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ORIGINAL ARTICLE – GASTROINTESTINAL ONCOLOGY

CD151 Gene and Protein Expression Provides Independent Prognostic Information for Patients with Adenocarcinoma of the Esophagus and Gastroesophageal Junction Treated by Esophagectomy

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ABSTRACT

Background. Esophageal and gastroesophageal junctional (GEJ) adenocarcinoma is one of the most fatal cancers and has the fastest rising incidence rate of all cancers. Identification of biomarkers is needed to tailor treatments to each patient's tumor biology and prognosis.

Methods. Gene expression profiling was performed in a test cohort of 80 chemoradiotherapy (CRTx)-naïve patients with external validation in a separate cohort of 62 CRTx-naïve patients and 169 patients with advanced-stage disease treated with CRTx.

Results. As a novel prognostic biomarker after external validation, CD151 showed promise. Patients exhibiting high levels of CD151 (≥median) had a longer median overall survival than patients with low CD151 tumor levels (median not reached vs. 30.9 months; $p = 0.01$). This effect persisted in a multivariable Cox-regression model with adjustment for tumor stage [adjusted hazard ratio (aHR), 0.33; 95 % confidence interval (CI), 0.14–0.78; $p = 0.01$] and was further corroborated through immunohistochemical analysis (aHR, 0.22; 95 % CI, 0.08–0.59; $p = 0.003$). This effect was not found in the separate cohort of CRTx-exposed patients.

Conclusion. Tumoral expression levels of CD151 may provide independent prognostic information not gained by conventional staging of patients with esophageal and GEJ adenocarcinoma treated by esophagectomy alone.

The most common esophageal malignancy in the Western world is esophageal and gastroesophageal junctional (GEJ) adenocarcinoma (EAC), which has shown a sixfold increase in incidence and thus has increased faster in incidence than any other cancer since the 1970s.[1–5] The EAC case fatality rates are particularly high, with population-based 5-year survival rates of approximately 15 %.[6,7] Even for patients referred to curative treatment, usually by neoadjuvant chemotherapy or chemoradiotherapy (CRTx) followed by esophagectomy, the 5-year survival rates still are mostly lower than 45 %.[8]

The current staging of EAC involves imaging and histopathologic examination of the tumor, and for patients undergoing esophagectomy, the resected lymph nodes. Unfortunately, staging by these means is inadequate for many patients because it fails to stratify patients in the same tumor-node-metastasis (TNM) stage reliably into

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those who will have a better or worse prognosis.[9,10] In contrast, molecular staging information has become routine for other epithelial malignancies to inform clinical management.[11] Although efforts are ongoing to refine current EAC clinicopathologic staging systems[12,13] and predictors of lymph-node involvement,[14,15] few molecular biomarkers have been externally validated for this cancer, and none to date have been introduced into routine clinical care.[10,16]

In this study, we performed gene expression profiling to identify and validate novel markers for prognosis in three separate cohorts of patients with EAC. More than 300 patients with EAC were included in the study, providing the opportunity for biomarker discovery followed by external validation in independent patient cohorts.

MATERIALS AND METHODS

Patients and Tissues

Institutional review board approval for this study was obtained at all collaborating institutions, and all patients provided written informed consent.

This study used three independent cohorts of EAC patients (total of 313) for discovery and validation of prognostic candidates, as described in supplementary information and summarized in Table 1. Briefly, for all in-house molecular analyses, tissue specimens were collected from a total of 249 patients enrolled in the population-based, case-control Australian Cancer Study (ACS) by the Queensland Institute of Medical Research (QIMR) and stratified according to stage and chemoradiotherapy exposure status (CRTx).[17]

The independent validation cohort was obtained from a publically available gene expression omnibus (GEO) microarray dataset (GEO-Accession GSE19417). This GEO external-validation cohort included 64 esophageal and GEJ adenocarcinoma patients with complete survival information. Similar to the discovery cohort, the patients were mainly (95 %) radiochemotherapy naïve and also had undergone esophagectomy with curative intent. This dataset has been used previously in other prognostic biomarker studies.[10]

RNA Isolation

All samples were formalin-fixed paraffin-embedded (FFPE) tissues from either surgical specimens or biopsies collected at endoscopy. Before RNA extraction, pathologic review was performed to confirm the correct diagnosis and to identify specimens with the highest tumor cellularity content. Wherever possible, analyses were performed on pretreatment FFPE specimens. Measurement of RNA extraction, yield, and quality was performed as described in Fisher et al.[18]

Gene Expression Quantification

As previously described,[18–20] multiplexed tandem polymerase chain reaction (MT-PCR) was used to quantitate mRNA expression levels of a panel of 21 genes of interest selected from published studies and our previous discovery studies (Supplementary Table 1). For quantification of CD151 gene expression in the ACS validation cohort, the hydrolysis-probe FFPE-optimized Quantifast Probe Assay quantitative polymerase chain reaction (qPCR) kit (Cat#QF00434147; QIAGEN, Valencia, CA, USA) was used with β-Actin (ACTB) as a reference gene according to the manufacturer's protocol.

Immunohistochemistry and Scoring

Tissue specimens were processed in a standard fashion with regular formalin fixation and paraffin embedding. In 5-µm tissue sections, CD151 was identified using a mouse monoclonal antibody (CD151 monoclonal antibody [RLM30], Cat#MAB9606; Sapphire Bioscience, Australia) as described previously.[18,21]

Sections were scored by two experienced investigators blinded to clinical information. Staining of each element (nucleus, cytoplasm, cell membrane and stroma) was evaluated using the following four-step scale[22]: 0 (no staining or equal to background), 1 (weak diffuse staining), 2 (moderate staining in at least 10 % of cells), and 3 (strong immunostaining in a majority of cells). Scores were tallied to allow for a separate analysis of the contribution of "tumoral" and/or "stromal" staining to possible survival differences. In cases of disagreement, consensus was reached after combined re-analysis on a multi-headed microscope.

Statistical Analyses

Continuous variables were compared using Student's *t* test, the Wilcoxon rank-sum test, one-way analysis of variance (ANOVA), and/or the Kruskal–Wallis test as appropriate. Where necessary, log 2 transformation of data was performed to achieve normal distribution. Differences between proportions derived from categorical data were compared using Pearson's chi-square or Fisher's exact test where appropriate. Data are reported as median with interquartile range (IQR) unless denoted otherwise. The Kaplan–Meier method was used for survival estimates, and differences in survival were analyzed using the log-rank test. Cox proportional hazards models were used for uni- and multivariable analysis, with overall survival defined from the date of diagnosis to death or censoring. A forward selection process was applied to identify genes with prognostic biomarker potential as described in "Methods"

TABLE 1 Demographic and clinicopathologic features of the internally available patient cohorts

Variable	All ($n = 249$) n (%)	Chemo-radiotherapy naïve discovery cohort ($n = 80$) n (%)	Chemo-radiotherapy exposed validation cohort ($n = 169$) n (%)	p-value[a]
Median age: years (IQR)	64 (56–64)	66 (58–73)	63 (57–63)	0.25
Sex[b]				
Males	225 (90)	72 (90)	153 (91)	0.89
Females	24 (10)	8 (10)	16 (9)	
Primary tumor location				
Distal esophagus	188 (76)	19 (24)	169 (100)	<0.001
Esophago-gastric junction (Siewert Type II)	61 (24)	61 (76)	–	
TNM (6th ed)				
T stage				<0.001
T1–2	88 (35)	80 (100)	18 (11)	
T3–4	87 (35)	0	87 (52)	
Missing T information[c]	64 (26)	0	63 (38)	
Nstage				0.04
N0	139 (56)	54 (67)	84 (50)	
N+	98 (39)	26 (33)	72 (43)	
Missing N information	13 (5)	0	13 (8)	
M-stage				<0.001
M0	159 (64)	80 (100)	95 (56)	
M+	32 (13)	0	32 (19)	
Missing M information	42 (17)	–	42 (25)	
R-stage				
R0	122 (49)	63 (79)	49 (29)	
R1	10 (4)	6 (7.5)	4 (2)	
R2	–	–	–	
Missing R information/R status not applicable as no surgery	157 (63)	11 (14)	144 (85)	
Tumor stage				<0.001
1A–B	34 (14)	28 (35)	6 (4)	
2A	66 (27)	26 (33)	40 (24)	
2B	32 (13)	26 (33)	6 (4)	
3A–C	38 (15)	0	38 (22)	
IV	32 (13)	0	32 (19)	
Unknown stage	47 (19)	0	47 (28)	
Chemo-radiotherapy				0.06
Only CTX	28 (11)	0	28 (17)	
Only RTX	16 (6)	2[d] (3)	14 (8)	
Both CTX and RTX	127 (51)	0	127 (75)	
Neither CTX nor RTX	78 (31)	78 (98)	0	
Chemo-radiotherapy time				<0.001
Pre surgery	53 (21)	0	53 (31)	
Post surgery	18 (7)	2[d] (3)	18 (11)	
Both pre and post surgery	10 (4)	0	10 (6)	
No surgery attempted	74 (30)	0	74 (44)	
Missing information	14 (6)	0	14 (8)	
Median survival (months)	24.9	72	18.6	<0.001
No. of deaths	167 (67)	34 (43)	129 (76)	–

TABLE 1 continued

Variable	All ($n = 249$) n (%)	Chemo-radiotherapy naïve discovery cohort ($n = 80$) n (%)	Chemo-radiotherapy exposed validation cohort ($n = 169$) n (%)	p-value[a]
5-year survival rate	31	58	18	–

IQR interquartile range, *TNM* tumor-node-metastasis, *CTX* chemotherapy, *RTX* radiotherapy

[a] *Note:* Percentages may not equal 100 due to rounding

[b] Despite extensive chart review and data collection efforts, some staging information from patients in this population-based cohort study was not accessible

[c] For differences in stage comparisons, missing data points were excluded for calculations

[d] Two patients received radiotherapy for metachronous bone metastasis detected 6 and 12 months after surgery

section in supplementary information. All p values lower than 0.05 were regarded as statistically significant, and all analyses were performed using R Statistical Packages.[23]

RESULTS

Patients and Tissues

After application of inclusion criteria based on qPCR quality control metrics, 80 (87 %) of the 92 ACS discovery and 165 (97 %) of the 169 ACS validation cohort patients could be included for gene expression and survival analyses. Complete gene expression and survival data for final prognostic candidates were available for 64 of the 77 tumors in the GEO external-validation cohort.

A summary of the ACS patient clinicopathologic features is provided in Table 1. Details of the GEO validation cohort have been described previously.[10] As expected, the TNM stage was higher in the patient group exposed to multimodality therapy. Due to incomplete staging information provided in the GEO validation cohort, only a comparison of positive nodal status was possible. Compared with the ACS discovery and validation cohort, the GEO cohort had a significantly higher proportion of patients with positive lymph nodes (ACS discovery: 75 vs. 33 %; ACS validation: 75 vs. 43 %; $p < 0.001$).

Patient Survival

The overall median survival period for all three cohorts ($n = 313$) was 24 months, with a 5-year survival rate of 28.3 % (95 % CI, 23.3–34.5 %). In the ACS discovery cohort, early-stage CRTx-naïve patients had a significantly longer median overall survival (72 months) than the advanced-stage CRTx-exposed ACS validation cohort (18.6 months) or the GEO validation cohort (18.8 months). The respective 5-year survival rates for these cohorts were 57.6 % (95 % CI, 47.6–69.9 %), 17.5 % (95 % CI, 11.4–27.0 %), and 21.9 % (95 % CI, 13.8–34.8 %) ($p < 0.001$; Fig. 1). The overall survival of the patients with positive resection margins ($n = 6$) in the ACS discovery cohort did not differ significantly (data not shown).

Global Gene Expression Pattern and Survival

Unsupervised hierarchical clustering of gene expression levels in the ACS discovery cohort identified two distinct clusters, with the one group of tumors showing globally upregulated gene expression levels (cluster 1: $n = 63$)

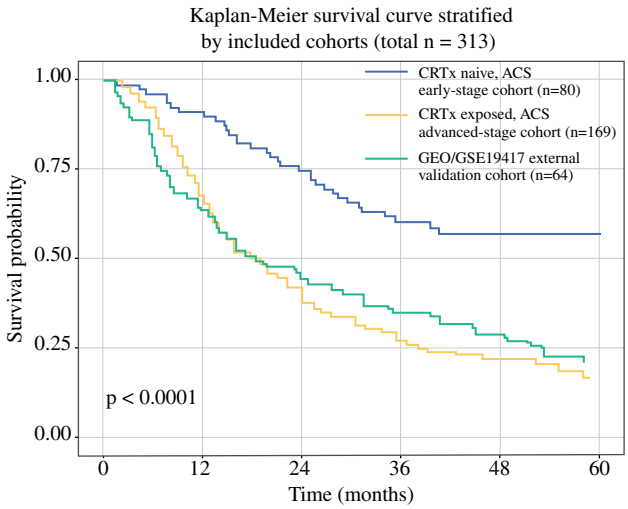

FIG. 1 Kaplan-Meier survival curve of overall patient survival stratified by the respective cohorts. Overall survival was significantly longer in the Australian Cancer Study (ACS) chemoradiotherapy (CRTx)-naïve cohort than in the ACS CRTx-exposed validation cohort. Overall patient survival of the externally accessed Gene Expression Omnibus (GEO) dataset (GEO Accession GSE19417) was significantly worse than that of the ACS CRTx-naïve discovery cohort but not significantly different from the ACS CRTx-exposed validation cohort

compared with the other group, which showed lower levels of the analyzed gene panel (cluster 2: $n = 17$; Supplementary Fig 1A). However, in both the unsupervised clustering and the contingency table analyses, no significant association could be identified for the clusters and frequency of deaths (cluster 1: 39.7 %; cluster, 2: 52.9 %; $p = 0.32$, chi-square), for the proportion of positive lymph nodes (cluster 1: 33.3 %; cluster 2: 29.4 %; $p = 0.75$ chi-square), or for the percentage of higher-stage distribution (cluster 1, stage 2B: 33.3 %; cluster 2, stage 2B: 29.4 %; $p = 0.94$ chi-square). In addition, no statistically significant differences in survival could be determined, although median survival was more than twice as long for the patients in cluster 1 (72 vs. 30.6 months; $p = 0.18$, log-rank; Supplementary Fig. 1B).

Levels of CD151 Gene Expression are Independently Associated with Survival in Chemoradiotherapy-Naïve Patients

Gene expression values were dichotomized at the median to determine the influence of gene expression levels on overall patient survival in the ACS discovery cohort. Kaplan–Meier survival curve comparison showed that 4 of the 21 genes (IMP3, PRNP, TSPAN1, and TYMS) had a borderline significant impact on patient overall survival, which was not corroborated when confounders such as tumor stage were adjusted (Supplementary Table 1). Equally, when the identified genes in the GEO validation cohort were tested, no significant association with overall survival could be determined (data not shown). Consequently, these candidates were not taken forward for further analysis.

The patients with high CD151 gene expression levels ($n = 39/79$, 49 %) displayed a significantly longer overall survival than the patients with low CD151 levels (median survival not reached vs. 30.9 months; $p = 0.01$; Fig. 2), corresponding to 5-year survival rate of 71 % (95 % CI, 58–87 %) versus 43 % (95 % CI, 30–62 %). In a subsequent multivariable Cox regression analysis with adjustment for patient age, sex, tumor location, stage, and resection margin status, high levels of CD151 were an independent prognostic marker of improved overall survival (aHR, 0.31; 95 % CI, 0.15–0.67; $p = 0.003$; Table 2).

Consequently, this gene was tested in the GEO external-validation cohort by also dichotomizing expression levels at the median. Elevated tumoral CD151 gene expression levels ($n = 32/64$, 50 %) also were associated with an almost threefold longer overall survival compared with tumors displaying low gene expression levels, but this difference was not statistically significant (31.2 vs. 11.2 months; $p = 0.09$). However, in a multivariable Cox regression analysis that adjusted for patient sex, tumor differentiation, and nodal positivity, elevated levels of CD151 were a significant, independent predictor of lower mortality (aHR, 0.33; 95 % CI, 0.14–0.78; $p = 0.01$; Supplementary Fig. 2).

Tumoral CD151 Protein Expression is Associated with Survival for Chemoradiotherapy-Naïve Patients

We subsequently performed immunohistochemistry to quantify CD151 protein expression levels in 62 primary tumor sections of the ACS discovery cohort. Tumors that showed elevated protein levels of CD151 (stratified as ≥moderate-strong immunostaining; $n = 34/62$, 55 %) were significantly associated with improved patient survival (median survival not reached vs. 28.6 months; $p = 0.04$, log-rank). A multivariable Cox-regression model that adjusted for patient age, sex, tumor location, stage, and resection margin status also showed that elevated tumoral protein levels of CD151 were a significant independent predictor of improved patient survival (aHR, 0.22; 95 % CI, 0.08–0.59; $p = 0.003$; Fig. 2b; Supplementary Table 2). Stromal CD151 staining was not associated with any differences in survival (data not shown). An example of tumoral staining patterns is provided in Supplementary Fig. 3.

CD151 Does Not Predict Survival for Advanced-Stage EAC Patients Who Have Been Exposed to Multimodality Therapy

In a final analysis, gene expression levels of CD151 were measured in the ACS validation cohort of CRTx-treated patients. No differences in overall survival could be determined for CD151 high-expressing tumors versus CD151 low-expressing tumors (median survival, 15.6 vs 20.1 months; $p = 0.4$; Fig. 3). This finding was irrespective of the patients receiving multimodality therapy combined with surgery or palliative CRTx alone (data not shown). Similarly, no significant prognostic effect of CD151 expression was found in a multivariable Cox proportional hazard analysis (aHR, 1.09; 95 % CI, 0.72–1.65; $p = 0.67$).

DISCUSSION

In this study investigating three independent cohorts of more than 300 esophageal and GEJ adenocarcinoma patients, we performed a gene expression profiling study of 21 genes that identified elevated tumoral CD151 gene and protein levels as a significant, independent predictor of improved patient survival. However, this effect could be documented only in patients who were chemoradiotherapy naïve because CD151 gene expression levels did not

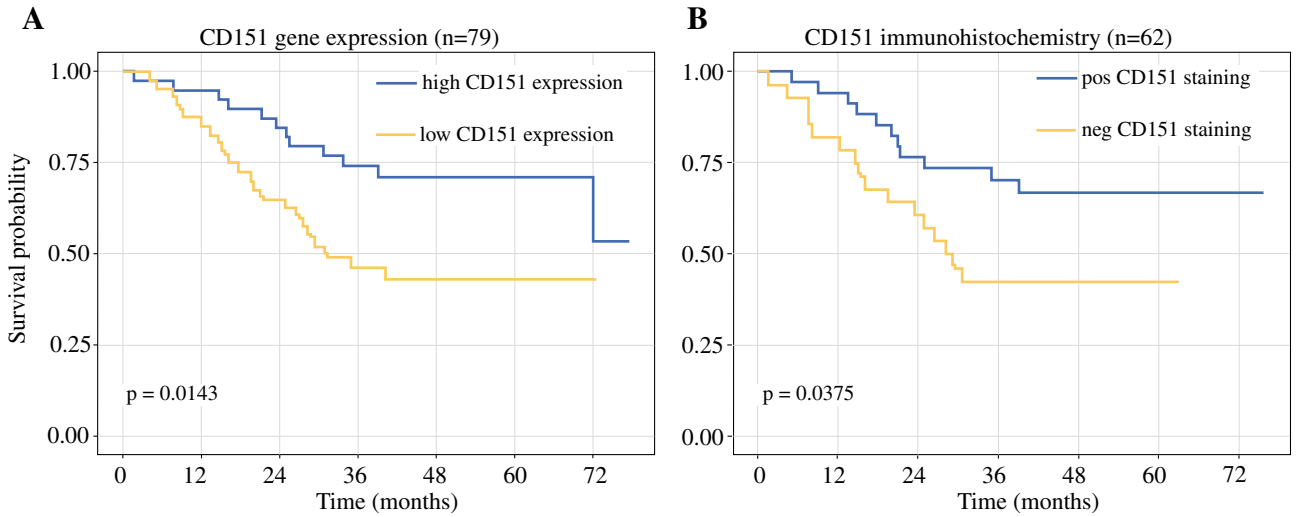

FIG. 2 Overall survival of the patients in the Australian Cancer Study (ACS) chemoradiotherapy (CRTx)-naïve discovery cohort stratified by CD151 gene (2A) and protein (2B) expression levels. Patients with high gene and protein expression levels (stratified by median expression) exhibit significantly longer overall survival times

TABLE 2 Uni- and multivariable analysis of factors contributing to mortality according to Cox proportional hazards modeling in chemoradiotherapy-naïve patients (discovery cohort, $n = 80$)[a]

	Univariable analysis			Multivariable analysis[a]		
Variable	HR	95 % CI	p-value	HR	95 %CI	*p* value
Age	1.05	1.0–1.1	0.02	1.04	0.09–1.08	0.10
Sex						
Female	Reference	–	–	Reference	–	–
Male	1.03	0.31–3.38	0.96	1.79	0.49–6.55	0.38
Tumour location						
Distal esophagus	Reference	–	–	Reference	–	–
Esophago-gastric junction (Siewert Type II)	3.81	1.16–12.46	0.03	4.80	1.09–21.06	0.04
Stage						
I	Reference	–	–	Reference	–	–
2A	7.81	2.3–27.33	0.001	8.28	2.15–31.82	0.002
2B	8.44	2.50–28.84	<0.001	7.81	2.10–28.99	0.002
Resection margin status						
Negative	Reference	–	–	Reference	–	
Positive	2.18	0.75–6.32	0.15	1.54	0.48–4.91	0.46
CD151 expression[b]						
Low CD151 expression	Reference	–	–	Reference	–	–
High CD151 expression	0.42	0.21–0.86	0.018	0.33	0.14–0.78	0.01

HR hazard ratio, *CI* confidence interval

[a] One patient had insufficient qPCR data for reliable CD151 gene expression quantification and was subsequently excluded from the multivariable Cox proportional hazards analysis

[b] Stratified as above or below median expression

correlate with survival among EAC patients undergoing multimodality therapy. To our knowledge, this is the first study to document CD151 as an independent prognostic marker for this disease.

The CD151 protein (also known as TSPAN24) is a member of the tetraspanin family of transmembranous proteins. Tetraspanins are widely expressed on a variety of epithelial cells, where they can mediate cell adhesion,

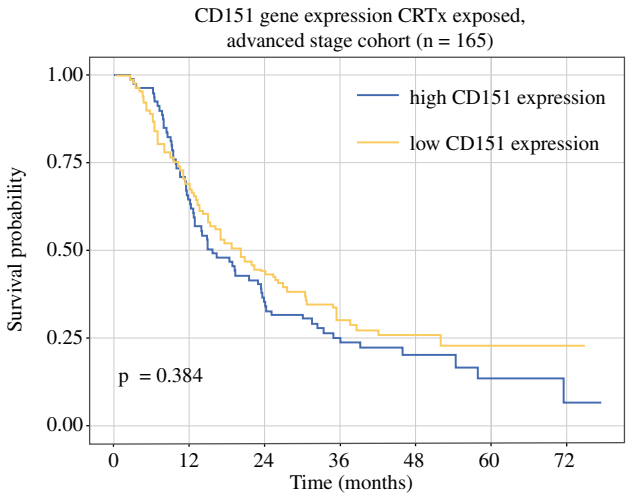

FIG. 3 Overall survival of the patients in the Australian Cancer Study (ACS) chemoradiotherapy (CRTx)-exposed validation cohort. In this cohort, no significant differences in overall patient survival could be identified when the patients were stratified by median tumoral CD151 gene expression levels

migration, invasion, cell signaling, and survival.[24] Most studies investigating other cancers point to a central role of CD151 in promoting progression, invasion, metastasis, and consequently poor prognosis.[24–30] In colon cancer, however, the role for CD151 seems more complex because CD151 levels are downregulated in colonic adenocarcinomas compared with normal tissue.[31] Equally, reduced CD151 levels correlate with advanced disease stage in urothelial cancer.[32] Furthermore, low CD151 protein expression is significantly and independently associated with worse survival for patients with endometrial cancer.[33]

Our analysis had several limitations. First, only differences in stage distribution could be accurately determined in the ACS discovery and validation cohorts because the publically available GEO dataset included only data on tumor differentiation grade and positive lymph node status. However, this dataset has been extensively characterized,[10] and 95 % of the included GEO dataset patients were radiochemotherapy naïve, thus permitting an analysis of the prognostic impact of CD151 levels unperturbed by the effects of (neo-)adjuvant therapy. Both of the CRTx-naïve cohorts included a large proportion of GEJ tumors, whereas the cohort of CRTx-exposed patients was mainly EAC. But no difference in CD151 expression depending on tumor location could be found, and CD151 expression provided survival information independent of tumor location in our multivariable analysis. Furthermore, because the ACS was conducted during the mid 2000s,[17,34] the sixth edition of American Joint Committee on Cancer (AJCC) staging was used for the included patients. This needs to be taken into account when our findings are interpreted because stage migration may occur in more recent staging systems, and the seventh edition allows for better prognostication of patients.[35]

Finally, our analysis of gene expression levels in the ACS validation cohort was performed using a different PCR technique. But the validation of our findings at the protein level as well as in an external cohort that also used another technique for gene-expression quantification increases our confidence that we are reporting a true prognostic effect unlikely to be explained by measurement error due to differences in CD151 quantification methods. Overall, we believe that the assessment of CD151 expression levels across three independent cohorts and by various analysis methods is a strength of this study because it allows for a robust assessment of its true prognostic impact.

The mechanisms by which high CD151 levels may exert a protective impact on tumor patient survival are unclear. It has been hypothesized that E-cadherin (CDH1) expression may be inhibited through downregulation of CD151 expression via hypoxia-inducible factor 1-alpha.[31] This would in turn lead to a reduction in cell–cell adhesion and consequently an increase in invasion and metastastic potential. Supporting this hypothesis, we found a strong correlation between CD151 and CDH1 gene expression levels in EAC (Supplementary Fig. 4), indicating that elevated levels of CD151 may exert a protective effect through E-cadherin. Furthermore, the main binding partners for CD151 are integrin α3β1, which mediates rapid tumor cell migration/invasion, and integrin α6β4, which mediates stable cell attachment.[36] Consequently, CD151 has the potential either to restrain or to promote tumor cell invasion and metastasis. In our analysis, we found a significant positive correlation of CD151 gene expression levels with integrin alpha 6 (Supplementary Fig. 5), thus suggesting a possible tumor invasion-restraining effect of CD151 in this cancer. Further studies investigating the molecular mechanisms by which CD151 may exert a prognostic role in EAC would be valuable.

We found that the independent prognostic effect of CD151 expression was not seen in patients with advanced stage EAC who had been treated by multimodality therapy. Findings have shown CD151 to increase chemoresistance, possibly through cell adhesion-mediated drug resistance, whereas CD151-ablated cells show increased sensitivity to DNA-damaging agents and some targeted therapies (e.g., ErbB2-antagonists).[24,37] It is therefore possible that the effect of chemo- and/or radiotherapy in patients expressing low tumor levels of CD151 may be enhanced, thereby diminishing any differences in survival outcomes based on examination of gene expression levels alone.

In summary, our findings suggest that survival is significantly better among EAC patients with high tumor levels of CD151 than among those with low levels if they have not been exposed to multimodality therapy.

Consequently, pre-therapeutic measurements of tumor CD151 levels could provide clinically valuable information to guide treatment decisions. Future studies should aim at elucidating the clinical importance of this finding in the setting of neoadjuvant treatment protocols, as well as how this novel biomarker may be exploited from a therapeutic perspective.

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DISCLOSURE None to declare.

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