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Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Mitotic Checkpoints and Chromosome Instability Are Strong Predictors of Clinical Outcome in Gastrointestinal Stromal Tumors

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Abstract

Purpose: The importance of *KIT* and *PDGFRA* mutations in the oncogenesis of gastrointestinal stromal tumors (GIST) is well established, but the genetic basis of GIST metastasis is poorly understood. We recently published a 67 gene expression prognostic signature related to genome complexity (CINSARC for Complexity INdex in SARComas) and asked whether it could predict outcome in GISTs.

Experimental Design: We carried out genome and expression profiling on 67 primary untreated GISTs.

Results: We show and validate here that it can predict metastasis in a new data set of 67 primary untreated GISTs. The gene whose expression was most strongly associated with metastasis was *AURKA*, but the *AURKA* locus was not amplified. Instead, we identified deletion of the *p16* (*CDKN2A*) and retinoblastoma (*RB1*) genes as likely causal events leading to increased *AURKA* and CINSARC gene expression, to chromosome rearrangement, and ultimately to metastasis. On the basis of these findings, we established a Genomic Index that integrates the number and type of DNA copy number alterations. This index is a strong prognostic factor in GISTs. We show that CINSARC class, *AURKA* expression, and Genomic Index all outperform the Armed Forces Institute of Pathology (AFIP) grading system in determining the prognosis of patients with GISTs. Interestingly, these signatures can identify poor prognosis patients in the group classified as intermediate-risk by the AFIP classification.

Conclusions: We propose that a high Genomic Index determined by comparative genomic hybridization from formalin-fixed, paraffin-embedded samples could be used to identify AFIP intermediate-risk patients who would benefit from imatinib therapy. *Clin Cancer Res; 18(3); 826–38. ©2011 AACR.*

Introduction

Gastrointestinal stromal tumors (GIST) are the most frequent mesenchymal tumors of the gastrointestinal tract and account for approximately 25% of soft tissue sarcomas. They are thought to arise from the intestinal cells of Cajal (1) or from a common progenitor cell (2). Most GISTs (80%) have activating mutations in the *KIT* tyrosine kinase receptor gene, but 8% have platelet-derived growth factor receptor α (*PDGFRA*) mutations (3, 4) and a few of the remainder have *BRAF* mutations (5). In addition to these mutations, the most frequently reported genetic changes are 14q, 22q, and 1p losses (6).

Clinical management of GISTs consists mainly of surgical resection and adjuvant targeted therapy with imatinib mesylate (Gleevec, Novartis Pharma AG), which targets mutationally activated KIT or PDGFRA signaling (7). Around 20% to 40% of patients relapse, with distant liver metastasis being the most common manifestation of recurrence. It is mainly patients with these aggressive GISTs who benefit from imatinib therapy. Precise evaluation of metastatic risk is therefore highly desirable.

Many pathologic criteria based on tumor site, tumor size, cell type, degree of necrosis, and mitotic rate have been proposed for predicting the outcome of patients with GISTs. A consensus grading scheme based on tumor size and mitotic count was proposed by the U.S. NIH in 2001 to estimate the prognosis of GIST patients (8). In 2006, the Armed Forces Institute of Pathology (AFIP) proposed an updated system taking into account also tumor location (9). Both systems are based on histopathologic assessment of

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Gastrointestinal stromal tumors (GIST) are the most frequent mesenchymal tumors of the gastrointestinal tract and are among the rare tumors to benefit from a targeted therapy. Thus, the development of a method for GIST prognostication has become essential for the proper clinical management of GIST patients, especially in the context of adjuvant treatment, in which many patients are exposed to a drug although only a small proportion will likely benefit from such treatment. Here, we show that mitotic checkpoint expression and chromosome complexity are strong predicators of metastatic outcome in GISTs. Of particular interest, these signatures can distinguish good from poor prognosis patients classified as intermediate-risk by the current histologic method for risk assessment (which represent around 25% of diagnoses). Comparative genomic hybridization technique is already used in pathology laboratories with formalin-fixed paraffin-embedded samples. Genomic profiling could therefore be a powerful tool to manage imatinib therapy for intermediate-risk GIST patients.

tumor aggressiveness. Cutoff values defining risk groups have been determined empirically but generate a large intermediate-risk group for which adjuvant imatinib is controversial because the real metastatic risk is poorly defined. Hence, there is a need to better understand GIST biology to identify biomarkers causally linked to poor outcome.

To address this need, multiple DNA copy number and gene expression studies have been carried out but, for a variety of reasons including small sample size and availability of clinical data, the results were generally inconclusive. It has been shown that the number and complexity of genomic rearrangements increase with tumor stage but no threshold has been defined (6, 10–16). At the expression level, Yamaguchi and colleagues reported a gene expression signature based on 32 GISTs that predicts outcome, but only in gastric GISTs (17).

We recently established a 67 gene prognostic signature related to chromosome integrity, mitotic control, and genome complexity in sarcomas (CINSARC for Complexity INdex in SARComa; ref. 18). To assess the effectiveness of this signature in GISTs, we have used it to score 67 fully annotated primary untreated GISTs. To identify the underlying mechanisms leading to high CINSARC scores, we have carried out genome-wide DNA copy number and gene expression analyses of these tumors.

Materials and Methods

Tumor samples

Frozen samples from 67 resected primary GISTs untreated until tumor recurrence were selected from the European GIST database CONTICAGIST (www.conticagist.org). Dates of diagnosis range from June 1995 to February 2009. Information regarding tumors and patients are summarized in Table 1.

Array-comparative genomic hybridization analysis

DNA was hybridized to 8 × 60 K whole genome Agilent arrays (G4450A) according to the manufacturer's protocol. The ADM-2 algorithm of comparative genomic hybridization (CGH) Analytics v4.0.76 software (Agilent) was used to identify DNA copy number anomalies at the probe level. A low-level copy number gain was defined as a $\log_2$ ratio more than 0.25, and a copy number loss was defined as a $\log_2$ ratio less than −0.25. A high-level gain or amplification was defined as a $\log_2$ ratio more than 1.5, and a homozygous deletion was suspected when the ratio was below −1.

Gene expression profiling

Gene expression analysis was carried out by Agilent Whole human 44K Genome Oligo Array (Agilent Technologies) according to the manufacturer's protocol. All microarrays were simultaneously normalized with the Quantile algorithm. *t* Tests were carried out using Genespring (Agilent Technologies), and *P* values were adjusted by the Benjamini–Hochberg procedure. The *P* value and fold change cutoff for gene selection were 0.001 and 3, respectively. Gene ontology (GO) analysis was conducted to establish statistical enrichment in GO terms using Genespring (Agilent Technologies). MIAME-compliant data have been deposited at Array Express [Experiment name: Prediction of clinical outcome in GISTs (Gastro Intestinal Stromal Tumours); ArrayExpress accession: E-MTAB-373; Reviewer login: E-MTAB-373_Reviewer; Password: Gc3giN7].

Quantitative genomic and reverse transcription PCR

The copy number status of *p14*, *p15*, and *p16* was determined as previously described (19). A normal status was assigned to a ratio of 0.8 or more and 1.2 or less. A ratio of more than 0.1 and less than 0.8 was scored as a hemizygous deletion. When ratio was below 0.1, the deletion was scored as homozygous.

Reverse transcription (RT) and quantitative PCR (qPCR) for *p14*, *p16*, *AURKA*, and *RB1* were carried out as previously described (19). A reference C_t (threshold cycle) for each sample was defined as the average measured CT of the 3 reference genes, *GAPDH*, *ACTB*, and *RPLP0*. Relative mRNA level in a sample was defined as: $\Delta C_t = C_t$ (gene of interest) $- C_t$ (mean of the 3 reference genes).

Immunohistochemistry

Immunohistochemistry experiment was realized on tissue microarrays (TMA) containing 15 cases from the present series and carried out as previously described (20). Antigen retrieval was achieved using the Dako Target Retrieval Solution, pH 9 for 20 minutes at 98°C. Slides were incubated for 1 hour with the AURKA antibody used at a dilution

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Table 1. Description of patients

Follow-up (y)	3.7
95% CI	3.08–4.4
Sex	
Male	27 (40)
Female	40 (60)
Location	
Stomach	43 (64)
Small intestine	12 (18)
Other	12 (18)
Histological subtype	
Spindle	52 (77.5)
Epithelioid	5 (7.5)
Mixed	10 (15)
Tumor size – 1	
≤2 cm	5 (7.5)
2–5 cm	25 (37)
5–10 cm	21 (31.5)
>10 cm	15 (22.5)
nd	1 (1.5)
Tumor size – 2	
<3 cm	9 (13.5)
≥3 cm	57 (85)
nd	1 (1.5)
Mitotic index	
≤5	42 (63)
>5	25 (37)
AFIP risk	
Very low	15 (22)
Low	16 (24)
Intermediate	16 (24)
High	19 (28.5)
nd	1 (1.5)
Surgery margin	
R0	46 (69)
R1	4 (6)
nd	17 (25)
Mutations	
KIT	**52 (77.5)**
Ex 9	2 (3)
Ex 11	48 (71.5)
Ex 13	1 (1.5)
EX 17	1 (1.5)
PDGFRA	**12 (18)**
Ex 12	2 (3)
Ex 14	1 (1.5)
Ex 18	9 (13.5)
WT	**3 (4.5)**
Relapse events	
Local	7 (10)
Distance	18 (27)

NOTE: Percentages are indicated in brackets.
Abbreviation: nd, not determined.

of 1:50 (Novocastra, NCL-L-AK2, clone JLM28). Each case was spotted in triplicate on the TMA, and we used the average value of the 3 spots.

Statistical analysis

The CINSARC centroids are mean-centered reference profiles for the CINSARC signature genes in the 310 metastatic and nonmetastatic sarcomas from our previous study (18). Each GIST was allocated to the prognostic class with the highest Spearman correlation to the reference centroids.

Metastasis-free survival (MFS) was calculated by the Kaplan–Meier method from the date of initial diagnosis to the date of first metastasis, relapse, last follow-up or death for patients without diagnosis of metastasis. Survival curves were compared with the log-rank test. All survival analyses were conducted using R software (version 2.11.11) and the "survival" package. HRs and multivariate analysis were conducted with the Cox proportional hazard model or Cox regression with the Firth's correction (R software, "coxph" package) depending on occurrence or not of events in the reference group.

Results

Is CINSARC a significant prognostic factor for GISTs?

To test whether the CINSARC signature has prognostic value in GISTs, we carried out gene expression profiling on a series of 60 of 67 (89.5%) GISTs with mRNA of sufficient quality (Table 1). We assigned these tumors to prognostic groups based on correlation with the published CINSARC centroids from our previous series of 310 sarcomas (18). Survival analysis (Fig. 1) revealed that the CINSARC classification split the tumors into 2 groups with very different MFS ($P = 1.4 \times 10^{-5}$). No metastasis or other relapse event occurred in the good prognosis group.

Is it possible to derive a better signature specifically for GISTs?

The CINSARC signature is based on several different types of sarcoma. To test whether it is possible to derive a better signature that is specific for GISTs, we analyzed the *GIST* gene expression profiles to identify genes differentially expressed by the metastatic and nonmetastatic tumors. Among the 297 differentially expressed genes (Supplementary Table S1), 70 (86 probe sets) were downregulated and 227 (252 probe sets) were upregulated in metastatic cases (FC > 3 and $P < 0.001$). GO analysis identified no significantly enriched pathways for the 70 downregulated genes. In contrast, GO analysis revealed that 32 of the 40 (80%) pathways containing upregulated *GIST* genes were also identified by GO analysis with the *CINSARC* genes (Supplementary Table S2). Indeed, 45 of the 227 upregulated *GIST* genes belonged directly to the CINSARC signature. Moreover, GO analysis of the remaining 182 differentially regulated genes not included in CINSARC signature showed enrichment for the same pathways as for the *CINSARC* genes (Supplementary Table S3).

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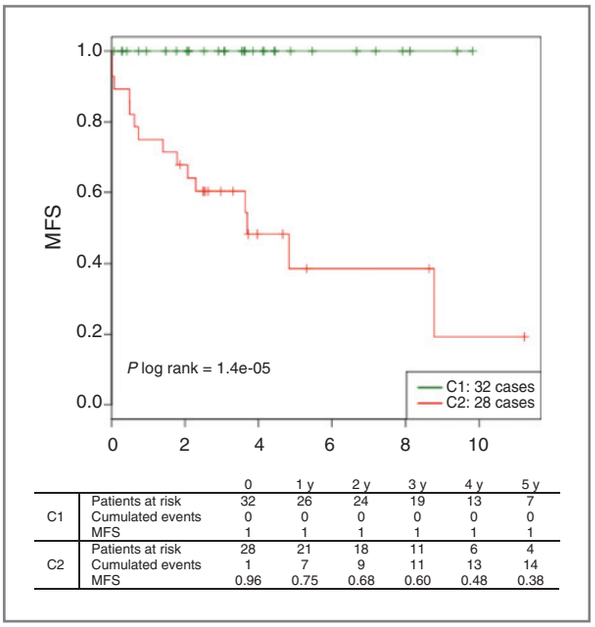

		0	1 y	2 y	3 y	4 y	5 y
C1	Patients at risk	32	26	24	19	13	7
	Cumulated events	0	0	0	0	0	0
	MFS	1	1	1	1	1	1
C2	Patients at risk	28	21	18	11	6	4
	Cumulated events	1	7	9	11	13	14
	MFS	0.96	0.75	0.68	0.60	0.48	0.38

Figure 1. Kaplan–Meier analysis of MFS of 60 GISTs stratified by CINSARC class. C1, patients with low expression of *CINSARC* genes; C2, patients with high expression of *CINSARC* genes; *x*-axis, time in years.

Among the top-ranked differentially expressed genes identified by *t* test, *AURKA* (Aurora kinase A, previously called *STK6* or *STK15*) was the highest ranked gene belonging to the CINSARC signature (Supplementary Table S1). We validated this result by qRT-PCR, which showed that there was a high correlation between the microarray and PCR data (Pearson correlation coefficient $= 0.94$; $P < 1 \times 10^{-15}$), and by immunohistochemistry on a TMA containing 15 of the GISTs from the present series (Supplementary Fig. S3). To test the hypothesis that AURKA alone has prognostic value, we stratified samples by *AURKA* expression. We used the mean *AURKA* level (9.15) as a cutoff (Table 2). Survival analysis showed that *AURKA* expression splits the tumors into 2 groups with very different outcomes (MFS: $P = 5.31 \times 10^{-11}$; Fig. 2A). To validate the result, we studied *AURKA* expression in the GISTs from the study by Yamaguchi and colleagues (17). This confirmed that AURKA splits GISTs into groups with a large difference in MFS ($P = 9.5 \times 10^{-4}$; Fig. 2B).

Is there a genomic explanation for AURKA overexpression?

To test the hypothesis that *AURKA* amplification could account for the *AURKA* overexpression, we carried out CGH to determine the genomic profile of 66 GISTs for which DNA of sufficient quality was available. No *AURKA* amplification was detected. We therefore examined the CGH data for other alterations that could potentially explain the increased *AURKA* expression and poor clinical outcome. The CGH profiles ranged from simple, that is, without any detectable changes, to complex, with multiple full chromosome and segmental gains and losses (Fig. 3A). We compared the frequency of gains and losses for each probe between GISTs with and GISTs without metastatic outcome (Supplementary Fig. S1). No significant difference in gains was observed, whereas several probes showed significant differences in losses. Among the top-ranked losses, the biggest difference was observed for 8 probes on 9p21 deleted in 78.9% and 9.6% of the metastatic and nonmetastatic cases, respectively (Supplementary Fig. S1). All these probes target either the *CDKN2A* (3 probes), *CDKN2B* (3 probes), or *MTAP* (2 probes) loci. 9p21 deletions were observed in 18 patients (18 of 66 = 27%), of whom 13 developed metastases (13 of 18 = 72%). The deletions involved either the whole 9p arms or they were restricted to the *CDKN2A/B* loci (Supplementary Fig. S2). They were scored as homozygous in 7 cases (6 of 7 with metastatic outcome) because of the very low CGH ratios (Supplementary Fig. S2). These homozygous deletions allowed us to define more precisely the genes of interest because in 2 tumors the homozygous deletion excluded *MTAP* (GISTs #5 and #17). We checked the *CDKN2A* and *CDKN2B* copy number status by genomic qPCR and fully confirmed the exclusion of *MTAP* from the minimal deleted region. Interestingly, qPCR showed that the minimal deleted region included *CDKN2A* but not *CDKN2B* (GIST #5; Table 2). As the Agilent gene expression probes target sequences common to the *p14* and *p16* mRNA, we carried out RT-qPCR with primers specific for the individual transcripts (Supplementary Table S4). In all 7 tumors lacking both copies of *CDKN2A*, and in 3 tumors with only 1 copy, both the *p14* and *p16* transcripts were absent or nearly absent. However, in 2 cases with *CDKN2A* deletion but without downregulation of p14/p16 expression in the gene expression microarray data, we observed a specific decrease of p16 but not p14 expression, indicating that the target gene of the *CDKN2A* deletions is likely to be *p16*. To explain metastatic cases without *CDKN2A* deletion, we sought other possible genomic alterations that could interfere with Restriction point control. We identified 1 homozygous deletion and 13 hemizygous deletions at the *RB1* locus (Table 2). Nine of these deletions occurred in recurrent or metastatic tumors and none of them were observed in cases with *CDKN2A* homozygous deletions. RT-qPCR analysis confirmed that deleted tumors had significant downregulation of *RB1* expression (*t* test: $P = 3.5 \times 10^{-4}$; Supplementary Table S4). Comparison of tumors with and without *p16/RB1* alterations showed that tumors with *p16/RB1* alterations overexpressed 235 probe sets (FC > 3; $P < 10^{-3}$) including 42 genes from the CINSARC signature, one of which was *AURKA* (Supplementary Table S5).

Do genomic changes predict GIST outcome?

The CGH profiles of the tumors that did not undergo metastasis had no or few losses or gains, generally involving whole chromosomes, whereas the tumors that developed metastases harbored more frequently segmental alterations. We therefore decided to test whether genome

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Table 2. Results summary of the CINSARC analysis, *AURKA* expression (A: 9.15 as cutoff), CGH analysis (GI: 10 as cutoff), and *CDKN2A/2B* and *RB1* copy number determined by genomic qPCR and array-CGH, respectively (2, without detectable deletion; 1, hemizygous deletion; 0, no copy)

	Expression (Agilent)			CGH				CDKN2A/2B and RB1 copy number					Histology	Annotations			*KIT* and *PDGFRA* mutations	
GIST	CINSARC grading	AURKA	ARUKA stratification	Number of Alt	Nbr Chr	Alt^2 /Nbr chr	Genomic Index	p14	p16	p15	RB1	Pathway	AFIP	Site of primary tumor	Local recurrence	Metastasis	Mutated gene	Mutation
GIST10	C1	8.56	A1	5	4	6.25	GI1	2	2	2	2	N	Low risk	Small intestine	No	No	*K11*	p.V560D
GIST13	C1	8.05	A1	2	2	2	GI1	2	2	2	2	N	Intermediate	Stomach	No	No	*K11*	p.W557R
GIST15	C1	7.89	A1	4	3	5.33	GI1	2	2	2	2	N	Low risk	Stomach	No	No	*K11*	p.V559D
GIST21	C1	8.66	A1	2	2	2	GI1	2	2	2	2	N	Intermediate	Stomach	No	No	*K11*	p.L576P
GIST23	C1	8.39	A1	0	0	0	GI1	nd	nd	nd	2	N	Low risk	Small intestine	No	No	*P12*	p.Y555C
GIST24	C1	8.23	A1	4	4	4	GI1	2	2	2	2	N	Low risk	Peritoneum	No	No	*K11*	p.T574_R586insK
GIST27	C1	7.75	A1	1	1	1	GI1	2	2	2	2	N	High risk	Stomach	No	No	*K11*	p.K581_S590dup
GIST30	C1	7.62	A1	2	2	2	GI1	2	2	2	2	N	Intermediate	Stomach	No	No	*K11*	p.L576_R588dup
GIST32	C1	8.09	A1	1	1	1	GI1	2	2	2	2	N	Intermediate	Stomach	No	No	*K11*	p.W557R
GIST33	C1	8.55	A1	3	3	3	GI1	2	2	2	2	N	Very low	Stomach	No	No	*P18*	p.D842V
GIST36	C1	7.61	A1	1	1	1	GI1	2	2	2	2	N	Very low	Stomach	No	No	*K11*	p.V559D
GIST40	C1	7.8	A1	1	1	1	GI1	2	2	2	2	N	Low risk	Stomach	No	No	*K11*	p.P573_T574dup; T574dup; Q575_R586dup
GIST43	C1	8.01	A1	1	1	1	GI1	2	2	2	2	N	Very low	Stomach	No	No	*K11*	p.T574_L589dup
GIST44	C1	8.41	A1	5	3	8.33	GI1	2	2	2	2	N	Low risk	Stomach	No	No	*K11*	p.Q556_V559del
GIST46	C1	8.6	A1	5	3	8.33	GI1	2	2	2	2	N	Very low	Small intestine	No	No	*K11*	p.Q556_V559del
GIST48	C1	8.14	A1	8	7	9.14	GI1	2	2	2	2	N	Low risk	Small intestine	No	No	*K11*	p.M552_E561del
GIST49	C1	8.93	A1	7	5	9.8	GI1	2	2	2	2	N	Very low	Stomach	No	No	*K11*	p.E554_K558del
GIST51	C1	8.33	A1	0	0	0	GI1	2	2	2	2	N	Very low	Stomach	No	No	*K11*	p.W557R
GIST55	C1	7.72	A1	5	4	6.25	GI1	2	2	2	2	N	Very low	Stomach	No	No	*K11*	p.D572_D579dupinsL
GIST60	C1	8.77	A1	1	1	1	GI1	2	2	2	2	N	Very low	Stomach	No	No	*P18*	p.D842V
GIST62	C1	8.3	A1	1	1	1	GI1	2	2	2	2	N	Very low	Stomach	No	No	*K11*	p.N566_P573del
GIST8	C1	7.71	A1	1	1	1	GI1	2	2	2	2	N	Low risk	Stomach	No	No	*K11*	p.W557_K558del
GIST29	C1	8.48	A1	2	2	2	GI1	2	2	2	2	N	Intermediate	Stomach	No	No	*K11*	p.D572_T574dup
GIST31	C1	8.51	A1	3	3	3	GI1	2	2	2	2	N	Low risk	Stomach	No	No	*P18*	p.I843_D846del
GIST41	C1	8.97	A1	1	1	1	GI1	2	2	2	2	N	Low risk	Stomach	No	No	*P12*	p.D561V

(Continued on the following page)

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	Expression (Agilent)			CGH				CDKN2A/2B and RB1 copy number					Histology	Annotations			*KIT* and *PDGFRA* mutations	
GIST	CINSARC grading	AURKA	ARUKA stratification	Number of Alt	Nbr Chr	Alt^2 /Nbr chr	Genomic Index	p14	p16	p15	RB1	Pathway	AFIP	Site of primary tumor	Local recurrence	Metastasis	Mutated gene	Mutation
GIST50	C1	8.36	A1	7	6	8.17	GI1	1	1	1	2	1	High risk	Small intestine	No	No	*K11*	p.M552_E554delinsK
GIST66	C1	8.82	A1	7	6	8.17	GI1	1	1	1	2	1	Low risk	Duodenum	No	No	*K11*	p.V559G
GIST1	C1	8.12	A1	3	3	3	GI1	2	2	2	1	1	High risk	Stomach	No	No	*P18*	p.D842V
GIST54	C1	9.11	A1	2	2	2	GI1	2	2	2	1	1	Very low	Stomach	No	No	*P18*	p.D842V
GIST59	C1	7.31	A1	8	6	10.7	GI2	2	2	2	2	N	Very low	Stomach	No	No	*K11*	p.N567_L576delinsKE homo
GIST67	C1	7.35	A1	11	6	20.17	GI2	2	2	2	2	N	Low risk	Stomach	No	No	*K11*	p.V560D
GIST65	C1	8.69	A1	20	11	36.36	GI2	1	1	1	2	1	Intermediate	Small intestine	No	No	*K13*	p.K642E
GIST52	C2	8.32	A1	nd	nd	nd	nd	2	2	2	nd	nd	Very low	Stomach	No	No	*K11*	p.P573_H580ins
GIST18	C2	9.05	A1	6	4	9	GI1	2	2	2	2	N	Intermediate	Duodenum	No	No	*K11*	p.L576P
GIST64	C2	8.6	A1	5	5	5	GI1	2	2	2	2	N	Low risk	Small intestine	No	No	*K11*	p.V560D
GIST12	C2	8.66	A1	0	0	0	GI1	2	2	2	2	N	High risk	Retroperitoneum	No	No	*WT*	WT
GIST4	C2	9.06	A1	2	2	2	GI1	2	2	2	2	N	Low risk	Stomach	No	No	*K11*	p.V559D
GIST45	C2	8.84	A1	2	2	2	GI1	2	2	2	2	N	Very low	Stomach	No	No	*P18*	p.D842V
GIST35	C2	8.85	A1	6	5	7.2	GI1	2	2	2	1	1	Intermediate	Stomach	No	No	*P14*	p.N659K
GIST20	C2	9.02	A1	9	5	16.2	GI2	2	2	2	2	N	High risk	Abdominal wall	No	No	*K11*	p.W557R
GIST39	C2	8.88	A1	12	11	13.09	GI2	1	1	1	2	1	Intermediate	Stomach	No	**Yes**	*K11*	p.W557_V559delins F
GIST22	C2	9.71	A2	5	4	6.25	GI1	2	2	2	2	N	Intermediate	Stomach	No	No	*P18*	p.D842V
GIST42	C2	9.5	A2	2	2	2	GI1	1	1	1	2	1	Low risk	Stomach	No	No	*WT*	WT
GIST53	C2	10.1	A2	4	4	4	GI1	**0**	**0**	**0**	2	**0**	Intermediate	Stomach	No	No	*K11*	p.Q556_I563del
GIST5	C2	9.92	A2	5	4	6.25	GI1	**0**	**0**	1	2	**0**	High risk	Stomach	No	**Yes**	*K11*	p.W557_K558 del
GIST63	C2	10.7	A2	5	4	6.25	GI1	1	1	1	2	1	High risk	Rectum	No	**Yes**	*K11*	p.V560D
GIST11	C2	9.73	A2	9	8	10.13	GI2	nd	nd	nd	2	N	Low risk	Duodenum	No	No	*K11*	p.V560A
GIST6	C2	12.11	A2	13	11	15.36	GI2	2	2	2	**0**	**0**	High risk	Small intestine	**Yes**	No	*K11*	p.E554_K558del
GIST14	C2	11.95	A2	11	8	15.13	GI2	2	2	2	1	1	Intermediate	Mesenterium	**Yes**	**Yes**	*K17*	p.N822K
GIST16	C2	9.7	A2	8	6	10.67	GI2	2	2	2	1	1	High risk	Jejunum	No	**Yes**	*K9*	p.A502_Y503dup
GIST19	C2	12.01	A2	29	17	49.47	GI2	2	2	2	1	1	Intermediate	Colon	**Yes**	**Yes**	*K9*	p.A502_Y503dup

(Continued on the following page)

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Table 2. Results summary of the CINSARC analysis, *AURKA* expression (A: 9.15 as cutoff), CGH analysis (GI: 10 as cutoff), and *CDKN2A/2B* and *RB1* copy number determined by genomic qPCR and array-CGH, respectively (2, without detectable deletion; 1, hemizygous deletion; 0, no copy) (Cont'd)

GIST	Expression (Agilent)			CGH				CDKN2A/2B and RB1 copy number				Histology	Annotations			*KIT* and *PDGFRA* mutations	
	CINSARC grading	AURKA	ARUKA stratification	Number of Alt	Nbr Chr	Alt[2] /Nbr chr	Genomic Index	p14	p16	p15	RB1	Pathway	AFIP	Site of primary tumor	Local recurrence	Metastasis	Mutated gene	Mutation
GIST2	C2	10.22	A2	12	11	13.09	GI2	2	2	2	1	1	High risk	Small intestine	No	Yes	*K11*	p.Y553_Q556del
GIST38	C2	10.8	A2	31	17	56.53	GI2	1	1	1	1	1	High risk	Stomach	No	Yes	*K11*	p.W557_V560delinsF
GIST9	C2	11.67	A2	16	10	25.6	GI2	1	1	1	1	0	High risk	Stomach	Yes	Yes	*K11*	p.V560D
GIST61	C2	12.89	A2	26	17	39.76	GI2	1	1	1	1	0	High risk	Stomach	No	Yes	*P18*	p.D842V
GIST56	C2	13.11	A2	21	13	33.92	GI2	1	1	1	2	0	High risk	Small intestine	Yes	Yes	*WT*	WT
GIST37	C2	11.2	A2	29	15	56.07	GI2	1	1	1	2	1	Intermediate	Stomach	Yes	Yes	*K11*	p.W557_K558del
GIST28	C2	10.76	A2	14	9	21.78	GI2	0	0	0	2	0	High risk	Stomach	No	Yes	*K11*	p.W557_V559delinsF
GIST47	C2	9.64	A2	22	12	40.33	GI2	0	0	0	2	0	High risk	Stomach	No	Yes	*K11*	p.E554_D572delinsF
GIST58	C2	10.19	A2	17	8	36.13	GI2	0	0	0	2	0	High risk	Stomach	No	Yes	*K11*	p.W557_K558delinsFP
GIST57	nd	nd	nd	13	10	16.9	GI2	0	0	0	2	0	High risk	Small intestine	No	Yes	*K11*	p.V559D
GIST17	nd	nd	nd	26	13	52	GI2	0	0	0	2	0	nd	Duodenum	Yes	Yes	*K11*	p.V569_L576del
GIST3	nd	nd	nd	16	10	25.6	GI2	2	2	2	1	1	High risk	Stomach	No	Yes	*K11*	p.V560D
GIST26	nd	nd	nd	11	7	17.29	GI2	2	2	2	1	1	Intermediate	Mediastinum	No	No	*K11*	p.K558_V559delinsN homo
GIST34	nd	nd	nd	11	8	15.13	GI2	2	2	2	1	1	Very low	Small intestine	No	No	*K11*	p.V560D
GIST25	nd	nd	nd	0	0	0	GI1	2	2	2	2	N	Very low	Stomach	No	No	*P18*	p.D842V
GIST7	nd	nd	nd	1	1	1	GI1	2	2	2	2	N	Intermediate	Stomach	No	No	*K11*	p.W557_E561del

NOTE: The "pathway" column indicates the p16/RB1 pathway status: N, normal; 1, one copy of one gene is altered; 0, one gene is completely inactivated. Tumors are sorted according to CINSARC, AURKA expression, and GI stratification.

Abbreviations: P, *PDGFRA*; K, *KIT*; WT, wild type; nd, not done; Nbr Chr, number of involved chromosomes; Alt, alterations.

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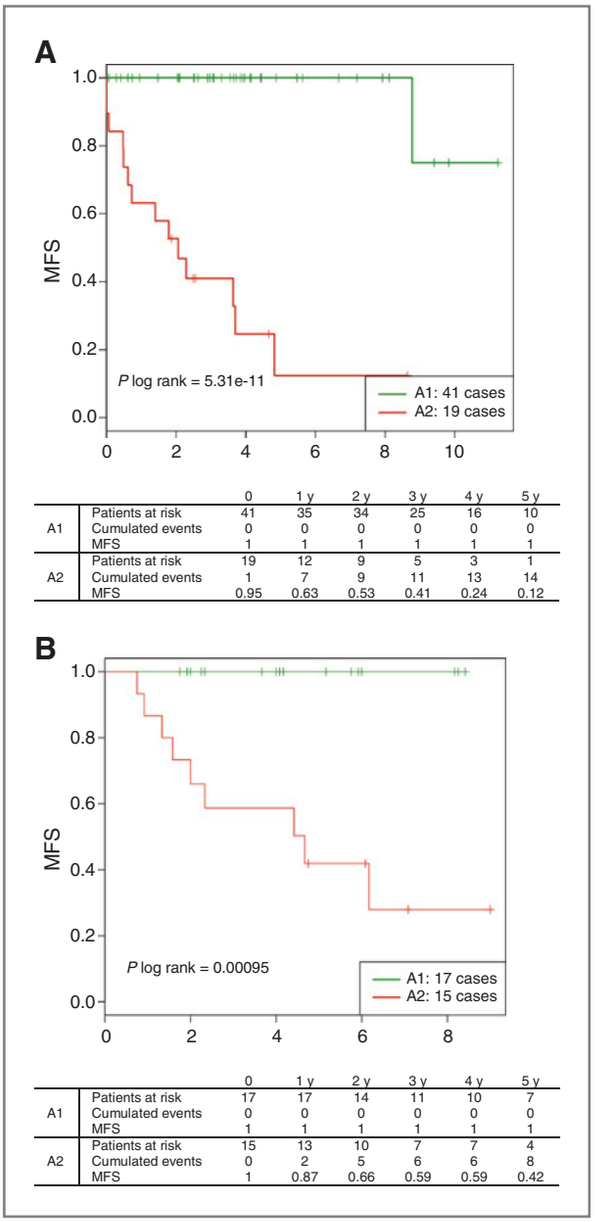

		0	1 y	2 y	3 y	4 y	5 y
A1	Patients at risk	41	35	34	25	16	10
	Cumulated events	0	0	0	0	0	0
	MFS	1	1	1	1	1	1
A2	Patients at risk	19	12	9	5	3	1
	Cumulated events	1	7	9	11	13	14
	MFS	0.95	0.63	0.53	0.41	0.24	0.12

		0	1 y	2 y	3 y	4 y	5 y
A1	Patients at risk	17	17	14	11	10	7
	Cumulated events	0	0	0	0	0	0
	MFS	1	1	1	1	1	1
A2	Patients at risk	15	13	10	7	7	4
	Cumulated events	0	2	5	6	6	8
	MFS	1	0.87	0.66	0.59	0.59	0.42

Figure 2. Kaplan–Meier analysis of MFS according to *AURKA* expression. A, *AURKA* has prognostic value in the 60 GISTs described here. B, it has prognostic value in an independent set of 32 GISTs reported by Yamaguchi and colleagues. A1 (green) and A2 (red) correspond to tumors with below-average and above-average *AURKA* expression, respectively. *x*-axis, time in years.

complexity could predict metastatic outcome (Fig. 3A). To take into account the number and the type of changes, a Genomic Index (GI) was calculated for each profile as follows: GI = A^2/C, where A is the total number of alterations (segmental gains and losses) and C is the number of involved chromosomes. The Genomic Index across the entire series ranged from 0 to 56. The proportion of metastatic cases increased with Genomic Index. Metastatic cases predominated when the Genomic Index was over 10 (Table 2 and Fig. 3B). Stratification by Genomic Index at a cutoff of 10 split the tumors into 2 groups with very different outcomes (Table 2, Fig. 4A). Interestingly, Genomic Index was able to predict metastatic outcome in GISTs in the intermediate-risk group of the AFIP classification (9; Fig. 4B and C).

Do these signatures outperform AFIP grading system?

To assess this issue independency of CINSARC signature, AURKA expression and Genomic Index were evaluated together with AFIP grading system in a multivariate analysis (Table 3). AURKA expression seems to be the stronger prognostic marker (HR = 11.97, 95% CI = (1.60–1406); Table 3, a), and the AFIP is not significant when compared with AURKA expression. As suspected, this analysis showed that CINSARC, AURKA (which belongs to CINSARC), and Genomic Index correlate making the former and the later not significant face to AURKA expression. We thus carried out multivariate analyses comparing each molecular signature with AFIP grading system (Table 3, b) and showed that each signature is superior to AFIP grading system to predict metastasis outcome. Of interest, AFIP intermediate statue against each of the molecular signature has no more significant prognostic value.

Discussion

The development of a valid and reliable, investigator-independent method of GIST prognostication is essential for the proper clinical management of GIST patients, especially in the context of adjuvant treatment, in which many patients are exposed to imatinib, whereas only a small proportion will likely benefit from such treatment (21).

The main conclusion from this study is that the CINSARC score is a strong and validated predictor of metastasis in patients with GISTs. Remarkably, none of the patients assigned to the good prognosis group developed metastases or relapsed. Prognostic expression signatures have showed their experimental efficacy in several other tumor types, but their clinical application has been complicated by technical issues such as weak reproducibility across array platforms. Importantly, we show here that CINSARC scoring is platform independent: the signature we developed on Affymetrix data was applied and validated here on Agilent data. Furthermore, the CINSARC score was prognostic for both the nontranslocation related sarcomas on which it was originally developed (18) and for the GISTs in this study.

The CINSARC signature comprises 67 genes involved in maintenance of chromosome integrity and mitotic control, indicating that these processes play a crucial role in the development of metastasis in sarcomas (18). Supervised analysis showed that 45 of the 227 genes prognostic in GISTs were common to the CINSARC signature. The top-ranked gene common to both approaches was

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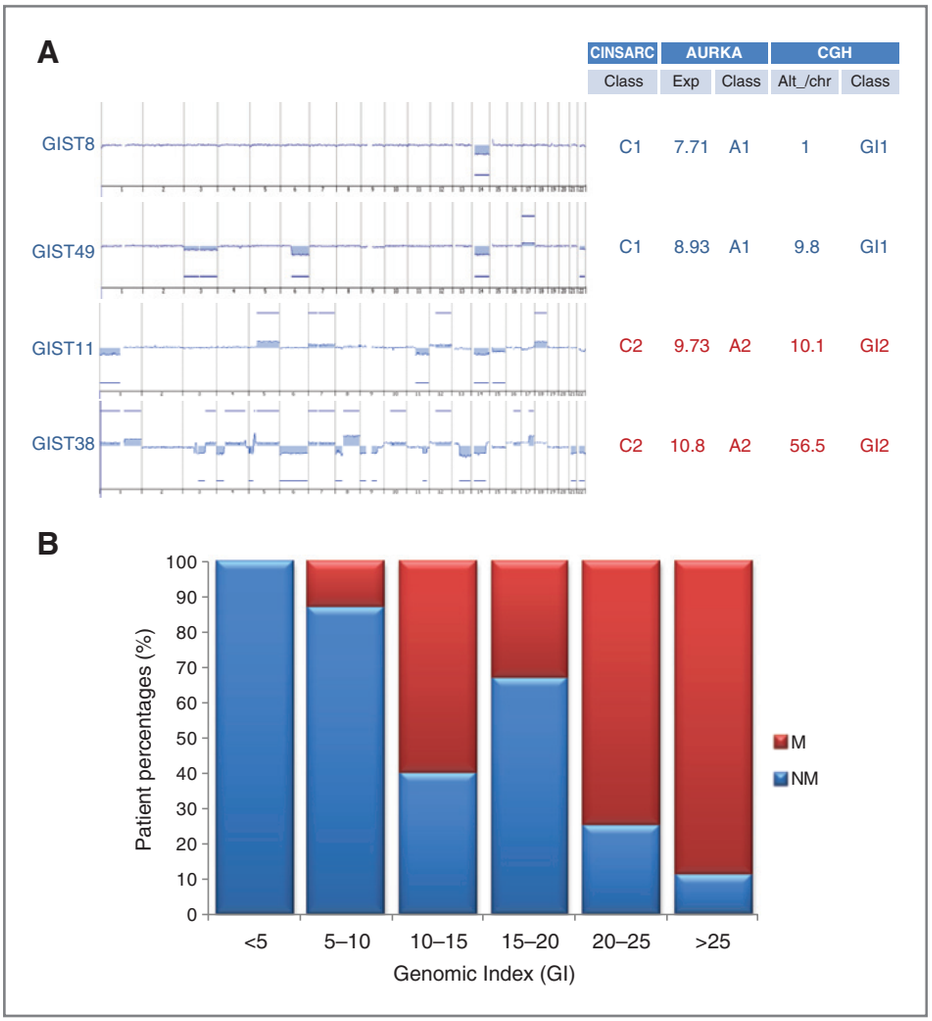

Figure 3. Array-CGH analysis. A, CGH profiles, CINSARC grading, *AURKA* expression (Exp), and Genomic Index of 4 cases representing GISTs with very few rearrangements (GIST #8), GISTs moderately rearranged (GISTs #49 and #11), and GISTs highly rearranged (GIST #38). Genomic alterations are presented and organized from chromosome 1 to 22 on the *x*-axis and log ratio values are reported on the *y*-axis. Significant gains or losses are indicated by blue lines and blue areas above or below each profile, respectively. Expression values are $\log_2$ transformed. B, cumulated proportions of metastatic (M, red) and nonmetastatic (NM, blue) patients according to GI. *x*-Axis corresponds to GI classes and *y*-axis to patient percentages.

AURKA. The AURKA protein is a mitotic centrosomal protein kinase amplified in many cancer types (22–25). Increased AURKA expression is associated with poor prognosis in breast carcinoma (26), colon carcinoma (27, 28), neuroblastoma (29), and head and neck squamous cell carcinoma (30). AURKA overexpression induces centrosome duplication and segregation abnormalities leading to aneuploidy and malignant transformation (25). Whole chromosome losses are the most frequently observed alterations in GISTs and are assumed to originate from unequal chromosome segregation, which can be induced by AURKA overexpression (31). Contrary to the mechanism seen in other cancers, AURKA overexpression in GISTs is not explained by gene amplification, but is instead a secondary change we postulate to be caused by defects in Restriction point control. Our results point to AURKA being a very interesting potential therapeutic target in GISTs. With this in mind, it is noteworthy that immunohistochemistry shows that AURKA mRNA overexpression translates into AURKA protein overexpression (Supplementary Fig. S3). AURKA inhibitors have entered clinical trials (32–36) and could be particularly useful for imatinib-resistant GISTs that have not yet disseminated because AURKA could be an essential event leading to acquisition of metastatic potential.

Previous copy number studies identified few aberrations in GISTs, deletions being more common than gains (6, 10–12, 37–39). The authors concluded that chromosome 14, 22, and 1p deletions were the most frequent aberrations. Two studies noted that copy number changes were commoner in high-risk GISTs but did not identify a

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Table 3. Multivariate analyses comparing prognostic value of CINSARC signature, AURKA expression, GI, and AFIP grading system

	Univariate		Multivariate	
	P	**HR (95% CI)**	***P***	**HR (95% CI)**
CINSARC	1.4×10^{-5}	2×10^{9} (0–inf)	0.445	3.8 (0.122–707)
AURKA	5.3×10^{-11}	4×10^{9} (0–inf)	0.009	11.9 (1.6–1406)
GI	8.1×10^{-9}	21.34 (4.86–93.67)	0.248	2.1 (0.61–11.88)
AFIP low		Reference		Reference
AFIP intermediate	3.2×10^{-7}	5.25 (0–inf)	0.459	2.8 (0.22–396)
AFIP high		2.16×10^{9} (0–inf)	0.055	7.7 (0.96–1011)
CINSARC	1.4×10^{-5}	2×10^{9} (0–inf)	3.9×10^{-4}	26.5 (3.11–3570)
AFIP low		Reference		Reference
AFIP intermediate	3.2×10^{-7}	5.25 (0–inf)	0.37	3.4 (0.27–396)
AFIP high		2.16×10^{9} (0–inf)	0.0055	14.4 (1.83–1867)
AURKA	5.3×10^{-11}	4×10^{9} (0–inf)	6.37×10^{-7}	43.5 (5.5–5652)
AFIP low		Reference		Reference
AFIP intermediate	3.2×10^{-7}	5.25 (0–inf)	0.28	4.01 (0.37–545)
AFIP high		2.16×10^{9} (0–inf)	0.028	9.7 (1.2–1280)
GI	8.1×10^{-9}	21.34 (4.86–93.67)	5×10^{-4}	7.8 (2.29–41)
AFIP low		Reference		Reference
AFIP intermediate	3.2×10^{-7}	5.25 (0–inf)	0.18	5.6 (0.49–779)
AFIP high		2.16×10^{9} (0–inf)	0.004	17.7 (2.05–2333)

Abbreviation: Inf, infinite.

clear cutoff delineating the high-risk group (6, 10). At the expression level, most studies were designed to facilitate diagnosis (40, 41) or to predict *KIT* or *PDGFRA* mutation status (42–44). Yamaguchi and colleagues (17) carried out gene expression profiling on 32 GISTs and identified CD26 as a prognostic marker, but only in GISTs of gastric origin. They concluded that CD26 might not be the cause of malignant progression of gastric GISTs. In contrast, our study shows that CINSARC score, *AURKA* expression, and Genomic Index are prognostic irrespective of the tumor location (Supplementary Fig. S4). Furthermore, the biological meaning of CINSARC score and its association with genomic changes strongly indicate that *CINSARC* genes are implicated in malignant progression and are not just a consequence of the process. This hypothesis is supported by the association we observe between *CDKN2A* deletion, *RB1* deletion, AURKA expression, CINSARC score, and metastasis. *CDKN2A* encodes 2 key tumor suppressor proteins, $p16^{INK4a}$ and the $p14^{ARF}$, which regulate the Restriction point and p53, respectively. Previous studies on GISTs have linked 9p21 alterations to tumor progression (11–16, 45), but the driver gene was not positively identified (*CDKN2A*, *CDKN2B*, or *MTAP*; refs. 37, 39, 46–48). Here, we have shown that homozygous deletions target *CDKN2A* and more specifically $p16^{INK4a}$.

RB1 deletions associated to reduced Rb expression in tumors with high AURKA expression, but normal *CDKN2A* loci, are consistent with the known cooperation between p16 and RB1 in control of the Restriction point. Most of the *CINSARC* genes are known to be under the transcriptional control of E2F. RB1 sequesters E2F, which is released from the complex upon RB1 phosphorylation by CDK4. CDK4 is, in turn, inhibited by $p16^{INK4a}$. Hence, we hypothesize that alteration of the *p16 INK4a* or *RB1* genes in GISTs is likely to be a causative event that leads to the overexpression of *CINSARC* genes, which in turn induce chromosome instability and ultimately metastasis. Although this model requires experimental validation in cellular and mouse models of GIST, the strong association between *CINSARC* gene expression and p16/Rb pathway alteration make it an attractive hypothesis.

Both the AFIP (9) and NIH (8) histologic-based grading systems are widely accepted as "gold standards" in determining metastatic risk and to determine whether a GIST patient should receive adjuvant therapy with imatinib. Adjuvant imatinib is now recommended for localized GISTs of more than 3 cm in the United States or for high-risk or intermediate-risk localized GISTs in Europe. Many patients at AFIP-intermediate risk or with a tumor more than 3 cm will not benefit from imatinib. The

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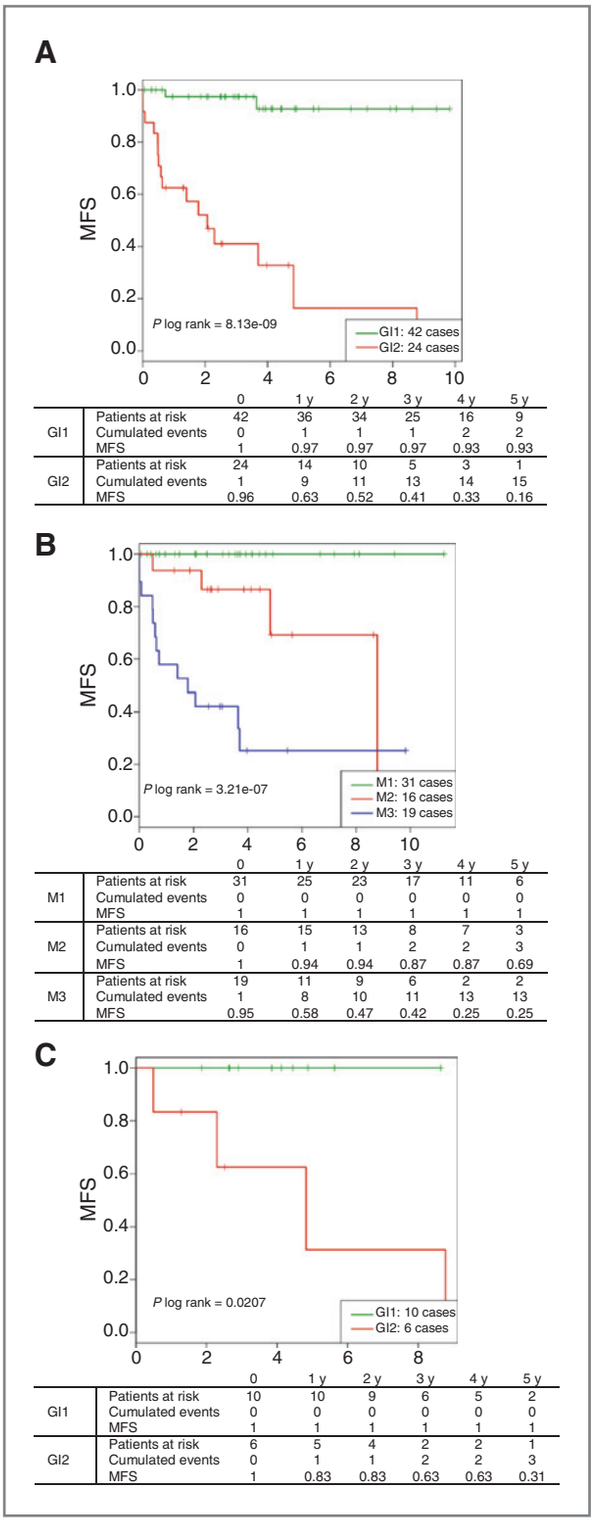

		0	1 y	2 y	3 y	4 y	5 y
GI1	Patients at risk	42	36	34	25	16	9
	Cumulated events	0	1	1	1	2	2
	MFS	1	0.97	0.97	0.97	0.93	0.93
GI2	Patients at risk	24	14	10	5	3	1
	Cumulated events	1	9	11	13	14	15
	MFS	0.96	0.63	0.52	0.41	0.33	0.16

		0	1 y	2 y	3 y	4 y	5 y
M1	Patients at risk	31	25	23	17	11	6
	Cumulated events	0	0	0	0	0	0
	MFS	1	1	1	1	1	1
M2	Patients at risk	16	15	13	8	7	3
	Cumulated events	0	1	1	2	2	3
	MFS	1	0.94	0.94	0.87	0.87	0.69
M3	Patients at risk	19	11	9	6	2	2
	Cumulated events	1	8	10	11	13	13
	MFS	0.95	0.58	0.47	0.42	0.25	0.25

		0	1 y	2 y	3 y	4 y	5 y
GI1	Patients at risk	10	10	9	6	5	2
	Cumulated events	0	0	0	0	0	0
	MFS	1	1	1	1	1	1
GI2	Patients at risk	6	5	4	2	2	1
	Cumulated events	0	1	1	2	2	3
	MFS	1	0.83	0.83	0.63	0.63	0.31

Figure 4. Kaplan–Meier analysis of MFS according to Genomic Index (A), AFIP classification (B), and Genomic Index in the subgroup of AFIP intermediate-risk cases (C). GI1 and GI2 are low and high Genomic Index patients, respectively. M1, M2, and M3 are AFIP low-, intermediate-, and high-risk GISTs.

ability to select patients likely to benefit from imatinib would be an important advance in the management of GISTs. Here, we show that CINSARC score, *AURKA* expression, and Genomic Index all outperform the AFIP classification (Table 3b and Fig. 4) and do so independently of tumor location (gastric versus nongastric GISTs; Supplementary Fig. S4). Even if *AURKA* expression (mRNA level) seems in multivariate analysis as the best predictor of metastasis outcome, its clinical application is limited due to weak quality of mRNA extracted from formalin-fixed paraffin-embedded (FFPE). CGH is a technique applicable to FFPE samples already used in routine diagnostic pathology laboratories, and the Genomic Index is nearly as much effective as AURKA and CINSARC to distinguish good from poor prognosis patients particularly in AFIP-intermediate risk GISTs (which represent around 25% of diagnoses). Genomic Index is therefore potentially the best overall tool to manage imatinib therapy for intermediate risk GIST patients. We recommend carrying out a clinical trial comparing these molecular signatures to the AFIP/NIH methods to validate this hypothesis in a prospective clinical context.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

R. Sciot, P. Schöffski, M. Debiec-Rychter, A. Neuville, and J.-M. Coindre supplied tumor tissue, did the central pathology review, and collected the clinical follow-up data. V. Dapremont carried out DNA and RNA extractions. F. Chibon supervised the laboratory experiments. P. Lagarde and G. Pérot carried out laboratory experiments. A. Wozniak, M. Debiec-Rychter, and I. Hostein carried out *KIT* and *PDGFRA* mutational analysis. A. Kauffmann and C. Brulard calculated centroid scores and conducted survival analysis. P. Lagarde, G. Pérot, and F. Chibon analyzed the data. F. Chibon designed the study. F. Chibon wrote the report. A. Aurias, J.-M. Coindre, and F. Chibon obtained funding for the project. All investigators reviewed and approved the final report.

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