Absence of HER2 Expression of Circulating Tumor Cells in Patients with Non-Metastatic Esophageal Cancer

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Abstract. *Background/Aim:* The clinical significance of circulating tumor cells (CTC) in non-metastatic esophageal cancer (EC) remains controversial and the cellular and molecular characteristics of CTCs are poorly understood. Especially the frequency and oncological impact of HER2 expression in CTCs in patients with EC have not been evaluated until now. *Materials and Methods:* In this single-center, prospective study, peripheral blood samples were obtained preoperatively from 45 patients who were diagnosed with resectable EC. CTC detection and HER2 expression were performed using the CellSearch System. Data were correlated with clinicopathological parameters and patient outcomes. *Results:* The study included 13 patients with squamous cell carcinomas (SCC) and 32 patients with adenocarcinomas (AC). HER2 gene amplification in the primary tumor was detected in 9.1% of patients. One or more CTCs were detected in 15.6% (SCC 1/13; AC 6/32) of the patients. None of the detected CTCs showed HER2 expression. Patients with CTCs showed significantly shorter relapse-free ($p < 0.001$) and overall survival ($p = 0.015$) than CTC-negative patients. *Conclusion.* This is the first study analyzing HER2 expression and the clinical significance of CTCs in patients with non-metastatic EC using an automated immunomagnetic detection system. HER2 expression in CTCs is very rare in patients with non-metastatic EC and seems to have a low clinical and oncological impact.

Cancer of the esophagus, despite technical advances, is still a highly lethal malignancy. Most esophageal cancers (EC) are squamous cell or adenocarcinomas. Although the incidence of squamous cell carcinoma (SCC) is decreasing, the incidence of adenocarcinoma (AC) arising from Barrett's esophagus is dramatically increasing.

More than two thirds of all patients with esophageal cancer develop metastases or local recurrence and circulating tumor cells (CTCs) or disseminated tumor cells are supposedly playing a key part in this development (1-3). Previous studies showed that the presence of CTCs is correlated with shorter overall survival in patients with metastatic disease (1, 4, 5). In EC, CTC detection is an independent prognostic marker of overall and recurrence-free survival. However, its significance in non-metastatic EC remains controversial and the molecular biological characteristics of CTCs are poorly understood (4, 6).

The human epidermal growth factor receptor gene HER2 is known to play a role in the development of malignancies and is often linked to a poor prognosis, for example in breast cancer (7). The HER2-inhibitor trastuzumab has been shown to be very effective against breast cancer overexpression HER2 (8, 9). In EC however, there is no correlation between the presence of HER2 in the primary tumor and patient survival (10-12). Stoecklein *et al.* showed that HER2 gain in at least a single disseminated tumor cell (DTC) in bone marrow was a prognostic factor of poor survival (13). However, the frequency and oncological impact of HER2 expression in CTCs in patients with EC has not been evaluated until now.

Materials and Methods

Patients. Forty-seven patients with non-metastatic EC were prospectively enrolled in this single-centre study. All patients underwent surgery at the University Hospital Hamburg-Eppendorf, and in all cases no evidence of metastatic disease was evident in preoperative staging. Forty-five patients had an *en-bloc* esophageal resection with radical lymph node dissection with curative intention.

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Table I. Patients' and tumor characteristics.

	Patients (n=45)	%	CTC pos.	%	p-Value	HER2 pos. CTC
All	45	100	7	15.6		
Gender					0.583	
Male	35	77.8	6	17.1		0
Female	10	22.2	1	10		0
Age (years)					0.826	
≤65	24	55.3	4	16.7		0
>65	21	44.7	3	14.3		0
pT stage					0.152	
T1	12	26.7	2	16.7		0
T2	9	20.0	0	0		0
T3	20	44.4	3	15.0		0
T4	4	8.9	2	50.0		0
pN stage					0.999	
N0	25	55.6	4	16.0		0
N1	7	15.6	1	14.3		0
N2	6	13.3	1	16.7		0
N3	7	15.6	1	14.3		0
Stage groups (UICC)					0.581	
IA	11	24.4	2	18.2		0
IB	4	8.9	0	0		0
IIA	9	20.0	2	22.2		0
IIB	4	8.9	0	0		0
IIIA	3	6.7	0	0		0
IIIB	4	8.9	0	0		0
IIIC	10	22.2	3	30.0		0
Histology					0.354	
SCC	13	28.9	1	7.7		0
AC	32	71.1	6	18.8		0
Barrett's esophagus					0.100	
No	36	80.0	4	11.1		0
Yes	9	20.0	3	33.3		0
HER2 status of primary tumor				0.051		
Negative	40	90.9	5	12.5		0
Positive	4	9.1	2	50.0		0

In two patients, esophagectomy was not performed due to the presence of distant tumor spread detected intraoperatively. These two patients were excluded from the study. Histopathological analysis was performed in accordance with the seventh edition of the tumour-node-metastasis classification (TNM) of the International Union against Cancer (14).

The study was approved by the Ethics Committee of the Hamburg Chamber of Physicians and informed consent was obtained from all patients. Postoperative follow up was conducted at 3-month intervals for the first 2 years and afterwards at 6-month intervals.

Clinical samples. Preoperatively, peripheral blood samples (7.5 ml) were collected in CellSave (Menarini Silicon Biosystems, Castel Maggiore, Italy, former Janssen Diagnostics, Raritan, NJ, USA) preservative tubes, stored at room temperature and processed within 48 h.

CTC enumeration and HER2 determination. CTC enumeration by the CellSearch system (Menarini) was performed using the CellSearch Epithelial Cell Kit (Menarini). The blood samples were

enriched for cells that expressed epithelial cell adhesion molecules (EpCAM) with antibody-coated magnetic beads. The nucleus was labelled with fluorescent nucleic acid dye 4,2-diamidino-2-phenylidole (DAPI). To distinguish epithelial cells from leukocytes, fluorescently labelled monoclonal antibodies specific for leukocytes (CD45) and epithelial cells (keratins [KER] 8, 18, and 19) were used. The samples were measured by CellTracks Analyzer II (Menarini) and an event was classified as a tumor cell with the phenotype KER+, DAPI+, CD45- and a round or oval cell morphology. The cut-off value for CTC positivity was 1/7.5 ml.

In order to analyze HER2 expression in the detected CTCs, the CellSearch Tumor Phenotyping Reagent HER-2 (Menarini) was used following manufacturers' instructions. Immunoscoring of HER2 expression by fluorescence intensity was conducted as described by Riethdorf *et al.* (15).

Statistical analysis. SPSS 22 software (SPSS Inc., Chicago, IL, USA) was used. Histological characteristics were expressed as descriptive statistics. The χ^2 test was used to investigate the

Figure 1. Kaplan-Meier curves for overall (A) and relapse-free (B) survival according to CTC status.

association between CTCs and histopathological parameters. Univariate survival analysis was plotted by the Kaplan-Meier method and analyzed using the log-rank test. The results were presented as the median survival in months with the 95% confidence interval (CI) and number of patients at risk. Significance was indicated by p -values of less than 0.05 in 2-tailed tests.

Results

Patient characteristics. Overall, 13 patients with non-metastatic SCC and 32 patients with non-metastatic AC of the esophagus were enrolled. Patient and tumor characteristics are shown in Table I. The mean patient age was 63.3 years, 35 (77.8 %) were males and 10 (22.2 %) were females (Table I).

CTC detection and HER2 expression. CTCs were detected in 15.6 % of all patients. Of note, CTCs were detected in only 1 of 13 patients with SCC; in this sample 3 CTCs were identified. However, 6 out of 32 patients with AC had 1 CTC or more; in one patient 31 CTCs were detected and in the other five patients 1 CTC was detected. None of the detected CTCs expressed HER2. HER2 gene amplification in the primary tumor was seen in 9.1% of patients, two of whom were CTC positive and HER2-negative (Table I).

Survival analysis. The median survival time was 37 months. The median follow-up time of the surviving patients was 33 months. Patients with CTCs showed significantly shorter relapse-free ($p<0.001$) and overall survival ($p=0.015$) compared to CTC-negative patients (Figure 1). Patients with

HER2-positive primary tumors had no significant difference in overall survival compared to patients with HER2-negative primary tumors ($p=0.846$).

Discussion

Preoperative staging in EC remains inaccurate, despite several diagnostic tools, such as endoscopic ultrasound and computed tomography (16, 17). Several studies indicated that CTCs play an important role in the metastatic cascade (18-20) and the detection of CTC in peripheral blood was shown to be a useful prognostic indicator of tumor progression and prognostic outcome (1). Thus, analysis of CTCs is a valuable addition to preoperative staging in EC and may give an insight into intra-tumoral and inter-metastatic heterogeneity (21). Recently, Iwatsuki *et al.* showed that in patients with metastatic EC the HER2 oncogene is amplified in 32.3% of patients and its expression is augmented in disseminated and circulating tumor cells (22). The HER2 oncogene encodes for a transmembrane glycoprotein receptor with intracellular tyrosine kinase activity and belongs to the epidermal growth factor receptor family, controlling epithelial cell growth and differentiation (23, 24). In metastatic breast cancer, HER2 is differentially expressed in the primary tumor compared to metastatic foci in 5% up to 48% of cases (15, 25). This discordance might be useful as a prognostic marker for the clinical outcome of patients with metastatic versus non-metastatic disease. It is also posing a problem in therapeutically targeting HER2 (26). As this treatment will specifically target cells expressing HER2 and not the tumor cells that do not, such a treatment could be

ineffective (25, 27). HER2 expression in metastatic gastrointestinal cancer has been shown to range between 11-73% of the cases (10, 22). In addition, HER2 has been suggested as a prognostic marker for metastatic esophageal cancer disease (13, 22). However, there are no studies on patients with non-metastatic EC.

This is the first study evaluating HER2 expression of CTCs in non-metastatic EC patients by the CellSearch system. This system is the gold standard for selecting and identifying circulating epithelial cells in small amounts (7.5 ml) of blood samples with single-cell sensitivity (28, 29). We observed, that the overall and relapse-free survival of patients with detectable CTCs was significantly lower than the survival of CTC-negative patients. In agreement with previous studies, HER2 amplification in the primary tumor did not have a prognostic impact (13, 30). In a cohort of 7 CTC positive patients including two with HER2-positive primary tumors, no HER2-positive CTCs were detected. This might be due to the fact, that esophageal cancer cells share many chromosomal changes and are well known to have histological heterogeneity. Furthermore, the cut-off value for HER2 positivity in the primary tumor was 10% and therefore HER2 negative cells may have been present, as well (13, 22, 31).

Conclusion

This is the first study analyzing HER2 expression and the clinical significance of CTCs in patients with non-metastatic EC by an automated immunomagnetic detection system. In conclusion, HER2 expression in CTCs is very rare in patients with non-metastatic EC and has no prognostic value, clinical and oncological impact.

Conflicts of Interest

The Authors declare no conflicts of interest.

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