

Patterns of gene amplification in gastrointestinal stromal tumors (GIST)

Luigi Tornillo¹, Giacomo Duchini¹, Vincenza Carafa¹, Alessandro Lugli¹, Stefan Dirnhofer¹, Dolores Di Vizio², Amedeo Boscaino³, Rosanna Russo⁴, Coya Tapia¹, Regine Schneider-Stock⁵, Guido Sauter¹, Luigi Insabato² and Luigi M Terracciano^{1,2}

¹Institute of Pathology, University of Basel, Switzerland; ²Department of Biomorphological and Functional Sciences, University 'Federico II' of Naples, Italy; ³Operative Unit of Pathology, 'Antonio Cardarelli' Hospital, Naples, Italy; ⁴Operative Unit of Pathology, 'San Leonardo' Hospital, Salerno, Italy and ⁵Department of Pathology, Otto-von-Guericke University, Magdeburg, Germany

Gastrointestinal stromal tumors (GIST) are the most common primary mesenchymal tumors of the gastrointestinal tract (GIT). They represent a wide clinico-pathological spectrum of tumors. No single histological or clinical parameter can predict the prognosis while the response to therapy is related to the type of KIT or PDGFRA mutation. Cytogenetic and CGH studies have identified frequent gross chromosomal aberrations but the target genes of these changes are unknown. To determine whether known oncogenes take part in genomic rearrangements and to investigate the potential clinical significance of their amplifications, nine known oncogenes (CMYC, MDM2, GLI1, CDK4, HER2, EGFR1, CCND1, FGF3, EMS) were analyzed by fluorescent *in situ* hybridization (FISH) on a tissue microarray (TMA) containing 94 primary GIST. Clinical follow-up information was available for 57 of these patients. Amplification was found for CMYC in three of 90 (3.3%), for MDM2 in five of 94 (5.3%), for EGFR1 in five of 94 (5.3%), and for CCND1 in seven of 79 (8.9%) evaluable cases. No amplifications were seen for HER2, GLI1, CDK4, FGF3, and EMS. Amplifications of MDM2 and CCND1 were associated with clinical and histological malignancy. In conclusion, our data show that gene amplification does occur in a subset of GIST. Identification of MDM2/CCND1 amplification may represent another molecular feature that could help in the evaluation of the behavior of GISTs.

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Gastrointestinal stromal tumors (GIST) occur in about 20/100 000 individuals per year. Despite their overall rarity, GIST are the most common primary mesenchymal tumors of the gastrointestinal tract (GIT).^{1–5} They share morphologic, immunophenotypic and genetic characteristics with the interstitial cells of Cajal (ICCs), the pacemaker cells of the gut^{1,5–7} from which they are thought to be derived. Both ICCs and GISTs show diffuse strong KIT (CD117) protein expression,^{1,4–6,8,9} which is considered a diagnostic hallmark of GIST.^{4,10} Since KIT is one of the targets for the tyrosine kinase inhibitor Imatinib mesylate, successful treatments of GIST with this drug have recently been reported.^{11–13}

Moreover, it has been shown that some GISTs may show mutations in the gene coding for platelet-derived-growth factor alpha (PDGFRA). Tumors expressing KIT or PDGFRA oncoproteins were indistinguishable with respect to activation of downstream signaling intermediates and cytogenetic changes associated with tumor progression. KIT and PDGFRA mutations appear to be alternative and mutually exclusive oncogenic mechanisms in GISTs.^{14,15} From a clinico-pathological point of view GISTs represent a spectrum of tumors including very low-risk, low-risk, intermediate-risk and high-risk variants. However, no single morphologic, immunohistochemical or genetic feature allows to predict the behavior of an individual tumor.^{10,16–27} Some authors suggested that presence of KIT mutations is associated with malignancy;^{21,28,29} however, these results have not been confirmed.^{25,30} Recently, it has been shown that the type of KIT and PDGFRA mutation may predict the response to therapy with Imatinib.^{31,32} Other molecular alterations have been

Correspondence: Dr L Tornillo, MD, Institute of Pathology, University of Basel, Schönbeinstrasse 40, CH-4003 Basel, Switzerland.

E-mail: tornillo@uhbs.ch

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reported in GIST. Comparative genomic hybridization (CGH) and fluorescent *in situ* hybridization (FISH) studies have identified numerous chromosomal regions with frequent losses such as 1p, 9p, 11, 13q, 14q, 15q, 19q, or 22q or over-representations including 5p, 8q, 17q, and 20q.^{2,3,33–39} Little is known about the target genes of these alterations and their possible associations with clinical behavior. In particular, information is lacking on the role of individual oncogenes and the prevalence of their amplifications in GIST.

In an attempt to investigate the prevalence and clinical significance of gene amplifications in GIST, we brought well-documented cases of GIST of 94 patients in a tissue microarray (TMA) format as previously described.⁴⁰ In this procedure hundreds of minute tissue samples (diameter: 0.6 mm) can be simultaneously analyzed on one microscope glass slide by all types of *in situ* techniques. Nine different oncogenes (CMYC, MDM2, GLI1, CDK4, HER2, EGFR1, CCND1, FGF3, and EMS) were analyzed by FISH. Apart for EGFR1, based on our previous experience (unpublished data), we decided not to perform additional immunohistochemical stainings for the products of these oncogenes. Our data may suggest that oncogene amplifications occur at significant frequency in GIST.

Materials and methods

Patients

All tumors were retrieved from the files of the Institutes of Pathology of the Universities of Basel and of Naples, 'Federico II' and of the Hospitals 'Antonio Cardarelli' of Naples and 'S. Leonardo' of Salerno. Two pathologists (LT and LI) re-examined representative slides of each tumor. The diagnosis of GIST was confirmed in case a mesenchymal spindle cell or epithelioid tumor of the GIT showed unequivocal positivity for CD117.⁴¹ In all, 100 tumors (94 primary tumors and six metastasis) of 94 patients met these criteria and were included in this study. The 94 GIST patients comprised 50 males and 44 females with a mean age of 61 years (range 26–91, SD 14). Tumor localization was the stomach in 56 (56%), small bowel in 27 (27%), mesentery in six (6%), duodenum in four (4%), rectum in three (3%), esophagus in one (1%), colon in one (1%), abdominal soft tissues in one (1%) and pancreas in one (1%). The mean tumor size was 67.3 mm (range 5–250). A total of 37 tumors (37%) were <50 mm in diameter.

Assessment of Behavior

To distinguish tumors of different degrees or probabilities of malignancies we first reviewed medical charts that were available from 59 of 94 patients (63%). Of these 59 patients, 11 (19%) died

of the disease, and 12 (20%) were alive with recurrent/metastatic disease. These 23 tumors were considered clinically malignant.

Additional 35 patients (61%) were alive without disease, and one (2%) died of other causes. As the follow-up of these patients was generally not long enough to fully exclude malignancy (median 23 months), these tumors were not classified according to their clinical information. Together with these, 35 primary tumors for which clinical data were lacking were classified into the following categories: malignant, high risk, intermediate risk, low risk and very low risk of malignancy. Tumors were considered malignant if the presence of metastasis at time of operation or later was observed ($n=14$); tumors of any size and with mitotic index (MI) >10/50HPF or tumors >10 cm in size and with any MI or tumors 5–10 cm in size and MI >5/50 HPF were considered as high risk ($n=12$); tumors <5 cm in size and with MI 6–10/50 HPF or tumors 5–10 cm in size and with MI ≤5/50 HPF were considered intermediate risk ($n=19$); tumors with diameter between 2 and 5 cm and MI ≤5/50 HPF were considered as low risk ($n=21$); tumors with diameter <2 cm and MI ≤5/50 HPF were considered as very low risk ($n=5$).⁴ The characteristics of our series are shown in Table 1.

TMA Construction

Representative areas of each tumor were identified on a HE-stained slide. One sample each with a diameter of 0.6 mm was taken from 147 tissue blocks and placed in our TMA using a semiautomated tissue arraying device. The tissue collection included 94 primary GIST, six GIST metastases and a control section composed of 33 leiomyomas, seven leiomyosarcomas, and two schwannomas of the gastrointestinal tract as well as five samples of normal gastric mucosa (Figure 1).

FISH

Five micrometer sections were made from TMA blocks using an adhesive-coated slide system (Instrumedics Inc., Hackensack, NJ, USA). Sections were treated according to the Paraffin Pretreatment Reagent Kit protocol before hybridization (Vysis, IL, USA). FISH was performed with Spectrum Orange-

Table 1 Evaluation of relative risk of malignant behavior

Relative risk	N (%)
Very low risk	5 (5.3)
Low risk	21 (22.3)
Intermediate risk	19 (20.2)
High risk	12 (12.8)
Malignant	37 (39.3)
Total	94 (100)

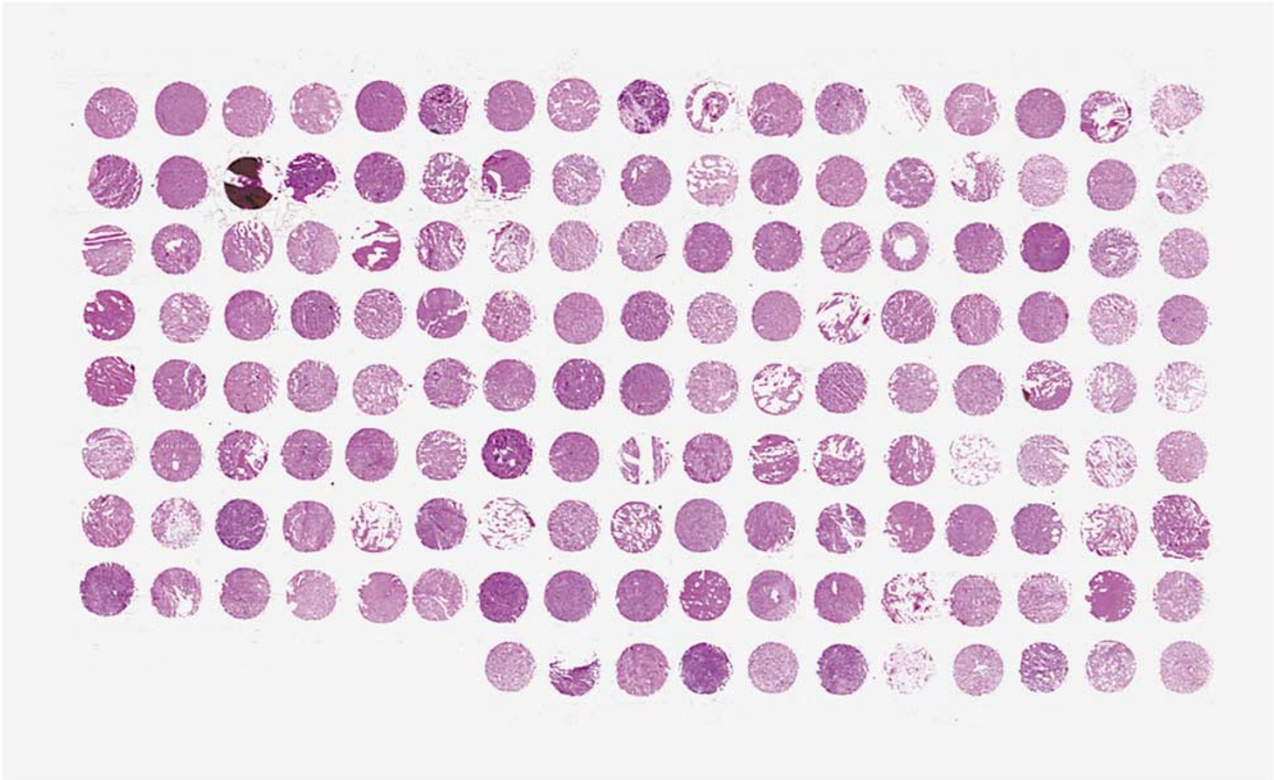


Figure 1 Tissue array for FISH analysis. Overview of hematoxylin- and eosin-stained sections from one block containing 147 specimens.

labeled CMYC, MDM2, GLI1, CDK4, HER2, EGFR1, Cyclin D1, FGF3, and EMS probes, each in combination with Spectrum Green-labeled centromere probes for the corresponding chromosomes^{8,12,17,7,11} as reference (Vysis, IL, USA). Hybridization and posthybridization washes were performed according to the 'LSI procedure' (Vysis, IL, USA). Slides were then counterstained with 125 ng/ml 4',6-diamino-2-phenylindole in antifade solution. The green and red signals were counted in 20 nuclei per tissue sample. Amplification was defined as a gene probe/centromere probe ratio of ≥ 2.0 . To assess the samples for polysomy of the analyzed centromere probes, we first determined the average copy numbers of centromere probes in benign control tissues (smooth muscle and normal gastric epithelium). A tumor was then classified as polysomic for a chromosome if the average centromere copy number exceeded the average copy number detected in 10 normal control tissues plus 3 SD (Table 1). As positive controls we performed FISH analysis on sections of tissue with known amplifications.

Immunohistochemistry

Four micrometer thick sections of TMA blocks were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, NJ, USA) supporting the cohesion of 0.6 mm array elements on glass. Standard indirect immunoperoxidase procedures

were used for immunohistochemistry. After pretreatment with proteinase K (DAKO, Carpinteria, USA) monoclonal mouse antibody (dilution 1:50, DAKO Cytomation, Carpinteria, USA) was used for EGFR1 detection. A DAB chromogen was used. Nuclei were counterstained with hematoxylin.

For tumor and normal tissues the percentage of positive cells was estimated. Only membranous staining was considered positive in this setting.

KIT Sequence Analysis

Deparaffinization of the formalin-fixed tissues and DNA extraction were performed according to established protocols (Qiagen, Basel, Switzerland). The exons 9, 11, 13, and 17 of the KIT gene, where mutations were previously reported, were amplified using a seminested polymerase chain reaction approach. All exons were sequenced directly using Big Dye Terminators Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Primers designed for polymerase chain reaction and sequence reactions are listed below. Sequence products were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Exon 9

Forward 5-TCCTAGAGTAAGCCAGGGCTT-3

Reverse 5-TGGTAGACAGAGCCTAAACATCC-3

Nested forward 5-AGCCAGGGCTTTTGTCTTCT-3

Exon 11

Forward 5-CCAGAGTGCTCTAATGACTG-3

Reverse 5-AGCCCCTGTTTCATACTGAC-3

Nested reverse 5-ACTCAGCCTGTTTCTGGGAAA
CTC-3

Exon 13

Forward 5-GCTTGACATCAGTTTGCCAG-3

Reverse 5-AAAGGCAGCTTGGACACGGCTTTA-3

Nested forward 5-TGACATCAGTTTGCCAGTTG-3

Exon 17

Forward 5-TCCTTACTCATGGTCCGATC-3

Reverse 5-AAGAGACGAACTGTCAGGAC-3

Nested reverse 5-ACTGTCAAGCAGAGAATGGG-3

Statistics

Association of polisomies or amplifications with tumor phenotype and malignancy was investigated by a χ^2 test with correction for continuity as appropriate. For this analysis, very low and low

risk, tumor such as high risk and malignant tumors were considered as one group.

Results

Gene Amplification

The number of evaluable cases was 81 for HER2 (81%), 94 for EGFR1 (94%), 90 for CMYC (90%), 79 for CCND1 (79%), and 94 for MDM2 (94%). Reasons for nonevaluable cases were insufficient hybridizations or a lack of tumor tissue on the TMA. A total of 20 amplifications were detected in 16 cases (four tumors had multiple amplifications). Clear cut amplifications ($2.0 < \text{ratio} \leq 5$) were found for CMYC in three of 90 (3.3%), for MDM2 ($4 \leq \text{ratio} \leq 7$) in five of 94 (5.3%), for EGFR1 ($4 \leq \text{ratio} \leq 7$) in five of 94 (5.3%), and for CCND1 ($4 \leq \text{ratio} \leq 8$) in seven of 79 (7.7%) of evaluable cases (Figure 2). Coamplifications involved CCND1 and MDM2 in three cases and EGFR1 and CMYC in one case. Examples of amplified tumors are shown in Figure 2. No amplifications were observed for HER-2.

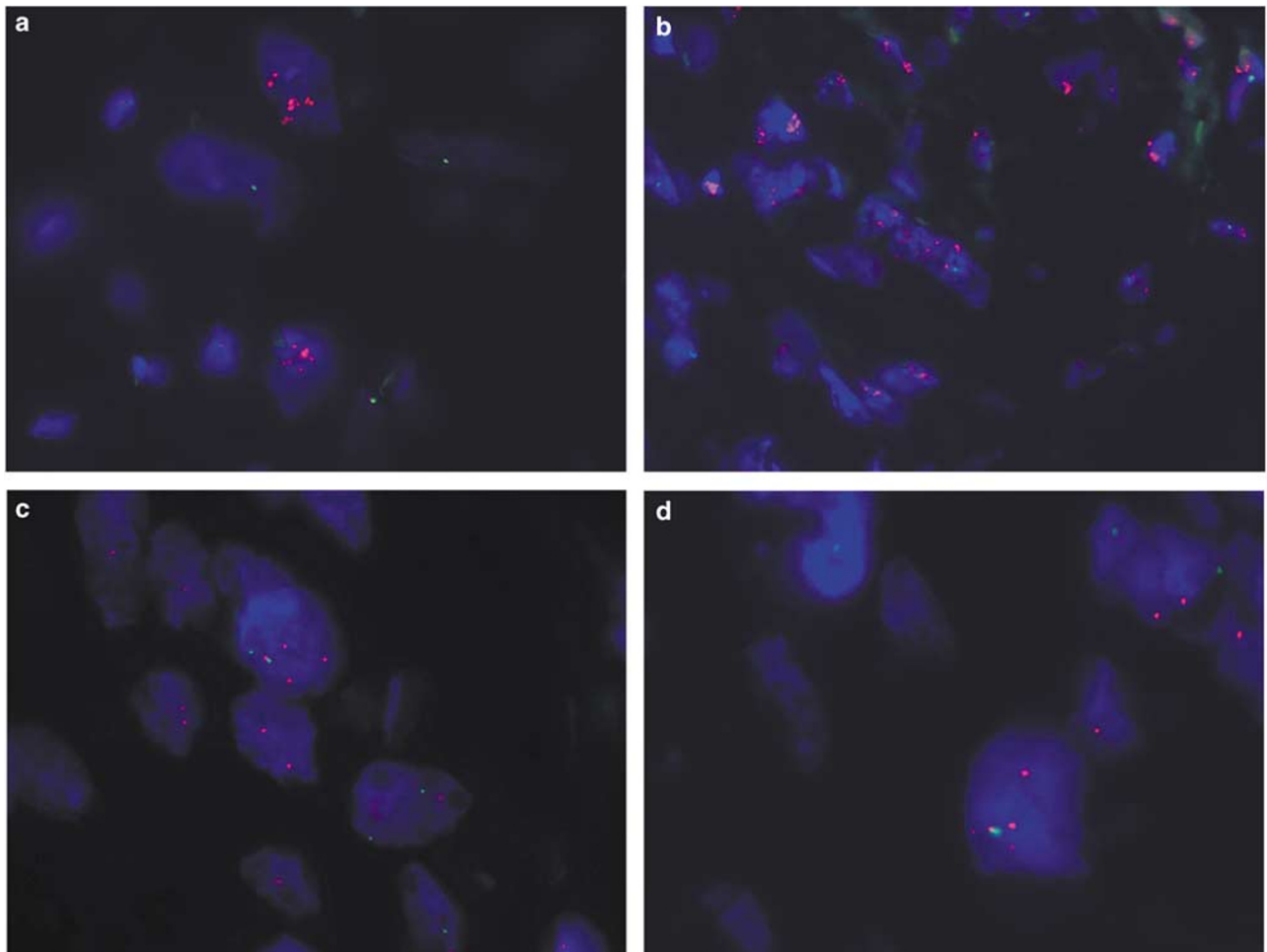


Figure 2 Examples of amplification of CCND1, MDM2, CMYC, and EGFR1. The red signals are from the gene probe, the green ones from the centromeric probe. There are at least two-fold red signals (sometimes in clusters). (a) CCND1, $\times 100$ (b) MDM2, $\times 100$ (c) CMYC, $\times 100$ (d) EGFR1, $\times 100$.

The presence of amplification was linked to malignancy (Table 2). Amplifications were seen in eight (22%) of 37 malignant primary tumors, five (42%) of 12 high-risk primary tumors, one (6%) of 18 intermediate risk primary tumors, two (10%) of 21 low-risk tumors. No amplification was found in very low-risk tumors. The analysis of individual genes revealed that CCND1 and MDM2 amplifications were detected only in high risk or malignant tumors while the other genes, which were only rarely amplified, showed no significant correlation with malignancy. In order to better define the state of the corresponding chromosomal regions (12q13–14, 11q13–14) we also performed additional FISH analyses with probes for FGF3 (11q13), EMS1 (11q13), GLI1 (12q13), and CDK4 (12q14). However, all these genes failed to show any amplification. Interestingly, if very low and low-risk tumors formed one group and high risk and malignant tumors another group, the distribution of amplifica-

tion reached statistical significance for CCND1 and MDM2 (Table 2). No significant association was seen between presence of gene amplification and histologic tumor type or tumor location.

Only occasional (three cases) low-level immunohistochemical positivity was observed for EGFR1, without any relationship with amplification or clinicopathologic parameters.

Polisomies

Between 83 and 94 tumors were interpretable for polysomy analysis. According to our definition, 22 tumors were found to be polysomic for at least one chromosome. Chromosome 17 (12 polisomies) was most frequently involved. Multiple polisomies were found in one tumor. No polysomy was observed for chromosomes 11 and 7. No association was observed between presence of polysomy and malignancy (Table 3) and as histologic tumor type or tumor location.

Table 2 Correlation of amplification with risk of malignant behavior

Gene (cases assessed)	Low or very low risk	Intermediate	High-risk malignant metastasis	P ^a
<i>CMYC</i> (90)				
Normal	24/24 (100%)	14/15 (93%)	49/51 (96%)	0.49
Amplification	0/24 (0%)	1/15 (7%)	2/51 (4%)	
<i>MDM2</i> (94)				
Normal	22/22 (100%)	17/17 (100%)	50/55 (91%)	0.05
Amplification	0/22 (0%)	0/17 (0%)	5/55 (9%)	
<i>HER2</i> (81)				
Normal	20/20 (100%)	17/17 (100%)	44/44 (100%)	NA ^b
Amplification	0/20 (0%)	0/17 (0%)	0/44 (0%)	
<i>EGFR1</i> (94)				
Normal	21/23 (91%)	15/16 (94%)	53/55 (96%)	0.67
Amplification	2/23 (9%)	1/16 (6%)	2/55 (4%)	
<i>CCND1</i> (79)				
Normal	19/19 (100%)	15/15 (100%)	38/45 (84%)	0.05
Amplification	0/19 (0%)	0/15 (0%)	7/45 (16%)	
<i>All genes</i> (97)				
Normal	22/24 (92%)	17/18 (94%)	42/55 (77%)	0.09
Amplification	2/24 (8%)	1/18 (6%)	13/55 (23%)	

^a χ^2 test with Pearson's correction.

^bNA, not assessed.

Table 3 Association of the polisomies with tumor phenotype

	N	FISH results				
		Polisomies (97)	Chr. 8	Chr. 12	Chr. 17	
Behavior	Very low risk	5	0/3 (0%)	0/3 (0%)	0/2 (0%)	0/3 (0%)
	Low risk	21	4/21 (19%)	1/21 (5%)	1/21 (5%)	2/20 (10%)
	Intermediate risk	19	4/18 (22%)	1/15 (7%)	2/17 (12%)	3/18 (17%)
	High risk	12	1/12 (8%)	0/9 (0%)	1/12 (8%)	0/12 (0%)
	Malignant+Metastasis	43	13/43 (30%)	1/42 (2%)	5/43 (12%)	7/41 (17%)

NB: The sum of the polisomies in this table is 24 and not 22 because there are cases with more than one polysomy.

Sequence Analysis of KIT Gene

Informative results were obtained in 70 cases; in the remaining cases the quality of DNA was suboptimal. In all, 59 cases showed mutations in KIT gene, namely 56 in exon 11 (80%) and three in exon 9 (4.2%). No mutation was observed in exon 13 and 17. In all, 11 cases were wild-type KIT. Of the 56 cases with mutations in exon 11, 38 (68%) were deletions, 13 (23%) were point mutations, five (9%) were insertions. The clinicopathologic characteristics and molecular characteristics of exon-11-mutated cases are shown in Table 4. No relationship was found between type of mutation and risk classification ($P = 0.3993$). Cases with deletions however showed a significantly shorter survival than cases with insertions and point mutations ($P = 0.0406$, log-rank test, Figure 3). In all, 17 (45%) of the 38 deletions involved codons 557–8. In all, 11 of 17 (65%) were high-risk or clearly malignant tumors, but this result was not significant. In amplified cases, only point mutations and deletions of KIT exon 11 were observed (Table 4), but no relationship was observed between presence and/or type of mutation and presence of amplification. No case showed internal tandem duplications (ITD) corresponding to the duplication described by Lux and Hirota.^{42,43} All three cases with exon 9 mutations had six base

pairs insertion at codon 502, which were clinically malignant and localized in the small intestine.

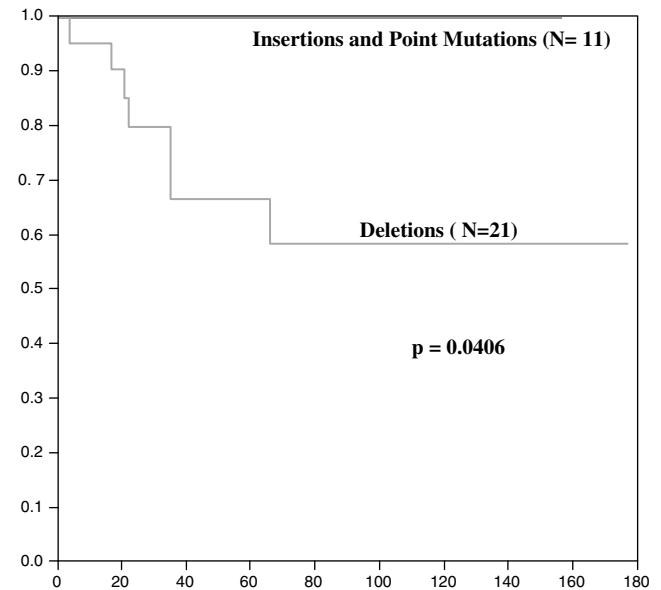


Figure 3 Survival curves according to type of mutation in KIT-exon-11-mutated cases (Kaplan–Meier method).

Table 4 Association of KIT exon 11 mutation with risk classification and presence of amplifications

Type of mutation	Del	Ins	Point mutation	P ^a
<i>Risk classification</i>				
Low and very low risk	11/38 (29%)	3/5 (60%)	2/13 (15%)	0.3993
Intermediate risk	6/38 (16%)	1/5 (20%)	3/13 (23%)	
High risk malignant metastasis	21/38 (55%)	1/5 (20%)	8/13 (62%)	
<i>All genes</i>				
Amplification	6/38 (16%)	0/5 (0%)	3/13 (23%)	0.4885
Normal	32/38 (84%)	5/5 (100%)	10/13 (77%)	
<i>CMYC</i>				
Amplification	1/37 (3%)	0/4 (0%)	0/12 (0%)	0.8022
Normal	36/37 (97%)	4/4 (100%)	12/12 (100%)	
<i>MDM2</i>				
Amplification	2/37 (5%)	0/5 (0%)	2/13 (15%)	0.3961
Normal	35/37 (95%)	5/5 (100%)	11/13 (85%)	
<i>HER2</i>				
Normal	32/32 (100%)	4/4 (100%)	13/13 (100%)	NA ^b
<i>EGFR1</i>				
Amplification	2/37 (5%)	0/5 (5%)	0/13 (0%)	0.6036
Normal	35/37 (85%)	5/5 (100%)	13/13 (100%)	
<i>CCND1</i>				
Amplification	2/31 (6%)	0/5 (0%)	2/12 (17%)	0.4297
Normal	29/31 (94%)	5/5 (100%)	10/12 (83%)	

^a χ^2 test with Pearson's correction.

^bNA, not assessed.

NB: The total number of amplified cases is nine and not 11 because there are cases with coamplifications.

Discussion

The results of this study show that oncogene amplification may be a relevant feature in GIST occurring in at least 10% of these tumors. Previous studies using CGH and other techniques have already suggested that gross chromosomal aberrations occur frequently in GIST. This includes reports of high-level over-representation of loci at 1q, 8q, and 17q.^{33–38,44} The present data demonstrate that oncogenes that are known to play a role in various epithelial and mesenchymal tumors can also be amplified in GIST. Among the five oncogenes examined, HER2 was the only one that was never found amplified. 17q+ has been reported to be frequently amplified in ‘malignant’ and metastatic GISTs, but the minimal overlapping region of amplification is 17q22-ter, distal to HER2 locus.³⁸ The lack of HER2 alterations fits well with data from Cai *et al*⁴⁵ who did not find any immunohistochemically detectable HER2 expression in a series of GIST. All together, these results do not suggest a possible role of trastuzumab (herceptin) therapy. The genes that were found amplified include EGFR1, CCND1, CMYC, and MDM2.

Presence of EGFR1 amplification in five GISTs is of interest. EGFR1 is a transmembraneous receptor kinase protein interacting with HER2. Similar to HER2, EGFR1 serves as a therapeutic target for several new cancer drugs. Our results are consistent with reports suggesting EGFR1 expression in at least a fraction of GIST.⁴⁵ Based on these data, it appears possible that anti-EGFR1 therapies could be another therapeutic alternative in GIST with amplified EGFR1. This is all the more true as the experience from the Her2/Herceptin story seems to suggest that tumors that do not only overexpress a protein but also have a gene amplification might respond particularly well to targeted therapies.^{46–48} An especially important predictive role of gene amplification could be explained by the fact that genes that are overexpressed because of structural DNA damage are more likely to represent a primary (driving) event for the affected cancer cell than overexpression alone.^{49,50} Simple overexpression is more likely to represent a less vital feature of the cell that can be compensated by alternative pathways if EGFR1 is disturbed by interaction with a drug compound.⁵¹ Moreover, overexpression without detectable DNA damage can always constitute a temporary phenomenon.⁵² We have indeed performed an immunohistochemical staining for EGFR1, but the results did not show any relationship either with prognosis or with amplification.

Amplification of CCND1 and MDM2 were both associated with malignancy. Interestingly, additional FISH analysis of neighboring loci did not show any amplification, suggesting that amplification should be restricted to these two genes. The CCND1 gene, located at 11q13,⁵³ is coding for a cyclin which is known to be translocated, amplified

and overexpressed in many different tumor types including mantle cell lymphoma, head and neck carcinoma, breast carcinoma, hepatocellular carcinoma, and various sarcomas.^{54–56} In GIST it was found in 25% of the high risk and in 16% of the malignant tumors but not in 24 very low, low or intermediate-risk tumors. CCND1 amplification may have additional importance as CCND1 has been shown to be essential for malignant transformation in mammary epithelial cells.⁵⁷

MDM2, localized on chromosome 12, has initially been found to be amplified in human sarcomas⁵⁸ and has been proposed as a marker of malignancy in liposarcomas.⁵⁹ It is involved in the Raf/MEK/MAP kinase pathway⁶⁰ and in the phosphatidylinositol 3-kinase/AKT/JNK pathway⁶¹ that are both triggered by KIT activation.^{62,63} In GIST, MDM2 amplification was present in 7% of the high risk/malignant tumors but absent in 39 very low/low/intermediate-risk tumors. If these results will be confirmed by RNA expression studies, it could be speculated that determination of CCND1 and MDM2 amplification may have clinical utility for the difficult assessment of behavior of GIST. In this case, evaluation of these amplifications could be included in clinical studies dealing with GIST.

CMYC is a well-known oncogene localized on chromosomes 8q24.12–13⁶⁴ and amplification has also been described in many different tumor entities.^{65–68} In a previous CGH study,³⁸ 8q was identified as a frequent site of amplification, above all in ‘malignant’ GISTs (until 57% of metastatic tumors). We have found amplification of CMYC only in three cases. That could imply that target of this amplification are other still unknown protooncogenes. Moreover, it should be also kept in mind that comparison with studies published before 2002 is hampered by the lack of universally accepted features suitable to predict the clinical behavior of these tumors. Increased CMYC expression has previously been described in GIST and it has been proposed to include immunohistochemical CMYC analysis in a scoring system to predict the clinical behavior of GISTs together with size and other parameters.⁶⁹ We have not performed immunohistochemical CMYC analysis in this study because in a previous study in bladder cancer we were unable to identify a CMYC antibody that would yield staining results, which show a significant relationship with high-level CMYC amplification (unpublished data). Instability of the MYC protein in formalin-fixed tissues may be the cause for these difficulties.

Interestingly, we found amplifications of multiple genes in four GIST. In three of these cases coamplification of CCND1 and MDM2 was observed. Both genes are related to the RB pathway. MDM2 inhibits the growth regulatory capacity of RB, interacting functionally and physically with it,⁷⁰ while CCND1, through cyclin-dependent kinases, may phosphorylate and inactivate RB. It is tempting to speculate that at least in a subset of GISTs the Rb-CCND1

pathway may play a key role in the acquisition of the features of malignancy. Apparently, there is a small group of GIST with a high level of genomic instability that are prone to development of genomic amplifications at multiple sites. The role of D-type cyclins in the genesis and progression of GIST is underlined by the recent report of high prevalence of cyclin D3 immunoreactivity in a small series of GISTs.⁷¹

Using a dual labeling FISH approach also including centromeric probes we also had an opportunity to investigate the prognostic significance of numeric chromosomal aberrations. Cytogenetic alterations in GISTs have previously been extensively studied by CGH and FISH.^{33–38,44,72} From these data it was suggested that GISTs of different grades may reflect a cytogenetic continuum with an increasing number of aberrations.⁶³ ‘Benign’ GISTs often only have an isolated loss of chromosome 14, while intermediate-risk-malignant GISTs have more changes, often including loss of chromosomes 1p, 9p, 11p or 22q.^{33,63} Other aberrations, such as deletion of 9p and gains of 8q and 9q have been described as associated with malignant GISTs.³⁸ Other authors have described, for instance, loss of 13q and gain of a whole chromosome 4,³⁶ correlating it with the behavior of the tumor. In our study, only chromosomes were examined that previously had not been suggested as potential prognostic markers. However, no evidence was found that these changes do have clinical significance.

The use of only one minute tissue sample (0.6 mm) per tumor has been criticized in the past because of the representativity. Many studies have addressed the question as to whether a higher concordance of TMAs and large section data can be obtained if multiple samples of each tumor are arrayed. As expected, they show that the results obtained on TMAs approach large section results as more samples are being analyzed.^{73–76} However, because large sections contain only a small fraction of the entire tumor mass (1/10 000),⁷⁷ probably reproducing the findings of large sections is not an optimal end point for the validation of the method. On the contrary, all previously well-established associations between molecular features and clinicopathological end point such as the prognostic importance of ER, PR, p53, and HER2 expression in breast cancer,^{74,78} or Ki67 labeling index in bladder cancer⁷⁵ have been confirmed in TMAs using one spot per tumor. It is probable that the high level of standardization and the greater objectivity of the staining interpretation on one small tissue piece are factors that may compensate for the disadvantage of the small size.⁷⁷

The earlier reported association between KIT exon 11 mutation and tumor prognosis^{10,21} has not been confirmed.^{25,30,79} However, it has been recently claimed that specific types of exon 11 mutations could predict the behavior of the tumor.^{80–82} In our series, tumors showing deletions in KIT exon 11 behaved significantly worse than those with inser-

tions or point mutations. This result is in keeping with the recently published data of Miettinen *et al*⁸³ that have found, in a large series of gastric GISTs, a strong association between deletions in exon 11 and a shorter survival. Most of the deletions involved codons 557–558, and were high-risk and clearly malignant tumors. Even if not significant, this is in agreement with the results of Wardelmann *et al*,⁸⁰ who have related codon 557–558 deletions to metastatic behavior. Internal tandem duplication (ITD) at the 3′ end have been described as possibly defining a subset of GISTs with favorable course.^{42,43,81} We have not found ITD, maybe due to the relatively small size of our series. The presence of exon 9 mutation has been claimed to be associated with localization in small intestine and malignant behavior.^{79,82,84,85} We found exon 9 mutation in three tumors (six base pairs insertion at codon 502) and they were all localized in the small intestine and clinically malignant (metastatic). No association was found between the type of mutation and the presence of amplifications.

In summary, we have performed a large scale FISH analysis of a series of 94 primary GISTs. Our data suggest that oncogene amplification is a frequent event in a subset of GIST. MDM2 and CCND1 amplification, both occurring in nearly 10% of the high risk/malignant samples, may have prognostic relevance in GIST.

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