551_Glu554del	deletion
60	Low	<i>KIT</i>	11	p.Lys550_Lys558del	deletion
67	High	<i>KIT</i>	11	p.Met552_Trp557del	deletion
69	High	<i>KIT</i>	11	p.Lys550_Glu554del	deletion
11	High	<i>KIT</i>	11	p.Asn567_Tyr578delinsGluAsp	delins
13	High	<i>KIT</i>	11	p.Tyr553_Leu576delinsAsnCysLeuHis LeuTyrSerSerGln	delins
15	nd	<i>KIT</i>	11	p.Gln556_Val559delinsHis	delins
19	Low	<i>KIT</i>	11	p.Val559_Ile571delinsVal	delins
32	High	<i>KIT</i>	11	p.Glu554_Lys558delinsGlu	delins
33	High	<i>KIT</i>	11	p.Trp557_Val559delinsPhe	delins
39	Low	<i>KIT</i>	11	p.Pro551_Gln556delinsThr	delins
41	High	<i>KIT</i>	11	p.Gln556_Val559delinsProThrVal	delins
43	Intermediate	<i>KIT</i>	11	p.Lys558_Thr574delinsAsnArgSer	delins
47	High	<i>KIT</i>	11	p.Asn567_Leu576delinsIle	delins
10	Intermediate	<i>KIT</i>	11	p.Asp572_His580dup	duplication
28	Intermediate	<i>KIT</i>	11	p.Leu576_Arg588dup	duplication

Patient ID	Risk Group	Gene	Exon	Mutation*	Type
36	Intermediate	<i>KIT</i>	11	p.Pro573_Tyr578dup	duplication
55	Intermediate	<i>KIT</i>	11	p.Thr574_Asp579dup	duplication
59	Intermediate	<i>KIT</i>	11	p.Asp572_Pro573dup	duplication
68	High	<i>KIT</i>	11	p.Ile571_Asp579dup	duplication
71	nd	<i>KIT</i>	11	p.Ile571_Asp579dup	duplication
70	Intermediate	<i>KIT</i>	11	p.Pro585_Arg586insThrThrGlnLeu ProTyrAspHisLysTrpGluPhePro	insertion
5	High	<i>KIT</i>	11	p.Trp557Arg	missense
14	High	<i>KIT</i>	11	p.Trp557Gly	missense
14 (met)	High	<i>KIT</i>	11	p.Trp557Gly	missense
25	High	<i>KIT</i>	11	p.Trp557Arg	missense
26	Intermediate	<i>KIT</i>	11	p.Val559Asp	missense
29	High	<i>KIT</i>	11	p.Val559Asp	missense
30	High	<i>KIT</i>	11	p.Val559Ala	missense
40	Low	<i>KIT</i>	11	p.Trp557Arg	missense
44	Low	<i>KIT</i>	11	p.Val559Asp	missense
52	High	<i>KIT</i>	11	p.Val559Asp	missense
54	nd	<i>KIT</i>	11	p.Val559Asp	missense
56	nd	<i>KIT</i>	11	p.Leu576Pro	missense
57	Low	<i>KIT</i>	11	p.Val559Asp	missense
58	High	<i>KIT</i>	11	p.Asp579del	missense
63	High	<i>KIT</i>	11	p.Val560Asp	missense
73	High	<i>KIT</i>	11	p.Val559Ala	missense
75	Very low	<i>KIT</i>	11	p.Trp557Arg	missense
76	Intermediate	<i>KIT</i>	11	p.Leu576Pro	missense
77	High	<i>KIT</i>	11	p.Val560Glu	missense
78	Intermediate	<i>KIT</i>	11	p.Trp557Arg	missense
5 (met)	High	<i>KIT</i>	13	p.Val654Glu	missense
56 (met)	nd	<i>KIT</i>	13	p.Val654Glu	missense
21	Low	<i>PDGFRA</i>	12	p.Ser566_Glu571delinsArg	delins
64	Low	<i>PDGFRA</i>	12	p.Val561Asp	missense
20	Intermediate	<i>PDGFRA</i>	14	p.Asn569Tyr	missense
7	Intermediate	<i>PDGFRA</i>	18	p.Met844_Ser847del	deletion
16	High	<i>PDGFRA</i>	18	p.Met844_Ser847del	deletion
6	Low	<i>PDGFRA</i>	18	p.Asp842Val	missense
9	Intermediate	<i>PDGFRA</i>	18	p.Asp842Val	missense
48	Intermediate	<i>PDGFRA</i>	18	p.Asp842Val	missense

Patient ID	Risk Group	Gene	Exon	Mutation*	Type
51	Low	<i>PDGFRA</i>	18	p.Asp842Val	missense
1	Very low	-			
8	Intermediate	-			
24	High	-			
31	Low	-			
37	High	-			
38	High	-			
61	High	-			
62	High	-			
65	Low	-			
74	nd	-			

*Mutant sequences at the protein level are deduced from the mutations identified at the DNA level. Mutation nomenclature followed the recommendations of Human Genome Variation Society (<http://www.hgvs.org>).
Abbreviations: nd, not determined; met, metastasis.

Table 4. Chromosomal imbalances and molecular alterations detected in 27 GIST submitted to CGH analysis.

Patient ID	Gene	Exon	Mutation*	Mutation type	CGH findings**
2	KIT	9	p.Ala502_Tyr503dup	duplication	rev ish dim(X)(p11p22), dim(1)(p13p36), enh(1)(q21), enh(1)(q32), dim(2)(q11q37), enh(3)(p21), enh(3)(q21), enh(3)(q28q29), enh(6)(p21), enh(7)(q22), enh(7)(q36), enh(8)(p11p23), enh(9)(p12p13), enh(9)(q22q34), enh(10)(q22q26), dim(11)(p14p15), enh(11)(q13q14), enh(12)(p13), enh(12)(q13), enh(12)(q22q24), dim(13)(q), dim(14)(q), dim(15)(q15q26), enh(16), enh(17), enh(19), enh(20)(q), dim(21)(q)
53	KIT	9	p.Ala502_Tyr503dup	duplication	rev ish dim(1)(p13p36), dim(6)(q12q27), dim(9)(p21p24), dim(14)(q), dim(18)(p11q21), dim(19)(p13), enh(20)(p12p13), enh(20)(q), dim(22)(q)
17	KIT	9	p.A502_Y503dup	duplication	rev ish dim(1)(p13p36), dim(2)(p25q13), dim(11), enh(12)(p12p13), dim(13)(q), dim(15)(q), enh(21)(q)
3	KIT	11	p.Trp557_Lys558del	deletion	rev ish enh(Y), dim(1)(p21p36), enh(1)(q21q23), enh(1)(q42q44), dim(2)(q11q37), enh(6)(p12p25), dim(6)(q24q27), enh(8)(p21p22), enh(12)(q21q24), dim(13)(q), dim(14)(q), dim(15)(q21q26), enh(17)(q11q24), dim(18)(q21q23), dim(22)(q)
4	KIT	11	p.Lys550_Gln556del	deletion	rev ish enh(6)(q26q27), dim(10), dim(14)(q), dim(15)(q), dim(22)(q)
42	KIT	11	p.Val559_Glu561del	deletion	rev ish dim(14)(q), dim(15)(q), dim(22)(q)
58	KIT	11	p.Asp579del	deletion	rev ish dim(14)(q)
60	KIT	11	p.Lys550_Lys558del	deletion	rev ish dim(11)(p), dim(14)(q), dim(22)(q)
13	KIT	11	p.Tyr553_Leu576delinsAsnCysLeuHisLeuTyrSerGln	delins	rev ish dim(14)(q)
33	KIT	11	p.Trp557_Val559delinsPhe	delins	rev ish dim(1)(p13p36), enh(1)(q21q44), dim(2)(p), enh(2)(q14q36), enh(3), enh(4), enh(5)(p12p15), enh(5)(q12q35), enh(6)(p12p25), dim(6)(q16q27), enh(7)(p13p22), enh(7)(q21q35), enh(8)(p22p23), enh(8)(q13q24), enh(10)(p13q22), enh(10)(q24), enh(11)(p14p15), dim(11)(q22q23), enh(12)(q14q22), dim(13)(q), dim(14)(q), dim(15)(q), dim(18)(q), dim(21)(q), dim(22)(q)
43	KIT	11	p.Lys558_Thr574delinsAsnArgSer	delins	rev ish dim(1)(p12p36), dim(2)(p22p23), dim(3)(q12q22), dim(4)(p12p16), dim(7)(p), dim(9)(q22q32)
10	KIT	11	p.Asp572_His580dup	duplication	rev ish dim(X)(q21q28), dim(14)(q)
70	KIT	11	p.Pro585_Arg586insThrThrGlnLeuProTyrAspHisLysTrpGluPhePro	insertion	rev ish dim(14)(q), enh(20)(q)
5	KIT	11	p.Trp557Arg	missense	rev ish dim(1)(p13p36), dim(3)(p21p25), dim(14)(q22q32), dim(15)(q12q22), enh(17)(q21q25), dim(22)(q)

Patient ID	Gene	Exon	Mutation*	Mutation type	CGH findings**
52	<i>KIT</i>	11	p.Val559Asp	missense	rev ish dim(14)(q),dim(22)(q)
14(met)	<i>KIT</i>	11	p. Trp557Gly	missense	rev ish dim(1)(p36q21),enh(10)(q22q23),dim(10)(q24q26),dim(14)(q),dim(15)(q21q26),dim(22)(q)
21	<i>PDGFRA</i>	12	p.Ser566_Glu571delinsArg	delins	rev ish dim(14)(q)
20	<i>PDGFRA</i>	14	p.Asn569Tyr	missense	No copy number changes
7	<i>PDGFRA</i>	18	p.Met844_Ser847del	deletion	rev ish dim(12)(p),enh(12)(q12q24),dim(14)(q)
6	<i>PDGFRA</i>	18	p.Asp842Val	missense	rev ish dim(14)(q),dim(22)(q)
9	<i>PDGFRA</i>	18	p.Asp842Val	missense	rev ish dim(14)(q22q32)
48	<i>PDGFRA</i>	18	p.Asp842Val	missense	rev ish enh(10)(q26),dim(14)(q)
51	<i>PDGFRA</i>	18	p.Asp842Val	missense	rev ish dim(1)(p),enh(5),dim(10)(q21q26),dim(14)(q12q32)
61	-				rev ish dim(X),dim(1)(p12p36),enh(1)(q21q44),dim(3)(q13q29),dim(9)(q22q34),enh(11)(p15),enh(11)(q13q25),dim(13)(q),dim(14)(q)
8	-				No copy number changes
31	-				No copy number changes
1	-				No copy number changes

*Mutant sequences at the protein level are deduced from the mutations identified at the DNA level. Mutation nomenclature followed the recommendations of Human Genome Variation Society (<http://www.hgvs.org>).

**CGH descriptions follow the guidelines proposed by the International System for Human Chromosome Nomenclature (ISCN) 2005.

Abbreviations: delins, deletion insertion mutation; met, metastasis; rev, reverse; ish, in situ hybridization; dim, diminished; enh, enhanced.