Nature and Significance of Stromal Differentiation, PD-L1, and VISTA in GIST: Shifting Current Paradigms

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Abstract

The nature and significance of stromal differentiation (SD), program death-ligand 1 (PD-L1), and v-domain Ig suppressor of T cell activation (VISTA) in gastrointestinal stromal tumor (GIST) is largely unknown. Looking forward, the assessment of SD and immune check point inhibition will become more ubiquitous in surgical pathology. Immature, myxoid stroma has been found to be a poor prognostic signature in many cancer subtypes (colon, breast, cervix, esophagus, stomach); although little is known regarding its significance in GIST. For immune check-point inhibition, studies have demonstrated high PD-L1 and expression to be associated with patient outcomes in numerous cancer subtypes. The present body of work aims to discover the role of SD, PD-L1 and VISTA: both in terms of its nature, and its significance in a clinical setting. Here we found PD-L1 expression in immune cells (IC) and immature SD to be associated with worse cancer free survival, while positive VISTA expression was found to be associated with improved outcomes. High-grade immature SD had the highest propensity for death/recurrence and was the only variable found to have prognostic significance on multivariate analysis. Our findings support the evaluation of SD, PD-L1 and VISTA in GIST, with clinical practice implications for pathologists. Ultimately, we hope our findings lead to improved prognostication, further optimization of therapeutics, and improved outcomes in a true clinical environment. For GIST, tumoral differentiation (TD) may be performed in isolation by pathologists, but cautiously; PD-L1 and VISTA could be on the cutting edge of an immunotherapeutic revolution, and SD may be the answer to clinical heterogeneity in GIST. We hope that this work can serve as a lexicon and a guide for discovering the essence of stroma in GIST, while also being a catalyst for pathologists to shoulder the adoption of SD in cancer.

Introduction

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal neoplasm of the gastrointestinal tract and harbors a propensity for malignant transformation¹. Risk stratification is based on tumor size, mitotic rate, location, and perforation status; however, stromal differentiation² (SD) and immune check-point inhibition³ are garnering significant attention in the world of oncology. Today, more than 35 years after Dvorak⁴ colorfully termed cancer “a wound that does not heal,” our understanding of the stroma may be coming of age.

It has been long understood that biological processes controlling wound response also control developing tumoral stroma⁵. Current research paradigms are transitioning to the extracellular matrix, deciphering cellular components of the tumoral microenvironment, and how they are influenced by each other. Now we are beginning to truly understand the significance of this biology, perhaps in the form of SD.

In the field of histopathology, SD has been recently described in many notable cancer subtypes: colon, breast, cervix, esophagus and stomach⁶⁻¹⁰. In the breast, SD has been found to predict clinical outcomes,

immune profiles and molecular phenotypes⁹. Overall, immature stroma is a bad player, being associated with reduced survival and higher pathologic stage².

The evaluation of immune check-point inhibition is becoming more and more ubiquitous to the practice of surgical pathology. Here protein expression is evaluated from immunohistochemically stained slides, and positive expression can determine therapeutic candidacy: such as seen with Atezolizumab targeting the program death-ligand 1 (PD-L1) in triple negative breast cancer¹¹.

In 2015, Bertucci et al.¹² was the first to evaluate PD-L1 expression in GIST, they found PD-L1 expression to predict metastatic relapse. Low PD-L1 expression was found to correlate with higher metastatic risk independent of the AFIP classification and the *KIT* mutational status¹². This suggests that evaluating PD-L1 in GIST may help improve outcomes and better tailor adjuvant imatinib; in the metastatic setting, Importantly, PD-L1 may be able to guide the use of immune check-point inhibition.

It is important to remember that immune escape in cancer is not restricted to the program death ligand axis. Markers such as v-domain Ig suppressor of T cell activation (VISTA) are on the cutting edge and as of now¹³. studies have described the suppressive effect of VISTA, with a presumed efficacy for anti-VISTA therapy. However, VISTA is highly controversial: it acts as a ligand on antigen-presenting cells, while also serving as a receptor on T cells¹³. Clinical trials with anti-VISTA are ongoing (Clinicaltrials.gov) and studies correlated with the expression and VISTA as a poor prognostic signature will be important in justifying its use.

This body of work is one of the larger studies on GISTs to date. Prior to this, no studies have evaluated SD in GIST, and for immune check point inhibitors we are living in the equivalent to the wild west era of the American frontier. It can certainly be said that the expression of VISTA in GIST and its relationship to the clinical profile is not understood, as it has never been evaluated before. Innovations here could lead to improved prognostication, further optimizing therapeutics and improved patient outcomes in a true clinical environment.

Looking forward, GIST patients may benefit from a more holistic diagnostic approach, one which incorporates the underlying tumoral microenvironment. The current state of art as proposed by Fletcher et al.¹⁴ is somewhat rudimentary in nature, being primarily based off mitotic indices, tumor size and kit immunopositivity¹⁴, these variables do not allow one to discover the essence of stroma, so we must shift current paradigms.

Materials And Methods

Institutional Review Board:

Institutional Review Board approval from the Office of the Human Research was obtained for all experiments. All methods were carried out in accordance with relevant guidelines and regulations. Patient

consent was not required by the institutional review board (IRB) committee due to the retrospective nature of the study (Northwell Health IRB #: 19-1066).

Design:

This study was retrospective in nature and included patients diagnosed at the Northshore University and Long Island Jewish Hospitals, part of the Northwell Health system. Routine hematoxylin and eosin (H&E) stained slides and representative cancer blocks were collected for GIST cases between 2010 to 2017. We collected resection specimens which lacked neoadjuvant chemo/radiation therapy. One representative block was selected per case from a single-slide containing the tumor's deepest invasion. Both spindle cell and epithelioid GIST cancers were collected (FIG 1). Clinicopathological data was collected and reviewed for each case including age, gender, tumor site, pathologic stage, tumor size, tumor site/surgery, as well as the histopathology. Patients were retrospectively followed for cancer free survival (CFS): defined as any recurrence, second primaries or death by the Northwell Health Cancer Registry.

Immunohistochemistry:

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tumor sections cut at 4-micron thickness and stained on a Ventana Bench Mark Autostainer (Ventana Medical System, Tucson, Arizona). The following rabbit monoclonal primary antibodies were used: PD-L1 (SP142, Ventana), VISTA (D1L2G, Cell Signaling Technology), PHH3 (7604591, Ventana). Antibodies came pre-diluted from the manufacturer and staining was performed following all protocols in collaboration with platform vendors. Technical methodologies and quality assurance were performed at the Immunopathology Laboratory of Long Island Jewish Medical Center (Northwell Health System, New Hyde Park, NY).

Digital Slides:

We utilized digital slides for the purpose of analyzing histology and immunohistochemistry for this study. Whole slide images were accessed through the Aperio vendor agnostic whole slide image viewer and slides were scanned on a Leica Aperio AT2 (Leica Biosystems, Buffalo Grove, Illinois, USA) whole slide scanner at 20×. Digital PHH3 was calculated on whole slide images (WSI) in the tumoral hotspot containing the greatest mitotic activity. The number of digital PHH3 positive cells was counted in 0.5 mm² automatically using the Ventana Virtuoso software (Roche Diagnostics, North America).

Stromal Differentiation:

Scoring was based on the 3-tier grading system proposed by Hacking et al.² (TAB 1). Mature stroma (SD1) was composed of mature collagen fibers, stratified into multiple fibrous bands at tumoral front. We assessed immature stroma in a semi-quantitative manner based on the degree of myxoid, amorphous SD. Immature, myxoid stroma contained basophilic to grey extracellular matrix and often intermingled with hyalinized collagen. Low grade stroma usually contains high variability in absolute difference in intensity between stromal matrix regions (mosaic pattern), less contiguous areas of myxoid stroma (FIG

2). Low grade should contain a 40x field of myxoid stroma and intermediate stroma containing “keloid like” can usually be categorized as low–grade. High grade usually contains low variability in absolute difference in intensity between stromal matrix regions, with stromal cells surrounded by contiguous regions of myxoid stroma, being the predominate stromal pattern (>50%) at the extramural tumor front.

PD-L1 and VISTA Expression:

PD-L1 and VISTA expression was evaluated in both tumoral cells (TC) and in immune cells (IC). Staining expression was scored as the proportion of tumor area occupied by VISTA positive TC and IC. Briefly, scoring was primarily based on the Allred system¹⁵; here the percentage of staining is scaled from 0 to 5: 0=0%, 1=1%, 2=>1%-10%; 3=11%–33%; 4=34%–66%; 5=67%–100%. Staining intensity was scored as none (0), mild at 2x (1), moderate at 10x (2), and strong at 4x (3). The combination of staining percentage and intensity yields a total score between 0 and 8. Scoring was performed in the tumoral microenvironment and in both lymphocytes and myeloid cells (macrophages, dendritic cells, and granulocytes). PD-L1 and VISTA expression can be seen in FIGURE 3.

Histologic Grade:

We based our grading off Fletcher et al.¹⁴ who creased the NIH classification, which was the first to classify GIST. It determined the risk of stratification based on a mitotic count higher than 5/50 high power fields (HPF). conventional counting of mitotic cells was performed consecutively in HPFs, which allowed for a mitotic rate per 5 mm² surface area calculated according to a 400x field diameter.

Statistical Analysis:

The primary objective of the statistical analysis was to assess the relative prognostic importance of stromal differentiation and immune checkpoint inhibition in GISTs. Descriptive statistics such as frequencies and percentages were calculated for the categorical variables. The Kaplan-Meier method was used to evaluate the disease-free survival rate as a function of time. The two tailed Fisher exact test was used to assess relationships between stromal differentiation, immune checkpoint inhibition and the clinicopathological profile. Statistical Analysis was performed using Prism graphpad 8.4.2. Cox-regression was performed on SPSS 1.0.0.1508. A p value of <0.05 was used to indicate statistical significance.

Results

Patient Characteristics:

This study comprised data from 105 patients with GIST who underwent surgical resection at our health system. Surgeries included gastrectomy (54), small bowel resection (33), colectomy (10), sigmoid and rectectomy (3) omentectomy (5). The mean age for our patient cohort is 70.5. 63 patients in our cohort were under the age of 70, while 42 patients were over the age of 70. Regarding gender, 56 patients were

male and 49 were female. The majority of GIST in our study occurred in the stomach (54), although 51 cases occurred outside the stomach: small bowel NOS (23), colon (10), duodenum (5), omentum (5), ileum (3), rectum (3), and jejunum (2). The minority of tumors were stage T2 and under (34), while 71 patients presented with higher stage tumors (T3-4). 84 patients were histological grade 1, while 21 patients were grade 2. For histological type 79 patients were spindled, 5 were epithelioid and 21 patients had a mixed morphology. 83 patients had a mitotic index of 5 or under, while 21 had a mitotic index of greater than 5. Necrosis was present in 17 patients and was absent in 79 patients. 88 patients had digital PHH3 counts of 5 or less, while 17 patients had counts greater than 5.

Stromal Differentiation:

70 patients in our cohort had mature SD. On the other hand, 35 patients were defined as having immature SD. 21 of these were designated as low-grade (SD2) and 14 as high-grade (SD3). When comparing SD to the clinical and IHC profile, we broke SD into both mature and immature, as well as into low and high-grade. Firstly, based on mature and immature status, immature SD was found to be associated with higher tumoral stage ($p=0.021$), higher histological grade ($P<0.001$), higher mitosis ($P<0.001$), a higher digital PHH3 ($P<0.001$), and lower PD-L1 TC ($P<0.001$), IC ($P<0.001$); as well as VISTA TC ($P<0.001$), and IC ($P<0.001$) expression, and worse cancer free survival ($P=0.001$). When immature stroma was broken up into low (SD2) and high-grade (SD3), higher histological grade ($P<0.001$), higher mitosis ($P<0.001$), and a higher digital PHH3 ($P<0.001$). High-grade immature SD had the highest propensity for death or recurrence ($P=0.001$). The results for Fisher Exact analyses can be viewed in TABLE 1.

PD-L1 and VISTA Expression:

PD-L1 expression scores were as follows: PD-L1 TC: 0 (41), 1(1), 2(13), 3(16), 4(14), 5(14), 6(1), 7(3), 8(2); PD-L1 IC: 0 (72), 1(0), 2(18), 3(7), 4(5), 5(1), 6(1), 7(0), 8(0). VISTA expression scores were as follows: VISTA TC: 0 (28), 1(0), 2(20), 3(14), 4(20), 5(17), 6(6), 7(0), 8(0); VISTA IC: 0 (53), 1(1), 2(22), 3(10), 4(10), 5(6), 6(3), 7(0), 8(0). Heatmaps are presented in Figure 4(a) and bar graphs for PD-L1 and VISTA scoring is presented in Figure 4(b) for both the TC and IC components. Based on non-linear regression data in Figure 4(c), a combined score of 3 was used as a cutoff for PD-L1 and VISTA.

PD-L1 was found to be associated with gender in the TC ($P=0.01$) and IC components ($P=0.01$); histologic grade TC ($P<0.001$) IC ($P<0.001$) components. Tumor size was also found to be associated with TC ($P=0.001$) and IC ($P=0.001$) positivity. PD-L1 TC positivity correlated with VISTA TC ($P=0.001$), IC ($P=0.001$); while PD-L1 IC positivity correlated with PD-L1 TC ($P=0.01$) and IC ($P=0.001$) positivity. The remaining variables were not statistically significant (TAB 3).

VISTA was found to be associated with gender in the TC ($P=0.01$) and IC components ($P=0.01$); stage ($P=0.024$) in the TC ($P=0.011$) and IC ($P=0.001$); as well as histologic grade ($P=0.009$), mitosis ($P=0.009$) and digital PHH3 ($P=0.024$) in VISTA ICs. VISTA TC positivity correlated with PD-L1 TC ($P=0.001$), IC ($P=0.001$); while VISTA IC positivity correlated with PD-L1 TC ($P=0.01$) and IC ($P=0.001$) positivity. The remaining variables were not statistically significant (TAB 4).

Cancer Free Survival:

Cancer free survival (CFS) data was collected for all of the 105 patients with a mean follow up time of 1913 days. Out of our patients, 16 had recurrence and 12 patients died. After setting the positivity cutoff to 3 for PD-L1 and VISTA, we found that IC positivity was associated with KM survival for PD-L1 ($P=0.01$) and VISTA ($P=0.045$). KM survival analysis revealed SD ($P=0.005$), stage ($P=0.029$), mitosis ($P=0.0003$), and digital PHH3 ($P=0.0005$) to be associated with CFS. PD-L1 ($P=0.23$) and VISTA ($P=0.23$) staining in TC components was not statistically significant (FIG 5).

Based upon cox-regression of cancer-free survival (CFS), high stage ($P=0.048$), histologic grade ($P=0.001$), mitosis ($P=0.01$), size ($P=0.032$), digital PHH3, PD-L1 IC ($P=0.028$) and VISTA IC ($P=0.048$) on univariate but not on multivariate analysis ($P>0.05$). Stromal differentiation was the only variable found to be significant on univariate ($P=0.002$) and multivariate ($P=0.024$) analysis. The remaining clinicopathological variables were not significant ($P>0.05$) on cox proportional hazard regression analysis (TAB 5).

Discussion

The present study elucidates the role of stromal differentiation and immune check-point inhibition (PD-L1 and VISTA) in GISTs. In addition to having clinical practice implications for patients with GIST, our findings may also be transferable to other cancer subtypes.

The differentiation of the extracellular matrix leads to characteristic immature, myxoid stroma seen on routine histologic evaluation. In the present study we found immature stroma to be associated with higher anatomical extent of disease, higher tumoral grade, higher mitosis, negativity for and worse CFS. This is reminiscent of what was seen in breast cancer⁹, where mature stroma was also found to be associated with PD-L1 expression. Importantly, high grade differentiation (SD3) was found to have the highest risk of recurrence and was the only variable in GIST found to be statistically significant on multivariate analysis. Looking forward, the *Hacking Classification of Stromal Differentiation* could be used in clinical practice, identifying patients with poor prognostic outcomes, allowing them to be treated more aggressively.

Understanding the peculiarities of the ECM in GIST could lead to therapeutic opportunities; myxoid degeneration of the ECM has been demonstrated to decrease the physical barrier in the cancer microenvironment, which can improve the delivery of therapeutics, nutrients, and immune cells¹⁶. Therefore, mature SD could act as barrier, which is supported by preclinical studies showing ECM degradation to improve drug uptake and response¹⁶. In this setting, myxoid stromal degeneration could be good and bad: while it may have an improved therapeutic response, these patients do have the highest risk of recurrence.

For PD-L1 and VISTA, this is the second thorough study to explore PD-L1¹² and the first to explore VISTA in GIST. Similar to Bertucci et al.¹² we also found PD-L1 expression to be associated with poor prognostic outcomes and recurrence in GIST. In mouse models, PD-1 expression on T cells has been found correlate with imatinib treatment in GIST¹⁷. Meanwhile, treatment with imatinib inhibited the IFN γ -induced upregulation of PD-L1 via STAT1 inhibition¹⁷, suggesting a role for PD-L1 based combination therapy.

It is interesting that VISTA was found to be protective in GIST when positive in ICs. While interpreting these prognostic findings, it is important to understand the complexity of VISTA as an immune check point regulator and the tumoral microenvironment in general. VISTA is expressed on both CD4+/Foxp3+ regulatory T cells and myeloid-derived suppressor cells (MDSCs)¹⁸. Loeser et al. demonstrated significant VISTA expression on CD4+ T cells to result in improved outcomes in esophageal adenocarcinoma¹⁹.

These findings may be secondary the effect of VISTA in converting naïve T cells into FoxP3 expressing T cells²². FoxP3 has been shown to improve survival in patients with colorectal cancer^{2,23} but the prognostic effect varies in other cancer types²⁰. FoxP3 plays a role as a transcriptional repressor for the proto-oncogene SKP2, and in-turn regulates the cell cycle in the G2/M phase^{2,24}. Decreased FoxP3 expression promotes SKP2 and cell proliferation. This may explain why manual mitotic count and digital PHH3 was decreased in patients with significant VISTA expression in immune cells.

Our findings do support that VISTA may be immune-protective for patients with GIST. However, VISTA was found to be a poor prognostic signature in melanoma²¹, suggesting a multifaceted role of VISTA in different tumor subtypes.

PD-L1 and VISTA are molecules that can be targeted by synthesized antibodies and for GIST, clinical trials using immune checkpoint inhibition in combination with anti-KIT and chimeric antigen receptor (CAR) T-cells have shown promising results²². The treatment of GIST and the use of immunotherapy is evolving; robust, well-designed clinical trials will have the ultimate say in determining therapeutic response.

Our study did not come without pitfalls, firstly this study was retrospective, which allows for the potential of bias and secondly we were unable to perform molecular testing for SD, PD-L1, and VISTA. Thirdly, we were unable to perform multiplex immunolabeling, this would have allowed us to identify PD-L1 and VISTA expression in different immune cell subtypes.

The use of digital image analysis has been shown to outperform manual analysis²³; in our study we also found digital PHH3 to be comparable to manual mitotic count. Coupling our whole slide images with deep learning²⁴ approaches could have improved prognostication; future studies should integrate computational approaches to access the tumoral microenvironment.

To conclude, SD, PD-L1 and VISTA evaluation may help tailor the need for adjuvant imatinib therapy, which can be given alone or in combination with immune check point inhibition. The status of a patients SD may be the most important factor for predicting recurrence, and for GIST patients with high-grade SD, clinical trials will be needed to determine whether aggressive therapeutics are beneficial. For GIST, tumoral differentiation may be performed in isolation by some pathologists, but cautiously: PD-L1 and VISTA could be on the cutting edge of an immunotherapeutic revolution, and SD may finally be the answer to clinical heterogeneity in GIST we have been searching for.

Declarations

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Author Contributions

MN, DW, and SH developed the theoretical formalism. SH, DW, TV, LL, and HC contributed to the acquisition of data. DW performed the analytic calculations and performed the numerical simulations. SH, DW, and MN contributed to the final version of the manuscript.

Data Availability

Pathology data and the statistical analyses for the current study are available from the corresponding author upon reasonable request.

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Disclosure

The authors report no conflicts of interest in this work.

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Tables

TABLE 1: Hacking Classification of Stromal Differentiation.

Hacking Classification of Stromal Differentiation ²		
1	<i>Mature, SD1 Stromal Differentiation</i>	Mature stroma consists of fine collagen fibers stratified into multiple layers in fibrous zones and less than a 40x focus of immature (myxoid) stroma
2	<i>Immature, SD2 Stromal Differentiation Low Grade, Minimal</i>	Low grade stroma contains high variability in intensity between stromal matrix regions (mosaic pattern), less contiguous areas of myxoid stroma with admixed collagenous stroma and a minimum amount of myxoid stroma (40x field)
3	<i>Immature, SD3 Stromal Differentiation High Grade, Predominant</i>	High grade contains low variability in absolute difference in intensity between stromal matrix regions, stromal cells surrounded by contiguous regions of myxoid stroma with myxoid stroma being the predominate stromal pattern (>50%) at the extramural tumor front

Table 2. Fisher Exact Analysis for Stromal Differentiation. Significant features ($P \leq 0.05$) are shown in bold. T, Stage; SD, Stromal differentiation; PHH3, Phosphohistone H3; PD-L1, Program Death-Ligand 1; VISTA, V-domain immunoglobulin suppressor of T cell activation. TC, Tumor Cell; IC, Immune Cell.

Variable	Stroma		P-Value	Stromal grade			P-Value
Cutoff	Mature	Immature		SD1	SD 2	SD3	
Age			0.582				0.961
<=70	42	21		42	13	8	
>70	28	14		28	8	6	
Gender			0.72				0.620
Male	37	19		37	10	9	
Female	33	16		33	11	5	
Site			0.3				0.251
Stomach	33	21		33	11	10	
Other than stomach	37	14		37	10	4	
Stage			0.021				0.08
≤ T2	16	18		16	10	8	
T3-T4	54	17		54	11	6	
Histologic grade			<0.001				<0.001
1	65	19		65	11	8	
2	5	16		5	10	6	
Histologic type			<0.001				0.250
Spindle	50	29		50	16	13	
Epithelioid	3	2		3	1	1	
Mixed	17	4		17	4	0	
Mitosis			<0.001				<0.001
<=5	65	19		65	11	8	
>5	5	16		5	10	6	
Size			<0.001				0.390
<=2	41	24		41	16	8	
2-5	15	5		15	1	4	
5-10	12	4		12	3	1	
>10	2	2		2	1	1	
Necrosis			0.143				0.249
Yes	9	8		9	5	3	
No	58	21		58	13	8	
PHH3			<0.001				<0.001
<=5	67	21		67	13	8	
>5	3	14		3	8	6	
PD-L1 TC			0.05				0.168
Negative	24	18		24	12	6	
Positive	46	17		46	9	8	
PD-L1 IC			<0.001				0.08
Negative	43	29		43	17	12	
Positive	27	6		27	4	2	
VISTA TC			<0.001				0.387
Negative	36	18		36	11	7	
Positive	34	16		34	9	7	
VISTA IC			<0.001				0.376
Negative	58	28		58	16	12	
Positive	12	6		12	4	2	
Status			0.001				0.001
Cancer free	59	18		59	13	5	
Recurrence/Death	11	17		11	8	9	

Table 3. Fisher Exact Analysis for PD-L1. Significant features ($P \leq 0.05$) are shown in bold. PD-L1, Program Death-Ligand 1; T, Stage; SD, Stromal differentiation; VISTA, V-domain immunoglobulin suppressor of T cell activation; PHH3, Phosphohistone H3; TC,

Tumor Cell; IC, Immune Cell. *Extragastric sites include colon, duodenum, omentum, ileum, rectum, and jejunum.

Variable	PD-L1 TC		P-Value	PD-L1 IC		P-Value
Cutoff	neg	pos		neg	pos	
Age			0.08			0.202
<=70	21	42		40	23	
>70	21	21		32	10	
Gender			0.01			0.01
Male	23	26		34	15	
Female	19	37		38	18	
Site			0.554			0.410
Gastric	20	34		35	19	
*Extragastric	22	29		37	14	
Stage			0.137			0.286
≤ T2	18	14		25	7	
T3-T4	24	49		47	26	
Histologic grade			0.807			0.487
1	33	51		57	27	
2	9	12		15	6	
Histologic type			0.001			0.001
Spindle	35	44		57	22	
Epithelioid	1	4		3	2	
Mixed	6	15		12	9	
Mitosis			0.807			0.487
<=5	33	51		57	27	
>5	9	12		15	6	
Necrosis			0.588			0.403
Yes	7	10		13	4	
No	35	53		59	29	
Size			0.001			0.001
<=2	23	42		40	25	
2-5	9	11		15	5	
5-10	7	9		13	3	
>10	3	1		4	0	
SD			0.168			0.05
SD1	24	46		43	27	
SD2	12	9		17	4	
SD3	6	8		12	2	
Digital PHH3			0.746			0.545
<=5	35	53		60	28	
>5	7	10		12	5	
VISTA TC			0.001			0.001
Negative	23	31		38	16	
Positive	18	32		33	17	
VISTA IC			0.001			0.001
8Negative	35	51		62	24	
Positive	6	12		9	9	
Status			0.928			0.637
Cancer free	31	46		54	23	
Recurrence/Death	11	17		18	10	

Table 4. Fisher Exact Analysis for VISTA. Significant features ($P \leq 0.05$) are shown in bold. VISTA, V-domain immunoglobulin suppressor of T cell activation; TC, Tumor Cell; IC, Immune Cell; T, Stage; PHH3, Phosphohistone H3; PD-L1, Program Death-Ligand

1. Significant features ($P \leq 0.05$) are shown in bold. *Extragastric sites include colon, duodenum, omentum, ileum, rectum, and jejunum.

Variable	VISTA TC		P-Value	VISTA IC		P-Value
Cutoff	neg	pos		neg	pos	
Age			0.549			0.663
<=70	34	28		52	10	
>70	20	22		34	8	
Gender			0.01			0.01
Male	24	32		44	12	
Female	30	18		42	6	
Site			0.539			0.577
Gastric	27	27		45	9	
*Extragastric	27	23		41	9	
Stage			0.011			0.001
≤ T2	9	22		16	15	
T3-T4	45	28		70	3	
Histologic grade			0.278			0.009
1	40	43		65	18	
2	14	7		21	0	
Histologic type			<0.001			<0.001
Spindle	42	36		67	11	
Epithelioid	3	2		4	6	
Mixed	9	12		15	1	
Mitosis			0.278			0.009
<=5	40	43		65	18	
>5	14	7		21	0	
Necrosis			0.123			0.740
Yes	13	4		15	2	
No	41	46		71	16	
Size			<0.001			<0.001
<=2	32	33		55	10	
2-5	8	12		15	5	
5-10	10	5		13	2	
>10	4	0		3	1	
Stromal Differentiation			0.387			0.376
Grade 1	36	34		58	12	
Grade 2	11	9		16	4	
Grade 3	7	7		12	2	
Digital PHH3			0.746			0.024
<=5	44	43		69	18	
>5	10	7		17	0	
PD-L1 TC			0.001			0.001
Negative	23	18		35	6	
Positive	31	32		51	12	
PD-L1 IC			0.001			0.001
Negative	38	33		62	9	
Positive	16	17		24	9	
Status			0.815			0.463
Cancer free	39	37		61	15	
Recurrence	15	13		25	3	

TABLE 5. Univariate and Multivariate analyses of Cancer Free Survival Using the Cox Proportional-Hazard Regression. CI, Confidence Interval; HR, Hazard Ratio; PD-L1, Program Death-Ligand 1. VISTA, V-domain immunoglobulin suppressor of T cell activation; TC, Tumor Cell; IC, Immune Cell. Significant features ($P \leq 0.05$) are shown in bold. *Extragastric sites include colon, duodenum, omentum, ileum, rectum, and jejunum.

Variable	Frequency	Univariate HR	CI	P Value	Multivariate HR	CI	P Value
Age		1.270	0.604-2.670	0.528	1.870	0.705-4.962	0.209
<=70	63 (60%)						
>70	42 (40%)						
Gender		0.857	0.401-1.831	0.690	1.082	0.429-2.729	0.867
Male	56 (53.3%)						
Female	49 (46.7%)						
Site		1.205	0.573-2.534	0.623	1.128	0.437-2.913	0.804
Gastric	54 (51.4%)						
Extragastric	51 (48.6%)						
Stage		1.415	1.004-1.996	0.048	1.369	0.830-2.256	0.218
<=T2	34 (32.4%)						
T3-T4	71 (67.6%)						
Histologic grade		3.625	1.723-7.626	0.001	2.631	0.430-2.997	0.410
1	84 (80%)						
2	21 (20%)						
Histologic type		0.613	0.326-1.151	0.128	0.593	0.312-1.125	0.110
Spindle	79 (75.2%)						
Epithelioid	5 (4.8%)						
Mixed	21 (20%)						
Mitosis		3.625	1.723-7.626	0.001	2.789	0.299-6.039	0.368
<=5	84 (80%)						
>5	21 (20%)						
Size		0.458	0.224-0.937	0.032	0.502	0.229-1.103	0.086
<=2	65 (61.9%)						
2-5	20 (18.1%)						
5-10	16 (15.2%)						
>10	4 (3.8%)						
Necrosis		2.034	0.881-4.695	0.096	1.154	0.369-3.603	0.806
Yes	17 (16.2%)						
No	88 (83.8%)						
Digital PHH3		3.503	1.657-7.408	0.001	0.849	0.094-8.002	0.887
<=5	88 (83.8%)						
>5	17 (16.2%)						
PD-L1 TC		1.014	0.930-1.311	0.243	1.064	0.855-1.325	0.577
Negative	42 (40%)						
Positive	63 (60%)						
PD-L1 IC		1.129	1.001-1.412	0.028	1.321	0.924-1.889	0.127
Negative	72 (68.6%)						
Positive	33 (31.4%)						
VISTA TC		0.888	0.715-1.104	0.286	1.063	0.823-1.373	0.641
Negative	54 (51.9%)						
Positive	50 (48.1%)						
VISTA IC		0.844	0.674-0.995	0.048	0.841	0.577-1.226	0.368
Negative	86 (82.7%)						
Positive	18 (17.3%)						
Stromal Differentiation		2.155	1.322-3.514	0.002	2.229	1.112-4.468	0.024
Grade 1 (SD1)	70 (66.7%)						
Grade 2 (SD2)	21 (20%)						
Grade 3 (SD3)	14 (13.3%)						

Figures

Figure 1

Morphology in GIST. (a) Spindle Cell Features in a Collagenous Stroma (20x); (b) Epithelial Cell Features in a Myxoid Stroma (20x).

Figure 2

A Semi-Quantitative Approach to Stromal Differentiation. Low grade should contain a minimum amount of myxoid stroma ($\times 40$ field), while high-grade is the predominate stromal pattern ($>50\%$) at the extramural tumor front.

Figure 3

PD-L1 and VISTA Expression Patterns. Staining intensity was scored as none (0), mild at $\times 2$ -0 (1), moderate at $\times 10$ (2), and strong at $\times 4$ (3). PD-L1, Program Death-Ligand 1; VISTA, V-domain immunoglobulin suppressor of T cell activation. TC, Tumor Cell; IC, Immune Cell.

Figure 4

PD-L1 and VISTA Expression Scoring. (a) Heatmaps for PD-L1 and VISTA; (b) Bar Graph showing Allred scores for PD-L1 and VISTA TCs and ICs. (c) Linear Regression for PD-L1 and VISTA Scoring and Cancer Free Survival. PD-L1, Program Death-Ligand 1; VISTA, V-domain immunoglobulin suppressor of T cell activation. TC, Tumor Cell; IC, Immune Cell.

Figure 5

Kaplan-Meier Survival Analysis. (a) Cancer free survival between PD-L1 positive and negative tumor cell groups. (b) Cancer free survival between PD-L1 positive and negative immune cell groups. (c) Cancer free survival between VISTA positive and negative tumor cell groups. (d) Cancer free survival between VISTA positive and negative immune cell groups. (e) Cancer free survival between stroma 1 (mature, SD1); stroma 2 (Immature, low-grade, SD2); and stroma 3 (Immature, high-grade, SD3). (f) Cancer free survival between low (T1/2) and high (T3/4) tumoral stage. (g) Cancer free survival between manual mitosis low (P< 5) and high (>5) groups. (h) Cancer free survival between digital PHH3 low (P< 5) and high (>5) groups. PD-L1, Program Death-Ligand 1; T, Stage; SD, Stromal differentiation; VISTA, V-domain immunoglobulin suppressor of T cell activation. TC, Tumor Cell; IC, Immune Cell.