Increase of CD45-positive Immune Cells in Liver Parenchyma Indicates a More Favorable Prognosis for Patients With Barrett's Cancer

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Abstract. *Background:* Despite advances in perioperative management, the 5-year survival rate of patients with esophageal adenocarcinoma (Barrett's cancer) is poor. Adjuvant immunotherapies are currently the subject of clinical trials. The prognostic role of tumor-infiltrating T-lymphocytes (TILs) expressing CD45 has only been investigated in primary tumors. The significance of TILs in the target organs of distant metastases, in particular the liver, is unclear. This study examined the influence of CD45-positive cells in liver parenchyma and primary tumors on cumulative survival. *Materials and Methods:* The density of CD45-positive cells was analyzed immunohistochemically using tissue microarrays. Sixty-five patients for whom a liver biopsy was available in addition to the primary tumor were included in the study. Liver metastases were found in 21 patients. The results of the immunohistochemical analysis were correlated with patient's outcomes. The Cox proportional hazard model was used to compute mortality hazard ratio in consideration of clinical variables. *Results:* Elevated density of CD45-positive cells in the liver biopsy corresponded with a better cumulative survival rate ($p < 0.001$), while no significant differences were found for primary tumors. Multivariate Cox regression analysis showed that a high density of CD45-positive cells in the liver parenchyma was an independent prognostic parameter of longer overall survival (hazard ratio(HR)=0.432, $p=0.048$). *Conclusion:* The density of CD45-positive cells in the liver

parenchyma is an easily measured prognostic biomarker that can identify patient subgroups with a better prognosis. In addition, the density of CD45-positive cells in the liver may assist as a criterion for selecting patients with a high potential for response to adjuvant immunotherapy.

The incidence of adenocarcinoma of the esophagus (Barrett's cancer) has increased across the globe (1, 2). Adenocarcinoma of the esophagus is most commonly associated with initial Barrett's esophagus due to gastroesophageal reflux disease (3). In most cases where the curative multimodal approach to treatment is taken, a radical *en bloc* esophageal resection with radical lymph node dissection is performed with perioperative chemotherapy (4). Depending on the individual tumor type, neoadjuvant therapy may make sense (5). In recent years, it has been possible to reduce postoperative mortality and morbidity through improved perioperative management, the development of minimally invasive techniques, and the centralization of specialized centers. Despite this technical progress, the 5-year survival rate remains low at less than 15%. Thus, innovative therapies are urgently needed (6).

Tumor growth behavior is determined not only by tumor biology, but also by the immune reaction of the host. This consists of humoral and cellular components and interaction with the tumor cells and tumor stroma. In recent years immunotherapy has seen substantial advancement with immune checkpoint inhibition in the treatment of solid tumors, especially malignant melanoma (7, 8). The efficacy of programmed cell death protein 1 (PD1) blockade for adenocarcinoma of the esophagus is presently the aim of clinical trials (9-11). The current challenge lies in using biomarkers to identify which patients would respond to adjuvant immunotherapy.

CD45 (protein tyrosine phosphatase, receptor type C; PTPRC) is a pan-leukocyte marker that is simple to measure. This signaling molecule is an important regulator of T- and B-cell antigen receptor signaling. Activated, memory and

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cytotoxic tumor-infiltrating T-lymphocytes (TILs) belong to the cellular mechanisms of the immune reaction and express CD45 on their cell surface (12).

The aim of our study was to investigate the influence of CD45-positive immune cells in liver parenchyma and primary tumors on the prognosis of patients with adenocarcinoma of the esophagus.

Materials and Methods

Study design and patients. A group of 65 patients who underwent radical *en bloc* esophageal resection with radical lymph node dissection with metachronous or synchronous liver biopsy at the University Medical Centre Hamburg-Eppendorf between 1992 and 2009 were included in this study. Of these patients, 56 (86%) were male and nine (14%) female. Ongoing follow-up data were available for 60 patients. The mean age of patients at time of surgery was 63.4 years; none of the patients received neoadjuvant therapy and all patients underwent surgery for curative purposes. Tumor stage and degree of differentiation were classified according to the seventh edition of the TNM Classification of Malignant Tumours published by the Union for International Cancer Control (UICC) (13). All tumor stages ranging from carcinoma *in situ* to advanced stages with distant metastases were included. Liver biopsies were performed to rule out or confirm metastases. Liver metastases were found in 21 patients, while the suspicion of liver metastases was not confirmed in 44 patients.

The use of the tissue samples in this study was approved by the Ethics Committee of the Hamburg Chamber of Physicians (approval number: PV3548) and written consent was obtained from the patients.

Tissue microarray (TMA) construction. The tissue punches were taken from patients with histologically confirmed adenocarcinoma of the esophagus. The areas of the primary tumors and suspected liver metastases were marked on hematoxylin and eosin-stained tissue sections of the samples. These marked tissue sections served to indicate the sites for making sampling from the donor paraffin blocks. TMA construction was performed as described previously (14). The tissue punches (0.6 mm diameter) were taken from the marked regions using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD, USA). Tissue cylinders from each of the 65 primary tumors with liver biopsy and liver parenchyma were inserted into the 25×35 mm paraffin block to create the TMA used in this study. The TMA block was cut into 4-µm sections and mounted on slides using Paraffin Sectioning Aid System (Instrumedics, Hackensack, NJ, USA).

Immunohistochemistry and analysis. CD45 was marked immunohistochemically on the TMA sections. Briefly, after antigen retrieval of the deparaffinized tissue sections, the primary antibody to CD45 (Abcam, Cambridge, UK) was used according to the manufacturer's instructions. All samples were incubated overnight at 4°C. Staining was performed according to the labeled (strept)avidin-biotin method (HRP-AEC System; R&D Systems, Minneapolis, MN, USA). Evaluation of the density of CD45-positive cells within the spots on the stained TMA sections was carried out using light microscopy. Differentiation was made between the following cell densities: none (score 0), low (score 1), moderate (score 2) and high (score 3). For

Table 1. *Clinical and pathological characteristics of the patients according to the density of CD45-positive cells.*

Characteristic	CD45, n (%)		p-Value
	Low (n=14)	High (n=40)	
Gender			
Female	2 (3.7%)	7 (13.0%)	0.781
Male	12 (22.2%)	33 (61.1%)	
Tumor stage			
pT1	1 (2%)	11 (22%)	0.104
pT2	3 (6%)	11 (22%)	
pT3	8 (16%)	15 (30%)	
pT4	1 (2%)	0 (0%)	
Lymph node metastasis			
pN0	2 (4.2%)	16 (33.3%)	0.081
pN1	11 (22.9%)	18 (37.5%)	
pN2	0 (0%)	1 (2.1%)	
Distant metastasis			
M0	1 (1.8%)	36 (65.5%)	<0.001
M1	13 (23.6%)	5 (9.1%)	
Residual tumor			
R0	6 (11.5%)	33 (63.5%)	0.002
R1	3 (5.8%)	4 (7.7%)	
R2	5 (9.6%)	1 (1.9%)	
Grading			
1	0 (0%)	3 (6%)	0.404
2	4 (8%)	16 (32%)	
3	9 (18%)	18 (36%)	
UICC* classification			
I	0 (0%)	5 (11.9%)	<0.001
II	0 (0%)	10 (23.8%)	
III	1 (2.3%)	6 (14.3%)	
IV	13 (31.0%)	7 (16.7%)	

*UICC: Union for International Cancer Control (13).

further analysis, cell densities with scores of 0 and 1 were summarized as low CD45 density and spots with scores of 2 and 3 as high CD45 density. This evaluation was carried out by two experienced investigators without knowledge of the patient data.

Statistical analysis. For statistical analysis SPSS 22 (IBM, Armonk, NY, USA) was applied. Descriptive statistics were used to describe patient baseline characteristics. Correlations between parameters were performed using Pearson's correlation for continuous variables, and for discrete variables the Chi-square test or Fisher's exact test was used. Events considered for survival analysis were death due to cancer diagnosis. When no event was recorded, the patients were censored at the last contact for statistical evaluation. Survival curves for overall survival of the patients were plotted (Kaplan–Meier method) and analyzed by implementing the log-rank test. Results are presented as median survival in months with 95% confidence interval (CI) and number of patients at risk. The overall survival (OS) was computed as the time period from the date of surgery to either the date of death or last follow-up, whichever occurred first. The Cox proportional hazards model was used to examine multiple variables in one model, using low CD45 density as baseline hazard

Figure 1. Analysis of overall survival in histologically confirmed adenocarcinoma of the esophagus with synchronous or metachronous liver biopsy. Survival is shown according to the density of CD45-positive cells in the liver biopsy (A) and primary tumor (B). Differentiation was made between low and high density of CD45-positive cells. A high density of CD45-positive cells in the liver biopsy significantly corresponded with improved cumulative survival ($p=0.001$), while no significant association was found for primary tumors. Each tick mark represents one patient censored. p -Values were calculated by log-rank tests.

of 1.0. In this analysis the variables age, gender, pT, pN, and resection status were included. Statements of significance refer to p -values of two-tailed tests that were less than 0.05.

Results

The prepared TMA encompassed samples from 65 primary tumors and 65 liver biopsies, in 21 of which liver metastases were confirmed. Only samples with discernible immunohistochemical results were included in the analysis. The median age of the patients was 63.4 years. Clinicopathological associations of the CD45-positive cell density are summarized in Table I. Briefly, patients with a high density of CD45-positive cells in the liver samples less frequently had distant metastasis (pM). Moreover, a high CD45-positive cell density was associated with a lower UICC classification. R2 resections were more frequent in patients with low density of CD45-positive cells in the liver sample. No significant differences in CD45 density with regard to gender, pT, pN and grading were found.

A statistically significant difference was seen for the liver biopsy in regard to cumulative survival depending on the extent of CD45-positive cells in the liver biopsy. Patients with high density of CD45-positive cells in the liver biopsy had a statistically significant longer time of survival ($p=0.001$; Figure 1A). The median survival of patients with low density of CD45-positive cells in the liver sample was 6.4 months, whereas patients with high CD45-positive cell density had a prolonged median survival of 25.2 months. No significant differences concerning length of survival in

Table II. Multivariable analysis of CD45-positive cell density, age, sex, tumor stage (pT), lymph node metastasis (pN) and residual tumor (R).

		HR (95% CI)	p -Value
CD45	Low vs. high	0.432	0.048
Gender	Female vs. male	1.583	0.419
Age	Year increase	0.999	0.949
pT	Category increase	1.469	0.175
pN	Category increase	1.653	0.277
R	Category increase	1.504	0.248

CI: Confidence interval; HR: hazard ratio.

connection with density of CD45-positive cells of primary tumors was found (Figure 1B).

Multivariate Cox regression analysis showed that a high density of CD45-positive cells in the liver parenchyma was an independent prognostic parameter of longer overall survival (hazard ratio=0.432, $p=0.048$) (Table II).

Discussion

Adenocarcinoma of the esophagus is an aggressive malignant tumor of increasing significance and has often already metastasized at time of diagnosis. Tumor progression of esophageal adenocarcinoma is influenced not only by tumor biology, but also by the immune reaction of the host (15). The aim of our study was to evaluate a correlation between survival and the number of inflammatory cells in the liver parenchyma

and primary tumor. The pan-inflammatory cell marker CD45 was immunohistochemically stained on the TMA created for this study. Here, CD45 antibody was used to detect tumor-infiltrating, activated memory and cytotoxic T-lymphocytes (TILs), as before (16). The underlying assumption is that the presence of TILs in the tumor environment and within the primary tumor or metastasis lead to a quicker and more effective immune reaction to the expressed tumor antigens. A differentiation between intratumoral and peritumoral infiltrating inflammatory cells did not appear worthwhile, because of the small section in the TMA punches used for the study. In accordance with recently published studies, the stained immune cells were evaluated as being tumor-associated because their presence in the tissue most probably was induced by disseminated tumor cells (17). In addition, CD45 is not expressed in normal liver parenchyma (18).

The results showed that patients with high density of CD45-positive immune cells in the examined liver parenchyma had a significantly longer length of survival ($p=0.001$, Figure 1A), while there was no significant influence of the density of CD45-positive cells in the primary tumor on length of survival (Figure 1B). The strong correlation of a greater number of CD45-positive cells with favorable prognosis was confirmed by the Cox proportional hazard model. Our data postulate that T-cells expressing CD45 in the liver tissue inhibit micrometastasis and ultimately extend patient survival.

Similar observations have been made concerning histological examination of the local immune reaction to other tumors of the gastrointestinal tract, indicating that tumor infiltration with CD45-positive TILs is associated with a benefit for patients with gastric and colorectal cancer (19, 20).

In previous studies on Barrett's cancer, it has been shown that having a low number of intratumoral CD45-positive immune cells was an unfavorable risk factor for disease-free survival in patients with UICC stages I-IIa. In the case of other advanced carcinomas (stages IIB-IV), the study by von Rauser *et al.* showed no correlation between survival and CD45-positive immune cells in the primary tumor (16). This allows the conclusion that TILs in the early primary tumor stage are of great importance, but that as the disease progresses, less immunogenic tumor cell clones assert themselves and the significance of TILs in the primary tumor declines. However, the data here show that CD45-expressing T-cells in the liver parenchyma have a positive influence on patient survival regardless of primary tumor stage.

In recent years, substantial advancement has been seen in immunotherapy, with immune checkpoint inhibition in the treatment of solid tumors, especially malignant melanoma (8, 10). The correlation between the density of TILs and the response to immunotherapeutic active substances is of growing interest for the purpose of identifying which patients will profit most from immunotherapy and determining the optimal point in time for therapy.

PD1 and programmed cell death 1 ligand 1 (PD-L1) are critical immune checkpoint molecules that negatively affect T-cell activation (21, 22). The antibody-mediated blockade of PD1/PD-L1 effectively increases T-cell activity and consequently the antitumor immune response (8). The antibody-mediated blockade of these molecules has shown remarkable efficacy against malignant melanoma, non-small cell bronchial carcinoma and kidney cell carcinoma, and is already being used in the clinical setting (8, 23-26).

In regard to Barrett's cancer, Loss *et al.* demonstrated that high PD-L1 expression is associated with a poor chance of survival (23). The efficacy of PD1 blockade for esophageal adenocarcinoma is presently the subject of clinical trials (10, 24, 25). The current challenge in clinical practice when using PD1 blockade in esophageal carcinoma lies not only in increasing its efficacy by combining it with surgical resection, chemotherapy, radiation therapy and other biological agents, but also in identifying those patients who will most benefit from adjuvant immunotherapy in terms of a personalized therapy (26). The density of CD45-positive cells in the liver parenchyma is an easily measured prognostic biomarker that can identify subgroups of patients with a better prognosis. In addition, density of CD45-positive cells in the liver can assist as a criterion for selecting patients with great potential for response to adjuvant immunotherapy.

Additional Information

Ethics approval and consent to participate: The use of the tissue samples in this study was approved by the Ethics Committee of the Hamburg Chamber of Physicians and written consent was obtained from the patients. The study was performed in accordance with the Declaration of Helsinki.

Conflict of Interest

All Authors declare no conflict of interest in regard to this study.

Availability of Data and Material

Additional data and material are available upon request.

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Authors' Contributions

M.K., M.R., D.B. and M.B. developed the concept and designed experiments. T.S. and K.G. performed the experiments. T.S., K.G., M.K., M.R. and T.G. collected and analyzed the data. M.K. and M.R. wrote the article. J.I., G.S. and K.B. gave technical support and conceptual advice. All Authors contributed extensively to the work presented here.

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