

PDL1 expression is an independent prognostic factor in localized GIST

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Abbreviations: AFIP, Armed Forces Institute of Pathology; FDR, false discovery rate; GEO, gene expression omnibus; GES, gene expression signatures; GIST, gastrointestinal stromal tumors; GO, gene ontology; IHC, immunohistochemistry; ISH, *in situ* hybridization; MFS, metastasis-free survival; MHC, major histocompatibility complex; NCBI, National Center for Biotechnology Information; NK cells, natural killer cells; PCA, principal component analysis; PD1, programmed cell death 1; PDL1, programmed cell death ligand 1; PDGFRA, platelet-derived growth factor receptor α ; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; REMARK, REcommendations for tumor MARKer; RMA, robust multichip average; ROC, receiver operating characteristic; TILs, tumor-infiltrating lymphocytes; Treg, regulatory T cells; WT, wild type

Gastrointestinal stromal tumors (GIST) are the most frequently occurring digestive sarcomas. The prognosis of localized GIST is heterogeneous, notably for patients with an Armed Forces Institute of Pathology (AFIP) intermediate or high risk of relapse. Despite imatinib effectiveness, it is crucial to develop therapies able to overcome the resistance mechanisms. The immune system represents an attractive prognostic and therapeutic target. The Programmed cell Death 1 (PD1)/programmed cell death ligand 1 (PDL1) pathway is a key inhibitor of the immune response; recently, anti-PD1 and anti-PDL1 drugs showed very promising results in patients with solid tumors. However, PDL1 expression has never been studied in GIST. Our objective was to analyze PDL1 expression in a large series of clinical samples. We analyzed mRNA expression data of 139 operated imatinib-untreated localized GIST profiled using DNA microarrays and searched for correlations with histoclinical features including postoperative metastatic relapse. *PDL1* expression was heterogeneous across tumors and was higher in AFIP low-risk than in high-risk samples, and in samples without than with metastatic relapse. *PDL1* expression was associated with immunity-related parameters such as T-cell-specific and CD8⁺ T-cell-specific gene expression signatures and probabilities of activation of interferon α (IFN α), IFN γ , and tumor necrosis factor α (TNF α) pathways, suggesting positive correlation with a cytotoxic T-cell response. In multivariate analysis, the PDL1-low group was associated with a higher metastatic risk independently of the AFIP classification and the *KIT* mutational status. In conclusion, *PDL1* expression refines the prediction of metastatic relapse in localized GIST and might improve our ability to better tailor adjuvant imatinib. In the metastatic setting, *PDL1* expression might guide the use of PDL1 inhibitors, alone or associated with tyrosine kinase inhibitors.

Introduction

Gastrointestinal stromal tumors (GIST) are the most frequently occurring digestive sarcomas.^{1,2} They are an exemplary model for molecular-based treatment within solid tumors because of the presence of activating *KIT* or *PDGFRA* oncogenic mutations in ~85% of cases³ and the resulting high sensitivity to *KIT* and platelet-derived growth factor receptor α (PDGFRA) tyrosine kinase inhibitors (imatinib or sunitinib).^{4,5} In advanced stages, first-line imatinib increases both the response rate (70% vs. <10% with chemotherapy) and the median overall survival (76 months in the recent BFR14 clinical trial⁶ vs. <10 months

with chemotherapy). In localized stages treated by complete surgical resection,² adjuvant imatinib decreases the relapse rate^{7,8} and improves overall survival⁸ and is recommended for patients with intermediate or high metastatic risk according to the Armed Forces Institute of Pathology (AFIP) prognostic classification.⁹

Nevertheless, the current prognostic classifications remain imperfect with substantial heterogeneity within each class: for example, the 2-year relapse-free survival without adjuvant imatinib is ~75% in the AFIP intermediate-risk patients and ~50% in the high-risk patients.⁷ Clearly, these classifications, based on histoclinical features need to be refined.^{4,10-13} Another crucial objective is to develop new therapies able to overcome the

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molecular mechanisms of primary or secondary resistance to imatinib. Indeed, if not present initially, imatinib-resistant tumor clones emerge in most cases because of the mutagenic capacity of cancer cells, thus limiting the duration of tumor responses.

In this context, and even if our current understanding of the immune response in GIST remains limited when compared with other cancers, several data suggest that the exploration of the immune system is an interesting strategy. First, immune cells such as T cells, CD8⁺ T-cells, regulatory T cells (Treg), natural killer (NK) cells, and macrophages are present in clinical GIST samples.¹⁴⁻¹⁷ Second, their presence and/or activation have been associated with prognosis¹⁶ and/or response to imatinib.^{14,18,19} Third, and as observed with certain chemotherapy drugs,²⁰ the antitumor action of imatinib is also due in part to indirect effects on immune cells, notably NK cells;²¹ and CD8⁺ T cells¹⁴; in the same line, the concurrent CTLA-4 blockade augments the efficacy of imatinib in mouse GIST by increasing IFN γ -producing CD8⁺ T cells.¹⁴ Finally, by contrast with targeted therapies, immunotherapy can adapt to the emergence of resistant clones thanks to the high adaptability of the immune response.

Like CTLA-4, the Programmed cell Death 1 (PD1) pathway is a key inhibitor of the immune response, regulating the balance between activation and inhibition signals. PD1 is expressed at the surface of various immune cells including T cells. PD1 activation by its ligand Programmed cell Death 1 ligand (PDL1), expressed by antigen-presenting cells (APCs) such as macrophages or B cells, regulates lymphocyte activation²²⁻²⁴ and promotes Treg cell development and function, allowing termination of the immune response. Cancer cells from different locations have acquired the capacity to express PDL1.²⁵ The PD1-PDL1 pathway has, thus, been involved in cancer progression.^{26,27} Anti-PD1 and anti-PDL1 drugs²⁸ are being tested in phase 3 clinical trials after very promising results in phase 2 trials.²⁹⁻³² Durable responses have been observed, notably in melanoma and renal and lung carcinomas,^{33,34} and a relationship between PDL1 expression on cancer cells and objective response has been evidenced.^{34,35} PDL1 expression has never been studied in GIST.

Here, we analyzed PDL1 expression in 139 imatinib-untreated localized GIST and searched for correlations with histoclinical features including metastasis-free survival (MFS) after surgery. We show that PDL1 expression is heterogeneous and is an independent prognostic factor in multivariate analysis.

Results

Patients characteristics and *PDL1* expression

Of the 159 available GIST samples, a total of 139 represented localized tumors from patients treated with primary complete surgery without adjuvant imatinib. Their characteristics are summarized in Table 1. Sixty-four percent of patients were male. The median patient age was 63 y. The most frequent anatomical site was the stomach (78%), followed by the small intestine (15%). The mutational status was available for 138 samples: as expected, the most frequent mutations were *KIT* mutations, most frequently located in exon 11, followed by *PDGFRA*

Table 1. Histoclinical characteristics of patients and tumors

Characteristics ^a	N (%)
Sex (139)	
Female	50 (36%)
Male	89 (64%)
Age, years (79)	
≤60	31 (39%)
<60	48 (61%)
median (range)	63 (26–85)
Site (139)	
Gastric	109 (78%)
Small intestine	21 (15%)
Other	9 (6%)
Mutation ^a (138)	
<i>KIT</i> exon 11	88 (64%)
<i>KIT</i> exon 9	8 (6%)
<i>PDGFRA</i> exon 18	16 (12%)
Wild type (WT)	17 (12%)
Other ^b	9 (7%)
AFIP risk (139)	
Low	75 (54%)
Intermediate	25 (18%)
High	39 (28%)
Metastatic relapse (138)	
No	111 (80%)
Yes	27 (20%)

^aThe number of patients with available information is shown between brackets; ^b*KIT* exons 13 ($n = 1$) and 17 ($n = 2$), *PDGFRA* exons 12 ($n = 5$), and 14 ($n = 1$).

mutations, most frequently located in exon 18. No *KIT* and *PDGFRA* mutation was observed in 12% of samples (*KIT* and *PDGFRA* wild type, WT). Regarding the relapse risk defined according to the AFIP classification, 54% of samples were predicted as low risk, 18% as intermediate risk, and 28% as high risk. Twenty percent of patients experienced a metastatic relapse during follow-up. As shown in Figure S1A, *PDL1* mRNA expression varied among these 139 tumors with a wide range of intensities over 3 decades in a log₂ scale, suggesting a heterogeneous expression across clinical samples of GIST.

PDL1 expression and histoclinical and immune features

We searched for correlations between *PDL1* expression (continuous value; Student's *t*-test) and histoclinical features (Table 2). *PDL1* expression was not significantly associated with patients' sex and age or with tumor site and mutational status. By contrast, *PDL1* expression correlated with the AFIP classification and metastatic relapse. *PDL1* expression was higher in low-risk samples than in high-risk samples ($P = 0.0194$), and in samples without metastatic relapse than in samples with metastatic relapse ($P = 0.0029$), suggesting favorable prognostic value.

We then studied whether *PDL1* expression was associated (Student's *t*-test) with immunity-related parameters in clinical GIST samples. First, we found a correlation with T–cell-specific, CD8⁺ T–cell-specific, and B–cell-specific gene expression signatures³⁶; samples with higher expression of these signatures over-expressed *PDL1* ($P < 0.001$; Fig. 1A). Second, *PDL1* expression was higher ($P < 0.001$) in samples showing higher expression of

Table 2. Correlations of *PDL1* expression with histoclinical features

Characteristics*	N	Average <i>PDL1</i> expression (min–max)	P-value
Sex (139)			0.07
Female	50	0.27 (–1.32–3.15)	
Male	89	–0.07 (–2.13–2.24)	
Age, years (79)			0.649
≤60	31	0.16 (–1.5–2.24)	
<60	48	0.05 (–1.73–3.15)	
Site (139)			0.37
Gastric	109	0.09 (–2.13–3.15)	
Small intestine	21	–0.21 (–1.61–1.21)	
Other	9	0.23 (–0.41–1.19)	
Mutation (129)			0.89
<i>KIT</i> exon 11	88	0.08 (–2.13–2.36)	
<i>KIT</i> exon 9	8	–0.18 (–0.67–0.48)	
<i>PDGFRA</i> exon 18	16	–0.01 (–1.15–1.63)	
Wild type (WT)	17	–0.01 (–1.61–3.15)	
AFIP risk (139)			0.0194
Low	75	0.2 (–1.75–3.15)	
Intermediate	25	0.19 (–1.13–2.36)	
High	39	–0.32 (–2.13–1.67)	
Metastatic relapse (138)			0.0029
No	111	0.18 (–1.75–3.15)	
Yes	27	–0.46 (–2.13–1.19)	

*The number of patients with available information is shown within parentheses.

an immune kinase signature reflecting the immune response and, particularly, cytotoxic T-cell response.³⁷ Finally, we found that the probability of activation of IFN α , IFN γ , and tumor necrosis factor α (TNF α) pathways³⁸ was associated ($P < 0.001$) with higher *PDL1* expression (Fig. 1B). Altogether, these results suggested that *PDL1* expression in samples of GIST is associated with antitumor T-cell response.

PDL1 expression and metastatic relapse

The follow-up was available for 138 patients: 27 experienced a metastatic relapse and 111 did not. As shown in Table 1, lower *PDL1* expression was associated with more frequent metastatic relapse. We repeated the analysis using *PDL1* expression as a binary variable. We first defined the optimal expression cut-off associated with the occurrence of metastatic relapse in a learning set of 92 samples: measured at 5.08, it resulted into an odds ratio (OR) for relapse of 4.74 ([1.30–17.68], $P = 0.0083$; Fisher's exact test) in the PDL1-low group versus the PDL1-high group. We confirmed its discriminatory prognostic value in the validation set of 46 samples with an OR for relapse of 8.80 ([1.04–115.54], $P = 0.023$; Fisher's exact test) in the PDL1-low vs. PDL1-high groups (Table S1).

Of the 138 samples, 27 were in the PDL1-low group (20%) and 111 in the PDL1-high group (80%). In the univariate analysis (Table 3), PDL1-low group ($P = 0.0002$; logit test), AFIP intermediate-risk classification ($P = 0.0065$), and AFIP high-risk classification ($P < 0.0001$) were associated with a higher risk of relapse, whereas patient age, anatomical site, and *PDGFRA* exon 18 mutations were not. A trend ($P < 0.10$) toward worse prognosis was observed for *KIT* exon 11 and *KIT* exon 9 mutations,

and male gender. In the multivariate analysis, PDL1-low group ($P = 0.0056$; logit test), AFIP intermediate-risk classification ($P = 0.0226$), and AFIP high-risk classification ($P < 0.0001$) remained significant, whereas gender and mutations did not, even if a trend existed for *KIT* exon 11 mutation versus WT status (Table 3). Of note, and to verify the absence of overfitting with regard to the independent prognostic value of PDL1, we repeated the same multivariate analysis in the validation set only: 2 variables – the PDL1 group ($P = 0.0097$; logit test) and the AFIP high-risk classification ($P = 0.0004$) remained significant (data not shown). Stratification of patients according to the PDL1 group and, respectively, the AFIP risk and the mutational status identified subgroups with different relapse rates (Table 2). For example, *PDL1* expression affected the clinical outcome of AFIP high-risk patients: the PDL1-low group showed a higher rate of relapse (79%) than the PDL1-high group (46%). Similarly, *PDL1* expression affected the clinical outcome of patients with a *KIT* exon 11 mutation (56% relapse rate in the PDL1-low group vs. 16% in the PDL1-high group) and patients with wild-type (WT) GIST (20% relapse rate in the PDL1-low group versus 0% in the PDL1-high group), leading to similar relapse rate between *KIT* exon 11-PDL1-high GIST (16%) and WT-PDL1-low GIST (20%).

PDL1 expression and associated biological processes

Supervised analysis comparing the whole-genome expression profiles of the PDL1-low ($n = 27$) and PDL1-high ($n = 111$) samples identified 303 differentially expressed genes, which were all overexpressed in the PDL1-high samples (Table S3). Ontological analysis (Table S4) showed that these genes were particularly involved in immune response regulation, and more specifically in T-cell activation. Indeed, many top genes (correlated with *PDL1*) were major histocompatibility complex (MHC)-related molecules, that is, involved in the processing of endogenous antigens and presentation to cytotoxic and helper T cells. Among them, we found not only numerous human leukocyte antigen (HLA)-I or HLA-I-related molecules (*HLA-A-C*, *HLA-E*, *HLA-G*, *HLA-F*, all members of the BTN3A family, *KLRG1*, etc.), but also HLA-II molecules (*HLA-DR*, *HLA-DP*, *HLA-DQ*, *HLA-DM*, *CD74*, etc.) and molecules involved in the degradation of cytosolic peptides across the endoplasmic reticulum into the membrane-bound compartment where class I molecules assemble (*TAP1*, *TAPBP*, *PSMB8–10*, *CTSS*, etc.). This “antigen presentation” signature was associated with a strong antitumor response, as suggested by the numerous genes testifying of TH1 activation (many IFN-related genes, *IL12RB1*, *IL18BP*, *IL18*, *IL2RB*, *IL2RG*, *IL15*, *IL7* and *IL7R*, *STAT1*, *ITK*, *LCK*, *JAK2*, *LAG3*, *CD69*, etc.) and cytotoxic effector molecules (*GZMA*, *GZMK*, *GZMH*, *PRF1*, C1-complement members, *CD52*, *FASLG*, etc.). This signature suggested that cytotoxic T cells are major mediators of such antitumor response, which was further confirmed with the increased expression of transcripts related to CD8⁺ T-cells (*CD160*, *CD2*, *CD53*, *CD8A*, *CD3D*, *CD247*, *PTPRC*, etc.). Other actors of the immune system, notably APCs, certainly play an important role as well (especially, macrophages or related APCs expressing CD163, CD209,

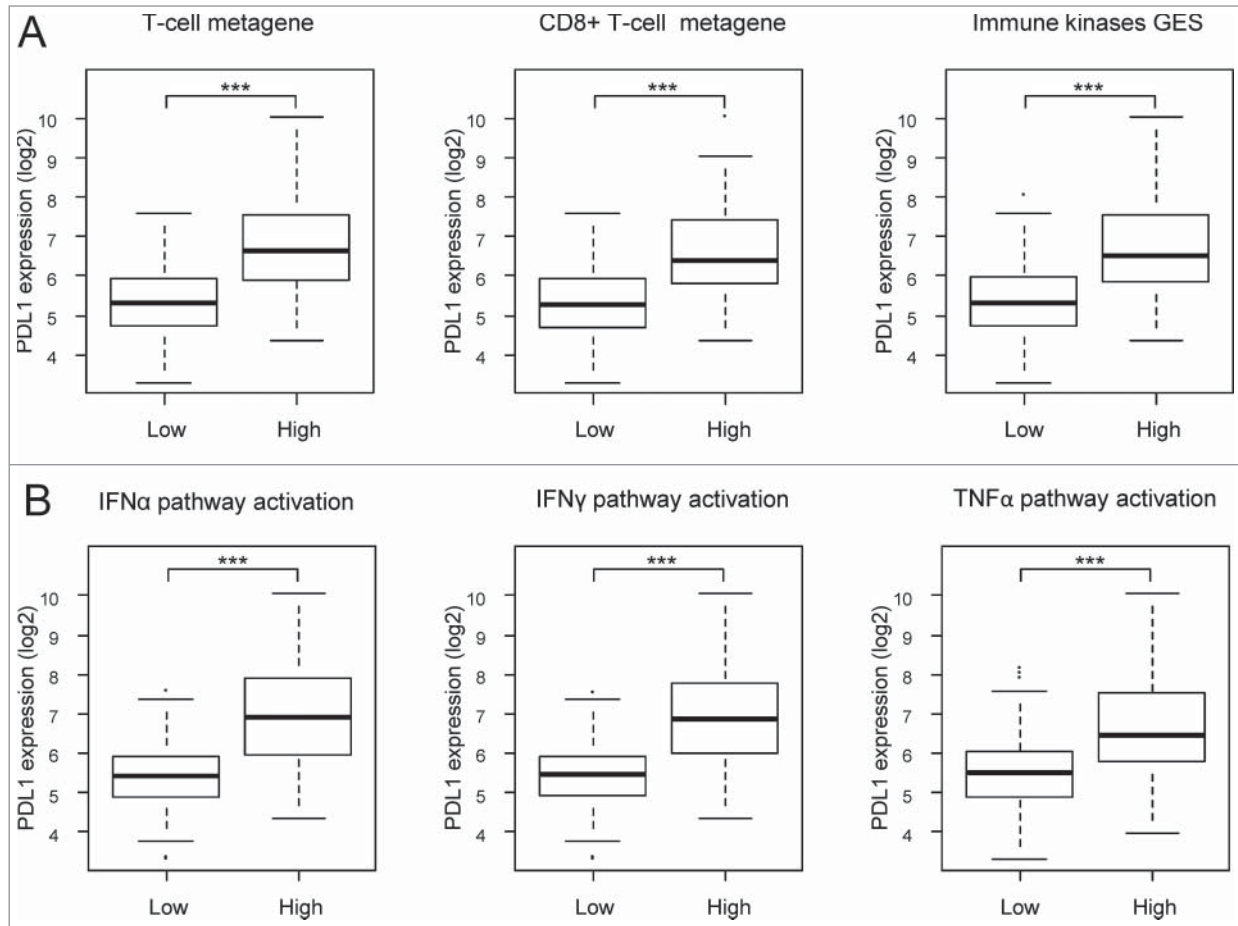


Figure 1. Correlations of *PDL1* expression with immune features. *PDL1* expression levels reported as a box plot according to sample classifications based on gene expression signatures (A) including T cells (left) and CD8⁺ T cells (middle) metagenes and a prognostic immune kinase gene expression signature (right), and based on the probability of activation of immune pathways (B) including IFN α (left), IFN γ (middle), and TNF α (right). The *P*-values are indicated (Student's *t*-test) are indicated as follows: ***, *P* < 0.001.

Table 3. Univariate and multivariate prognostic analyses for metastasis-free survival

	Univariate analysis			Multivariate analysis		
	<i>N</i>	Odds ratio [95%CI]	<i>P</i> -value	<i>N</i>	Odds ratio [95%CI]	<i>P</i> -value
Sex,						
Male vs. Female	138	1.13 [1.00–1.26]	0.09	128	1.08 [0.98–1.19]	0.20
Age,						
≤60 vs. >60 years	78	0.94 [0.81–1.08]	0.44			
Site,						
Other vs. Gastric	138	1.18 [0.94–1.48]	0.23			
Small intestine vs. gastric	138	1.13 [0.96–1.32]	0.21			
Mutation,						
<i>KIT</i> exon 11 vs. WT	128	1.20 [1.01–1.43]	0.09	128	1.15 [1.00–1.32]	0.10
<i>KIT</i> exon 9 vs. WT	128	1.37 [1.03–1.82]	0.07	128	1.30 [1.04–1.63]	0.06
<i>PDGFRA</i> exon 18 vs. WT	128	1.00 [0.80–1.26]	0.98	128	1.08 [0.90–1.30]	0.48
AFIP risk,						
Intermediate vs. low	138	1.22 [1.08–1.38]	0.0065	128	1.19 [1.05–1.35]	0.0226
High vs. low	138	1.78 [1.61–1.98]	< 0.0001	128	1.66 [1.49–1.86]	< 0.0001
<i>PDL1</i> expression, High vs. low	138	0.74 [0.64–0.84]	0.0002	128	0.81 [0.72–0.92]	0.0056

CD14, TLR3, or TLR8, etc). Both protagonists were probably recruited within the tumor through the following cytokines/cytokine receptors whose genes were found included in the signature: CCR5, CXCR6, CCL4 and CCL5, CXCL9–11, CD97, LTB, etc Altogether, this PDL1-high signature indicated a strong ability of PDL1-high GIST microenvironment to trigger an efficient antitumor response, principally conducted by cytotoxic CD8⁺ cells.

Discussion

We analyzed *PDL1* expression in 139 imatinib-untreated, operated, localized GIST and showed that high expression was an independent favorable prognostic factor for metastatic relapse. To our knowledge, this is the first study analyzing PDL1 expression in GIST.

Our analysis was based on mRNA expression measured using DNA microarrays rather than on immunohistochemistry (IHC). In recent years, PDL1 expression in cancer has most often been studied at the protein level using IHC, but discordant results have been reported across studies, notably in prognostic studies.³⁹ The main reason for these divergences is the absence of standardization of PDL1 IHC, an issue that remains unsolved today. Many antibodies are available but lack specificity and reproducibility,^{40,41} the optimal positivity cut-off is not defined, and staining interpretation suffers from subjectivity. These limitations led to the use of alternative methods, such as mRNA analysis based on *in situ* hybridization (ISH),⁴² DNA microarrays,⁴³ or quantitative real-time polymerase chain reaction (qRT-PCR).⁴⁴ A positive relationship between protein and mRNA expression has been reported.⁴² One limitation of DNA microarray- or qRT-PCR-based measurements is that they quantify expression level of both tumor and non-tumor cells present in the sample. This is particularly critical for carcinomas, although results are consistent with those reported using ISH.⁴² Our mRNA analysis based on DNA microarrays allowed us to avoid the limitations of IHC and to work on a relatively large pooled series of samples.

We showed that *PDL1* expression was heterogeneous in GIST samples with a range of values over 3 decades on logarithmic scale, providing the opportunity to search for correlations with histoclinical features. Significant association was found with the AFIP classification and metastatic relapse. *PDL1* expression was higher in AFIP low-risk samples than in high-risk samples. In literature, other immune tumor features have been found associated with the AFIP classification: in a series of 57 localized GIST, NK tumor-infiltrating lymphocytes (TILs) were more frequent and Tregs were less frequent in low/intermediate-risk samples than in high-risk samples,¹⁶ whereas no correlation was evidenced with CD3⁺ cells. Such association and ours with *PDL1* expression suggest that the immune microenvironment may be, in part, driven by GIST cell-intrinsic features. In our series, *PDL1* expression was also higher in samples without metastatic relapse than in samples with metastatic relapse, suggesting favorable prognostic value. This was confirmed in univariate and multivariate analyses,

which identified not only the AFIP classification but also the PDL1-based tumor classification as independent prognostic variables. Patients of the PDL1-low group experienced more metastatic relapses than patients in the PDL1-high group. PDL1-based classification added prognostic information to the AFIP classification: for example, whereas AFIP high-risk patients displayed a 58% relapse rate, those included in the PDL1-low group displayed a 79% rate vs. 46% in the PDL1-high group. Such favorable prognostic value of high *PDL1* expression has already been reported in other cancers, such as breast cancer,⁴² lung cancer,³⁹ colorectal cancer,⁴⁵ and Merkel cell carcinoma,⁴⁶ but has never been studied in GIST.

The prognostic classifications, currently based on features such as anatomical site, pathological tumor size, mitotic count, tumor rupture, and mutational status, are imperfect and efforts are ongoing to improve them.^{4,10–13} Several tumor cell-intrinsic molecular prognosticators have been suggested, mainly based on proteins^{47–51} or gene signatures^{52,53} related to cell proliferation. Potential immune prognostic/predictive parameters have been more recently reported. In metastatic patients treated with imatinib, the NK-cell IFN γ production after 2 months of treatment¹⁸ and an alternative transcript of NKp30 gene¹⁹ were predictors for survival. In localized GIST, a low blood neutrophil-to-lymphocyte ratio was an independent favorable prognostic parameter,⁵⁴ as were strong tumor infiltrates of CD3⁺ T cells and of NK cells.¹⁶ The favorable prognostic value of high *PDL1* expression that we observed is in agreement with these data. Our observation, *a priori* counterintuitive given the immunosuppressive function of PDL1, may be explained by the fact that *PDL1* expression is a consequence of a strong cytotoxic immune response (associated with lower rate of metastatic relapse¹⁶), and is induced in response to a homeostatic negative feedback loop, such as the one associated with IDO overexpression. For example, in breast cancer, high *PDL1* mRNA expression is induced in tumor microenvironment by activated TILs^{42,55} through the massive release of IFN γ .^{43,56} Our results seem to corroborate this hypothesis because *PDL1* expression positively correlated with expression signatures reflecting the immune response, and particularly cytotoxic T-cell response^{36,37}, and the probability of activation of IFN α , IFN γ , and TNF α pathways.³⁸ Furthermore, we identified a robust cytotoxic immune response signature in the PDL1-up GIST group, which involved differentiated CD8⁺ T cells, but also other actors of antitumor immunity such as $\gamma\delta$ -T cells, NK cells, macrophages and related APCs, B cells, etc. These cells were differentiated TH1-biased cells (eomesodermin, IL-12 and IFN-induced pathways), clearly endowed with cytotoxic effector functions (CD160, granzymes, perforin, complement-related molecules, etc).

Cancer immunosurveillance relies on effector/memory tumor-infiltrating CD8⁺ T cells with a TH1 profile. Our results suggest a major role of the immune microenvironment, notably cytotoxic cells, in influencing the clinical outcome of GIST patients independently from other tumor cell-intrinsic features, such as mitotic index or mutational status. This natural immunosurveillance system, which essentially involves activated cytotoxic cells, most probably due to an efficient antigen presentation, might

control the PDL1-high group of GIST. The composition of the immune environment, and particular cytotoxic T cells and related APCs, might shape the tumor microenvironment in the early stages. Further studies linking *PDL1* transcript and PDL1 protein expression should now be launched to establish whether PDL1 could be a new biomarker able to refine the current methods of risk stratification in GIST. Finally, the use of anti-PDL1 or anti-PD1 antibodies might help reactivate an inhibited antitumor response or enhance the efficiency of the TH1 response already initiated. This might be particularly interesting in combination with imatinib administration, as shown in murine models with CTLA-4 blockade.¹⁴

In conclusion, we showed that *PDL1* mRNA expression is heterogeneous in GIST samples and is associated with AFIP classification and metastatic relapse. Samples with low expression are associated with a higher risk of metastatic relapse independently from the AFIP classification and the mutational status, suggesting that *PDL1* expression cooperates with tumor cell-intrinsic features to influence survival. The strength of our results lies in its originality (the first one describing PDL1 expression in GIST), the analysis of a homogeneously treated population (surgery without adjuvant imatinib), the biological and clinical relevance of *PDL1* expression and its independent prognostic value in multivariate analysis. Limitations include its retrospective nature, the size of our series, although relatively important for a rare tumor and when compared to other biological prognostic studies published to date, and the analysis at the mRNA level on whole tissue samples rather than protein level. Analysis of larger imatinib-untreated patients series, retrospective, then prospective, is warranted to confirm our observation, as well as protein analyses when reliable antibodies are commercially available. IHC analyses will help determine whether PDL1 is predominantly expressed in tumor or immune cells in clinical GIST samples. If confirmed, PDL1 expression might refine the prediction of metastatic relapse and improve our ability to better tailor adjuvant imatinib. In the metastatic setting, PDL1 expression might guide the use of PDL1-inhibitors alone or in association with tyrosine kinase inhibitors.

Patients and Methods

Tumor samples

We collected histoclinical and gene expression data of clinical GIST samples from our 2 databases^{57,58} and 3 public data sets^{53,59,60} from the National Center for Biotechnology Information (NCBI)/Genbank GEO and ArrayExpress databases. The 5 datasets are described in Table S5. Samples were profiled using whole-genome DNA microarrays: Affymetrix U133 Plus 2.0⁵⁷⁻⁵⁹, and Agilent 44K.^{53,60} The pooled data set contained 159 samples, including 139 with localized GIST treated with primary surgery without adjuvant imatinib. The study was approved by our institutional board.

Gene expression data analysis

Data analysis required pre-analytic processing. The first step was to normalize each data set separately: we used quantile

normalization for the available processed Agilent data and Robust Multichip Average (RMA)⁶¹ with the non-parametric quantile algorithm for the raw Affymetrix data. Normalization was done in R using Bioconductor and associated packages. Then, hybridization probes were mapped across the 2 technological platforms represented as previously reported.⁶² When multiple probes mapped to the same GeneID, we retained the 1 with the highest variance in a particular dataset. We then merged the 5 data sets by using COMBAT (empirical Bayes)⁶³ as batch effects removal method, included in the inSilicoMerging R/Bioconductor package.⁶⁴ The final merged set included 16 759 genes in log2-transformed data. The accuracy of normalization was controlled by principal component analysis (PCA) (Fig. S1B).

PDL1 (*CD274*) tumor expression was measured by analyzing different probe sets whose identity and specificity were verified using the NCBI program BLASTN 2.2.29+ (Table S6). Analysis was done by using both continuous and binary values. Because of the involvement of PDL1 in immunity, we searched for correlations of its expression with other immune features of tumors. We thus applied different immune multigene classifiers to each tumor in each dataset separately, including metagenes associated with different immune populations such as T cells, CD8⁺ T cells, and B cells³⁶, gene expression signatures (GES) of immune pathway activity such as IFN α , IFN γ , and TNF α pathways³⁸ and a prognostic immune kinase GES.³⁷ To define the optimal cut-off of *PDL1* expression associated with the occurrence of metastatic relapse, we divided our population into 2 randomly selected sets: a learning set to define the cut-off by using a ROC curve ($n = 92$) and a validation set to validate it in independent samples ($n = 46$). Once validated, the cut-off was applied to all samples to define the PDL1-low group (expression inferior to the cut-off) and the PDL1-high group (expression superior or equal to the cut-off). Finally, to explore the biological pathways linked to *PDL1* expression in GIST, we applied a supervised analysis to the 139 samples to compare the whole-genome expression profiles of 14 546 filtered (expression level above background) genes between the PDL1-high versus PDL1-low groups. We used Significant Analysis of Microarrays (SAM)⁶⁵ algorithm and *P*-values, corrected for multiple comparisons, were considered significant only if the false discovery rate (FDR) was smaller than 0.001. Ontological analysis of the resulting gene list was based on GO biological processes of the Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/>) and on the BioCarta database (<http://www.biocarta.com/support/howto/path.asp>).

Statistical analyses

Correlations between *PDL1* expression and histoclinical factors were calculated with the Student's *t*-test or One-way ANOVA when appropriate for expression assessed as continuous variable and the Fisher's exact test for expression assessed as binary variable (PDL1-low and PDL1-high). The primary endpoint was the occurrence of metastatic relapse during follow-up, the delay of relapse and follow-up being not available in 2 data sets.^{53,60} Univariate and multivariate analyses were done using a logistic regression analysis using the *lm* function (R's statistical

package) (significance estimated by specifying a binomial family for model with a logit link). The variables tested in univariate analysis included the sample groups based on *PDL1* expression (PDL1-low and PDL1-high), patient age and gender, tumor site and mutational status, and the AFIP classification (high vs. intermediate vs. low-risk). Multivariate analysis incorporated all variables with a *P*-value inferior to 10% in univariate analysis. All statistical tests were 2-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). We wrote the article in accordance with the criteria specified in the reporting recommendations for tumor marker prognostic studies (REMARK).⁶⁶

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No potential conflicts of interest were disclosed.

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Supplemental Material

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