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Prognostic Role of a Multimarker Analysis of Circulating Tumor Cells in Advanced Gastric and Gastroesophageal Adenocarcinomas

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Key Words

Circulating tumor cells · Gastric cancer · Gastroesophageal adenocarcinoma · Epithelial cell adhesion molecule · Mucin 1 · Survivin · Carcinoembryonic antigen · Keratin 19

Abstract

Objective: We aimed to assess the prognostic value of circulating tumor cells (CTC) in patients with advanced gastric and gastroesophageal adenocarcinomas. **Methods:** The presence of CTC was evaluated in 62 patients with advanced gastric and gastroesophageal adenocarcinomas before systemic therapy and at follow-up through immunomagnetic enrichment for mucin 1- and epithelial cell adhesion molecule (EpCAM)-positive cells, followed by real-time RT-PCR of the tumor-associated genes *KRT19*, *MUC1*, *EPCAM*, *CEACAM5* and *BIRC5*. **Results:** The patients were stratified into groups according to CTC detection (CTC negative: with all marker genes negative; CTC positive: with at least 1 of the marker genes positive). Patients who were CTC positive at baseline had a significantly shorter median progression-free survival (PFS; 3.5 months, 95% CI: 2.9–4.2) and overall survival (OS; 5.8

months, 95% CI: 4.5–7.0) than patients lacking CTC (PFS 10.7 months, 95% CI: 6.9–14.4, $p < 0.001$; OS 13.3 months, 95% CI: 8.0–18.6, $p = 0.003$). Alterations in the marker profile during the course of chemotherapy were not predictive of clinical outcome or response to therapy. Yet, a favorable clinical response depended significantly on CTC negativity ($p = 0.03$). **Conclusion:** Our data suggest that the presence of CTC is a major predictor of outcome in patients with gastric and gastroesophageal malignancies.

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Background

Gastric cancer, amounting to approximately 1 million new cases per year, is still the fourth most common cancer worldwide, with great gender and geographic differences in incidence. Adenocarcinomas of the gastroesophageal junction possess a lower but rising incidence [1]. Despite advances in diagnosis and treatment, the prognosis for these cancer patients is dismal, with a 5-year survival rate below 27% for all stages, making this cancer the second

most common cause of cancer-related death worldwide [2]. This is primarily due to a long asymptomatic course and usually diagnosis at an advanced stage, precluding curative resection. Current prognostic assessment of both cancers is mainly based on morphological criteria categorized in the International Union against Cancer Tumor-Node-Metastasis (TNM) staging system [3] and the Lauren classification [4]. In early-stage gastric cancer, endoscopic ultrasound aides in decisions for immediate resection or neoadjuvant chemotherapy [5]. However, this evaluation cannot address the molecular and genetic heterogeneity of the cancer, precluding an accurate assessment of the individual patient's prognosis. Over the last few years, analysis of human epidermal growth factor receptor 2 (HER2/neu) gene expression has been established as a predictive tool in a first step towards a targeted therapy for these cancers [6]. However, there is an urgent need to develop more broadly applicable parameters in order to improve outcome prediction and therapy monitoring of patients. While back in 1869 Ashworth [7] provided a first report on cells in peripheral blood that resembled those discovered in the primary tumor, refined technologies developed in the last decades have unambiguously demonstrated the presence of circulating tumor cells (CTC) in the peripheral blood of patients with gastric cancer [8, 9].

We previously conducted studies demonstrating that immunomagnetic enrichment followed by real-time PCR analysis of the tumor-associated genes keratin 19 (*KRT19*), mucin 1 (*MUC1*), epithelial cell adhesion molecule (*EPCAM*), carcinoembryonic antigen-related cell adhesion molecule 5 (*CEACAM5*) and survivin (*BIRC5*) can be used to detect CTC as independent predictors of progression-free survival (PFS) in patients with pancreatic [10] and colon cancer [11].

Here, we present a study using this unique technique for the first time to investigate the role of CTC as a prognostic marker in patients with advanced-stage cancer of the stomach and the esophagogastric junction.

Subjects and Methods

Patient Selection

Between January 2010 and December 2011, all patients with histologically proven advanced or metastatic adenocarcinomas of the stomach and the gastroesophageal junction at the Department of Internal Medicine of Chemnitz Central Hospital, one of the largest tertiary care centers in Germany, were enrolled in the study. The cohort included patients initiating a first-line or a second-line chemotherapy and displaying an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0–2, but excluded pa-

tients with previous or secondary carcinoma and severe infection. The patients' characteristics are detailed in table 1.

The therapy regimes were FLO (5-fluorouracil, leucovorin and oxaliplatin), FLOT (5-fluorouracil, leucovorin, oxaliplatin and docetaxel), TOGA (trastuzumab, 5-fluorouracil and cisplatin), PLF (5-fluorouracil, leucovorin and paclitaxel), DCF (docetaxel, cisplatin, 5-fluorouracil and leucovorin), FUFIRI (5-fluorouracil, leucovorin and irinotecan) and AIO (5-fluorouracil and leucovorin).

Before starting new systemic treatment, the patients underwent a clinical evaluation including a physical examination, laboratory analyses, computed tomography scanning of the abdomen and the chest and a baseline 2×10 ml peripheral blood draw for CTC analysis. In order to prevent contamination of epithelial cells, we discarded the first 10 ml of blood and used the following 10 ml of blood for CTC analysis. Along with clinical follow-up, another blood draw was performed for follow-up CTC analysis. The stage of disease was reevaluated after 10–14 weeks depending on the treatment regimen using the Response Evaluation Criteria in Solid Tumors (RECIST) [12] and classified as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). We subdivided the patients into a group with non-PD (including CR/PR/SD) and one with PD or death. Surviving patients were followed up for at least 12 months. The study was approved by the local ethics committee and conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all patients.

CTC Isolation from Blood Samples, mRNA Isolation and DNA Synthesis

CTC were isolated from peripheral blood by using 200 μ l immunomagnetic Dynabeads® (Invitrogen, Karlsruhe, Germany) coated with the antibodies BM7 and VU1D9 (targeting mucin 1 and EpCAM, respectively). After performing ablation (5 times) with phosphate-buffered salt solution, the obtained mucin 1 and EpCAM-positive cells were lysed in 400 μ l Tris-HCl buffer. The cell lysates were stored at -85°C until further processing.

The following mRNA isolation was performed with the Dynabeads® mRNA DIRECT Kit according to the manufacturer's guidelines. For reverse transcription of the purified mRNA into cDNA, we used Sensiscript® Reverse Transcriptase (Qiagen, Hilden, Germany) in combination with Dynabeads® oligo(dT)25 (Invitrogen, Germany).

The resulting cDNA was the template for tumor cell detection and characterization by real-time PCR. Using real-time RT-PCR, epithelial tumor-associated genes found to be expressed in adenocarcinomas of the stomach and the gastroesophageal junction (*KRT19*, *MUC1* [13], *EPCAM* [14], *CEACAM5* [15] and *BIRC5* [16]) were analyzed.

Primers were selected from the Universal ProbeLibrary (table 2) and were designed to be intron spanning (exon specific) so as to eliminate reactivity with genomic DNA. We used the amplification of *ACTB* (primers: forward 5'-GAAGAGCCAAGGACAGGTAC-3'; reverse 5'-CAACTTCATCCACGTTTACC-3') as the internal control as well as to verify the integrity of the RNA and the quality of the samples. PCR amplifications were performed on a Rotor-Gene device in a total volume of 25 μ l containing 12.5 μ l reaction buffer (MESA FAST qPCR MasterMix Plus for SYBR® Assay; Eurogentec, Köln, Germany), 0.1 μ l of each primer, 2 μ l of cDNA and 10.3 μ l of RNase-free H_2O . We used the following ther-

Table 1. Demographic and clinical characteristics of the patients

Evaluated patients	62	Tumor size	
Median age at baseline (range), years	64 (47–81)	T1	3 (4.8)
Sex		T2	9 (14.5)
Female	23 (37)	T3	29 (46.8)
Male	39 (63)	T4	21 (33.9)
Baseline ECOG PS		Node	
0	26 (41.9)	N0	4 (6.5)
1	27 (43.6)	N1	25 (40.3)
2	9 (14.5)	N2	26 (41.9)
Primary tumor		N3	7 (11.3)
AEG I	14 (22.6)	Site of metastasis	
AEG II	9 (14.5)	Liver	22 (35.5)
AEG III	2 (3.2)	Lymph node	29 (46.8)
AEG (all)	25	Peritoneal	26 (41.6)
Gastric cancer	37 (59.7)	Lung	10 (16.1)
Stage at primary diagnosis		Adnexa	6 (9.7)
III	4 (6.5)	Bone	4 (6.5)
IV	58 (93.5)	Spleen	1 (1.6)
Laurén type (gastric cancer only)		Her2 status	
Intestinal	17 (45.9)	Positive	5 (13.5)
Diffuse	20 (54.1)	Negative	15 (40.5)
Grading		Not tested	17 (45.9)
G1	7 (11.3)	Type of therapy at study entry	
G2	19 (30.6)	5-Fluorouracil used	57 (91.9)
G3	28 (45.2)	Cisplatin used	19 (30.7)
G4	8 (12.9)	Oxaliplatin used	33 (53.2)
Line of therapy		Docetaxel used	29 (46.8)
First	55 (88.7)	Irinotecan used	6 (9.7)
Second	7 (11.3)	Trastuzumab used (TOGA)	2 (3.2)

Data are presented as n (%) unless specified otherwise. AEG = Adenocarcinoma of the esophagogastric junction.

Table 2. Intron-spanning primer pairs for each selected gene

Marker	NM reference	Primer sequence	Location	Product size, bp
<i>KRT19</i>	NM_002276.2	forward: GCCACTACTACACGACCATCC reverse: CAAACTTGGTTCGGAAGTCAT	525–545, exon 1 650–630, exon 2/3	126
<i>MUC1</i>	NM_002456.4	forward: TCGTAGCCCCTATGAGAAGG reverse: CCACTGCTGGGTTTGTGTAA	795–814, exon 7/8 865–846, exon 8	71
<i>EPCAM</i>	NM_002354.2	forward: CGTCAATGCCAGTGTACTTCA reverse: TTTCTGCCTTCATCACCAAA	448–508, exon 2 575–553, exon 3	88
<i>CEACAM5</i>	NM_004363.2	forward: ACCACAGTCACGACGATCAC reverse: CTCCACGGGGTTGGAGTT	1052–1071, exon 4 1129–1112, exon 5	78
<i>BIRC5</i>	NM_001168.2	forward: GCCCAGTGTTTCTTCTGCTT reverse: CCGGACGAATGCTTTTATG	284–303, exon 2 369–350, exon 3	86

mocycler protocol for real-time PCR: initial 5 min denaturation at 95°C; 40 cycles carried out by denaturation at 95°C for 5 s, annealing at 59°C for 20 s and extension at 72°C for 12 s. Further technical details, and in particular the calculation of the accuracy, precision, linearity and reproducibility of the PCR procedure, have been described in a previous study [17]. Considering the limitations presented by relative and absolute quantification, we used real-time RT-PCR to verify the presence of the gene rather than to quantify it precisely.

According to the results obtained in our previous investigation, the Cq cutoff below which a marker gene is considered to be positive was defined as 36.0 for *KRT19*, 37.1 for *MUC1*, 36.0 for *EPCAM*, 37.8 for *CEACAM5* and 35.0 for *BIRC5*. A sample was considered to be CTC positive when at least 1 of the marker genes was positive.

Statistical Analysis

PFS was measured as the time elapsed between the baseline CTC assessment (at initiation of the therapy) and the first documentation of progress, death or the last follow-up (in the event that no progression and/or death occurred during the follow-up period). Overall survival (OS) was measured as the time elapsed between the baseline CTC assessment and either the date of death or the last follow-up (in case no death occurred during the follow-up period).

PFS and OS were compared between the CTC-positive and the CTC-negative group with the Kaplan-Meier method, and differences were tested with the log-rank test. Furthermore, a multivariate Cox regression was calculated. The distribution of patients with positive and negative CTC and the clinical response was compared using Fisher's exact test.

All tests were two-sided, and the significance level required was at $p < 0.05$. Statistical analyses were carried out using SPSS (version 19.0; SPSS, Chicago, Ill., USA).

Results

Patient Characteristics

Between January 2010 and December 2011, a total of 62 patients (39 men and 23 women; 37 with gastric and 25 with gastroesophageal cancer) were enrolled. The median age at first blood draw was 64 years (range 47–81). Table 1 provides detailed information on the patients' baseline characteristics.

After a median follow-up of 17.2 months with a range of 1–35 months (95% CI: 14.2–20.3), 61 of the 62 patients had progressed and 55 had died. Median PFS from baseline was 5.9 months (95% CI: 2.8–6.9), and median OS was 7.6 months (95% CI: 5.5–9.8).

CTC at Baseline

At baseline, 69.4% of the patients tested positive for CTC (detection of at least 1 tumor-associated transcript). The detection rate for each marker was as follows: 43.4%

for *KRT19*, 51.6% for *MUC1*, 50.0% for *EPCAM*, 27.4% for *CEACAM5* and 24.2% for *BIRC5*. As previously described, the healthy controls ($n = 40$) did not show any amplification of the marker genes [17].

CTC at Follow-Up

At follow-up, 85.5% of the remaining 61 patients tested positive for CTC (detection of at least 1 tumor-associated transcript). The detection rate for each marker was as follows: 37.1% for *KRT19*, 67.7% for *MUC1*, 59.7% for *EPCAM*, 22.6% for *CEACAM5* and 27.4% for *BIRC5*.

CTC as a Prognostic Marker

Identifying CTC at baseline strongly predicted a significantly shorter median PFS (3.5 months, 95% CI: 2.9–4.2) and OS (5.8 months, 95% CI: 4.5–7.0) as compared with patients lacking CTC at baseline (median PFS: 10.7 months, 95% CI: 6.9–14.4, log-rank $p < 0.001$; median OS: 13.3 months, 95% CI: 8.0–18.6, log-rank $p = 0.003$). The corresponding Kaplan-Meier curves are shown in figures 1 and 2.

As described above, a sample was considered to be CTC positive when at least 1 of the 5 marker genes was positive. We also examined whether instead of a 5-marker panel, the sole detection of a single marker or a combination of 2 or 3 markers has prognostic value. Detection of the transcripts *KRT19* ($p = 0.025$), *MUC1* ($p = 0.007$) or *EPCAM* ($p = 0.009$) alone is sufficient to predict a significantly shorter PFS, while the sole detection of the transcripts *CEACAM5* or *BIRC5* could not predict significant effects on PFS. Regarding a combination of 2 markers (positive if at least 1 of the 2 marker genes was positive), we were able to demonstrate that all possible combinations of 2 markers predicted a significantly shorter PFS (table 3).

When calculating OS, it could be shown that only the sole detection of the transcript *CEACAM5* ($p = 0.006$) was associated with a significantly shorter OS, while the sole detection of the transcripts *KRT19*, *MUC1*, *EPCAM* or *BIRC5* was not associated with a significantly shorter OS. Regarding a combination of 2 markers (positive if at least 1 of the 2 marker genes was positive), we were able to demonstrate that all possible combinations of 2 markers, except the combination of *EPCAM-BIRC5* ($p = 0.05$), predicted a significantly shorter OS.

Predictors of PFS and OS

Univariate Cox regression analysis demonstrated ECOG PS, type of therapy as well as CTC detection (at

Fig. 1. Kaplan-Meier plot of PFS in advanced gastric and gastroesophageal cancer patients with positive versus negative CTC at baseline.

Fig. 2. Kaplan-Meier plot of OS in advanced gastric and gastroesophageal cancer patients with positive versus negative CTC at baseline.

baseline and follow-up) to be significantly associated with both PFS and OS. No significance was found for degree of differentiation of the tumor, site of metastasis, line of therapy, initial involvement of lymph nodes or age. The univariately significant associated factors were included in a multivariate Cox regression analysis. Expression of the tumor-associated marker genes of CTC, irrespective of their nature, at baseline as well as at follow-up remained a strong and independent predictor of PFS and OS (table 4). However, this analysis must be interpreted with caution because the number of covariates exceeds the number of events necessary to get a robust estimate of the regression coefficients.

Correlation between CTC and Radiographic Response

Sixty-one of the 62 patients underwent follow-up imaging in order to be assessed by the RECIST at weeks 10–14 (mean: week 12). One of the patients (1.6%) had a PR, 33 (54.1%) SD and 27 (44.6%) PD. All 27 patients with PD (100%) had tested positive for CTC at the beginning of the study. The sensitivity and specificity of CTC detection were 100 and 22.85%, respectively, and the positive and negative predictive values for the indi-

vidual prognosis were 49 and 100%, respectively. Alterations within the marker profile during the course of chemotherapy were not associated with clinical outcome or response to therapy. Yet, a favorable clinical response depended significantly on CTC negativity ($p = 0.03$, Fisher's exact test; table 5). This can be explained by the fact that our CTC study was based on the presence/absence of CTC rather than on the use of real-time PCR for quantification of these cells. Therefore, alterations in the marker profiles could not be precisely assessed.

Discussion

We demonstrated that a combination of immunomagnetic separation of CTC followed by a real-time RT-PCR analysis of *KRT19*, *MUC1*, *EPCAM*, *CEACAM5* and *BIRC5* can serve as a prognostic tool for PFS and OS in patients with advanced cancer of the stomach and the gastroesophageal junction.

Despite an increased understanding of the pathomechanisms of carcinogenesis and advances in diagnosis and

Table 3. PFS and OS prediction according to different models of tumor-associated transcript positivity**a** PFS

	CTC detection	Estimator, months	Standard error	95% CI		log-rank	
				lower limit	upper limit	sig. (p)	χ^2
<i>Sole marker</i>							
<i>KRT19</i>	neg.	7.41	1.02	5.40	9.42	0.025	5.05
	pos.	4.54	0.56	3.44	5.64		
<i>MUC1</i>	neg.	7.57	2.51	2.66	12.48	0.007	7.27
	pos.	3.90	0.54	2.85	4.95		
<i>EPCAM</i>	neg.	7.41	0.96	5.52	9.30	0.009	6.856
	pos.	3.90	0.40	3.12	4.68		
<i>CEACAM5</i>	neg.	7.34	1.60	4.20	10.48	0.055	3.684
	pos.	3.87	0.74	2.42	5.32		
<i>BIRC5</i>	neg.	5.98	1.85	2.35	9.61	0.239	1.389
	pos.	3.90	1.06	1.83	5.97		
<i>Combination of at least 2 markers (at least 1 positive)</i>							
<i>MUC1-EPCAM</i>	neg.	9.64	1.73	6.24	13.04	0.001	12.057
	pos.	3.90	0.42	3.07	4.73		
<i>KRT19-EPCAM</i>	neg.	7.71	1.43	4.91	10.51	0.001	11.088
	pos.	3.90	0.22	3.46	4.34		
<i>KRT19-BIRC5</i>	neg.	7.71	1.32	5.13	10.29	0.002	9.215
	pos.	3.91	0.37	3.18	4.64		
<i>MUC1-BIRC5</i>	neg.	7.71	1.57	4.64	10.78	0.001	11.41
	pos.	3.80	0.42	2.98	4.62		
<i>EPCAM-BIRC5</i>	neg.	7.57	0.33	6.92	8.22	0.004	8.319
	pos.	3.90	0.41	3.09	4.71		
<i>CEACAM5-BIRC5</i>	neg.	7.41	0.59	6.25	8.57	0.009	6.901
	pos.	3.87	0.34	3.21	4.53		
<i>KRT19-MUC1</i>	neg.	9.71	1.62	6.53	12.89	0.000	12.682
	pos.	3.90	0.22	3.46	4.34		
<i>KRT19-CEACAM5</i>	neg.	7.71	1.32	5.13	10.29	0.004	8.213
	pos.	4.16	0.38	3.41	4.91		
<i>MUC1-CEACAM5</i>	neg.	7.71	1.57	4.64	10.78	0.001	10.425
	pos.	3.87	0.31	3.27	4.47		
<i>EPCAM-CEACAM5</i>	neg.	7.71	1.93	3.93	11.49	0.003	9.087
	pos.	3.90	0.44	3.04	4.76		
<i>KRT19-MUC1-EPCAM</i>	neg.	9.74	1.13	7.53	11.95	0.001	15.493
	pos.	3.90	0.37	3.18	4.62		
All markers (at least 1 positive)	pos.	10.70	2.13	6.90	14.40	0.001	16.414
	neg.	3.50	0.41	2.90	4.20		
	total	4.82	1.03	2.79	6.85		

treatment, the overall prognosis remains dismal and selecting the most beneficial therapy for advanced gastric and gastroesophageal adenocarcinomas is challenging. Although patients with metastatic gastric cancer have benefited from the introduction of the first predictive marker *erbB-2* [18], there is an urgent need for other prognostic and predictive molecular markers to better elucidate the heterogeneity of gastric cancer. The addition of CTC analysis to the standard evaluation of these

cancers could lead to more reliable and accurate disease prediction and help identify possible novel therapeutic targets.

Currently, a variety of methods for detecting CTC from peripheral blood are available, including flow cytometry, immunomagnetic separation, CTC microchip technology, PCR-based approaches and the FDA-approved and widely accepted CellSearch® [19]. All of these techniques have distinct advantages and limitations. In

Table 3 (continued)**b OS**

	CTC detection	Estimator, months	Standard error	95% CI		log-rank	
				lower limit	upper limit	sig. (p)	χ^2
<i>Sole marker</i>							
<i>KRT19</i>	neg.	8.75	0.86	7.06	10.44	0.078	3.102
	pos.	6.21	0.83	4.58	7.84		
<i>MUC1</i>	neg.	9.71	1.93	5.93	13.49	0.102	2.680
	pos.	6.24	0.79	4.70	7.78		
<i>EPCAM</i>	neg.	9.64	1.59	6.53	12.75	0.108	2.577
	pos.	5.44	0.74	4.00	6.88		
<i>CEACAM5</i>	neg.	9.64	0.99	7.70	11.59	0.006	7.438
	pos.	5.44	0.66	4.15	6.73		
<i>BIRC5</i>	neg.	8.52	1.32	5.93	11.11	0.081	3.054
	pos.	6.31	0.68	4.99	7.64		
<i>Combination of at least 2 markers (at least 1 positive)</i>							
<i>MUC1-EPCAM</i>	neg.	11.87	2.24	7.47	16.27	0.021	5.295
	pos.	5.98	0.64	4.72	7.24		
<i>KRT19-EPCAM</i>	neg.	11.34	2.44	6.57	16.12	0.027	4.894
	pos.	5.44	0.72	4.04	6.85		
<i>KRT19-BIRC5</i>	neg.	9.71	1.84	6.11	13.31	0.009	6.729
	pos.	5.44	0.63	4.21	6.67		
<i>MUC1-BIRC5</i>	neg.	11.34	2.10	7.22	15.46	0.012	6.261
	pos.	5.44	0.70	4.07	6.81		
<i>EPCAM-BIRC5</i>	neg.	9.97	2.32	5.42	14.52	0.050	3.844
	pos.	5.44	0.76	3.95	6.93		
<i>CEACAM5-BIRC5</i>	neg.	9.97	2.02	6.01	13.93	0.001	10.381
	pos.	5.44	0.77	3.94	6.94		
<i>KRT19-MUC1</i>	neg.	11.87	2.42	7.12	16.62	0.031	4.665
	pos.	6.21	0.58	5.07	7.35		
<i>KRT19-CEACAM5</i>	neg.	9.71	1.85	6.08	13.34	0.024	5.130
	pos.	6.21	0.57	5.09	7.33		
<i>MUC1-CEACAM5</i>	neg.	11.34	2.10	7.22	15.46	0.021	5.288
	pos.	5.98	0.68	4.65	7.31		
<i>EPCAM-CEACAM5</i>	neg.	11.34	1.88	7.66	15.02	0.049	3.875
	pos.	5.98	0.61	4.79	7.17		
<i>KRT19-MUC1-EPCAM</i>	neg.	13.18	1.14	10.95	15.41	0.007	7.334
	pos.	5.98	0.56	4.89	7.07		
<i>All markers (at least 1 positive)</i>	pos.	13.30	1.05	11.12	15.24	0.003	7.183
	neg.	5.80	0.61	5.01	7.41		
	total	7.57	1.00	5.61	9.53		

pos. = Positive; neg. = negative; sig. = significance.

contrast, our assay combines the advantage of dual (mucin 1- and EpCAM-based) immunomagnetic enrichment of disseminated tumor cells and subsequent real-time RT-PCR of a spectrum of cancer-associated transcripts. Our dual immunomagnetic enrichment procedure was based on the specific antibodies VU1D9 and BM7 captur-

ing EpCAM and mucin 1 antigens, which are highly expressed in gastric [20, 21] and esophageal [22, 23] cancer tissue.

However, it has been proven that all EpCAM-based enrichment systems share the limitation that they could miss CTC [24–26] or capture other than cancer cells. By

Table 4. Multivariate Cox regression analysis for prediction of PFS and OS among univariately significant parameters

Parameters	Categories		PFS risk from blood draw				OS risk from blood draw			
	pos.	neg.	HR	95% CI	p	patients, n	HR	95% CI	p	patients, n
CTC at baseline	yes	no	4.96	2.2–11.2	0.000	62	4.1	1.7–9.5	0.001	62
EOCG PS	0/1	2	0.31	0.15–0.63	0.001		0.2	0.9–0.42	0.000	
Oxaliplatin used in regime	yes	no	5.67	1.8–18.2	0.004		5.86	1.9–18.6	0.003	
Cisplatin used in regime	yes	no	4.86	1.4–15.4	0.014		6.6	1.9–23.6	0.004	
Docetaxel used in regime	yes	no	0.4	0.2–0.8	0.012		0.66	0.3–1.3	0.246	
Irinotecan used in regime	yes	no	3.36	0.8–14.2	0.099		6.8	1.5–31.8	0.015	
Trastuzumab used in regime	yes	no	1.8	0.2–15.4	0.595		2	0.23–17	0.531	
CTC at follow-up	yes	no	3.84	1.6–9.3	0.003	61	6.5	2.0–21.3	0.002	61
EOCG PS	0/1	2	0.28	0.13–0.59	0.001		0.21	0.09–0.47	0.000	
Oxaliplatin used in regime	yes	no	1.8	0.51–6.5	0.359		3.2	0.8–12.1	0.094	
Cisplatin used in regime	yes	no	2.01	0.49–8.3	0.332		3.4	0.75–15.1	0.114	
Docetaxel used in regime	yes	no	0.9	0.45–1.8	0.763		0.69	0.33–1.4	0.691	
Irinotecan used in regime	yes	no	2.2	0.43–11.6	0.345		2.7	0.48–14.9	0.265	
Trastuzumab used in regime	yes	no	1.6	0.19–13.6	0.669		1.8	0.2–14.9	0.611	

pos. = Positive; neg. = negative; HR = hazard ratio.

Table 5. CTC and correlation with response assessment by imaging (computed tomography) at follow-up using Fisher's exact test

	Non-PD (by RECIST)			PD (by RECIST)			Fisher's exact p value
	total	CTC neg.	CTC pos.	total	CTC neg.	CTC pos.	
Patients	34	9	25	27	0	27	0.003

pos. = Positive; neg. = negative.

selecting an additional antigen such as mucin 1 that is ubiquitously and abundantly present on tumor cells [27] but that is absent on cells of hematopoietic origin, we enhanced the possibility of successful CTC isolation.

The obtained and isolated CTC were analyzed for transcripts that play an important role in gastric cancer biology. High mRNA levels of *BIRC5* (survivin) – a gene that encodes an antiapoptotic protein belonging to the class of inhibitors of apoptosis proteins – in peripheral blood has prognostic value in gastric cancer [28]. Studies using RT-PCR for the detection of mucin 1, keratin 19 and CEACAM5 in the peripheral blood of patients with gastric cancer are heterogeneous and controversial. Although *MUC1* [29], *CEACAM5* [30] and, in particular, *KRT19* [31] were found to predict postoperative recurrence, Bertazza et al. [28] demonstrated that the latter 2

markers (*KRT19* and *CEACAM5*) did not correlate with survival. Using nested RT-PCR, Matsumura et al. [32] detected EpCAM positivity in the peripheral blood of 30% of gastric cancer patients without validating them as prognostic parameters.

At baseline, we detected *EPCAM*, *MUC1*, *KRT19*, *CEACAM5* and *BIRC5* in 50, 51.6, 43.4, 27.4 and 24.2% of our blood samples, respectively. A total of 69.4% of the peripheral blood samples of our patients showed at least 1 tumor-associated marker on mRNA amplification. This high proportion, as compared to most other studies [33], is due to our patient cohort, which presented exclusively with advanced-stage carcinomas. Our results demonstrate that testing positive for at least 1 of 5 tumor-associated marker mRNAs strongly predicts PFS as well as OS. This is novel and indicates that the ability of an ad-

vanced cancer to progress does not depend on a few but on several major functional molecular alterations.

However, some limitations have to be considered. Our study cohort was relatively small and exposed to various types and lines of chemotherapy that may introduce bias. Moreover, undifferentiated CTC subpopulations that emerge from epithelial mesenchymal transition or cancer stem cells may have distinct patterns of gene expression, and therefore might have escaped our current detection methods. As a consequence for CTC separation and analysis, we have to target further tumor-associated antigens and also determine additional genes representing the variety of metastatic spreading in order to illuminate the mutational heterogeneity of cancer.

Conclusion

Our data suggest an independent prognostic value of CTC as identified by our method of magnetic bead selection using 2 common tumor surface markers, followed by PCR quantification of 5 key cancer-associated transcripts. Further investigation should help in establishing the role of CTC in clinical practice, especially as a parameter of (immediate) therapy response.

Disclosure Statement

The authors declare that they have no competing interests.

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