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ORIGINAL RESEARCH

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The novel negative checkpoint regulator VISTA is expressed in gastric carcinoma and associated with PD-L1/PD-1: A future perspective for a combined gastric cancer therapy?

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ABSTRACT

A combined blockade of V-domain Ig suppressor of T-cell activation (VISTA) and PD-1 is a promising new cancer treatment option, which was efficient in murine tumor models and is currently tested in first phase I studies. Here, we analyzed the VISTA expression in a large and well-characterized gastric cancer (GC) cohort on 464 therapy-naive GC samples and 14 corresponding liver metastases using immunohistochemistry. Staining results were correlated with clinico-pathological characteristics, genetic alterations and survival. VISTA expression in tumor cells was detected in 41 GCs (8.8%) and 2 corresponding liver metastases (14.3%). Moreover, VISTA expression in immune cells was observed in 388 GCs (83.6%) and 6 liver metastases (42.9%). VISTA expression was associated with the Laurén phenotype, tumor localization, Epstein–Barr virus infection, *KRAS*- and *PIK3CA*-mutational status and PD-L1 expression. There was no significant correlation with patient outcome. Moreover, a change of VISTA expression in immune cells during tumor progression was observed. The co-incidence of VISTA and PD-L1 expression indicates a dual immune evasion mechanism of GC tumor cells and makes GC an interesting target for novel combined immune checkpoint inhibitor treatments.

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Introduction

Cancer immunotherapy that targets immune checkpoints is a promising new treatment option in several tumor entities and is currently developing in a breathtaking speed. In gastric cancer (GC), targeting of the immune checkpoint pathways such as cytotoxic T-lymphocyte associated protein-4 (CTLA4), programmed cell death protein-1 (PD-1) and its ligand PD-L1 is currently investigated extensively in several clinical trials.[1] GC is a heterogeneous disease with a marked genetic complexity that has been recently shown in an integrative genomic analysis including whole-genome sequencing. A molecular classification was proposed, which categorizes four subtypes, i.e., Epstein–Barr-virus-associated (EBVa), microsatellite instable (MSI), chromosomal instable (CIN) and genomically stable (GS) GCs.[2,3] Two of the four molecular subtypes, EBVa and MSI GC, are significantly associated with an increased PD-L1 expression and are thereby suggested to be particular suitable for an immune checkpoint therapy.[4] Moreover, MSI GCs are characterized by a high-mutational load, which is associated with a better response to anti-PD-1/PD-L1 immune checkpoint therapies in several tumor entities.[5] Nevertheless, all kind of tumors, including microsatellite stable and EBV-negative GCs, need strategies to evade immune attacks.[6] This leads to the conjecture that malignant tumors apply additional or supplementary immune escape mechanisms.

V-domain Ig suppressor of T-cell activation (VISTA), also known as PD1 homolog (PD1H), C10orf54 or Dies1, belongs to the B7 family. VISTA encodes for a type I membrane protein and is expressed predominantly on hematopoetic cells, e.g., myeloid, granulocytic and T cells.[7] Although the exact VISTA binding partners are not yet known, several studies have demonstrated that VISTA serves both as a ligand (for antigen presenting cells) and as a receptor (for T cells), and that VISTA suppresses T-cell activation. In murine tumor models, anti-VISTA monoclonal antibodies (mAbs) lead to an increased number and elevated function of intratumoral T cells and thereby boost antitumor immunity.[8] Interestingly, VISTA-induced T-cell activation seems to be non-redundantly from the PD-1/PD-L1 pathway, which indicates that a combined VISTA/PD-1 blockade might be a promising new cancer treatment option, as it was efficient in murine tumor models.[9,10] One phase I study regarding an anti-VISTA mAb (JNJ-61610588; NCT02671955) and one phase I study that addresses both VISTA and PD-L1/PD-L2 in solid tumors using a small molecule (CA-170; NCT02812875) have recently started but to date, nothing is known about the expression and impact of VISTA in human GCs. To fill this gap of information, we systemically investigated the expression of VISTA in a large and thoroughly characterized cohort of therapy-naive GCs.

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Supplemental data for this article can be accessed on the publisher's website.

Table 1. VISTA expression and clinico-pathological characteristics.

				VISTA-positive immune cells per mm^2				VISTA-positive immune cells per 200 immune cells				VISTA-positive tumor cells			
		Total		Low		High		Low		High		Negative		Positive	
		N	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
Gender	*n p*[a]	464		464		0.028**		464		0.055		464		0.500	
Female		175	(37.7)	98	(56.0)	77	(44.0)	98	(56.0)	77	(44.0)	162	(92.6)	13	(7.4)
Male		289	(62.3)	131	(45.3)	158	(54.7)	134	(46.4)	155	(53.6)	261	(90.3)	28	(9.7)
Laurén phenotype	*n p* [a]	464		464		<0.001*		464		<0.001*		464		0.006*	
Intestinal		239	(51.5)	98	(41.0)	141	(59.0)	99	(41.4)	140	(58.6)	210	(87.9)	29	(12.1)
Diffuse		145	(31.3)	99	(68.3)	46	(31.7)	101	(69.7)	44	(30.3)	141	(97.2)	4	(2.8)
Mixed		30	(6.4)	14	(46.7)	16	(53.3)	13	(43.3)	17	(56.7)	28	(93.3)	2	(6.7)
Unclassified		50	(10.8)	18	(36.0)	32	(64.0)	19	(38.0)	31	(62.0)	44	(88.0)	6	(12.0)
Localization	*n p* [a]	459		459		0.009**		459		0.003*		459		<0.001*	
Proximal		146	(31.8)	59	(40.4)	87	(59.6)	58	(39.7)	88	(60.3)	121	(82.9)	25	(17.1)
Distal		313	(68.2)	168	(53.7)	145	(46.3)	173	(55.3)	140	(44.7)	297	(94.9)	16	(5.1)
T-category	*n p* [a]	463		463		0.324		463		0.188		463		0.729	
T1a		10	(2.2)	6	(60.0)	4	(40.0)	6	(60.0)	4	(40.0)	10	(100.0)	0	(0.0)
T1b		45	(9.7)	21	(46.7)	24	(53.3)	22	(48.9)	23	(51.1)	43	(95.6)	2	(4.4)
T2		54	(11.7)	21	(38.9)	33	(61.1)	21	(38.9)	33	(61.1)	47	(87.0)	7	(13.0)
T3		187	(40.4)	90	(48.1)	97	(51.9)	94	(50.3)	93	(49.7)	171	(91.4)	16	(8.6)
T4a		128	(27.6)	67	(52.3)	61	(47.7)	63	(49.2)	65	(50.8)	116	(90.6)	12	(9.4)
T4b		39	8.4	24	(61.5)	15	(38.5)	26	(66.7)	13	(33.3)	35	(89.7)	4	(10.3)
EBV status	*n p* [a]	450		450		<0.001*		450		<0.001*		450		0.240	
Negative		430	(95.6)	218	(50.7)	212	(49.3)	221	(51.4)	209	(48.6)	389	(90.5)	41	(9.5)
Positive		20	(4.4)	2	(10.0)	18	(90.0)	2	(10.0)	18	(90.0)	20	(100)	0	(0.0)
MSI status	*n p* [a]	451		451		0.164		451		0.056		451		0.233	
MSS		415	(92.0)	206	(49.6)	209	(50.4)	210	(50.6)	205	(49.4)	375	(90.4)	40	(9.6)
MSI		36	(8.0)	13	(36.1)	23	(63.9)	12	(33.3)	24	(66.7)	35	(97.2)	1	(2.8)
Her2/neu	*n p* [a]	431		431		0.009**		431		0.016**		431		0.769	
Negative		394	(91.4)	199	(50.5)	195	(49.5)	200	(50.8)	194	(49.2)	357	(90.6)	37	(9.4)
Positive		37	(8.6)	10	(27.0)	27	(73.0)	11	(29.7)	26	(70.3)	33	(89.2)	4	(10.8)
KRAS	*n p* [a]	457		457		0.007*		457		0.007*		457		0.213	
Wildtype		439	(96.1)	220	(50.1)	219	(49.9)	223	(50.8)	216	(49.2)	401	(91.3)	38	(8.7)
Mutated		18	(3.9)	3	(16.7)	15	(83.3)	3	(16.7)	15	(83.3)	15	(83.3)	3	(16.7)
PIK3CA (exon 9 or 20)	*n p* [a]	457		457		0.006*		457		0.019**		457		0.465	
Wildtype		433	(94.7)	218	(50.3)	215	(49.7)	220	(50.8)	213	(49.2)	395	(91.2)	38	(8.8)
Mutated		24	(5.3)	5	(20.8)	19	(79.2)	6	(25.0)	18	(75.0)	21	(87.5)	3	(12.5)
PD-L1 in TC	*n p* [a]	458		458		<0.001*		458		<0.001*		458		0.002*	
Negative		347	(75.8)	206	(59.4)	141	(40.6)	208	(59.9)	139	(40.1)	325	(93.7)	22	(6.3)
Positive		111	(24.2)	21	(18.9)	90	(81.1)	22	(19.8)	89	(80.2)	92	(82.9)	19	(17.1)
PD-L1 in IC	*n p* [a]	458		458		0.006*		458		0.003*		458		0.866	
Negative		294	(64.2)	160	(54.4)	134	(45.6)	163	(55.4)	131	(44.6)	267	(90.8)	27	(9.2)
Positive		164	(35.8)	67	(40.9)	97	(59.1)	67	(40.9)	97	(59.1)	150	(91.5)	14	(8.5)
PD-1 in IC	*n p* [a]	461		461		0.012**		461		0.009**		461		0.193	
Negative		215	(46.6)	120	(55.8)	95	(44.2)	122	(56.7)	93	(43.3)	200	(93.0)	15	(7.0)
Positive		246	(53.4)	108	(43.9)	138	(56.1)	109	(44.3)	137	(55.7)	220	(89.4)	26	(10.6)
Tumor-specific survival [mo] *p* [b]						0.278				0.242				0.931	
Total/events/censored		423/289/134		208/144/64		215/145/70		211/147/64		212/142/70		387/264/123		36/25/11	
Median survival		16.8 ± 1.6		16.8 ± 2.3		16.6 ± 2.1		16.8 ± 2.3		17.1 ± 2.0		17.1 ± 1.5		13.6 ± 3.1	
95% C.I.		13.7–19.9		12.3–21.3		12.5–20.7		12.2–21.3		13.1–21.0		14.0–20.1		7.6–19.6	

[a]Fisher's exact test.
[b]Log-rank test.
IC: immune cells; MSI: microsatellite instable; MSS: microsatellite stable; TC: tumor cells.
*Significant after multiple testing procedure.
**Not significant after multiple testing procedure.

Results

A total of 464 patients fulfilled all study criteria. The clinico-pathological characteristics of our patient cohort are summarized in Table 1. Tumor-specific survival data was available in 422 cases (90.9%). Mean follow-up period was 37.8 mo (range 0.2–135.3 mo).

VISTA expression

VISTA expression was observed in tumor, immune and endothelial cells, but not in nonneoplastic gastric epithelium. A total of 41 out of 464 cases (8.8%) showed a VISTA expression in tumor cells, which was exclusively cytoplasmatic. The percentage of stained tumor cells ranged from 0% to 90% (median

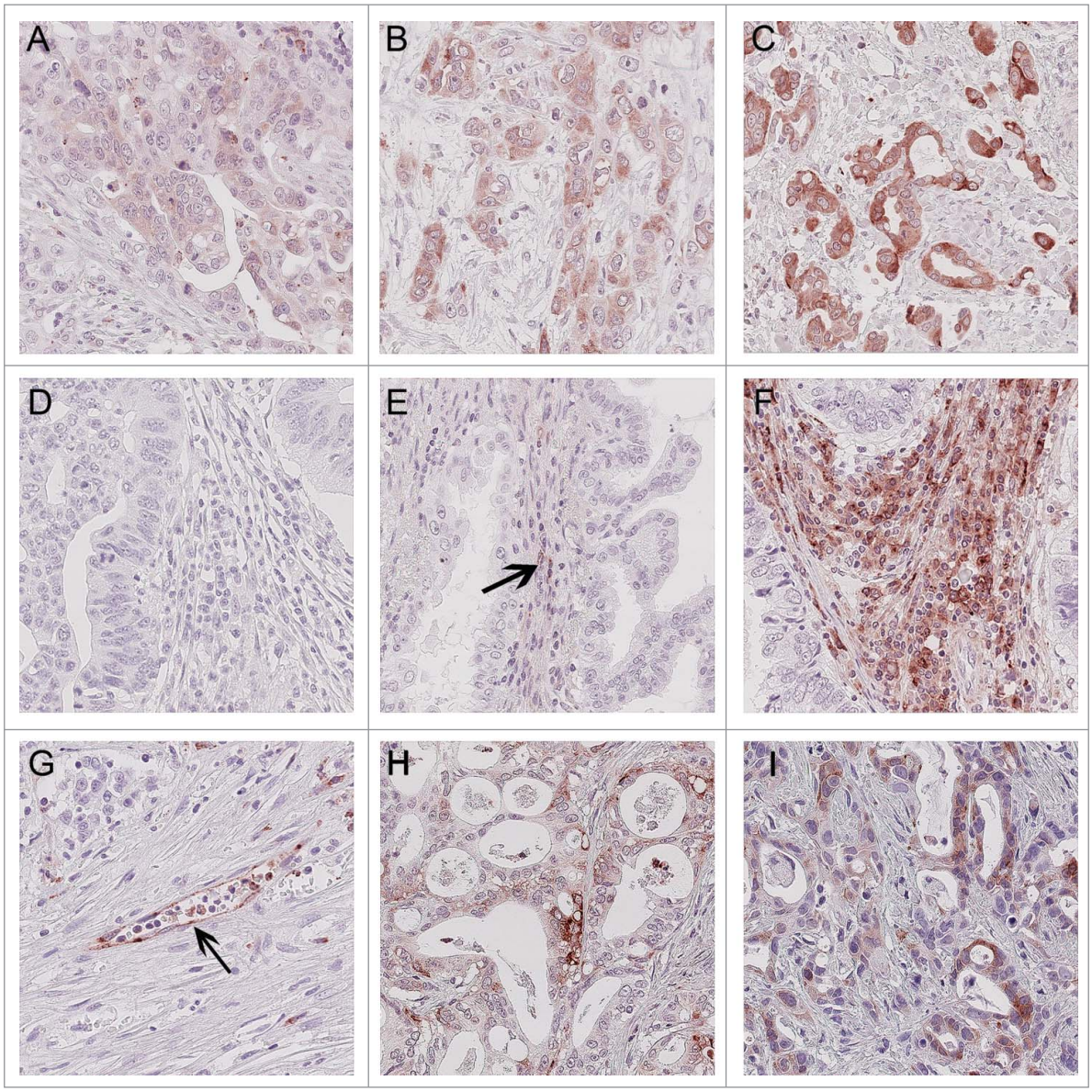

Figure 1. VISTA expression in gastric cancer and liver metastases. VISTA expression in tumor cells was observed in 41 of 464 gastric carcinomas (8.8%) and was graded as week (A), moderate (B) or strong (C). VISTA expression in immune cells ranged between absent (D), low (E) and high (F). VISTA expression in vessels was present in 110 GCs (23.7%) (G). VISTA expression in primary GCs (H) correlated in 85.7% with the corresponding liver metastases ($\kappa = 0.417$; $p = 0.119$) (I). Original magnifications 400-fold.

0%), the staining intensity ranged from negative (0) to strong (3; median 0; Fig. 1A–C). In cases of different staining intensities within the same tumor, only the highest intensity was indicated. Although we aimed for the HistoScore,[11] we recognized that the overall percentage of VISTA-positive tumor cells was mainly low (in 73.2% of the cases $\leq$ 10% positive tumor cells) and assessment of different percentages of three different staining intensities was indiscernible and impractical.

The overall number of intratumoral immune cells per mm^2 ranged from 14 to 841 (median 272). VISTA-positive intratumoral immune cells were found in 388 out of 464 cases (83.6%). The absolute number of VISTA-positive immune cells per mm^2 ranged from 0 (Fig. 1D) to 516 (median 35). GCs with $\leq$ 34 VISTA-positive immune cells were classified as VISTA-low in immune cells per mm^2 (Fig. 1E). Cases with more than 34 VISTA-positive immune cells were classified as VISTA-high in immune cells per mm^2 (Fig. 1F). The proportion of VISTA-positive immune cells per 200 immune cells ranged from 0 to 194 (median 36). GCs with $\leq$ 35 VISTA-positive immune cells were classified as VISTA-low in immune cells per 200 immune cells. Cases with more than 35 VISTA-positive immune cells were classified as VISTA-high in immune cells per 200 immune cells.

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 ($p < 0.001$; $r = 0.917$). Thus, a specification of the applied evaluation method is denoted below only if necessary.

VISTA-positive endothelial cells were observed in 110 of 464 cases (23.7%; Fig. 1G).

VISTA expression and clinico-pathological characteristics

VISTA expression in tumor cells and a high VISTA expression in immune cells were associated with the Laurén phenotype and

tumor localization: Pairwise testing demonstrated that VISTA was detected significantly more often in intestinal ($p = 0.001$ for VISTA expression in tumor cells; $p < 0.001$ for VISTA expression in immune cells) and unclassified GCs ($p = 0.019$ for VISTA expression in tumor cells; $p < 0.001$ for VISTA expression in immune cells) than in diffuse GCs, and significantly more often in GCs of the proximal than the distal stomach, respectively. Moreover, VISTA expression in immune cells was associated with the *KRAS*- and *PIK3CA*-mutational status: A high VISTA expression in immune cells was observed significantly more often in *KRAS*- and *PIK3CA*-mutant GCs (Table 1).

There was no association between VISTA expression in tumor cells or immune cells and gender, age, mucin phenotype, tumor stage (T-category), lymph node metastases (N-category), distant metastases (M-category), presence of liver metastases, UICC stage, lymph node ratio, lymph vessel invasion (L-category), blood vessel invasion (V-category), resection status (R-status), *Helicobacter pylori* infection, Her2/neu-, MET-status, *RHOA*- or *GNAS*-mutations, or no PD-1 expression in immune cells (Tables 1 and S1).

Although there was no association between VISTA expression in immune cells and overall T-category, the amount of VISTA-positive immune cells significantly increased ($p = 0.026$) from category T1 to T2 and decreased thereafter ($p = 0.016$; Fig. 2).

No association was found between the VISTA expression in endothelial cells and clinico-pathological patient characteristics (Table S2).

VISTA expression in PD-L1-positive, EBVa and MSI GCs

As shown in a previous study, a high PD-L1 expression in tumor cells was observed significantly more often in EBVa and MSI GCs.[4] In this study, high VISTA expression in immune cells was observed significantly more often in GCs with high PD-L1 expression and in EBVa GCs ($p < 0.001$), but not MSI GCs. Moreover, VISTA expression in tumor cells was associated with PD-L1 expression in tumor cells ($p = 0.002$), but not with the EBV-status ($p = 0.240$) or with the MSI-status ($p = 0.233$): VISTA-expressing tumor cells were found in only one MSI GC, and VISTA expression in tumor cells and EBV-association was mutually exclusive (Fig. 3A and Table 1).

Prognostic significance

Patient prognosis significantly depended on the Laurén-phenotype, T-, N-, M-, L-, V- and R-category, UICC-stage, lymph node ratio, MSI-, MET-, PD-L1- (tumor and immune cells) and PD-1-status (immune cells; data not shown). There was no significant correlation between VISTA expression in any tumor component and patient survival (Tables 1 and S2 and Fig. 3B–D).

Expression in liver metastases

Tissue specimens from liver metastases were available in 14 cases. Two metastases (14.3%) showed a cytoplasmatic VISTA expression in tumor cells, which accounted for 1–10%, respectively (Fig. 1H and I). Twelve of fourteen cases (85.7%) showed concordant staining results for the primary tumor and its corresponding metastasis ($\kappa = 0.417$; $p = 0.119$).

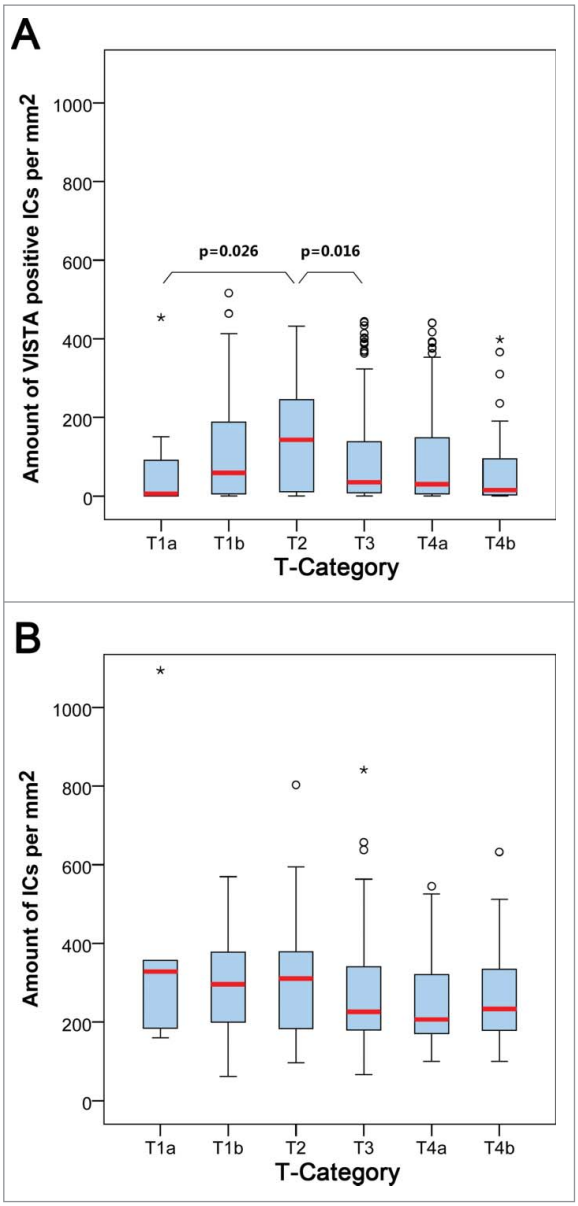

Figure 2. Amount of VISTA-positive and overall immune cells per mm^2 and association with tumor (T-) stage. The amount of VISTA-positive immune cells significantly increased from stage T1 to T2 ($p = 0.026$) and decreased from stage T2 to T3 ($p = 0.016$) (A), while there was no significant difference between the amount of immune cells and T-stage ($p = 0.130$) (B) or between T-stage and VISTA-positive immune cells per mm^2 ($p = 0.324$), respectively.

VISTA expression in immune cells was found in 10 of 14 liver metastases (71.4%). The total number of VISTA positive immune cells per mm^2 ranged from 5 to 201, the relative proportion of VISTA-positive immune per 200 immune cells ranged from 9 to 151. Dichotomized by the median, 6 cases (42.9%) were classified as VISTA-high in immune cells per mm^2, and 4 cases (28.6%) were classified as VISTA-high in immune cells per 200 immune cells. Twelve of the fourteen cases (85.7%) showed concordant staining results for the GC and its corresponding metastasis regarding VISTA expression in immune cells per mm^2 ($\kappa = 0.720$; $p = 0.005$), and eight cases (57.1%) showed concordant staining results regarding VISTA expression in immune cells per 200 immune cells ($\kappa = 0.276$; $p = 0.134$; Table S3).

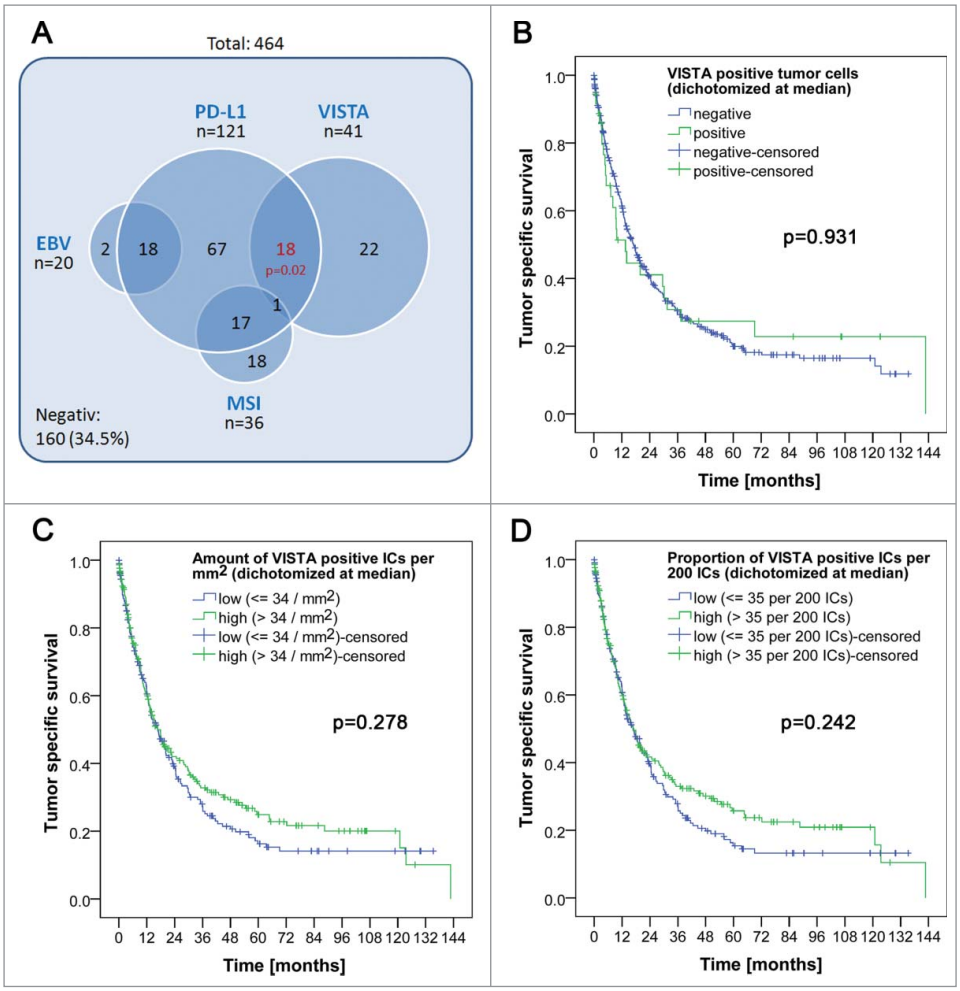

Figure 3. Association of VISTA expression in tumor cells with PD-L1, Epstein–Barr virus-association (EBV) and microsatellite instability (MSI) and prognostic significance of VISTA expression in tumor and immune cells. VISTA expression in tumor cells was associated with PD-L1 expression ($p = 0.02$), but not with EBV (p = 0.240) or MSI (0.233). VISTA expression was observed in one MSI GC, whereas VISTA expression and EBV-association were mutually exclusive (A). There was no significant correlation between tumor-specific survival and VISTA expression in tumor cells (median survival 17.1 vs. 13.6 mo; $p = 0.931$) (B), the amount of VISTA-positive immune cells per mm^2 (16.8 vs. 16.6 mo; $p = 0.278$) (C) or the proportion of VISTA-positive immune cells per 200 immune cells (16.8 vs. 17.1 mo; $p = 0.242$) (D).

Discussion

This study is the first evaluation of VISTA expression in a large cohort of human tumors. All previous studies have been done in small series of human tumor specimens, cell lines or murine models, and many results of these former studies cannot be directly transferred one-to-one to human subjects without verification. Yet, human VISTA shares 90% homology with murine VISTA, and similar suppressive functions and expression patterns are reported for both variants.[12]

VISTA is known to serve both as a receptor and a ligand.[13] Consistently with studies of murine models, in this study, VISTA was expressed in a substantial amount of immune cells. Additionally, a distinct cytoplasmatic VISTA expression in tumor cells was observed in a small subset of GCs. Interestingly, GCs with VISTA-positive tumor cells had the same clinicopathological characteristics like GCs with VISTA-positive immune cells, e.g., an intestinal phenotype and a proximal localization. One reason why VISTA expression in tumor cells was not found in a former GC cell line model of Oliveira et al.[14] might be its low frequency of 8%. Likewise, VISTA expression in GC tumor cells, most GC alterations apply to only a small subset of GCs. This necessitates validation studies on large cohorts. Moreover, it substantially aggravates the development of a personalized GC treatment. Hence, an appropriate case selection that considers tumor histology and intratumoral heterogeneity as well as other clinico-pathological characteristics is essential for a successful drug development, a practical testing and a reasonable therapy selection.

VISTA is expressed in GC and associated with PD-L1 expression

Although VISTA belongs to the B7 family, its functions are nonredundant with other Ig superfamily members.[7] In the present study, VISTA was significantly associated with PD-L1 expression. This might indicate that a subgroup of GCs seems to use a (at least) dual synergistic or successive VISTA/PD-L1/PD-1 related mechanism to escape immunity. Moreover, it is a highly interesting observation regarding a combined VISTA/PD-1 blockade as a new cancer treatment option.

It is well known that the composition of the immune cell infiltrates varies not only between different tumor entities, but

also within tumors of the same anatomic site. Lines et al. predicted that a frank myeloid infiltrate in tumor lesions would express high levels of VISTA.[13] In this study, a high level of VISTA expressing immune cells was indeed observed in EBVa GCs, but not in MSI GCs, which are both characterized by a marked lymphoid infiltrate. Contrarily to PD-L1, VISTA expression in tumor cells was associated neither with MSI nor with EBV status. This indicates that, besides EBVa and MSI GCs, GCs with VISTA/PD-L1 co-expression might represent a third GC subgroup that could benefit from a targeted immune checkpoint inhibitor treatment. A high VISTA expression was observed more often in intestinal GCs, according to Laurén, GCs of the proximal stomach, *KRAS*-mutated GCs and, although without statistical significance, in Her2/neu-positive GCs. These four clinico-pathological characteristics are in turn significantly correlated with the molecular subgroup of CIN GCs,[2] which leads to the consideration that GCs with high VISTA expression might be assigned to CIN GCs. To confirm this presumption, a correlation between VISTA expression and other putative markers of chromosomal instability, e.g., *TP53* mutations, should be evaluated.

Potential tumor biologic impact of VISTA expression in GC

VISTA expression in both immune cells and tumor cells was associated with the Laurén phenotype. A low VISTA expression was observed significantly more often in GCs with a diffuse phenotype according to Laurén, which is poorly differentiated and associated with a high recurrence rate and aggressive clinical behavior. The predominant expression of VISTA in intestinal GCs is in accordance with the finding that, apart from its immune modulating functions, VISTA is also known to be a regulator of differentiation.[15]

Although there was no association between VISTA expression and tumor stage, lymph node metastases, distant metastases, or UICC stage, interestingly, the amount of VISTA-positive immune cells significantly increased from tumor category pT1 to pT2 and significantly decreased from pT2 to pT3. Thus, VISTA expression in immune cells and immune evasion changes during tumor progression and hallmarks an adaptive process of GC. The fact that this applies only to the tumor category but neither to nodal or distant metastases (N-/M-category) nor the UICC stage leads to the conjecture that VISTA expression rather influences the local tumor progression than the distant tumor spread. In this context, it is interesting to note that we have previously made the same observation for PD-L1 expression in tumor cells, which was high in pT2-tumors and lower in pT3 and pT4-tumors. Thus, GCs may apply different strategies of immune evasion (PD-1/PD-L1 and/or VISTA), which take place during the transition between tumor stage T2 and T3.

In contrast to PD-L1 and PD-1, VISTA expression was not correlated with patient survival. Hence, VISTA expression is unsuitable as a predictor of GC prognosis. On the other hand, the incoherence between VISTA expression and survival might be beneficial in the future interpretation of therapy effects of an anti-VISTA cancer therapy. The results are not expected to be biased by treatment independent effects.

VISTA is concordantly expressed in corresponding liver metastases

In patients with a metastatic and/or unresectable GC, diagnostic tissue specimens might, e.g., be obtained from liver metastases. For Her2/neu, a discordance rate between the primary GC and the liver metastasis ranging from 9% to 16% is known.[16] In our study, VISTA expression in liver metastases was observed in only a small portion, but concordant staining patterns with the primary tumor were found in the majority of cases. Yet, VISTA-positive GCs had VISTA-negative liver metastases, and vice versa. Nevertheless, the presence of a VISTA expression in liver metastases alone is an interesting finding, which might be relevant for clinical trials regarding anti-VISTA immune checkpoint inhibitor treatment of metastatic disease.

Conclusion

Summing up, this is the first evaluation of VISTA expression in a large cohort of GC, and, moreover, the first description of VISTA expression in human tumor cells. We hereby show that VISTA expression changes during tumor progression. The coincidence of VISTA and PD-L1 expression in microsatellite stable and EBV negative GCs indicates a dual immune evasion mechanism and makes GC an interesting target for novel combined immune checkpoint inhibitor treatments. Further research in this field, including the impact of VISTA expression as a predictive biomarker, is urgently needed, and the results of ongoing anti-VISTA and anti-VISTA/anti-PD-L1 studies are eagerly awaited.

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). All patient data were pseudonymized before study inclusion. All experimental work was complied with all mandatory laboratory health and safety procedures.

Study population

From the archive of the Institute of Pathology, University Hospital Kiel, we sought all patients who had undergone either total or partial gastrectomy for adenocarcinoma of the stomach or esophago-gastric junction between 1997 and 2009. 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., lymphozytes, granulocytes, and 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. Patients were included if an adenocarcinoma of the stomach or esophago-gastric junction was histologically confirmed.

Exclusion criteria were defined as (1) histology identified a tumor type other than adenocarcinoma, and (2) patients had undergone a perioperative chemo- or radio-therapy. Each resected specimen had undergone gross sectioning and histological examination by the 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 re-examination of primary tissue sections was performed for all cases to ensure if inclusion criteria were met. Tumors were classified according to the Laurén classification[17] and re-examined by two surgical pathologists. pTNM-stage of all study patients was determined according to the 7th edition of the UICC guidelines.[18]

VISTA immunohistochemistry

FFPE tissue sections were pretreated in citrate buffer (pH 6) for antigen retrieval and incubated with hydrogen peroxide block and Ultra V Block (both Thermo Scientific, Braunschweig, Germany) to avoid unspecific reactions. Immunostaining was performed using a rabbit monoclonal anti-VISTA antibody (dilution 1:500, clone D1L2G, Cell Signaling, Danvers, USA) that recognizes endogenous levels of total VISTA protein. For visualization, the ImmPRESS-HRP-Universal-Antibody Polymer and the NovaRED substrate kit (both VectorLabs, Peterborough, United Kingdom) were applied. Counterstaining was done with hematoxylin (Dr. K. Hollborn & Söhne GmbH and Co KG; Leipzig, Germany).

Evaluation of immunostaining

Percentage, intensity and intracellular distribution of stained tumor cells (TC), the amount of positive immune cells (IC; e.g., lymphozytes, granulocytes, and macrophages) and positivity of endothelial cells were evaluated separately by two pathologists (CB and CR). The intensity of immunostaining of tumor cells was graded as negative (0), weak (1+), moderate (2+) or strong (3+). 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 IC. Hotspot regions were defined as those areas with the highest density of VISTA-positive immune cells. Immunostaining of endothelial cells was graded as present or absent.

Assessment of phenotypic and genotypic characteristics of the study cohort

The *H. pylori*-, Her2/neu-, MET-, EBV-, MSI-, PD-L1- and PD-1-status as well as the *KRAS*-, *PIK3CA*-, *RHOA*-, and *GNAS*-genotype was assessed as described previously.[4,11,19-23]

Study design

Whole tissue sections from GCs and corresponding liver metastases were stained with an antibody directed against VISTA. The staining results were correlated with clinico-pathological and survival data.

Statistical analysis

Statistical analyses were done using SPSS 20.0 (IBM Corporation, New York, USA). VISTA expressions within the different tumor components (tumor cells, immune cells and endothelial cells) were first examined as raw score values and then dichotomized by the median into "negative" and "positive" (tumor cells, endothelial cells) and "low" and "high" (immune cells), respectively. Cross tabulations of clinical data and marker expressions were tested for independence using Fisher's exact test. For cross tabulations of nominal variables having more than two categories and significant associations according to Fisher's exact test, an additional pairwise Fisher's exact test was performed. 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. The correlation between VISTA expression in GCs and corresponding metastases was calculated by using Cohen's kappa. A kappa value of 0.20 was considered to be poor, of 0.21–0.40 to be fair, of 0.41–0.60 to be moderate, of 0.61–0.80 to be good and of 0.81–1.00 to be very good. 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 associations between clinical variables and biomarkers was controlled by applying the explorative Simes (Benjamini–Hochberg) procedure. [24] All p-values are given unadjusted, but those having lost significance under the explorative Simes procedure are marked appropriately.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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