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Genomic Grade Index predicts postoperative clinical outcome of GIST

F Bertucci*[,1,2,3], P Finetti[1], J Ostrowski[4], WK Kim[5], H Kim[5], MA Pantaleo[6], A Astolfi[7], M Polkowski[8] and D Birnbaum[1]

[1]Department of Molecular Oncology, Centre de Recherche en Cancérologie de Marseille; UMR1068 Inserm; Institut Paoli-Calmettes, 232 Boulevard Sainte Marguerite, 13273 Marseille Cedex 09, France; [2]Department of Medical Oncology, Institut Paoli-Calmettes, Centre de Recherche en Cancérologie de Marseille, UMR1068 Inserm, Marseille, France; [3]Aix-Marseille University, Marseille, France; [4]Department of Oncological Genetics, M Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland; [5]Department of Pathology, Yonsei University College of Medicine, Seoul, Korea; [6]Department of Hematology and Oncological Sciences, L.A. Seragnoli, S. Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy; [7]Interdepartmental Centre for Cancer Research G. Prodi, University of Bologna, Bologna, Italy; [8]Department of Gastroenterology, Medical Center of Postgraduate Education, Warsaw, Poland

BACKGROUND: Prognosis of localised gastrointestinal stromal tumour (GIST) is heterogeneous, notably for patients with AFIP intermediate or high risk of relapse, who are candidates to adjuvant imatinib. We hypothesised that gene expression profiles might improve the prognostication and help to refine the indications for imatinib.

METHODS: We collected gene expression and histoclinical data of 146 pre-treatment localised GIST samples treated with surgery alone. We searched for a gene expression signature (GES) predictive for relapse-free survival (RFS) and compared its performances to that of three published prognostic proliferation-based GES (Genomic Grade Index (GGI), 16-Kinase, and CINSARC) and AFIP classification. We also analysed a data set from 28 patients with advanced GIST treated with neo-adjuvant imatinib.

RESULTS: We identified a 275-gene GES (gene expression signature) predictive of RFS in a learning set and validated its robustness in an independent set. However, the GGI outperformed its prognostic performances, and those of the two other signatures and the AFIP intermediate-risk classification in two independent tests sets in uni- and multivariate analyses. Importantly, GGI could split the AFIP intermediate/high-risk samples into two groups with different RFS. Genomic Grade Index 'high-risk' tumours were more proliferative and genetically unstable than 'low-risk' tumours, and more sensitive to imatinib.

CONCLUSION: GGI refines the prediction of RFS in localised GIST and might help tailor adjuvant imatinib.

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Gastrointestinal stromal tumours (GISTs) are the most frequent mesenchymal digestive tumours (Kitamura *et al*, 2003; Corless *et al*, 2011). Characterised by activating *KIT* or *PDGFRA* mutations, which rend them sensitive to tyrosine kinase inhibitors (imatinib and sunitinib), they represent a model for molecular-based diagnosis (West *et al*, 2004) and treatment (Antonescu, 2011; Joensuu and Dematteo, 2012). The mainstay of treatment for localised tumour is complete surgical resection (Casali and Blay, 2010). However, the postoperative clinical outcome is heterogeneous, and 20–40% of patients will relapse. *KIT* and *PDGFRA* mutations are early molecular events in GIST development, but those that accumulate during the disease progression are poorly elucidated. The current prognostic criteria – anatomical site, pathological tumour size, mitotic count and tumour rupture (Dematteo *et al*, 2008; Casali and Blay, 2010) – are combined in several risk classifications: NIH 2002 (Fletcher *et al*, 2002), AFIP 2007 (Miettinen and Lasota, 2006), and Joensuu's classification (Joensuu, 2008). Based on recent studies (Dematteo *et al*, 2009; Joensuu *et al*, 2011), adjuvant imatinib is recommended for patients with 'substantial risk of relapse' according to ESMO guidelines and with 'intermediate to high risk' according to AFIP. In Europe, the AFIP classification is widely used. However, the 2-year relapse-free survival (RFS) without adjuvant imatinib is close to 75% in the AFIP intermediate-risk patients and 50% in the high-risk patients (Dematteo *et al*, 2009), meaning that many of them are in fact cured by surgery alone.

Today, the imperfection of these risk stratification systems imposes to refine them to better tailor adjuvant treatment (Patel, 2011). The knowledge of molecular alterations may help achieve this. Prognostic value has been associated with the type of *KIT*/*PDGFRA* mutation (Heinrich *et al*, 2008a, b) and with a few markers (Schneider-Stock *et al*, 2005; Sabah *et al*, 2006; Schmieder *et al*, 2008). But the results are still in some cases contradictory, and these alterations, not currently considered as established independent prognostic factors, are not included in risk classifications. High-throughput gene expression profiling may improve the prognostic classification of cancers, as observed in breast cancer (Bertucci *et al*, 2006). Several DNA microarray-based studies of GIST samples (Allander *et al*, 2001; Antonescu *et al*, 2004; Koon *et al*, 2004; Subramanian *et al*, 2004; Kang *et al*, 2005; Yamaguchi *et al*, 2008; Ostrowski *et al*, 2009; Rink *et al*, 2009; Astolfi *et al*,

*Correspondence: Professor F Bertucci;
E-mail: BERTUCCIF@ipc.unicancer.fr
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2010; Yang *et al*, 2010; Arne *et al*, 2011; Ylipaa *et al*, 2011; Lagarde *et al*, 2012) have highlighted the transcriptional heterogeneity of the disease, notably related to the location, the mutational status, and the risk group. But these series are relatively small (60 cases for the largest one), only 4 directly addressed the prognostic issue (Koon *et al*, 2004; Yamaguchi *et al*, 2008; Arne *et al*, 2011; Lagarde *et al*, 2012), and none identified a multigene predictor that was tested in an independent validation set.

Here, we analysed expression data of 146 imatinib-untreated localised GIST profiled using whole-genome DNA microarrays, and searched for a GES predictive for RFS after surgery.

MATERIALS AND METHODS

Tumour samples

We collected from our databases (Kang *et al*, 2005; Ostrowski *et al*, 2009; Astolfi *et al*, 2010) and public databases (Yamaguchi *et al*, 2008; Rink *et al*, 2009; Lagarde *et al*, 2012) clinical and whole-genome expression data of pre-treatment samples from 146 non-metastatic GIST patients (Kang *et al*, 2005; Yamaguchi *et al*, 2008; Ostrowski *et al*, 2009; Astolfi *et al*, 2010; Lagarde *et al*, 2012) treated with complete primary surgery without adjuvant imatinib (Table 1), and from 28 patients with advanced GIST treated with neo-adjuvant imatinib in the Radiation Therapy Oncology Group Study 0132 (RTOG0132; Rink *et al*, 2009). Data sets are described in Supplementary Table S1. Samples were profiled using whole-genome DNA microarrays: Affymetrix U133 Plus 2.0 (Yamaguchi *et al*, 2008; Ostrowski *et al*, 2009; Astolfi *et al*, 2010), Agilent 44K (Rink *et al*, 2009; Lagarde *et al*, 2012), and home-made microarrays spotted at the Genome Institute of Singapore (Kang *et al*, 2005). The study was approved by our Institutional Board.

Table 1 Histoclinical characteristics of the 146 GIST samples

Characteristics (*N*)	*N* (%)
Sex (146)	
F	56 (38)
M	90 (62)
Age (86)	
$\leqslant 60$	38 (44)
>60	48 (56)
Site (146)	
G	116 (79)
SI	21 (14)
Other	9 (7)
Size (86)	
$\leqslant 5$	36 (42)
$\leqslant 10$	32 (37)
>10	18 (21)
AFIP (146)	
Low	77 (53)
Intermediate	30 (21)
High	39 (27)
Mutation (145)	
*KIT*_ex9	9 (6)
*KIT*_ex11	93 (64)
*KIT*_ex13	1 (1)
*KIT*_ex17	1 (1)
*PDGFRA*_ex12	5 (3)
*PDGFRA*_ex14	1 (1)
*PDGFRA*_ex18	18 (12)
Wild type	17 (12)
Relapse (146)	
0	114 (78)
1	32 (22)
5 Year RFS (86)	79% (0.69–0.90)
Median follow-up, months (range)	47 (2–165)

Abbreviations: ex = exon; F = female; G = gastric; GIST = gastrointestinal stromal tumour; M = male; RFS = relapse-free survival; SI = small intestine.

Gene expression data analysis

Before analysis, we first re-annotated all hybridisation probes present on the three types of microarrays (Affymetrix, Agilent, and Singapore's microarrays). Affymetrix gene chips annotations were updated using NetAffx Annotation files (www.affymetrix.com; release from 1 December 2008). Agilent gene chips annotations were retrieved and updated using both SOURCE (http://smd.stanford.edu/cgi-bin/source/sourceSearch) and EntrezGene (Homo sapiens gene information database, release from 09 December 2008, ftp://ftp.ncbi.nlm.nih.gov/gene/). Regarding the Singapore chips, the 18 664 probe sets representing 18 664 unique (LEADSt) genes were updated using both SOURCE and EntrezGene. All probes were thus mapped based on their EntrezGeneID. When multiple probes were mapped to the same GeneID, the one with the highest variance in a particular data set was selected to represent the GeneID.

Data sets were then processed as follows. For the Agilent-based sets and the Singapore set, we used the available processed data. Regarding the Affymetrix-based data sets, we applied Robust Multichip Average to the raw data before using distance weighted discrimination to make each set comparable to each other using the Yamaguchi's set as reference. Robust Multichip Average was done in R using Bioconductor (Seattle, WA, USA) and associated packages.

Unsupervised hierarchical clustering was applied to Affymetrix data only. Before clustering, a filtering retained the 12 619 genes with the most variable expression across all samples (s.d. $\geqslant 0.25$). Clustering was done with the Cluster program (Eisen *et al*, 1998) using Pearson's uncentered correlation and centroid linkage clustering. Results were displayed using TreeView program (Eisen *et al*, 1998). Biologically relevant gene clusters were identified and summarised as metagenes for prognostic analysis. Metagene expression value was the mean of the normalised expression values of all genes in the respective gene subset. Once computed, the metagenes were tested as continuous value for their prognostic incidence with respect to RFS using Cox regression univariate and multivariate analyses.

Supervised analysis searched for a GES associated with RFS. The data set was split into a learning set (64 Affymetrix samples; Yamaguchi *et al*, 2008; Ostrowski *et al*, 2009; Astolfi *et al*, 2010) and a validation set (82 independent, non-Affymetrix samples; Kang *et al*, 2005; Rink *et al*, 2009; Lagarde *et al*, 2012). Cox regression analysis (Wald test) was applied to the 17 870 genes using a FDR threshold of 1% after adjustment for testing of multiple hypotheses. The robustness of the signature was then tested in the independent validation set: by classifying samples according to the Pearson correlation coefficient of their expression profile with the mean profile of the samples with relapse from the learning set. The resulting gene list was interrogated using the Ingenuity Pathway Analysis (IPA) software (Redwood City, CA, USA) to assess significant representation of biological pathways (thresholds of significance for *P*-values were 0.001).

We also tested the prognostic value of three proliferation-based GES previously reported in breast cancer and soft tissue sarcoma: the Genomic Grade Index (GGI), which includes 128 probe sets (108 genes; Sotiriou *et al*, 2006), the 16-kinase signature (16-Kinase), which includes 16 kinase genes (Finetti *et al*, 2008), and the CINSARC signature, which includes 67 genes (Chibon *et al*, 2010). Each signature was applied in each of the five data sets separately to guarantee the largest number of common genes as

possible. We then strictly applied the same method (score or correlation, cutoffs, and scaling methods) as that reported in the original publications to classify each sample into the prognostic group 'low-risk' or 'high-risk'. More details are available in Supplementary Table S2. Another supervised analysis comparing the gene expression profiles of the two GGI-based sample groups was done in the Affymetrix data set using significance analysis of microarrays with a FDR threshold of 1%.

Statistical analyses

Correlations between sample groups and histoclinical factors were calculated with the Fisher's exact test for qualitative variables and the Mann–Whitney test for continuous variables. The primary endpoint was RFS calculated from the date of diagnosis until date of first relapse whatever its location using the Kaplan–Meier method. In one data set (Lagarde *et al*, 2012), the presence or absence of relapse was documented without information about the delay (60 samples). In the whole data set ($N=146$), 114 patients did not experience any relapse, whereas 32 relapsed. In the 86 cases with available time information, the median follow-up – measured from the date of diagnosis to the date of last news – was 47 months (range, 2–165) for the 71 relapse-free patients; the median time to relapse was 19 months (range, 5–74) for the 15 patients who relapsed, and the 5-year RFS was 79% (CI 95% (69–90)). Survival was compared between groups with the log-rank test. Univariate and multivariate analyses were done using either Cox regression analysis when the time information was available, or a logistic regression analysis using the *glm* function (R's statistical package) when it was not available (significance estimated by specifying a binomial family for model with a logit link). The variables tested in univariate analyses included the sample classification based on each GES 'high-risk' *vs* 'low-risk' and the AFIP classification (high *vs* intermediate *vs* low-risk). Multivariate analysis incorporated all variables with a *P*-value inferior to 1% in univariate analysis. The Cox multivariate analysis was done with the Cox proportional hazard model or Cox regression with the Firth's correction (R software, Bioconductor) depending on occurrence or not of relapse in the reference group. All statistical tests were two-sided at the 5% level of significance. Statistical analysis was done using the survival package (version 2.30) in the R software (version 2.9.1). The paper is written in accordance with reporting recommendations for tumour marker prognostic studies (REMARK) criteria (McShane *et al*, 2005).

RESULTS

Whole-genome gene expression profiles

Hierarchical clustering was applied to 64 samples and 12 619 most varying genes in the Affymetrix data set (Supplementary Figure S1). As reflected by the dendrogram, the samples displayed heterogeneous expression profiles (Supplementary Figures S1A and B). They were sorted into two main 32 sample clusters (I and II), which correlated with the anatomical site (69% gastric in cluster I *vs* 100% in cluster II, $P=8.5E-04$) and the mutation status (84% *KIT*-mutated in cluster II *vs* 48% in cluster I, and 3% *PDGFRA*-mutated in cluster II *vs* 29% in cluster I, $P=3.2E-04$). No significant correlation existed with the AFIP classification or clinical outcome, even if cluster II included more AFIP high-risk patients (34% *vs* 15% in cluster I; $P=0.17$), and more patients with relapse during follow-up (22% *vs* 9%; $P=0.3$; HR = 2.67).

Several gene clusters were evidenced. Some of them defined expression signatures related to specific cell types, biological functions, or chromosomal locations (Supplementary Figures S1A–C). A proliferation cluster included two classical proliferation markers (*MKI67* and *PCNA*) and many genes involved in the cell cycle and mitosis such as *AURKA/B*; cyclin genes, *TOP2A*, *TTK*, or *BUB1*. Visually, this cluster was more expressed in AFIP high-risk samples in agreement with a likely higher mitotic index. An immune cluster reflected variation in specific immune cell types within the tumours. It included genes encoding markers of B cells (immunoglobulins and HLA molecules), T cells (*CD3*, *ZAP70*, and *CD4*), NK cells (*NKG7* and *CD244*), or macrophages (*CD68*, *CD14*, and *CSF1R*). Visually, this cluster was more expressed in *PDGFRA*-mutated samples in agreement with their classically high lymphocyte infiltrate (Subramanian *et al*, 2004). An early response gene cluster, including for example *FOS*, *ATF3*, *DUSP2*, and *NR4A2,* appeared more expressed in *PDGFRA*-mutated samples as expected (Subramanian *et al*, 2004). A cluster, enriched in genes involved in angiogenesis (*VEGFA*, *TEK*, *FLT1/3*, *CDH5*, and *PECAM1*) was overall overexpressed in samples without *KIT* exon 11 mutation as expected (Imamura *et al*, 2007). Finally, a cluster of co-expressed genes represented a presumptive lost chromosomal region (9p loss cluster, of which 15% of genes are located on the 9p chromosomal arm); its expression was visually negatively correlated with that of the 'proliferation cluster' and associated with worse RFS as previously reported (Schneider-Stock *et al*, 2005; Haller *et al*, 2008a). We tested the prognostic value of these five clusters computed as metagenes. The proliferation, 9p loss, and early response metagenes were associated with poor RFS in Cox univariate analysis ($P<0.05$, Wald test), whereas the two other ones (immune and vascular) were not. In multivariate analysis including the three significant metagenes, only the proliferation metagene remained significant.

We also confirmed the overexpression of some individual control genes in relation with the mutation status (Subramanian *et al*, 2004; Chi *et al*, 2010; Arne *et al*, 2011): *KIT*, *CD34*, *ETV1*, *PROM1* (CD133), or *RPS6KA1* in *KIT*-mutated samples, *EPHA4* or *DSG2* for *KIT* exon 9 mutations, and *PDGFRA*, *IGFBP5*, *IGF1*, *SPON1*, or *PRKCA* for *PDGFRA* mutations.

GES for RFS

We searched for a GES associated with RFS. The data set was split into a learning set and a validation set. To avoid additional normalisation, we used the Affymetrix set as learning set ($N=64$) and the two other sets as independent validation set ($N=82$). In the learning set (10 relapses), Cox analysis identified 275 discriminating genes (FDR 1%; Supplementary Table S3), including 129 genes overexpressed and 146 underexpressed in samples with event. As expected, the resulting classification of learning set samples strongly correlated with clinical outcome ($P=8.5E-04$, Supplementary Figure S2A). More importantly, in the validation set (Supplementary Figure S2B), 39 out of 60 (65%) patients who did not relapse were classified by the signature as 'low-risk', and 21 out of 22 (95%) patients who relapsed were accurately predicted as 'high-risk'. The rate of accurate classification was 73%, suggesting the robustness of the signature ($P=5.4E-07$). When applied to the whole series of samples (learning and validation sets pooled), 71 out of 114 (63%) patients who did not relapse were classified as 'low-risk', and 31 out of 32 (97%) patients who relapsed were accurately predicted as 'high-risk' ($P=3.7E-10$). Considering the 86 patients with available RFS time, the 5-year RFS was 97% (CI 95% (92–100)) in the 'low-risk' group ($N=39$) and 64% (CI 95% (50–84)) in the 'high-risk' group ($N=47$) as defined using the signature ($P=0.0016$, Figure 1A).

The biological significance of the signature was interrogated using the IPA gene ontology software (Supplementary Table S4A). No significantly enriched canonical pathway was identified for the 146 underexpressed genes. By contrast, analysis found a significant overrepresentation of cell cycle-associated pathways within the overexpressed genes.

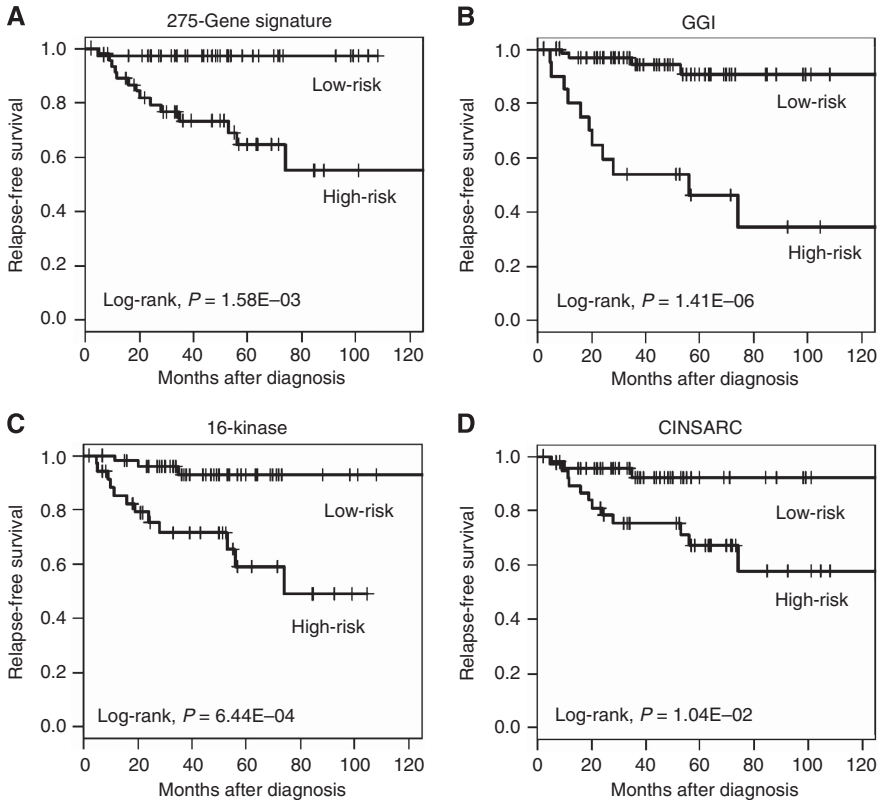

Figure 1 Time-dependent correlation between the classifications based on four prognostic GES and RFS in 86 GIST samples. Kaplan–Meier RFS curves of the two GIST groups (low-risk and high-risk) defined by the: (**A**) 275-gene signature, (**B**) GGI, (**C**) 16-Kinase signature, and (**D**) CINSARC.

Table 2 Correlation between the classifications based on three prognostic GES and RFS in GIST samples in the two tests sets, separately and pooled

	Test set no. 1 (*N* = 86; time to RFS available)				Test set no. 2 (*N* = 60; time to RFS not available)				Pooled test sets (*N* = 146; all samples)			
Prognostic	Relapse			*P*-value[a]	Relapse			*P*-value[a]	Relapse			*P*-value[a]
GES	No	Yes	Total	OR (95% CI)	No	Yes	Total	OR (95% CI)	No	Yes	Total	OR (95% CI)
GGI												
Low-risk	62	4	66	5.83E − 06	42	4	46	5.94E − 09	104	8	112	3.70E − 13
High-risk	9	11	20	17.9 (4.2–94.8)	1	13	14	114.2 (12.5–5653.5)	10	24	34	29.8 (10.0–100.2)
16-Kinase												
Low-risk	48	3	51	1.04E − 03	33	1	34	4.75E − 07	81	4	85	3.77E − 09
High-risk	23	12	35	8.1 (1.9–49.3)	10	16	26	48.75 (6.2–2246.3)	33	28	61	16.8 (5.3–71.1)
CINSARC												
Low-risk	45	3	48	3.34E − 03	32	0	32	5.54E − 08	77	3	80	2.30E − 09
High-risk	26	12	38	6.8 (1.6–40.8)	11	17	28	INF (9.4–Inf)	37	29	66	19.7 (5.6–107.7)

Abbreviations: CI = confidence interval; CINSARC = complexity index in sarcomas; GES = gene expression signature; GGI = Genomic Grade Index; GIST = gastrointestinal stromal tumour; OR = odds ratio; RFS = relapse-free survival. [a]Fisher's exact test *P*-value with OR and CI associated.

Prognostic value of three proliferation-based GES

Given the importance of proliferation genes in this signature, we tested whether three published proliferation-associated GES with prognostic value in breast cancer (GGI, 16-Kinase signature) and sarcomas (CINSARC) had prognostic value in GISTs. Analysis was done in two separated test sets (no. 1 and no. 2). We first analysed the 86 sample series (test set no. 1) where the time to RFS was available. Each GES divided samples into two groups 'low-risk' and 'high-risk' with different 5-year RFS (Figures 1B–D), respectively, 91% (CI 95% (82–100); $N = 66$) and 46% (CI 95% (28–77); $N = 20$) with the GGI ($P = 1.4E - 06$), 93% (CI 95% (85–100); $N = 51$) and 59% (CI 95% (42–84); $N = 35$) with the 16-Kinase signature ($P = 6.44E - 04$), and 92% (CI 95% (84–100); $N = 48$) and 67% (CI 95% (53–86); $N = 38$) with CINSARC ($P = 0.01$). Table 2 shows the corresponding contingency tables. Although each signature gave significant results, the largest difference in RFS was observed with the GGI.

Table 3 Univariate and multivariate analyses for RFS

	Univariate: Cox regression			Multivariate: Cox regression using Firth's bias reduction		
Test set no. 1 (*N* = 86; time to RFS available)	***N***	**HR (95% CI)**	***P*-value**	***N***	**HR (95% CI)**	***P*-value**
GGI						
High *vs* low	86	9.93 (3.13–32.3)	2.12E − 05	86	3.23 (1.12–10.83)	2.91E − 02
AFIP						
Intermediate *vs* low	86	3.84E08 (0–Inf)	3.38E − 08	86	11.2 (0.87–1.6E03)	0.06
High *vs* low	86	2.17E09 (0–Inf)		86	44.1 (5.23–5.8E03)	3.01E − 05
275-gene						
Poor *vs* good	86	12.64 (1.66–96.17)	1.43E − 02			
	Univariate: logistic regression			**Multivariate: logistic regression**		
Test set no. 2 (*N* = 60; time to RFS not available)	***N***	**Coefficient**	***P*-value**	***N***	**Coefficient**	***P*-value**
GGI						
High *vs* low	60	0.84	1.19E − 13	60	0.60	3.16E − 08
AFIP						
Intermediate *vs* low	60	0.29	9.17E − 03	60	0.11	0.19
High *vs* low	60	0.78	8.13E − 11	60	0.41	5.83E − 05
275-gene						
Poor *vs* good	60	0.63	1.22E − 09	60	0.27	7.14E − 04
	Univariate: logistic regression			**Multivariate: logistic regression**		
Pooled test sets (*N* = 146; all samples)	***N***	**Coefficient**	***P*-value**	***N***	**Coefficient**	***P*-value**
GGI						
High *vs* low	146	0.63	< 2.00E − 16	146	0.35	1.25E − 07
AFIP						
Intermediate *vs* low	146	0.20	< 2.00E − 16	146	0.12	0.05
High *vs* low	146	0.67	8.13E − 11	146	0.42	1.75E − 10
275-gene						
Poor *vs* good	146	0.41	3.62E − 10	146	0.15	2.88E − 03

Abbreviations: CI = confidence interval; GGI = Genomic Grade Index; HR = hazard ratio; RFS = relapse-free survival.

For further validation in an independent set, we repeated the analysis in the 60 Lagarde's samples. In this series, the time to relapse was not available (test set no. 2). The correlation between the resulting classification and the survival was thus analysed using contingency tables (Table 2). Correlation was significant for each signature, but again stronger for the GGI. When we pooled the two test sets ($N = 146$), the rate of accurate classifications was 88% for GGI, 75% for the 16-Kinase signature, and 73% for CINSARC (Table 2). We thus focused on the GGI in the next sections.

Univariate and multivariate analyses for RFS

We compared the prognostic performances of the classifications based on the 275-gene GES, GGI, and AFIP (Table 3). Analysis was done in the two test sets, separately then pooled.

In the test set no. 1 (86 samples with available time to RFS) Cox univariate analysis confirmed the prognostic value of the three classifiers. In multivariate analysis, the GGI and the AFIP high-risk classification remained significant, suggesting their independent prognostic value, but the AFIP intermediate-risk classification was not. Genomic Grade Index sorted the AFIP intermediate/high-risk samples into two groups 'low-risk' and 'high-risk' with respective 5-year RFS of 73% (CI 95% (52–100); $N = 22$) and 35% (CI 95% (17–70); $N = 16$; $P = 8.5E - 03$; Figure 2).

As independent validation, we repeated the analyses in the test set no. 2 (Lagarde's 60 sample series) using logistic regression. Results of univariate analysis were similar. In multivariate analysis, the GGI signature remained significant, whereas the AFIP intermediate-risk classification was not. Genomic Grade Index sorted the AFIP intermediate/high-risk samples into two groups with different relapse rate: 4 out of 17 patients (24%) in the 'low-risk' group and 13 out of 14 (93%) in the 'high-risk' (OR = 35.7; $P = 1.8E - 04$). When we pooled the two test sets ($N = 146$), similar results were observed (Table 3).

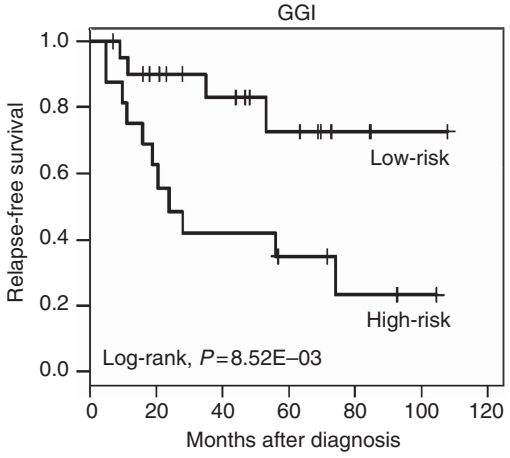

Figure 2 Correlation between the GGI-based classification and RFS in the AFIP intermediate/high-risk GIST samples. Kaplan–Meier RFS curves in the patients with available RFS time ($N = 38$).

Comparison of the two GGI-based GIST groups

The GGI was thus an independent predictor for RFS in two independent GIST tests sets. To explore the differences between the so-defined GGI 'low-risk' and 'high-risk' groups, we compared their histoclinical features ($N=146$; Supplementary Table S5). Significant differences existed for three parameters. Genomic Grade Index 'high-risk' samples were more frequently AFIP high-risk ($P=1.0E-05$) and superior to 10 cm ($P=1.22E-04$), and displayed more relapses ($P=3.6E-13$: OR = 29.84) despite a similar follow-up. The AFIP intermediate/high-risk samples were sorted by GGI into two groups with different rate of relapses: 8 out of 39 patients (21%) in the 'low-risk' group and 24 out of 30 (80%) in the 'high-risk' group (OR = 14.7; $P=8.8E-07$).

We then compared the whole-genome expression profiles of the two groups in the Affymetrix 64 sample series. Significance analysis of microarrays analysis identified 254 discriminating genes (FDR 1%), including 192 genes overexpressed and 62 underexpressed in the 'high-risk' samples (Supplementary Table S6). Ontology analysis found an overrepresentation of canonical pathways within the overexpressed genes only (Supplementary Table S4B). Most of them were associated with cell cycle control and genome stability: examples include 'mitotic roles of polo-like kinase', 'role of chk proteins in cell cycle checkpoint control', 'cell cycle: G2/M DNA damage checkpoint regulation', or 'cell cycle control of chromosomal replication'. Of note, 10 out of the 16 significant pathways (62.5%) were common to those identified by IPA analysis of the genes overexpressed in the 275-gene GES.

GGI-based classification and response to imatinib

A major and complementary issue is to determine whether the GGI 'high-risk' patients are more sensitive to imatinib. We analysed a set of 28 pre-treatment samples from patients with advanced primary and recurrent operable GIST treated in a phase II trial (RTOG0132) with 8–12 weeks neo-adjuvant imatinib and for whom the degree of clinical response (RECIST by comparing tumour measurements on computed tomography scans before and after imatinib) was available (Rink *et al*, 2009). The percentage of tumour shrinkage ranged from −76 to +21%.

We reproduced the two GGI-based groups. As expected given the advanced stage of disease, more patients were classified as 'high-risk' ($N=24$) than 'low-risk' ($N=4$). Despite the low number of samples, the correlation between those groups and the degree of response (continuous variable) was significant with greater tumour shrinkage in the 'high-risk' group than the 'low-risk' group (mean −29% *vs* −5%, $P=0.04$; Figure 3). By applying the response definition used in the original paper (binary variable), the correlation was high, even if not significant owing to the number of cases: 17 out of 24 'high-risk' samples (71%) were classified as rapid responders, whereas 3 out of 4 'low-risk' samples (75%) as non-responders ($P=0.116$), with a OR for response of 6.7 in the 'high-risk' group as compared with the 'low-risk' group. These results suggested that GGI 'high-risk' patients are more imatinib sensitive than are 'low-risk' patients.

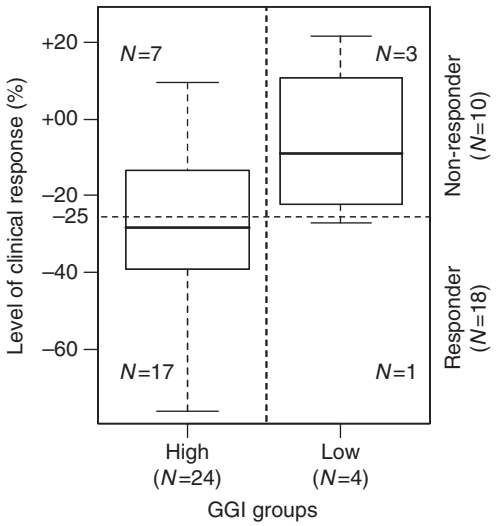

Figure 3 Correlation between the GGI-based classification and the response to neo-adjuvant imatinib. Correlation between the two GGI-based groups (high-risk and low-risk; $N=29$) and the response to imatinib assessed as a continuous variable (box plot). The horizontal dashed line indicates the cutoff of tumour shrinkage that defines the responder status. The figures within the box plot indicate the number of patients in each of the four categories.

DISCUSSION

The risk of postoperative relapse in GIST patients dictates the delivery or not of adjuvant imatinib and the follow-up. Risk stratification systems based on histoclinical features exist, but are not perfect to solve the actual prognostic heterogeneity of disease. In Europe, the current gold standard is the AFIP classification and all intermediate- or high-risk patients are candidate to imatinib although many of them are in fact cured by surgery alone and do not need any postoperative therapy. Better identifying them would avoid not only the imatinib-related toxicity, but also the financial cost related to at least 1–3 years of treatment. Efforts are ongoing to improve these stratification systems (Gold *et al*, 2009; Patel, 2011; Rossi *et al*, 2011; Joensuu *et al*, 2012). Although GIST is the most frequent digestive sarcoma, it has been only recently defined as an entity and the research on DNA microarrays and GIST prognosis has suffered from a lack of large clinically documented series (60 cases for the largest one), impeding the use of independent learning and validation sets. We analysed gene expression and histoclinical data of 146 localised GIST operated and untreated with adjuvant imatinib to assess the prognostic performance of gene expression profiles. To our knowledge, this is the largest series reported to date, and the first one that includes independent validation.

Unsupervised analysis confirmed the transcriptional heterogeneity of disease and expression differences associated with the mutation status. Supervised analysis searched for a GES associated with RFS. As recommended, our data set was split into two sets, learning and validation sets. We identified a 275-gene GES associated with RFS in a learning set and validated its robustness in an independent validation set. As this GES was mainly driven by proliferation genes, we hypothesised that three robust prognostic signatures associated with cell cycle control might be predictive for RFS in GIST. These signatures had been identified using hypothesis-driven approaches in breast cancer (by comparing histological grade III *vs* I for GGI, and basal *vs* luminal subtype for the 16-Kinase) and in sarcoma (CINSARC by comparing samples with high *vs* low number of genomic alterations and high *vs* low histological grade). Of note, none of the GIST samples had been used to generate these signatures. However, we divided our data set in two independent test sets, and interestingly, each signature had a prognostic value in each set. As GGI was the most significant one, we compared its prognostic performances with those of the 275-gene GES and the AFIP classification. We found that GGI and the AFIP high-risk classification were independent prognosticators in both test sets. Genomic Grade Index provided additional information to AFIP by discriminating within the intermediate/high-risk AFIP patients those with good prognosis GGI 'low-risk', who are not likely to need adjuvant imatinib, from those with poor prognosis GGI 'high-risk', who likely need imatinib.

Interestingly, we showed in a series of patients treated with neo-adjuvant imatinib for primary GIST (Rink *et al*, 2009) that GGI 'high-risk' patients were more frequently rapid responders to imatinib than 'low-risk' patients. If confirmed in a larger series, this correlation would further increase the clinical interest of GGI in GIST.

Ontologies associated with the genes discriminating the two GGI-based groups showed that the poor prognosis of 'high-risk' group was mainly related to alterations in cell cycle control and maintenance of genome stability, these tumours being more proliferative and more unstable at the chromosomal level. This was confirmed using GSEA and a GES of genome instability (Carter *et al*, 2006; normalised enrichment score = 1.86, $P < 2.12E-16$; data not shown). This higher instability likely confers to 'high-risk' tumours the ability to accumulate molecular alterations allowing them to metastasise. The prognostic importance of proliferation in GIST is well known. The mitotic index is a component of current risk stratifications systems, but is liable to interobserver and technical variability (Patel, 2011). The prognostic value of expression of cell cycle-regulatory genes has been reported (Haller *et al*, 2005; Schneider-Stock *et al*, 2005; Sabah *et al*, 2006; Schmieder *et al*, 2008; Haller *et al*, 2008a, b; Romeo *et al*, 2009; Dorn *et al*, 2010; Fujita *et al*, 2012; Okamoto *et al*, 2012). To date, four studies have analysed the prognostic value of gene expression profiles (Koon *et al*, 2004; Yamaguchi *et al*, 2008; Arne *et al*, 2011; Lagarde *et al*, 2012), but the largest one included 60 cases only. Here, using a stringent significance threshold and in our 146 sample series, *CCNB1* and *CENPF* (Koon *et al*, 2004) were included in our 275-gene list, but *CD133* (Arne *et al*, 2011) and *CD26* (Yamaguchi *et al*, 2008) were not; however, *CD26* expression was associated with RFS ($P = 0.0085$) using less stringent criteria. Recently, Lagarde *et al* (2012) showed the prognostic value of CINSARC and *AURKA* expression, and developed a Genomic Index defined upon array-CGH data as a score of genomic instability associated with metastasis-free survival. Unfortunately, this prognosticator was not validated by the authors in an independent sample set. Here, we confirmed the prognostic value of CINSARC, but showed that GGI was a stronger prognosticator in two independent test sets.

Several genes included in the GGI and/or overexpressed in the GGI 'high-risk' samples encode potential therapeutic targets involved in cell cycle regulation that could be, if functionally validated, targeted by new drugs in the adjuvant setting, alone or associated with imatinib: kinases (*AURKA/B*, *BUB1, CDC2*, *CDK4*, *CHEK1*, *NEK2*, and *PLK1/4*) and phosphatase (*CDC25*). Corresponding inhibitors have entered cancer clinical trials with promising results.

In conclusion, we show that a GGI-based classification of operated GIST outperforms the prognostic performances of three other GES and the AFIP intermediate-risk classification. The strength of our results lies in the size of our series (the largest one reported so far), the biological relevance of GGI, its independent prognostic value in two independent test sets through three different technological platforms. Of note, GGI sorts the AFIP intermediate/high-risk samples – current candidate to adjuvant imatinib – into two groups with different RFS. 'High-risk' tumours according to GGI are more proliferative and genetically unstable than 'low-risk' tumours, and more rapidly sensitive to imatinib. By refining the prediction of RFS, GGI might improve our ability to better tailor adjuvant imatinib. Further clinical validation of GGI is warranted in larger retrospective, then prospective series, as well as the functional validation of relevant genes that could provide new therapeutical targets.

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