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European Journal of Cancer 40 (2004) 689–695

European Journal of Cancer

www.ejconline.com

Use of *c-KIT*/*PDGFRA* mutational analysis to predict the clinical response to imatinib in patients with advanced gastrointestinal stromal tumours entered on phase I and II studies of the EORTC Soft Tissue and Bone Sarcoma Group

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Received 12 November 2003; accepted 21 November 2003

Abstract

Previous studies have shown that activating mutations of *c-KIT*/*PDGFRA*, potential therapeutic targets for imatinib mesylate, are implicated in the pathophysiology of gastrointestinal stromal tumours (GISTs). In this study, GISTs from 37 patients enrolled in an European Organisation for Research and Treatment of Cancer (EORTC) phase I/II clinical study of imatinib were examined for mutations of *c-KIT*/*PDGFRA* in order to explore whether the mutational status of the tumour predicts the clinical response to therapy. Mutations were screened by denaturing high-pressure liquid chromatography (DHPLC) and characterised by bi-directional DNA sequencing. Activating mutations of *c-KIT* or *PDGFRA* were found in 29 (78%) and 2 (6%) GISTs, respectively. Most *c-KIT* mutations involved exon 11 ($n=24$; 83%), all but one being an in-frame deletion; no isolated point mutations were found. The other *c-KIT* mutations included exon 9 AY 502–503 duplication ($n=4$; 14%) and exon 13 Lys→Glu^{642} missense mutation ($n=1$; 3%). Two tumours with no detectable *c-KIT* mutations demonstrated *PDGFRA* Asp→Glu^{842} amino acid substitutions. Patients with GISTs harbouring exon 11 mutations were more likely to achieve a partial response (PR) on imatinib therapy (83%) than all of the others (23%). The overall survival and progression-free survival rates for the entire group at 106 weeks were 78.3% and 46.9%, respectively. Based on a Kaplan–Meier analysis, patients with GISTs harbouring *c-KIT* mutations had longer median survival times and were less likely to progress than the other patients. These findings indicate that the mutational status of the c-KIT/PDGFRA oncoproteins could be useful to predict the clinical response of patients imatinib thereapy.

Keywords: Gastrointestinal stromal tumours; c-KIT; PDGFRA; Imatinib mesylate

1. Introduction

Most gastrointestinal stromal tumours (GISTs) express the receptor tyrosine kinase KIT oncoprotein and commonly have activating mutations in the *c-KIT* gene [1,7,11–13]. A subset of GISTs lacking *c-KIT* mutations carries intragenic activation mutations in a related receptor tyrosine kinase, platelet-derived growth factor receptor-alpha (PDGFRA) [10].

Imatinib mesylate, a selective inhibitor of ABL, ABL-BCR, KIT and PDGFR tyrosine kinases, produces high response rates in-patients with GIST [4,17,18]. Although

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0959-8049/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2003.11.025

the use of imatinib has revolutionised the clinical management of patients with advanced GISTs, the fundamental question regarding the likelihood of a positive response to the drug in the clinical setting has still to be addressed. Notably, *c-KIT*/*PDGFRA* mutations in GISTs differ in their form and in the protein domains involved. The presence, nature and location of the *c-KIT*/*PDGFRA* oncogenic mutations may translate into differences in tumour aggressiveness and influence the likelihood of a clinical response to imatinib [7,14,15]. Preliminary clinical observations linked responses to the presence of *c-KIT* mutations in the tumour, with the risk of progression during imatinib treatment being eight times higher in patients without *c-KIT* mutations than in patients with *c-KIT* mutations [8,9]. Moreover, patients with GISTs expressing mutant exon 11 isoforms responded extremely well compared with patients with GISTs harbouring mutations in exon 9, or without detectable *c-KIT* mutations [9]. Similarly, *PDGFRA* exon 12 mutations showed *in vivo* sensitivity to imatinib compared with *PDGFRA* exon 18 mutations.

In this study, we present the results of a mutational analysis from imatinib-treated GIST patients undergoing therapy in European Organisation for Research and Treatment of Cancer (EORTC) phase I and II trials [17–19]. Thirty-seven advanced GISTs were evaluated for mutations in the *c-KIT* exons 9, 11, 13 and 17, and *PDGFRA* exons 12 and 18. The results of the mutational analysis were then combined with the clinical data and the outcome of therapy, in order to determine whether the presence and type of *c-KIT*/*PDGFRA* mutations predicts the clinical response to the drug.

2. Patients and methods

2.1. Patients

The phase I and II studies were carried out in 13 centres of the EORTC Soft tissue and Bone Sarcoma Group. Adults with histologically-confirmed, unresectable or metastatic gastrointestinal stromal tumours that expressed CD117 antigen by immunohistochemical staining were eligible for the study. Criteria for inclusion, study design and procedures are detailed in recent publications from our group (see Refs. [17–19]). In short, patients in the Phase I trial were assigned to receive imatinib (Novartis Pharma AG, Basel, Switzerland) orally in one of four dose levels: 400 mg daily, 300 mg twice daily, and 400 mg twice daily. Patients in the Phase II study received 400 mg twice daily. Patients had regular physical examinations and evaluations of performance status. Both response and progression have been objectively assessed on the basis of the size of the lesions according to the Response Evaluation Criteria in Solid Tumors (RECIST). Dose escalation decisions were based on data from patients treated for at least 4 weeks. Patients whose tumour progressed despite treatment were withdrawn from the study. Patient, tumour, treatment and survival data were acquired prospectively and entered into our sarcoma database.

Histopathological examination and immunohistochemical analyses were performed on tissue fixed in 10% buffered formalin and embedded in paraffin. Immunohistochemical studies were performed using polyclonal antibodies against CD117 (A4502, dilution 1/250, DAKO, Denmark) and an avidin-biotin-peroxidase complex method without any antigen retrieval.

2.2. Sequence analysis

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue blocks, using the High Pure polymerase chain reaction (PCR) Template Preparation Kit (Roche, Mannheim, Germany). Exons 9, 11, 13 and 17 of the *c-KIT* gene, and exons 12 and 18 of the *PDGFRA* gene were amplified by PCR using an AmpliTaq Gold DNA polymerase and the GeneAmp® PCR System 2400 (Applied Biosystems, Foster City, CA, USA). The primers and the conditions used are listed in Table 1. Primers were design based on available *c-KIT*/*PDGFRA* sequences (http://www.ncbi.nlm.nih.gov, U63834, SEG_D50001S). The PCR products were purified (Microcon PCR, Millipore, MA, USA) and screened for mutations by denaturing high-pressure liquid chromatography (DHPLC) on a Transgenomic WAVE DHPLC system (DHPLC; Transgenomic, Ltd., UK). Samples showing an aberrant elution profile were re-amplified and sequenced in both directions using the BigDye® Terminator v.3.1 Cycle Sequencing Kit on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

2.3. Statistical analysis

Outcome variables considered in the present analysis were objective response to treatment, overall survival and time to first documented progression. Responses to treatment were classified as partial response (PR), stable disease (SD) or progressive disease (PD) and tabulated in contingency tables. Response rates have been compared in patients with versus those without *c-KIT* mutations, using the Fisher's exact test. Overall survival and event-free survival have been estimated within categories of interest by the Kaplan–Meier method. The survival and progression profile of patients with and without *c-KIT* mutations has been compared using the logrank test. The SAS software has been used to temporarily merge the clinical and mutation databases, and to perform all of the analyses.

Table 1
PCR primers sequence used, with the corresponding annealing temperatures (T_A) and expected PCR size products

Gene	Primers	Primer sequences 5′→3′	T_A (°C)	Product sizes
c-KIT	Ex9-F1	CCACATCCCAAGTGTTTTATG	56	352 bp
	Ex9-F2	CCCTCCTAGAGTAAGCCAGGGCTT		
	Ex9-R	GGTGTGATGCATGTATTACCAG		
	Ex11-F	GATGATTCTGACCTACAAAT	56	299 bp
	Ex11-R1	AGGAAGCCACTGGAGTTCCTT		
	Ex11-R2	CCCCGTCACTGTTATGTGTACCCA		
	Ex13-F1	GTATGGTACTGCATGCGCTT	56	294 bp
	Ex13-F2	GCTTGACATCAGTTTGCCAG		
	Ex13-R	GAGAACAACAGTCTGGGTAA		
	Ex17-F	TTCACTCTTTACAAGTTAAAATG	56	212 bp
	Ex17-R1	GAAACTAAAAATCCTTTGCAG		
	Ex17-R2	GGACTGTCAAGCAGAGAATG		
PDGFRA	Ex12-F	AAG CTC TGG TGC ACT GGG ACT T	65	251 bp
	Ex12-R	ATT GTA AAG TTG TGT GCA AGG GA		
	Ex18-F	TAC AGA TGG CTT GAT CCT GAG T	60	212 bp
	Ex18-R	AGT GTG GGA GGA TGA GCC TG		

PCR, polymerase chain reaction; Ex, Exon; bp, base pairs; F, forward; R, reverse.

3. Results

3.1. Clinical data

A total of 67 GIST patients were entered on the clinical trials. Tissue blocks were made available for mutational analysis from 37 patients from 3 institutions (Leuven, Belgium; London, United Kingdom (UK); Rotterdam, The Netherlands). There were 25 (68%) men and 12 (32%) women, with a median age of 54 years (range, 30–69 years) and median World Health Organisation (WHO) Performance Status (PS) 1 (range, 0–2). Most (77%) of the patients presented with liver metastases. The median time from the diagnosis to treatment was 16.4 months (range, 0–62 months) and the median time from the proven malignancy to treatment was 10.6 months (range, 0–58 months).

Of the 37 patients, 23 (62%) showed a partial response to treatment, 11 (30%) patients remained stable and three had progressive disease. Based on the Kaplan–Meier analysis, the overall survival for all patients at 730 days was 78.3% (Fig. 1a). One patient died from causes not related to malignancy. Seven patients discontinued therapy due to intolerance of the drug or progression of the disease, but were still alive.

3.2. Mutational analysis

All 37 specimens were analysed for *c-KIT* mutations, with the subsequent analysis of *PDGFRA* mutational status in 33 tumours. A combination of DHPLC and direct sequencing analysis disclosed *c-KIT* mutations in 29 (78%) tumour specimens. Among the GISTs with *c-KIT* mutations, 24 (83%) had exon 11 mutations, four (14%) had an exon 9 mutation, and one (3%) had a

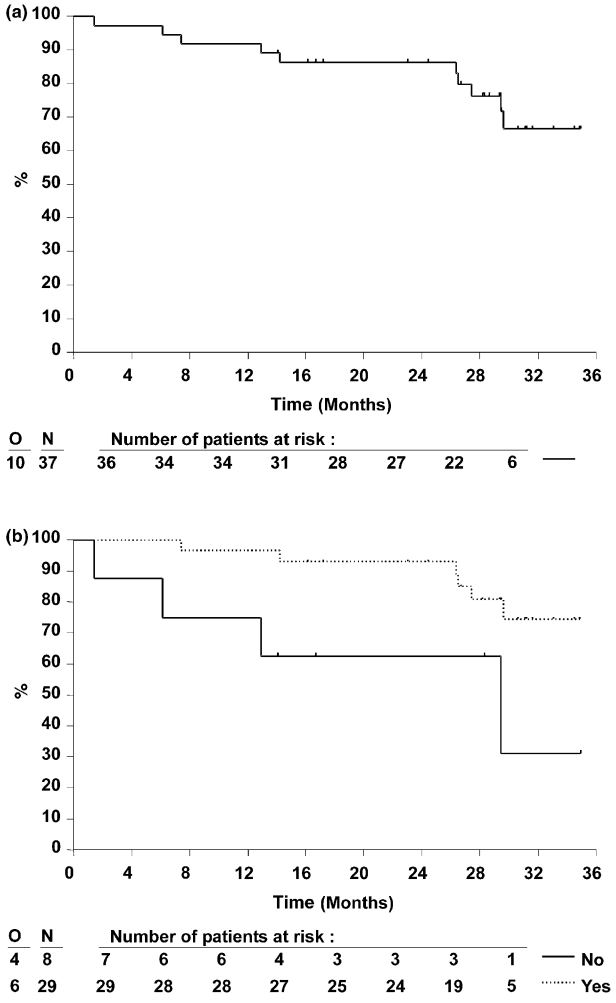

Fig. 1. Kaplan–Meier curves for the probability of overall survival for all patients in the study (A). Kaplan–Meier curves for the probability of survival for patients carrying tumours with *c-KIT* mutations versus all other patients (B).

mutation in exon 13. The exon 11 mutations included in-frame deletions (21 tumours; 57%), either alone (15 tumours; 41%) or associated with missense mutations or insertions (six tumours; 16%), and in-frame insertions (one tumour; 3%). Single base substitutions were not found. A case-by-case listing of these mutations is shown in Fig. 2. In 71% of cases (17 of 24), the mutations in exon 11 clustered in the region between codons 550 and 561, with the most common WK 557-558 deletion being present in five cases (21%). The involvement of one or both codons was also observed in 11 other tumours; codons 557 or 558 changes were identified in total in 67% (16 of 24) cases. Three tumours showed the same 569–576 deletion within exon 11, a finding that had been confirmed on the tumour specimens from the repeated biopsies during treatment. One tumour carried duplication of codons 574–586. Activating mutations in exons 9 and 13 were the same as those previously identified in GISTs [12,13], e.g. Ala-Tyr duplication between codons 502–503 and missense mutation Lys→Glu[642], respectively. Eight (27%) GISTs had no identifiable *c-KIT* sequence alteration. In six of these tumours, mutational analysis of *PDGFRA* was possible, disclosing missense mutation Asp→Val[842] in two cases.

3.3. Correlation of tumour genotype with the clinical response to therapy

Response data for the diverse tumour genotypes are shown in Table 2. Overall, 72% (21 out of 29) GISTs carrying *c-KIT* mutations showed a PR and 7 (24%) had stable disease. In addition, patients with GISTs harbouring exon 11 mutations were more likely to achieve a PR on imatinib therapy (20 of 24, 83%) than

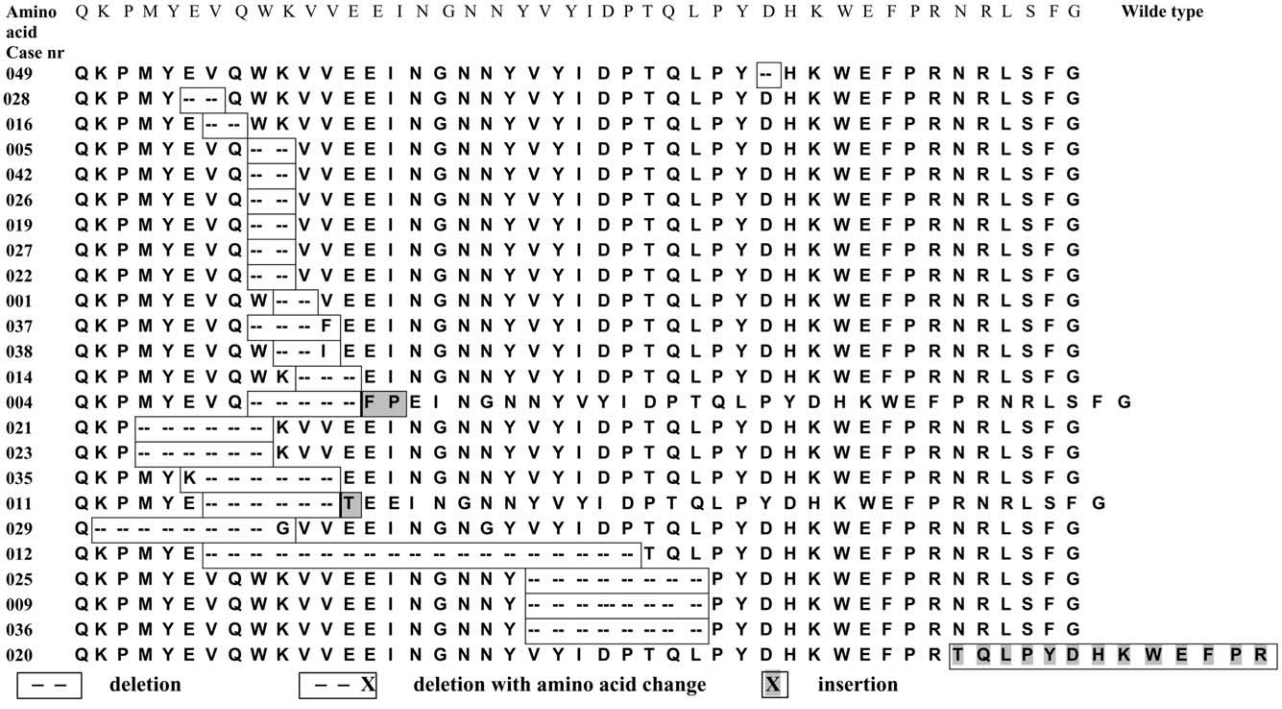

Fig. 2. Predicted amino acid sequences in *c-KIT* Exon 11 mutant-GISTs isoforms of 24 patients under the study.

Table 2
Clinical response versus GIST *c-KIT*/*PDGFRA* genotype

Best response (*n*)	Genotype *n* (%)				
	c-KIT exon 11	*c-KIT* exon 9	*c-KIT* exon 13	*PDGFRA* exon 18	No mutation
Partial response (23)	20 (83)	1 (25)	0 (0)	0 (0)	2 (33)
Stable disease (11)	3 (13)	3 (75)	1 (100)	2 (100)	2 (33)
Progressive disease (3)	1 (4)	0 (0)	0 (0)	0 (0)	2 (33)
Total (37)	24	4	1	2	6

Table 3
Duration of response versus *c-KIT*/*PDGFRA* genotype at week 104

Progression (*n*)	Genotype *n* (%)				
	c-KIT exon 11	*c-KIT* exon 9	*c-KIT* exon 13	*PDGFRA* exon 18	No mutation
Progression-free (16)	12 (50)	1 (25)	1 (100)	0 (0)	2 (33)
Progression (21)	12 (50)	3 (75)	0 (0)	2 (100)	4 (67)
Total (37)	24	4	1	2	6

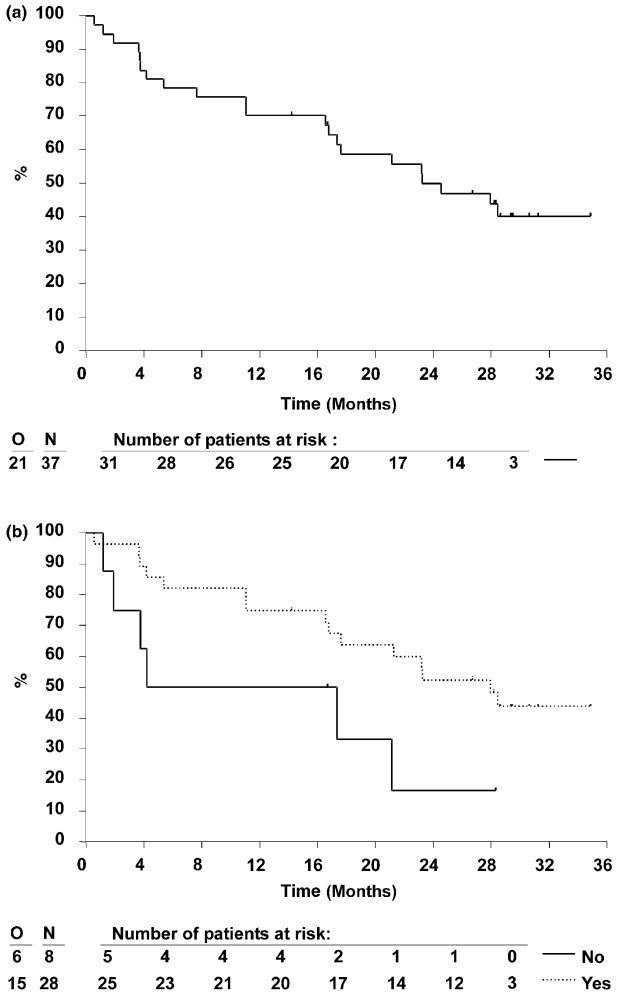

Fig. 3. Kaplan–Meier curves for the probability of event free survival for all patients in the study (A). Kaplan–Meier curves for the probability of progression free survival for patients with tumours carrying *c-KIT* mutations versus all other patients (B).

all of the others (3 of 13; 23%). One patient with a tumour harbouring *c-KIT* exon 11 deletion (D579) progressed initially in the course of treatment. Notably, this patient stopped receiving imatinib due to gross acceleration of disease on day 8 of therapy, but a computerized tomographic (CT) scan performed 2 months later showed a decline of their disease and they remain stable 30 months after stopping therapy. The number of patients with tumours containing *c-KIT* exon 9 or *PDGFRA* exon 18 mutations was too small to look for correlations between mutational status and response to therapy. Nevertheless, all four patients whose tumours showed an exon 9 *c-KIT* mutation responded to the drug. Two out of eight patients with tumours without *c-KIT* mutations had immense acceleration of their disease at the start of therapy; the others showed either partial responses or stable disease. Within this group, two patients harbouring a *PDGFRA* D842V mutation showed stable disease. Two other patients without *c-KIT* mutations in their tumours showed a PR—evaluation of the *PDGFRA* mutational status in these patients was hampered a lack of sufficient DNA for analysis.

3.4. Correlation of the tumour genotype with overall and event-free survival

Using a Kaplan–Meier analysis, the overall survival rate for all patients at 106 weeks was 78.3% (Fig. 1a). As can be seen in Fig. 1b, there was a significant difference in survival for patients with tumours containing *c-KIT*-mutations versus those patients with GISTs with no detectable *c-KIT* mutations ($P = 0.015$).

Duration of response for the diverse tumour genotypes at the time of analysis are shown in Table 3. The overall progression-free survival rate at 104 weeks was 46.9% (Fig. 3a) and the median event-free survival time for the entire group was 705 days. Based on Kaplan–Meier analysis, patients with GISTs harbouring *c-KIT* mutations were less likely to progress than other patients ($P = 0.03$) (Fig. 3b). The median event-free survival was over 2.5 times longer in patients with tumours expressing *c-KIT* exon 11 isoforms than in all others (849 versus 327 days, respectively).

4. Discussion

On the basis of screening solely by genomic sequencing, we detected oncogenic *c-KIT* and *PDGFRA*

mutations in 29 (78%) and 2 (5%) of 37 GISTs, respectively. Nearly 83% of *c-KIT*-mutant tumours carried exon 11 mutations. In 71% of these cases, the mutations clustered in the region between codons 550 and 561, with the vast majority having codons 557 and/or 558 deleted. The other 25% of cases harboured mutations in the more distal part of exon 11 (one showing internal tandem duplication at the 3′ end of exon 11). This type of *c-KIT* isoform has been rarely seen in other published series reporting on GISTs [1,12–14,16]. In addition, in contrast to previous reports, no missense exon 11 *c-KIT* mutations were observed in our series. Whether the observed differences are due to the selection of highly metastatic tumours in the study, reflect possible differences in geographical/ethnic distributions of particular genotypes or are simply coincidental is not clear and requires further study.

Previous *in vitro* studies demonstrated an increased sensitivity to imatinib therapy of tumours with *c-KIT* juxtamembrane mutations, and resistance to therapy of tumours with the kinase domain mutation, D816V, compared with patients with wild-type *c-KIT* [2,3,6,15]. Consistent with this finding, the likelihood of a clinical response to imatinib in our trial correlated with the *c-KIT* mutational status. Patients with GISTs harbouring exon 11 mutations were more likely to achieve a PR on imatinib therapy (83%) than all of the others (23%). This result is in agreement with the preliminary results obtained in the United States (US)-Finnish phase II trial of imatinib mesylate in the treatment of patients with advanced GIST [8,9]. In the latter study, patients with GISTs harbouring an exon 11 *c-KIT* mutation had a significantly higher partial response rate (72%) than patients whose tumour had an exon 9 *c-KIT* mutation (32%) or no detectable mutation (12%). The *in vivo* difference in sensitivity to imatinib of tumours expressing *c-KIT* exon 9 compared with exon 11 isoforms contrasts with *in vitro* data that shows an equal sensitivity to inhibition by the drug of all GIST-associated *c-KIT* mutant isoforms. The number of tumours with exon 9 *c-KIT* mutations in our series was too low to perform statistical comparison with other tumour genotypes. However, notably, only one of three patients with tumours expressing exon 9 *c-KIT* isoform showed a PR to treatment. In addition, consistent with the US-Finnish phase II trial, neither of the two patients in our study with the imatinib-resistant *PDGFRA* Asp-Val^{842} mutant isoform responded to treatment, but both showed stable disease.

Based on a Kaplan–Meier analysis, the overall survival time in our study was longer in GIST patients with *c-KIT*-mutations versus those without. However, the differences did not reach statistical significance when patients with exon 11 *c-KIT*-mutations were compared with all of the others. Most likely, this result was due to relatively long survival time of patients with mutant exon 9, 13 and wild-type *c-KIT* GISTs in our study. Interestingly, while the median event-free survival time for the whole group at 104 weeks of treatment was 705 days, it was 849 days for patients with tumours that harboured exon 11 *c-KIT* mutations versus 327 days for all of the others. It was substantially longer than in the US-Finnish phase II trial (median 687 versus 187 versus 82 days for tumours with an exon 11 *c-KIT* versus an exon 9 *c-KIT* versus no detectable kinase mutation, respectively). It is important to emphasise that in our series patients were treated in the Phase I trial at doses of up to 500 mg twice daily and in the Phase II study at 400 mg twice daily. Whether these somewhat higher doses than those used in the US-Finnish study group had any bearing on the longer event-free survival time is unknown and requires further investigation. Interestingly, the rate of progression in our series was relatively constant during the time of the study. Although it should be noted that imatinib exerts a selective activity against neoplastic cells, and higher doses of the drug might be counteracted by acquisition of a secondary *c-KIT* or *PDGFRA* mutation, with a subsequent KIT or PDGFRA protein re-activation [5]. On the other hand, accumulation of other genomic events is frequently involved in the progression of disease, and might drive the aggressive tumour behaviour despite adequate inhibition of KIT/PDGFRA tyrosine kinase activity by imatinib. Exposure of tumour cells to marginally active imatinib concentrations *in vivo*, as probably occurs with the lower doses commonly used at present, might favour the selection of such refractory clones, and thus the development of clinical resistance.

In conclusion, our results confirm that information derived from genomic testing may be useful to predict the clinical outcome to imatinib therapy in patients with advanced GISTs, thereby indicating the subset of patients that may have primary resistance to treatment. However, it is important to emphasise that the mutational status of *c-KIT*/*PDGFRA* in GISTs should not determine whether imanitib treatment is given, since even patients whose tumours had no detectable *c-KIT*/*PDGFRA* mutations showed benefit from the therapy. Moreover, this study focused on a small, selected group of patients. Analyses of a larger sample size are required for the adequate determination of the type and frequency of *c-KIT*/*PDGFRA* mutations among European populations of patients with advanced GISTs, and for the interpretation of the further clinical trials.

Acknowledgements

This study was partly supported by a research grant from Novartis (Basel, Switzerland). Bartosz Wasag is supported by Marie Curie European Community fellowship (contract HPMT-CT2001-00273).

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