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Therapy Resistance in Neoadjuvantly Treated Gastric Cancer and Cancer of the Gastroesophageal Junction is Associated with an Increased Expression of Immune Checkpoint Inhibitors—Comparison Against a Therapy Naïve Cohort

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Abstract

With recent studies uncovering the complex landscape of immune checkpoint regulators in gastric cancer (GC), we aimed to characterize the expression of the checkpoint proteins V-domain Ig suppressor of T-cell activation (VISTA), programmed cell death 1 ligand 1 (PD-L1), and programmed cell death protein-1 (PD-1) in a cohort of GCs following platinum-based neoadjuvant chemotherapy. A total of 141 GC samples, 93 lymph node metastases, and 15 distant metastases were assessed using immunohistochemistry. Staining results were correlated with clinicopathological patient characteristics, genetic alterations, and survival. The expression of VISTA was detected in tumor cells of 38 (30.9%) GCs and immune cells of 139 (98.6%) GCs. The expression of PD-L1 was detected in tumor cells of 27 (22.7%) GCs and immune cells of 134 (96.4%) GCs. The expression of PD-1 was only observed in lymphocyte aggregates/intratumoral lymphoid follicles of 123 (87.2%) GCs. VISTA and PD-L1 correlated in their expression and were associated with poor tumor regression. Compared with an ancient cohort of therapy naïve GCs, we observed a major increase in overall immune cell density accompanied by an over proportional increase in PD-1 and VISTA-positive immune cells. The frequency of VISTA expression in tumor cells was also found to be substantially increased. To the contrary, expression of PD-L1 was decreased in immune cells and tumor cells of neoadjuvantly treated GCs. As a result, a subset of GCs using a single (only VISTA or PD-L1) or combined (VISTA and PD-L1) immune evasion mechanisms might benefit from an anti-PD-L1/anti-VISTA—targeted therapy.

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Introduction

Gastric cancer (GC) is the fifth most common cancer in the world. In Western countries, the prognosis is dismal because of diagnosis at an advanced disease stage. Neoadjuvant (radio-)chemotherapy is standard of care for locally advanced GC in Europe [1]. Patients with limited metastatic GC or gastroesophageal junction cancer may benefit from a combination of fluorouracil, leucovorin, oxaliplatin, and docetaxel (FLOT) [2]. Whereas in the palliative setting, treatment options include antiangiogenic strategies (i.e.,

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ramucirumab) [3,4] or targeted therapies (i.e., trastuzumab) [5]. More recently, immune checkpoint inhibitors have gained considerable attention and have novel treatment options in the palliative setting. However, it becomes increasingly evident that not every patient responds to immune checkpoint inhibitors and patient selection is a pressing issue. Understanding the expression of immune checkpoint molecules has become a major research topic in recent years. These include, e.g., cytotoxic T-lymphocyte associated protein-4 (CTLA4), programmed cell death protein-1 (PD-1), its ligand PD-L1, and V-domain Ig suppressor of T-cell activation (VISTA, PD-1H).

Although PD-L1 is already established as a prime research topic across many tumor entities, VISTA has only recently come into focus with a suite of similar, but also some importantly different properties. PD-L1 and VISTA are part of the B7 family of immune checkpoint proteins [6]. As such they share the property of inhibiting proinflammatory T-cell interactions and promoting self-regulatory processes in the immune system [7]. PD-L1 interacts with its receptor PD-1. VISTA on the other hand has been shown to serve both as a receptor and a ligand with new interaction partners recently being discovered [8,9]. Most important in its implication for this study was the discovery of nonredundant pathways for VISTA compared with other B7-family members such as PD-L1, suggesting the usefulness of a combined targeted therapy [10].

Previously, we have shown that VISTA and PD-L1 are significantly associated with Epstein−Barr virus (EBV)−associated GC, whereas PD-L1 was also frequently expressed in microsatellite instable (MSI) GC [11,12]. Both, VISTA and PD-L1 shared a significant association with each other supporting their role in a dual immune evasion mechanism in GC. Nevertheless, the mechanism of immune evasion in GC, underlying the differential expression of immune checkpoint proteins, is complex and we hypothesize that apart from molecular subtypes of GC, neoadjuvant oncological treatment also affects the expression of immune checkpoint proteins. To test this hypothesis, we studied the expression of VISTA, PD-L1, and PD-1 in a cohort of neoadjuvantly treated GCs and compared the results with a previously published cohort of therapy naïve GCs.

Material and Methods

Ethics

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent or substitute for it was obtained from all patients for being included in the study. Ethical approval was obtained from the local ethical review board (D 453/10 and D 525/15). All patient data were pseudonymized before study inclusion. All experimental work was compliant with all mandatory laboratory health and safety procedures.

Study Population

From the archive of the Department of Pathology, University Hospital Kiel, we sought all patients who had undergone platinum--based neoadjuvant chemotherapy followed by either total or partial gastrectomy for adenocarcinoma of the stomach (distal) or esophagogastric junction (proximal) between 1998 and 2017. The following patient characteristics were retrieved: type of surgery, age at diagnosis, gender, tumor size, tumor localization, tumor type, number of immune cells (e.g., lymphocytes, granulocytes, macrophages) per 1 mm^2 in an intratumoral hot spot region, depth of invasion, residual tumor status, number of lymph nodes resected, and number of lymph nodes with metastases. Tumor regression was evaluated according to Becker et al. [13] into tumor regression grade (TRG) 1a (complete regression), TRG1b (<10% vital tumor cells), TRG2 (10−50% vital tumor cells), and TRG3 (>50% vital tumor cells).

Patients were included if an adenocarcinoma of the stomach or esophagogastric junction was histologically confirmed. Patients were excluded if a tumor type other than adenocarcinoma was histologically identified. Each resected specimen had undergone gross sectioning and histological examination by trained and board-certified surgical pathologists. Date of patient death was obtained from the *Epidemiological Cancer Registry* of the state of Schleswig-Holstein, Germany. Follow-up data of those patients who were still alive were retrieved from hospital records and general practitioners.

Histology

Tissue specimens were fixed in formalin and embedded in paraffin (FFPE). Deparaffinized sections were stained with hematoxylin and eosin. Histological reexamination of primary tissue sections was carried out for all cases to assure if inclusion criteria were met. Tumors were classified according to the Lauren [14] and reexamined by two surgical pathologists. pTNM-stage of all study patients was determined according to the 8th edition of the UICC guidelines [15].

Immunohistochemistry

Immunohistochemistry was carried out with antibodies directed against PD-L1 [dilution 1:100, E1L3N, Cell Signaling, Danvers, USA (catalog #13684)], PD-1 [dilution 1:100; clone MRQ-22, Cell Marque, Rocklin, USA (#315M-96)], and VISTA [1:500; clone D1L2G, Cell Signaling (#64953)]. Immunostaining of PD-L1 and PD-1 was performed with the autostainer Bond™ Max System (Leica Microsystems GmbH, Wetzlar, Germany). The immunoreaction was visualized with the Bond™ Polymer Refine Detection Kit [brown labeling; Novocastra; Leica Microsystems GmbH, Wetzlar, Germany (#DS9800)]. Immunostaining of VISTA was performed manually: Following antigen retrieval in citrate buffer (pH6), specimens were incubated with hydrogen peroxide block and Ultra V Block [both Thermo Scientific, Braunschweig, Germany (TA-125-HP and TP-125-HL)] to avoid unspecific reactions. The immunoreaction was visualized with the ImmPRESS-HRP-Universal−Antibody Polymer and the NovaRED substrate kit [both VectorLabs, Peterborough, United Kingdom (#SK-4800)]. Counterstaining was carried out with hematoxylin [Dr. K. Hollborn & Söhne GmbH & Co KG; Leipzig, Germany (#88663)].

Germinal centers of lymph follicles served as internal positive control for PD-L1 and PD-1.

Evaluation of Immunostaining

The evaluation of immunostaining results for VISTA and PD-L1 was mostly identical to our previous studies [11,12]. Any necessary deviations will be expressly denoted below.

Evaluation of VISTA Immunostaining. For evaluation of VISTA expression in tumor cells, an immunoreactivity score (IRS) was applied: Category A rated the percentage of immunoreactive tumor cells and was graded as 0 (negative), 1 (≤1% positive), 2 (2−10% positive), 3 (11−50%), and 4 (>50%). Category B rated the intensity

of immunostaining of tumor cells and was graded as 0 (negative), 1+ (weak), or 2+/3+ (strong). Different from our previous study by Böger et al. [12], we opted to use a three tiered instead of a four-tiered grading of the intensity (i.e., 0, 1+, 2+ and 3+), as only one case (three samples) was considered to exhibit a 3+ staining intensity. We therefore joined the 2+ and 3+ intensities into the category of strong (2+/3+) and considered its category B value to be 2. Category A and B were finally added together into the IRS of tumor cells with possible values from 0 to 6. We again recognized that the overall percentage of VISTA-positive tumor cells was mainly low and a HistoScore assessing different percentages of different staining intensities was indiscernible and impractical. Thus, the IRS representing VISTA status in tumor cells was dichotomized at the median into negative (no immunostaining at all) and any staining present (positive), aligning with the criteria described previously by Böger et al. [12].

The expression of VISTA in immune cells was identical with the procedure described by Böger et al. [12]. In brief, VISTA-positive immune cells were counted in intratumoral hot spot regions regarding (1) the absolute number of VISTA-positive immune cells per 1 mm^2 and (2) the proportion of VISTA-positive immune cells per 200 immune cells. Hot spot regions were defined areas with the highest density of VISTA-positive immune cells.

Immunostaining of endothelial cells was graded as present or absent. Tumor cells of available locoregional lymph nodes and distant metastases were assessed as described above. Immune cells in these metastases were assessed as described above in cases where the dimensions of the tumor allowed it, i.e., avoiding the assessment of surrounding lymphatic stroma.

Evaluation of PD-L1 and PD-1 Immunostaining. For the evaluation of the PD-L1 expression in tumor cells, only the membranous staining was evaluated and the following IRS was applied: Category A rated the percentage of immunoreactive cells and was graded as 0 (negative), 1 (≤1% positive), 2 (2−10% positive), 3 (11−50%), and 4 (>50%). Category B documented the intensity of immunostaining as 0 (no immunostaining), 1+ (weak), 2+ (moderate), or 3+ (strong). The addition of category A and B resulted in an IRS ranging from 0 to 7. Because of the overall lower expression of PD-L1 in neoadjuvantly treated GCs, and different from Böger et al. [11], any membranous staining of tumor cells was classified as PD-L1 positive, i.e., IRS ≥ 2.

For the evaluation of the PD-L1 expression in immune cells (lymphocytes, dendritic cells, macrophages), only the percentage of positive cells was considered, and cases were graded as 0 (negative), 1 (≤1 positive), 2 (2−10%) and 3 (>10%). PD-L1 in immune cells was considered positive if >1% of the immune cells showed an immunoreaction.

The immunostaining of PD-1 in immune cells was rated separately for tumor-infiltrating lymphocytes (TILs) and intratumoral lymph follicles as present or absent.

Assessment of Phenotypic and Genotypic Characteristics of the Study Cohort

The HER2-and MET-status was assessed as previously described [11,12,16−18] using immunohistochemistry [anti-Her2/neu antibody; clone SP3, Thermo Fisher Scientific; Fremont; USA (#MA5-14509); anti-MET antibody; clone SP44; Spring Bioscience; Pleasanton, California, USA (#M3444)] and in situ−hybridization [ZytoDot 2C SPEC HER2/CEN17 Probe (#C-3032-400), ZytoDot 2C SPEC MET/CEN7 Probe (#C-3057-400) and the ZytoDot 2C CISH Implementation Kit (#C-3044-40); ZytoVision GmbH, Bremerhaven, Germany)]. Epstein−Barr virus (EBV)−encoded RNA was detected using the EBER-probe (Novocastra, Leica Microsystems GmbH, Nussloch, Germany; #PB0589) and the BondMax-detection system according to the manufacturer's instructions (Leica Microsystems GmbH). MSI status was assessed by immunostaining using antibodies directed against MLH1 (clone G168-15, BD Biosciences, Heidelberg, Germany; #MA1-25669), PMS2 (clone MRQ-28; Cell Marque Corporation, Rocklin, USA; #288M-16-ASR), MSH2 (clone FE11; Calbiochem, Merck KGaA, Darmstadt, Germany; #MABE284), and MSH6 (clone 44, BD Biosciences; #610919) as well as by comparison of the allelic profiles of the mononucleotide repeat markers BAT-25, BAT-26, NR-21, NR-24, and NR-27 in tumor and corresponding normal tissue in cases with ambiguous immunostaining [16].

External Quality Assurance

The immunohistochemical staining of PD-L1, of the DNA-mismatch repair proteins (MSH2, MSH6, MLH1, and PMS2), the molecular biological MSI assay, and the HER2-assessment were certified successfully by the quality assurance program of the German Society of Pathology and the *Bundesverband Deutscher Pathologen e.V.*

Study Design

Whole tissue sections from GCs, corresponding lymph node metastases and distant metastases were stained with antibodies directed against PD-L1, PD-1, and VISTA. The staining results were correlated with clinicopathological characteristics, genetic alterations, and survival data.

Statistical Analysis

Statistical analyses were conducted using SPSS 20.0 (IBM Corporation, New York, USA). PD-L1, PD-1, and VISTA expression within the different tumor components (tumor cells, immune cells, endothelial cells) were dichotomized by their respective median into "negative" and "positive" (tumor cells, endothelial cells) or "negative/low" and "positive/high" (immune cells). Cross tabulations of clinical data and marker expressions were tested for independence using Kendall's tau or Fisher's exact test. The correlation between the number of VISTA-positive immune cells evaluated by two different methods (positive immune cells per mm^2 and per 200 immune cells) was calculated by Pearson correlation (r). An r value of −1 indicated a perfect negative linear correlation, and an r value of 1 indicated a perfect positive linear correlation. Median overall and tumor-specific survival were calculated using the Kaplan−Meier method. Log-rank test was used to determine significance of differences between survival curves. p-Values < 0.05 were considered as statistically significant. False discovery rate of correlations between clinical variables and biomarkers was controlled by applying the explorative Simes (Benjamini-Hochberg) procedure [19]. All p-values are given unadjusted, but are marked to highlight the outcome of the Simes procedure.

Results

Out of 141 cases with 93 corresponding lymph node metastases and 15 distant metastases that fulfilled all study criteria (Table 1), 16 cases exhibited full tumor regression of the primary tumor. Associations with clinicopathological patient characteristics are summarized in Suppl. Table 1 for VISTA and in Suppl. Table 2 for PD-L1 and PD-1. Overall and tumor-specific survival data was available in 135 cases.

Table 1. Patient Cohort

Patient Characteristic		Valid [*n*]	*n*	(%)
Sex	Female	141	28	(19.9)
	Male		113	(80.1)
Age	<66 years	141	70	(49.6)
	≥66 years		71	(50.4)
Localization	Proximal	141	90	(63.8)
	Distal		51	(36.2)
Laurén Phenotype	Intestinal	141	63	(44.7)
	Diffuse		26	(18.4)
	Mixed		26	(18.4)
	Unclassified		14	(9.9)
	N.A.		12	(8.5)
yT Category	ypT0	141	16	(11.3)
	ypT1(a/b)		20	(14.2)
	ypT2		20	(14.2)
	ypT3		75	(53.2)
	ypT4(a/b)		10	(7.1)
yN Category	ypN0	141	51	(36.2)
	ypN1		33	(23.4)
	ypN2		32	(22.7)
	ypN3(a/b)		25	(17.7)
yM Category	ypM0	141	119	(84.4)
	ypM1		22	(15.6)
UICC Stage	0/N+	139	15	(10.8)
	I(A/B)		21	(15.1)
	II(A/B)		24	(17.3)
	III(A/B/C)		57	(41)
	IV		22	(18.8)
yL Category	ypL0	141	99	(70.2)
	ypL1		42	(29.8)
yV Category	ypV0	141	131	(92.9)
	ypV1		10	(7.1)
yPn Category	ypPn0	141	111	(78.7)
	ypPn1		30	(21.3)
R Status	R0	141	126	(89.4)
	R1		13	(9.2)
	RX		2	(1.4)
MSI Status	MSS	121	113	(93.4)
	MSI		8	(6.6)
EBV Status	Negative	122	119	(97.5)
	Positive		3	(2.5)
MET Status	Negative	124	118	(95.2)
	Positive		6	(4.8)
HER2 Status	Negative	122	114	(93.4)
	Positive		8	(6.6)

VISTA Expression in Neoadjuvantly Treated Gastric Carcinomas

VISTA expression was observed in tumor, immune, and endothelial cells, but not in nonneoplastic gastric epithelium. The staining of tumor cells was exclusively cytoplasmatic.

Primary Tumor. Two cases did not allow for the interpretation of staining results. Out of 123 cases, 38 (30.9%) exhibited positive staining of tumor cells. The percentage of VISTA-positive tumor cells ranged from 0% to 80% (median 0%). The overall percentage was mainly low, with 89.4% of cases expressing VISTA in ≤10% of tumor cells. Staining intensity ranged from negative (0) to strong (2+/3+) (median 0) (Figure 1; A–C).

Overall immune cell density measured per mm^2 ranged from 214 to 3214 (median 946). Intratumoral immune cells of the primary tumor were found to express VISTA in 139 of 141 (98.6%) cases. VISTA-positive immune cell count ranged from 0 to 1479 per mm^2 (median 229). Cases with ≤229 were classified as having a negative/low VISTA immune cell count per mm^2 and any case with more than 229 as having a positive/high VISTA immune cell count per mm^2. The proportional amount of positive immune cells per 200 ranged from 0 to 178 (median 53). Cases with ≤53 VISTA-positive immune cells were classified as negative/low in immune cells per 200. Cases with more than 53 VISTA-positive immune cells were classified as positive/high in immune cells per 200.

The amount of VISTA-positive immune cells per mm^2 and the proportion of VISTA-positive immune cells per 200 immune cells correlated significantly with each other ($p < 0.001$; $r = 0.822$). Thus, a specification of the applied evaluation method is denoted below only if necessary.

Endothelial cells of the primary tumor exhibited the expression of VISTA in 124 of 141 cases (88%) (Figure 1; D).

Lymph Node and Distant Metastases. Ten cases of lymph node metastases did not allow for the interpretation of staining results. Eighteen cases (21.7%) showed the staining of tumor cells. The percentage of VISTA-positive tumor cells ranged from 0% to 80% (median 0%), staining intensity ranged from negative (0) to strong (2+/3+) (median 0). Endothelial cells in lymph node metastases exhibited the expression of VISTA in 76 of 93 cases (81.7%).

Distant metastases were available in 11 cases with a total of 15 samples mostly located in liver and peritoneum. Three cases (five samples) had VISTA-positive tumor cells. None of the positive distant metastases were concordant relative to their corresponding primary tumors. All seven remaining cases were concordant in VISTA negativity. The proportion of positive immune cells ranged from 0% to 36.2% in VISTA negative cases and 8.9–58.2% in VISTA-positive cases. Because of small sample size, no statistical testing was conducted.

PD-L1 Expression in Neoadjuvantly Treated Gastric Carcinomas

PD-L1 expression was observed in tumor cells and immune cells. PD-L1 positive tumor necrosis or nonneoplastic tissue was not evaluated. PD-L1 positive immune cells served as positive control for the negatively rated tumor cell cases.

Out of 119 GCs, 27 (22.7%) showed a membranous PD-L1 expression in tumor cells. The percentage of stained tumor cells ranged from 0 to 90% (median 0%), with the overall percentage of PD-L1 positive tumor cells being low (95.8% of the cases with <10% positive tumor cells). The staining intensities observed ranged from 0 to 3+ (median 0) (Figure 1; E-I).

Tumor cells in lymph node metastases showed PD-L1 expression in 19 (26.0%) of 73 cases. The percentage of stained tumor cells ranged from 0 to 50% (median 0%) and the intensity varied from 0 to 3+ (median 0).

PD-L1 expression in immune cells of the primary tumor was found in 134 (96.4%) of 139 cases. The percentage of positive cells ranged from 0 to 70% (median 1%). Out of 139 GCs, 51 (36.6%), with more than 1% PD-L1 positive immune cells, were classified as positive/high in immune cells.

PD-1 Expression in Neoadjuvantly Treated Gastric Carcinomas

Lymphocyte aggregates/intratumoral lymphoid follicles were present in 124 (89.9%) of 138 primary GCs. The expression of PD-1 was detected in 123 (99.2%) of 124 lymphocyte aggregates/intratumoral lymphoid follicles. Tumor-infiltrating lymphocytes (TILs) of the primary tumor expressed PD-1 in 126 (91.3%) of 138 cases.

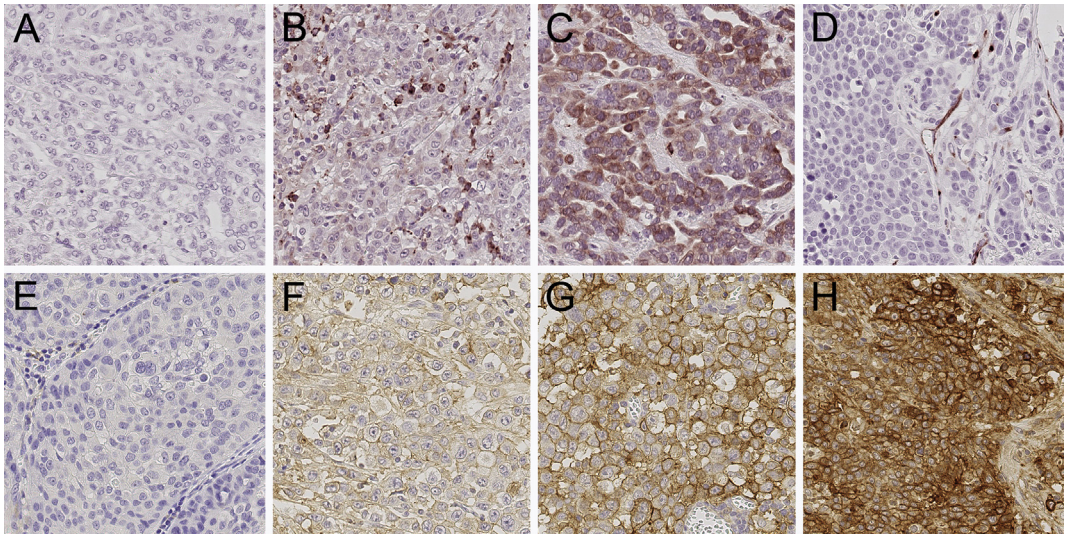

Figure 1. VISTA and PD-L1 expression in neoadjuvantly treated gastric cancer. VISTA expression was observed in 38 of 123 gastric carcinomas (30.9%). The intensity of VISTA immunostaining was graded as negative (A), weak (B) or strong (C). VISTA expression in vessels was present in 124 GCs (88%) (D). PD-L1 expression was observed in 27 of 119 GCs (22.7%). The intensity of PD-L1 immunostaining was graded as negative (E), weak (F), moderate (G) or strong (H). Original magnifications 400-fold.

Correlation Between VISTA and PD-L1 Expression in Neoadjuvantly Treated Gastric Carcinomas

Correlations between the expression of VISTA and PD-L1 in tumor cells and immune cells of the primary tumor and tumor cells of lymph node metastases are summarized in Table 2. The expression of VISTA in immune cells per 200 was significantly associated with the expression of PD-L1 in tumor cells of the primary tumor ($p = 0.004$) and immune cells ($p < 0.001$), respectively. The expression of VISTA in immune cells per mm^2 correlated significantly with the expression of PD-L1 in immune cells ($p = 0.005$).

Correlation of the Expression of VISTA, PD-L1, and PD-1 with Clinicopathological Patient Characteristics of Neoadjuvantly Treated Gastric Carcinomas

VISTA. VISTA expression in tumor cells of the primary tumor cells correlated significantly with TRG according to Becker ($p = 0.002$; Table 3) and the dichotomized amount of vital tumor residuals (Suppl. Table 1). VISTA-positive immune cells per 200 were significantly associated with TRG according to Becker ($p < 0.001$; Table 3), the dichotomized amount of vital tumor residuals ($p = 0.003$) and vital tumor residuals divided into quartiles ($p = 0.001$; Suppl. Table 1). VISTA-positive immune cells per mm^2 correlated significantly with the dichotomized amount of vital tumor residuals ($p = 0.005$; Suppl. Table 1).

No other clinicopathological patient characteristic showed a significant correlation with the expression of VISTA, neither in the primary tumor nor in lymph node metastases (Suppl. Table 1).

Although there was no significant correlation between VISTA expression in tumor cells of the primary tumor and UICC-stage, the amount of VISTA-positive cases significantly increased from stage I to III ($p = 0.028$) and decreased thereafter ($p = 0.013$; Suppl. Figure 1).

No correlation was found between the VISTA expression in endothelial cells and clinicopathological patient characteristics.

Table 2. Associations of VISTA and PD-L1 Expression in Tumor and Immune Cells

	Valid		VISTA in Tumor Cells (Primary Tumor)				VISTA in Immune Cells per 200 (Primary Tumor)				VISTA in Immune Cells per mm^2 (Primary Tumor)				VISTA in Tumor Cells (Lymph Node Metastases)			
			Valid		*p*-Value		Valid		*p*-Value		Valid		*p*-Value		Valid		*p*-Value	
			Negative		Positive		Negative/low		Positive/high		Negative/low		Positive/high		Negative/low		Positive/high	
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
PD-L1 in Tumor Cells (Primary Tumor)	119		118			0.057	118			0.004[a]	118			0.016	74			0.496
Negative	92	(77.3)	67	(73.6)	24	(26.4)	49	(53.8)	42	(46.2)	48	(52.7)	43	(47.3)	44	(74.6)	15	(25.4)
Positive	27	(22.7)	14	(51.9)	13	(48.1)	6	(22.2)	21	(77.8)	7	(25.9)	20	(74.1)	13	(86.7)	2	(13.3)
PD-L1 in Immune Cells (Primary Tumor)	139		121			0.236	138			<0.001[a]	138			0.005[a]	83			0.794
Negative/low	88	(63.3)	53	(73.6)	19	(26.4)	54	(62.1)	33	(37.9)	52	(59.8)	35	(40.2)	37	(77.1)	11	(22.9)
Positive/high	51	(36.7)	31	(63.3)	18	(36.7)	15	(29.4)	36	(70.6)	17	(33.3)	34	(66.7)	28	(80)	7	(20)
PD-L1 in Tumor Cells (Lymph Node Metastases)	73		68			0.048	72			0.185	72			0.282	69			0.193
Negative/low	54	(74)	34	(68)	16	(32)	27	(50.9)	26	(49.1)	26	(49.1)	27	(50.9)	42	(82.4)	9	(17.6)
Positive/high	19	(26)	7	(38.9)	11	(61.1)	6	(31.6)	13	(68.4)	6	(31.6)	13	(68.4)	12	(66.7)	6	(33.3)

[a] Significant after multiple testing procedure.

Table 3. Correlation of VISTA, PD-L1, and PD-1-Expression with Tumor Regression

	Valid		Tumor Regression Grade (TRG)				*p*-Value
			TRG1a	TRG1b	TRG2	TRG3	
	n		*n* (%)	*n* (%)	*n* (%)	*n* (%)	
Total	141		16 (11.3)	29 (20.6)	22 (15.6)	74 (52.5)	
VISTA in Tumor Cells (Primary Tumor)	123	Positive	N.A.	4 (14.8)	3 (13.6)	31 (41.9)	0.002[a]
		Negative	N.A.	23 (85.2)	19 (86.4)	43 (58.1)	
VISTA in Immune Cells per 200 (Primary Tumor)	140	Positive/high	4 (25.0)	10 (35.7)	9 (40.9)	47 (63.5)	<0.001[a]
		Negative/low	12 (75.0)	18 (64.3)	13 (59.1)	27 (36.5)	
VISTA in Immune Cells per mm^2 (Primary Tumor)	140	Positive/high	4 (25.0)	12 (42.9)	9 (40.9)	45 (60.8)	0.005
		Negative/low	12 (75.0)	16 (57.1)	13 (59.1)	29 (39.2)	
VISTA in Tumor Cells (Lymph Node Metastases)	83	Positive	1 (14.3)	3 (21.4)	3 (25.0)	11 (22)	0.837
		Negative	6 (85.7)	11 (78.6)	9 (75.0)	39 (78)	
PD-L1 in Tumor Cells (Primary Tumor)	119	Positive	N.A.	2 (8)	4 (18.2)	21 (29.2)	0.032
		Negative	N.A.	25 (92)	22 (81.8)	72 (70.8)	
PD-L1 in Immune Cells (Primary Tumor)	139	Positive/high	2 (12.5)	8 (27.6)	5 (22.7)	36 (50.0)	0.001[a]
		Negative/low	14 (87.5)	21 (72.4)	17 (77.3)	36 (50.0)	
PD-L1 in Tumor Cells (Lymph Node Metastases)	73	Positive	1 (25.0)	1 (10.0)	2 (20.0)	15 (30.6)	0.218
		Negative	3 (75.0)	9 (90.0)	8 (80.0)	34 (69.4)	
PD-1 in Tumor-infiltrating Immune Cells	138	Positive	13 (81.3)	26 (89.7)	21 (95.5)	66 (93.0)	0.234
		Negative	3 (18.8)	3 (10.3)	1 (4.5)	5 (7)	
Lymphocyte Aggregates Present	138	Positive	15 (93.8)	25 (86.2)	22 (100)	62 (87.3)	0.529
		Negative	1 (6.3)	4 (13.8)	0 (0)	9 (12.7)	
PD-1 in Lymphocyte Aggregates	138	Positive	15 (93.8)	25 (86.2)	22 (100)	61 (85.9)	0.910
		Negative	1 (6.3)	4 (13.8)	0 (0)	10 (14.1)	

[a] Significant after correction for multiple testing.

PD-L1. PD-L1 expression in immune cells of the primary tumor correlated significantly with TRG according to Becker ($p = 0.001$; Table 3) and the percentage of vital tumor residuals of the primary tumor divided into quartiles ($p < 0.001$; Suppl. Table 2). Similarly, we found that tumor cells of the primary tumor correlated with TRG according to Becker ($p = 0.032$; Table 3) and tumor cells both in the primary tumor as well as in lymph node metastases correlated with the percentage of vital tumor residuals of the primary tumor (primary tumor $p = 0.003$; lymph node metastases $p = 0.014$). However, these results lost their significance after Simes' multiple testing procedures (Suppl. Table 2). No significant correlation was found between PD-L1 expression in tumor or immune cells and other clinicopathological patient characteristic (Suppl. Table 2).

The expression of PD-L1 in tumor cells and immune cells of the primary tumor was significantly correlated ($p < 0.001$). The number of PD-L1 positive tumor cells in the primary tumor was also correlated with the number of positive tumor cells in the corresponding lymph node metastasis ($p < 0.001$).

PD-1. PD-1 expression in TILs of the primary tumor increased with T-category ($p = 0.024$) and UICC-stage ($p = 0.028$). However, these correlations lost significance after Simes' multiple testing procedures. No significant correlations between the PD-1 expression and other clinicopathological patient characteristics were found (Suppl. Table 2).

Prognostic Significance of the Expression of VISTA, PD-L1, and PD-1 in Neoadjuvantly Treated Gastric Carcinomas

There was no significant correlation between VISTA, PD-L1, or PD-1 expression in any tumor component and overall or tumor-specific patient survival in neoadjuvantly treated GCs (Figure 2).

Comparison of the Expression of VISTA, PD-L1, and PD-1 in Neoadjuvantly Treated with Therapy Naïve Gastric Carcinomas

Finally, we compared the expression of VISTA, PD-L1, and PD-1 in neoadjuvantly treated GC with our previously published data on therapy naïve GCs (Table 4) [11,12].

VISTA. The median intensity of VISTA-positive tumor cells was the same, i.e., 0 vs. 0, between therapy naïve and neoadjuvantly treated GCs. However, the percentage of VISTA-positive cases increased from 8.8% in therapy naïve GC to 30.9% in neoadjuvantly treated GC.

The median number of immune cells per mm^2 was almost three times higher in neoadjuvantly treated (946 per mm^2) compared with therapy naïve GCs (272 per mm^2). Among these, the percentage of VISTA-positive immune cells increased from 83.6% to 98.6% and the median of VISTA-positive immune cells per 200 immune cells increased from 36 to 53, and the median number of VISTA-positive immune cells per mm^2 increased from 35 to 229. Cases with VISTA expression in endothelial cells increased from 23.7% to 88%. Collectively, these data demonstrate an overall increased expression of VISTA in tumor, immune, and endothelial cells following neoadjuvant treatment.

PD-L1. With regard to PD-L1 and different from VISTA, the median IRS of tumor cells was lower in neoadjuvantly treated GCs compared with treatment naïve GCs (median IRS = 0 vs. median IRS = 2). The difference became evident, when the median of the treatment naïve GCs was applied to the neoadjuvantly treated GCs: only 15.1% of the GCs were classified as PD-L1-positive compared with 23.9% in the treatment naïve cohort (Table 4).

Similarly, the median of PD-L1 positive immune cells was lower in the neoadjuvantly treated cohort (1% vs. 5%). Again, the difference became evident when the cut-off of the treatment naïve GCs (10%; not identical with median) [11] was applied to the neoadjuvantly treated GCs: only 16.5% of the GCs were classified as PD-L1-positive in immune cells compared with 35.5% in the treatment naïve cohort (Table 4).

PD-1. Interestingly, and similar to VISTA, the percentage of PD-1 positive tumor-infiltrating immune cells was higher in neoadjuvantly treated GCs (91.1%) compared with therapy naïve GCs (53.8%) (Table 4). No difference was found with regard to PD-1 positive lymphocytic aggregates (88.8% vs. 89.1%).

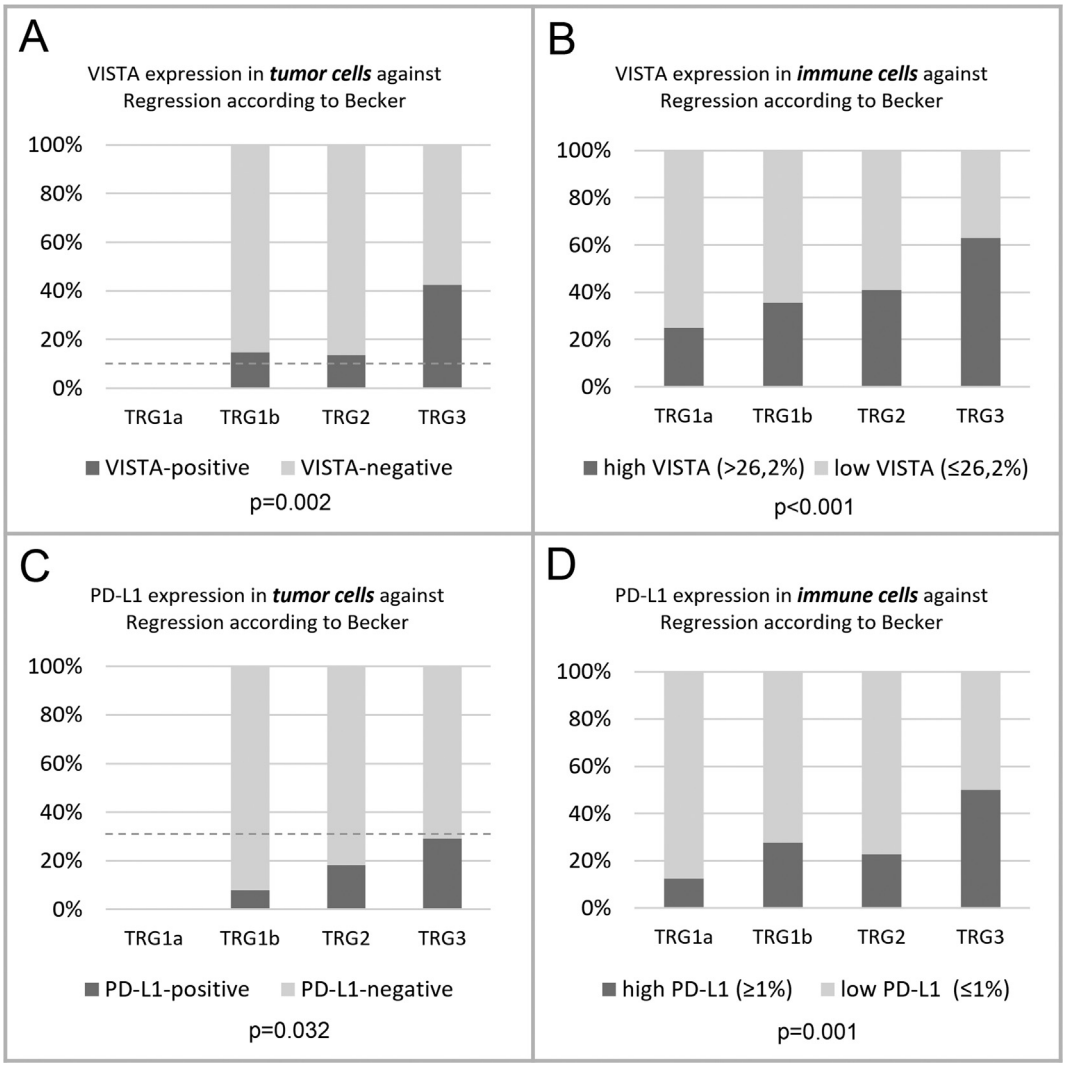

Figure 2. VISTA and PD-L1 expression in tumor and immune cells against tumor regression grade according to Becker. VISTA expression in tumor cells showed a significant increase in concordance with higher tumor regression grade (TRG) according to Becker ($p = 0.002$) and generally higher levels of VISTA than therapy naïve GC (dashed line; 8.8% [Böger et al. Oncoimmunology 2017; 6(4):e1293215]) (A). VISTA in immune cells divided into negative/low and positive/high expression by its median (26.2%) exhibited a steady increase with higher TRG ($p < 0.001$) (B). PD-L1 expression in tumor cells increased with increasing TRG ($p = 0.032$) (C) but did not reach the level found in therapy naïve GC (dashed line; 30.1% [Böger et al. Oncotarget 2016; 7(17):24269–83]). Positive/high PD-L1 expression in immune cells dichotomized at the median (1%) also increased with higher TRG ($p = 0.001$) (D).

Collectively these data provide evidence of a complex and differential response of VISTA, PD-L1, and PD-1 to neoadjuvant treatment.

Discussion

In this study, we explored the expression of the immune checkpoint proteins VISTA, PD-L1, and PD-1 in a cohort of neoadjuvantly treated GCs. A cohort of only patients after neoadjuvant treatment was chosen specifically to supplement previous findings of our research group regarding VISTA and PD-L1/PD-1 in therapy naïve GC published in 2016 and 2017 [11,12]. Although this study is not a follow-up of the same patients, it used the same antibodies and staining procedures, applied similar evaluation criteria, and examined patients from the same Central European catchment area. This similarity of geographic accrual was used as a basis to compare the characteristic expression with our two previous studies in an effort to allow for an approximation of the effect chemotherapy has on immune checkpoint proteins in GC. This also applies to the assessment criteria, which intentionally did not apply, e.g., the *combined positivity score* (CPS) [20], as the primary aim was not to find a predictive biomarker, but rather unravel the effects chemotherapy has on the expression of immune checkpoint proteins. It also illustrates that cut-off values (e.g., medians) can change as a result of therapy.

The two cohorts share similarities and differences, which reflect epidemiological developments of recent years. Both cohorts provide a male preponderance, are similar with regard to median patient age and the intestinal phenotype. However, the number of proximal tumors is twice as high in the neoadjuvantly treated cohort (63.8%) compared with the treatment naïve cohort (31.5%). Considering the difference in cohort size of 464 therapy naïve cases versus 141 preoperatively treated cases, the absolute number of proximal GCs is

Table 4. Comparison of the VISTA and PD-L1 Expression Between Therapy Naïve and Neoadjuvantly Treated Gastric Carcinomas

	VISTA in Tumor Cells (Primary Tumor)				VISTA in Immune Cells per 200 (Primary Tumor)				VISTA in Immune Cells per mm^2 (Primary Tumor)				PD-L1 in Tumor Cells (Primary Tumor)				PD-L1 in Immune Cells (Primary Tumor)				PD-1 in Tumor-infiltrating Immune Cells			
	Cut-off				Cut-off				Cut-off				Cut-off				Cut-off				Cut-off			
	Negative		Positive		Negative/low		Positive/high		Negative/low		Positive/high		Negative		Positive		Negative/low		Positive/high		Negative		Positive	
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
Therapy Naïve Gastric Cancer Cohort [11,12]	*IRS 0 vs. >0*				*≤36 vs. >36*				*≤35 vs. >35 per mm^2*				*IRS ≤2 vs. >2*[a]				*<10% vs. ≥10%*				*absent vs. present*			
	423	(91.2)	41	(8.8)	232	(50.0)	232	(50.0)	229	(49.7)	235	(50.3)	354	(76.1)	111	(23.9)	300	(64.5)	165	(35.5)	215	(46.3)	250	(53.8)
Neoadjuvantly Treated Gastric Cancer Cohort (Current Study)	*IRS 0 vs. >0*				*≤53 vs. >53*				*≤229 vs. >229 per mm^2*				*IRS 0 vs. ≥2*				*≤1% vs. >1%*				*absent vs. present*			
	85	(69.1)	38	(30.9)	70	(50.0)	70	(50.0)	70	(50.0)	70	(50.0)	92	(77.3)	27	(22.7)	88	(63.3)	51	(36.7)	12	(9.5)	126	(91.1)
													IRS ≤2 vs. >2[a]				*<10% vs. ≥10%*[a]							
													101	(84.9)	18	(15.1)	116	(83.5)	23	(16.5)				

Historical data for therapy naïve GC taken from Böger et al. [11,12] for VISTA and PD-L1 respectively.
[a] Cut-off used in Böger et al. [11].

representative in both cohorts (146 vs. 90). The difference in distal cases (313 vs. 51) suggests that patients with these tumors were more likely to undergo primary surgery than platinum-based neoadjuvant chemotherapy. Regarding tumor progression, the percentages of the different T-categories differed with ypT4 accounting for 7.1% in the neoadjuvantly treated cohort and 36% in the treatment naïve cohort. Minor differences were also found in ypT1 vs. pT1 (14.2% vs. 11.9%), ypT2 vs. pT2 (14.2% vs. 11.7%), and ypT3 vs. pT3 (53.2 vs. 40.4%) and may be related to therapy-induced down staging. Thus, while the comparison of the neoadjuvantly treated cohort with a "historical" treatment naïve cohort of GCs has limitations, it still may provide valuable clues about the effect; neoadjuvant treatment has on the expression of immune checkpoint proteins.

We abstained from the use of pretherapeutic biopsy samples for the following reasons. Firstly, the number of pretherapeutic biopsies was limited and would not have allowed a proper statistical analysis. More importantly, our previous studies on therapy naïve GC have shown that biopsies carry a significant risk of sampling errors [11] and are unsuitable for the reliable assessment of the expression patterns of immune checkpoint proteins. Additionally, we investigated the expression in lymph node and distant metastases to evaluate for differences chemotherapy has on primary tumors and metastases.

Overall, VISTA and PD-L1 were found to be expressed in a small subset of neoadjuvantly treated GCs as has been the case in previous investigations [12]. Interestingly immune cells expressed some amount of VISTA, PD-L1, or PD-1 in >90% of the cases.

VISTA is More Frequently Expressed in Gastric Carcinomas Exposed to Chemotherapy

First of all, we noticed that the percentage of primary tumors expressing VISTA is much higher in neoadjuvantly treated GCs (30.9% vs. 8.8%). This increase is even more pronounced in tumors with little to no response to neoadjuvant treatment (TRG3: 42%). The percentage of VISTA-positive GCs with a fair treatment response, i.e., TRG1b, was still higher compared with treatment naïve GCs (Figure 3; A). Interestingly, this pattern also applies to VISTA-positive immune cells (Figure 3; B). The change in the location of GCs contributes to this phenomenon only partly: In the treatment naïve cohort, VISTA was significantly more commonly expressed in proximal GCs [12]. However, the prevalence of VISTA in tumor cells of proximal treatment naïve GC was 17.1% and well below the prevalence of VISTA in proximal tumors of neoadjuvantly treated GCs (i.e., 40%). Thus, neoadjuvant chemotherapy leads to an overall increased prevalence of VISTA expression in both, tumor cells and immune cells, which is also associated with therapy resistance.

PD-L1 is More Commonly Expressed in Gastric Carcinomas with Poor Response

Different from VISTA, the overall expression of PD-L1 in tumor and immune cells appeared to be reduced. However, nonresponders seemed to behave differently (Figure 3; C and D). Earlier studies provided evidence that radiation and chemotherapy affect the tumor immune microenvironment, which might also impact on the expression of immune checkpoint proteins [21–24]. Therapy-induced destruction of tumor cells reduces antigen load, which in turn may reduce overall PD-L1 expression. This might explain the low prevalence of PD-L1 positive tumor cells in the TGR1b group.

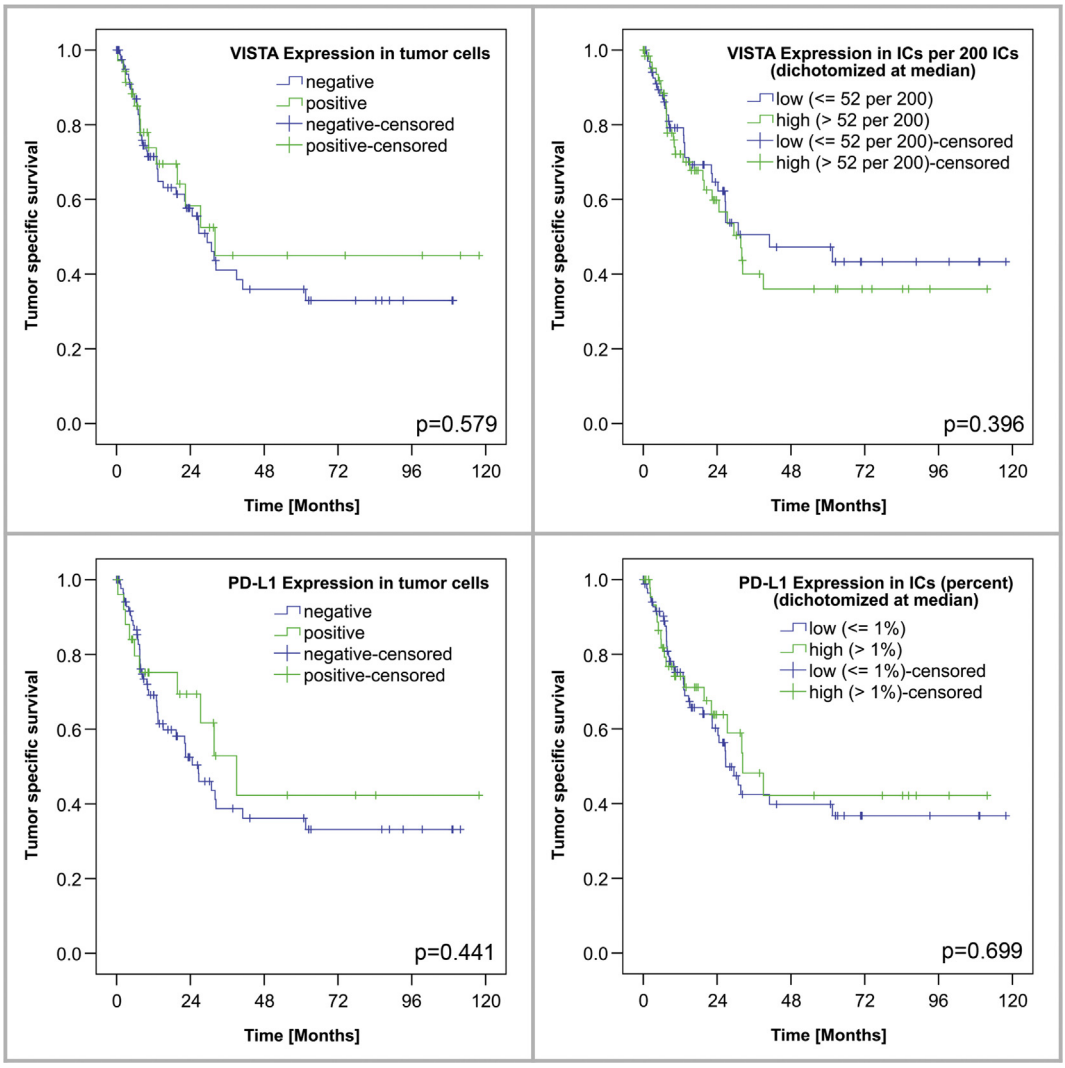

Figure 3. Prognostic significance of VISTA and PD-L1 in tumor and immune cells. No significant correlation was found between tumor-specific survival und VISTA in tumor cells (median survival 32.0 vs. 29.5 months; $p = 0.579$) (A) or the VISTA expression in immune cells (ICs) per 200 (median survival 31.7 vs. 41.0 months; $p = 0.396$) (B). There were also no significant correlations between tumor-specific survival and PD-L1 expression in tumor cells (median survival 26.6 vs. 39.0 months; $p = 0.441$) (C) or PD-L1 expression in ICs (median survival 26.8 vs. 32.3 months; $p = 0.699$) (D).

To the contrary, lack of tumor response to chemotherapy may be associated with an increased expression of immune checkpoint proteins: such as VISTA, the percentage of PD-L1 positive neoadjuvantly treated GCs with regard to tumor and immune cells increased steadily from TRG1b to TRG3, and the expression of PD-L1, and VISTA, might be a function of tumor regression (Table 2). It has been suggested that the local inflammatory response to chemotherapy is characterized by the infiltration of tumor-specific T-cells, which simultaneously could induce PD-L1 expression and hence immune evasion [21,22]. An observation supporting this assumption is the almost three times higher median number of immune cells per mm^2 in our cohort of neoadjuvantly treated (946 per mm^2) GCs compared with our therapy naïve GCs (272 per mm^2). This difference cannot be explained by demographic or ethnical differences, as all patients were recruited from the same catchment area. Thus, chemotherapy may induce a selection pressure and those GCs which are able to respond with an upregulation of VISTA and/or PD-L1 are not prone to immune destruction and hence show poor therapeutic response.

Our findings lead to the conjecture that neoadjuvant chemotherapy provokes a selection pressure and those tumors which are capable of upregulating VISTA and/or PD-L1 show a poor response. This treatment failure may in turn be used to select patients particularly eligible for immune checkpoint inhibitors.

PD-1 is More Frequently Expressed in Neoadjuvantly Treated Gastric Carcinomas

Like VISTA, the overall expression of PD-1 was increased in neoadjuvantly treated compared with treatment naïve GCs. However, and different from VISTA and PD-L1, it did not correlate with the tumor regression grade. Thus, the increased expression of PD-1 may be considered as a general response to chemotherapy, such as the increased number of immune cells, but not as a positive or negative indicator of treatment effect. There is some further evidence that the expression of PD-1 and PD-L1 is not homogenous. In the therapy naïve cohort, the expression of PD-1 in tumor cells correlated with the expression of PD-L1 in tumor cells, whereas in the neoadjuvantly treated cohort, this correlation was lost.

Correlations with Clinicopathological Patient Characteristics and Patient Survival

The comparison of VISTA, PD-L1, and PD-1-prevalences with clinicopathological characteristics found in therapy naïve and neoadjuvantly treated GCs reveals mostly similar patterns and some associations, which are absent in treated GC.

In therapy naïve GCs, VISTA status of tumor cells correlated significantly with localization, more commonly in proximal tumors, phenotype according to Laurén, and PD-L1 status in tumor cells [12]. Although similar findings were made in neoadjuvantly treated GCs with regard to tumor localization and histological phenotype, they lost significance after multiple testing probably because of small sample size (Suppl. Table 1). Interestingly, although VISTA status of immune cells in therapy naïve GCs correlated significantly with tumor localization, phenotype, EBV-, and HER2 status, no such correlation was found in neoadjuvantly treated GCs, even no tendency. These observations lead to the assumption that chemotherapy exerts different effects on VISTA expression in tumor and immune cells. Even though the expression in immune cells correlated with the expression in tumor cells ($p < 0.001$), the lack of correlations between VISTA-positive immune cells and clinicopathological characteristics might be attributable to the overall increase in immune cells and a disproportionate increase in VISTA-positive immune cells that mainly aligned with regression grade.

In therapy naïve GCs a pattern of significantly increasing expression of VISTA in immune cells from T1 to T3 and a decrease from T3 to T4 was observed [12]. Although this pattern was not found to hold any significance in our present study, a new and similar pattern was found in the association of VISTA in tumor cells and UICC-stage (Suppl. Figure 1). Thus, we can only reaffirm that the expression of VISTA is dynamic and may vary over "time" during tumor progression.

In treatment naïve GCs, PD-L1 expression was significantly more prevalent in men, GCs of the proximal stomach, unclassified, papillary, HER2 positive, EBV positive, and microsatellite unstable GCs. It also correlated with local tumor growth, lymph node ratio and, UICC-stage [11]. None of these clinicopathological patient characteristics correlated significantly with PD-L1 expression either in tumor or immune cells of neoadjuvantly treated GCs. This lack of correlation may in part be related to sample sizes, such as HER2-, EBV-, and MSI status. However, it is tempting to speculate that neoadjuvant therapy may suppress to some extent attributes untreated GCs have on PD-L1-expression patterns. However, the significant correlation between PD-L1 expression in tumor and immune cells was retained in neoadjuvantly treated GCs (therapy naïve cohort: $p < 0.001$; neoadjuvant cohort: $p < 0.001$) pointing towards a more "general" not cell-specific effect. In support of this contention, the prevalence of both, PD-L1 in tumor and in immune cells, steadily increased with decreasing response to chemotherapy (Suppl. Table 2).

With regard to PD-1 in immune cells, neither the treatment naïve nor the neoadjuvantly treated cohort showed any significant correlation with clinicopathological patient characteristics after Simes' multiple testing procedure.

Somewhat expectedly, the prognostic value of PD-L1/PD-1 in GC is influenced by chemotherapy. In the therapy naïve cohort, a high PD-L1/PD-1 expression was associated with a significantly better patient outcome, and PD-L1 turned out to be an independent survival prognosticator. No such correlation was found in the treated cohort and effects acting in untreated GCs are probably suppressed and modulated by neoadjuvant treatment. This can be either a result of the direct effect neoadjuvant therapy has on patient outcome, or the effect chemotherapy has on the expression of immune checkpoint proteins. It is once again tempting to speculate that in larger patient cohorts of neoadjuvantly treated GCs PD-L1 may even turn out to be a negative prognosticator, given its increased expression in nonresponders.

Regarding VISTA, there was no significant correlation between VISTA expression in any tumor component and patient survival, neither in the treatment naïve nor in the neoadjuvantly treated cohort.

Expression in Locoregional and Distant Metastases

By investigating VISTA, PD-L1, and PD-1 expression in both locoregional and distant metastases we were able to get an impression of the effects chemotherapy has on different manifestations of the same tumor within a single patient and in different immune environments. Different from PD-L1, the expression in locoregional and distant metastases did not align with VISTA positivity of the primary tumor. The expression of VISTA in tumor cells of lymph node metastases did not correlate with any clinicopathological patient characteristic and did not correlate with regression of the primary tumor. Distant metastases did neither express VISTA in any case the primary tumor expressed it, nor did the primary tumor express VISTA in any case the distant metastasis expressed VISTA. Concordance was only found in the lack of VISTA expression (7 of 11 cases). These findings suggest a difference between the tumor immunology of metastases and primary tumors for VISTA. This also entails that in our study biopsies of lymph node or distant metastases are not eligible as predictors of the primary tumor's VISTA status.

VISTA and PD-L1 are Expressed in a Subset of GC

Although VISTA and PD-L1 share many of their immune regulatory properties, VISTA's functions are known to be non-redundant with other B7-family members [10]. This feature becomes especially important in GC, as therapy naïve GC has been shown to coexpress PD-L1 and VISTA in a subset of cases [12,25]. A "monotherapy" with checkpoint proteins might therefor show only a limited or even no effect in tumors that use a dual evasion mechanism.

In the present study VISTA and PD-L1 expression in immune cells was found to be significantly correlated ($p < 0.001$) as well as VISTA in immune cells and PD-L1 in tumor cells ($p = 0.004$). The strong association in immune cells aligns with the association of PD-L1 and VISTA in therapy naïve GC and their coexpression in a subset of GC (Figure 4; A). We also uncovered a profound association of VISTA and PD-L1 expression in tumor cells and immune cells with high TRG and therefore poor tumor regression after chemotherapy (Figure 4; B) (Table 2). Patients with poor regression might therefor benefit from an adjuvant chemotherapy augmented by a targeted blockade of both, VISTA and PD-L1.

Study Limitations

Although this study reveals new information about the effect neoadjuvant treatment has on the expression of VISTA and PD-L1 in

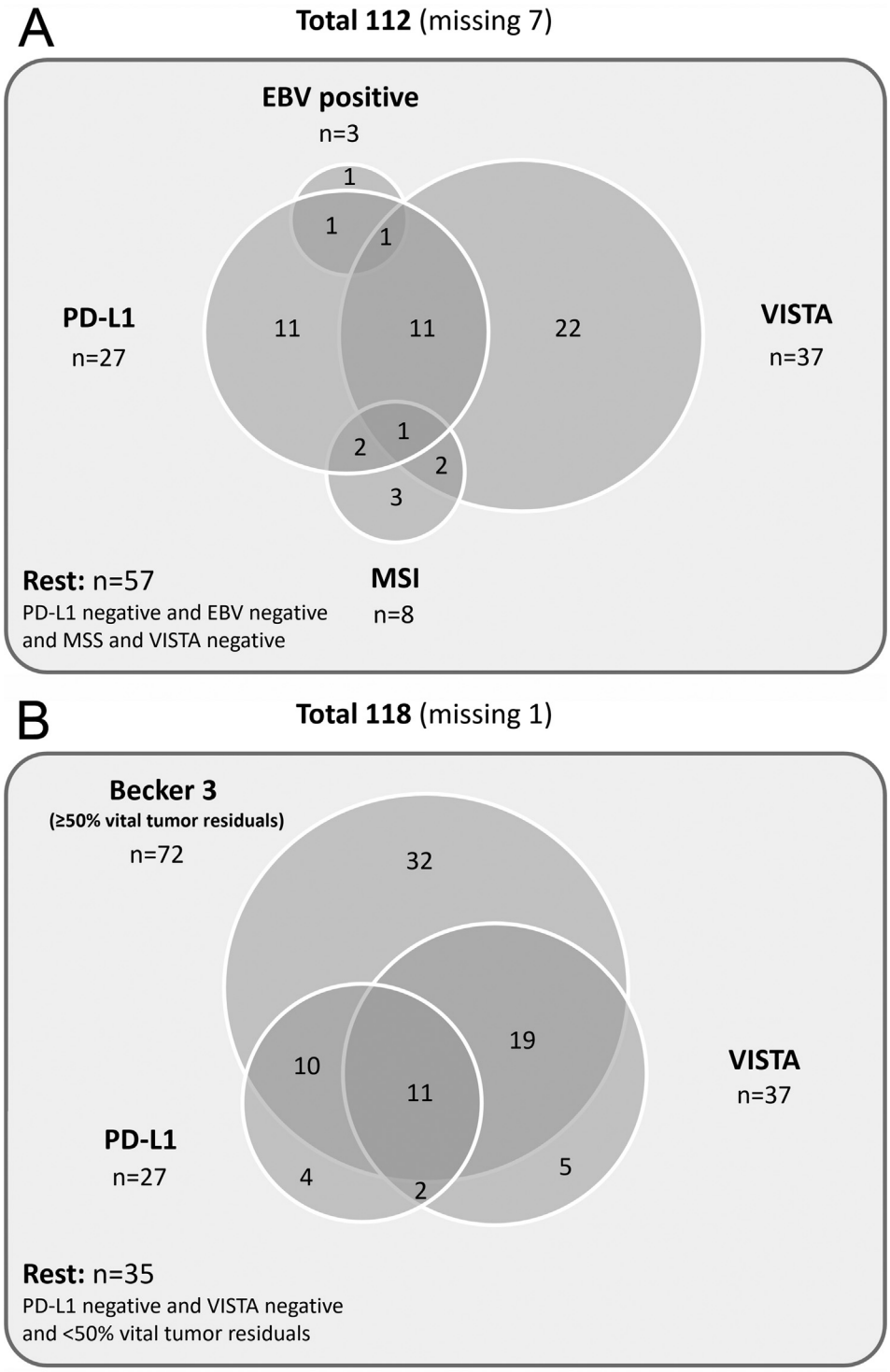

Figure 4. Association of VISTA expression, PD-L1 expression, Epstein–Barr positive status (EBV) and microsatellite instable (MSI) in tumor cells of the primary tumor. VISTA and PD-L1 expression were not significantly associated with each other ($p = 0.057$), nor with EBV positive status or MSI.

GC, some limitations do apply. Firstly, we dichotomized our neoadjuvant patient cohort at medians and may have opened ourselves up to the possibility of data driven bias. However, this approach facilitated the comparison with the therapy naïve cohort. In addition, we did not aim to develop a predictive biomarker, which may indeed require alternative dichotomization procedures and consideration of pre-specified outcome measures (e.g., survival or treatment response).

Secondly, the direct comparison with our "historical" therapy naïve cohort must take the inherit difference between the population that received neoadjuvant treatment and the one that received primary surgery into account, as outlined above. Because this study's scope was to retrospectively examine the expression in patients that have previously been assigned to a specific treatment regime, the results may only represent the differences between these two groups in the context of the assignment procedure.

Conclusion

Our study explored the effects of neoadjuvant chemotherapy on the expression of immune checkpoint proteins in GC. We found that nonresponders to platinum-based neoadjuvant chemotherapy show increased expression of VISTA, PD-L1, and PD-1 reaching prevalences >50% of the cases. VISTA and PD-L1 were also found to be coexpressed in a substantial number of GCs suggesting a dual evasion mechanism. However, response patterns of VISTA- and PD-L1/PD-1 expression in neoadjuvantly treated GCs are not completely uniform pointing towards a differential regulation. Collectively our data lead to the conjecture that immune checkpoint inhibitors are an important treatment option particularly in patients who failed to respond to neoadjuvant chemotherapy. Becker regression grade may be used as another surrogate marker for patient selection eligible for immune checkpoint inhibitor therapy because the expression of VISTA, PD-L1, and PD-1 is not limited to EBV-, and MSI status.

Financial Disclosures

None to disclose.

Conflict of Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.11.004.

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