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Cytopathological Heterogeneity of Circulating Tumor Cells in Non-metastatic Esophageal Adenocarcinoma

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Abstract. *Background/Aim: The presence of circulating tumor cells (CTC) has been reported to have an impact on prognosis in different tumor entities. Little is known about CTC morphology and heterogeneity. Patients and Methods: In a multicenter setting, pre-therapeutic peripheral blood specimens were drawn from patients with non-metastatic esophageal adenocarcinoma (EAC). CTCs were captured by size-based filtration (ScreenCell®), subsequently Giemsa-stained and evaluated by two trained readers. The isolated cells were categorized in groups based on morphologic criteria. Results: Small and large single CTCs, as well as CTC-clusters, were observed in 69.2% (n=81) of the 117 specimens; small CTCs were observed most frequently (59%; n=69), followed by large CTCs (40%; n=47) and circulating cancer-associated macrophage-like cells (CAMLs; 34.2%, n=40). Clusters were rather rare (12%; n=14). CTC/CAML were heterogeneous in the cohort, but also within one specimen. Neither the presence of the CTC subtypes/CAMLs nor the exact cell count were associated with the primary clinical TNM stage. Conclusion: Morphologically heterogenic CTCs and CAMLs are present in patients with non-metastatic, non-pretreated EAC.*

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Over the past decades the incidence of esophageal squamous cell carcinoma has decreased significantly in the U.S. and Europe. At the same time, the incidence of adenocarcinoma (EAC) has increased rapidly, especially among men (1). Although overall survival has improved over the years thanks to new treatment strategies, the prognosis is still limited (2). We still lack tools for better pre-therapeutic risk-stratification for cancer recurrence, which would enable us to design patient-adapted treatment strategies. Currently, treatment decisions are being made based on CT-scans, endoscopic findings, endoscopic ultrasound and tumor biopsy. This momentary pre-therapeutic "tumor-image" of the patient does not adequately reflect the actual tumor biology and aggressiveness. More than 50% of the patients initially considered curable will eventually relapse or develop metastasis after complete resection of the tumor (3). This relapse could be due to clinically invisible distant micro-metastases and tumor dissemination at an early time point, or due to lack of treatment response.

A promising tool to evaluate these occult metastases for risk stratification and treatment surveillance is the use of liquid biopsy (4). Liquid biopsy summarizes the analysis of tumor cells and tumor-derived products in body fluids, like circulating tumor cells (CTC) (5-7), circulating tumor DNA (ctDNA) (8, 9) and extracellular vesicles (EVs) (10-12). In the case of EAC, there are very limited studies available regarding CTC presence and their significance in general (5, 13-16). CTC have been found in about 20% of patients using epithelial surface antigen (EpCAM)-dependent isolation devices in EAC. These patients

showed significantly shorter relapse-free and overall survival (OS) in an obsolete surgery-alone treatment protocol. In addition, multivariate analysis identified the presence of CTC as a strong, independent prognostic marker of tumor recurrence and overall survival (5).

Furthermore, the presence of cancer-associated macrophage-like cells (CAML) has been described (17) in blood samples of patients with breast, pancreatic, and prostate cancers. It is hypothesized that CAMLs originate from the local tumor–associated macrophages (TAMs) and have spread into the blood circulation (17, 18). The increased CAML size and higher levels of CAML is correlated with shorter progression-free survival and worse OS in untreated breast cancer patients (19, 20).

CTC heterogeneity has been shown and described in other tumor entities (21-24). We also found a diverse population of CTC in a pilot study of 20 EAC patients undergoing multimodal treatment (14). The exact relevance of the CTC-subpopulations for the prognosis estimation is yet to be discovered.

The aim of this study was to assess the morphological presence, diversity and morphological subgroups of circulating cells in patients with non-metastatic non-treated EAC. To our knowledge no study has investigated the morphologic diversity of CTC in this entity.

Patients and Methods

Patients and study design. We included patients with diagnosed adenocarcinoma of the esophagus in a non-metastatic state (cT1N+ M0 or cT2-4a N0/N+, M0) and before the initiation of any treatment. Every patient was qualified for multimodal treatment and was considered curable. All participants gave full informed consent for material, data acquisition and the following experiments. The study was approved by the Ethics Committee of the Albert-Ludwigs University Freiburg (315/15 FF-MC), Freiburg, Germany. Patients had no history of squamous, adenosquamous or other non-adenocarcinoma tumors. Also, the patients had no history of chemotherapy for gastrointestinal cancer or no prior abdominal or thoracic radiotherapy.

The blood specimens were collected in transfix tubes (Circulating Tumor Cell TransFix/EDTA Vacuum Blood Collection Tubes 9ml, Firma: Cytomark, Caltag Medsystems Ltd, Buckingham, UK) right after the study enrolment. The tubes were sent within 24 hours after blood draw to the CTC laboratory at the Medical Center - University of Freiburg.

CTC analysis. The processing of the blood samples was performed in the laboratory of the University Hospital Freiburg within 96 hours after blood draw from the patient. The CTC enrichment was performed by cell size-based filtration using the ScreenCell® Cyto kit (ScreenCell, Sarcelles, France) as we have reported previously (14). This represents a surface marker independent CTC enrichment method. The SceenCell® system is fitted with microfilters that capture the cells on small metal-rimmed filters via low-pressure vacuum-filtration. The blood samples were processed through two ScreenCell® filtration devices (Paris, France), according to the manufacturer's instructions (3 ml blood per filtration device). The filter was then left to dry at room temperature and was subsequently stained with a standard May-Grünwald Giemsa staining. Every CTC was photographed, documented and categorized by two trained readers on bright field without immunofluorescence staining as previously described (25). Questionable interpretations were evaluated again until consensus was reached and were analyzed by two cytopathologists for verification. Circulating endothelial cells (CECs) also were classified by overall impression (negative, suspicious, or positive). The cut off for small CTC was set at 16 μm. Cells below this size were not counted as potential CTCs. The category "large CTC" was set for cells 25 μm or larger due to the gross quality and size across all specimens and according to previous descriptions (26). Clusters were rather smaller epithelial cells that occurred in clusters. The category CAML was included as described by Adams *et al*. as large cells >25 μm with multilobulated nuclei and a relatively low NC ratio (17). Differences were analyzed based on the initial clinical TNM classification (27).

Table I. *Patient characteristics and cTNM stage*.

Number of patients (n)	117
Gender (male/female) n (%)	103/14 (88.0/12.0)
Age in years (mean)	62.6
BMI in kg/m^2 (mean)	27.7
Clinical TNM stage, n (%)	
cT-Stage	
T1	2 (1.7)
T2	20 (17.2)
T3	85 (73.3)
T3-T4	3 (2.6)
T4	6 (5.2)
Tx	1
cN-Stage	
N0	30 (25.9)
N+	86 (74.1)
Nx	1
cM-Stage	
M0	117 (100)

BMI: Body mass index; T- Stage: size of the primary tumor; N-Stage: degree of spread in regional lymph nodes; M-Stage: presence of distant metastasis.

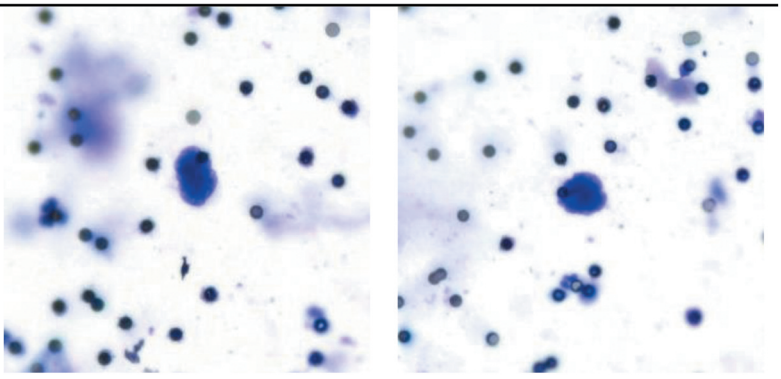
Statistics. No formal sample size calculation was performed. All analyses were performed using SAS 9.3. Data were analyzed descriptively. Categorical data were summarized by absolute and relative frequencies. Continuous data were summarized by mean, standard deviation, median, quartiles and range. The relationship between the prevalence of CTC and the cT and cN status were analyzed. The probability of any CTC was statistically compared between patients with cT1-2 versus cT3-4 and between patients with cN0 versus cN+ using chi-square tests each at a two-sided significance level alpha of 5%. The probability of any CTC was estimated with 95% confidence intervals in the whole study population and in subgroups defined by cT and by cN.

Results

Patients. We included 117 patients with non-metastatic EAC before the initiation of treatment. In two cases the exact cT and cN- stage were unknown. Therefore, these patients were

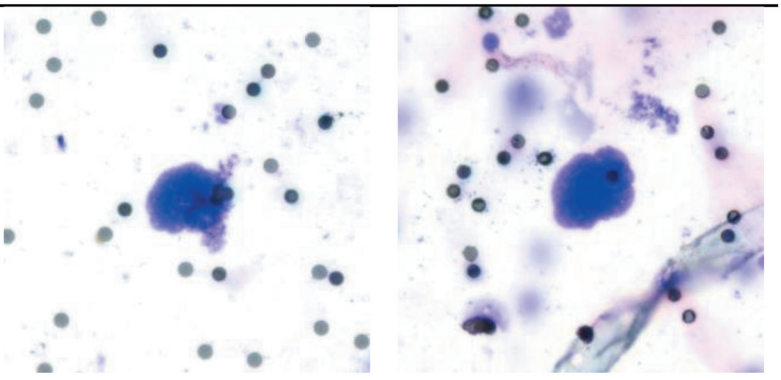
Small CTC

- Enlarged nuclei (≥ 16-24 μm)
- Hyperchromatic nuclei
- Irregular nuclear borders
- Increased NC ratio



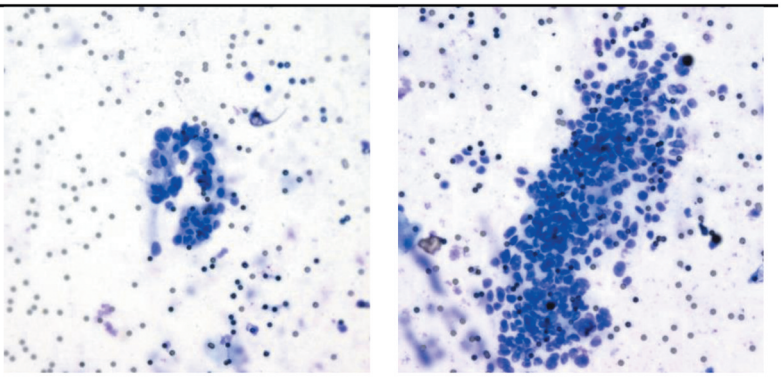
Large CTC

- Enlarged nuclei (≥ 25 μm)
- Hyperchromatic nuclei
- Irregular nuclear borders
- Increased NC ratio



Cluster CTC

- Anisonucleosis
- Hyperchromatic nuclei
- Irregular nuclear borders
- Increased isolated nuclei (>2 pore size)



CAML

- Enlarged, often multilobulated nuclei or separated polymorphic nuclei
- Large remarkable cytoplasm
- Relatively low NC ratio

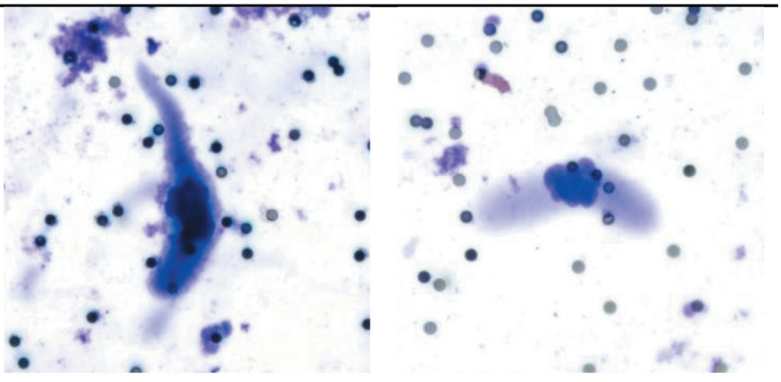


Figure 1. *CTC categorization: Exemplary pictures of the CTC categories. Cells were isolated by ScreenCell®, May-Grünwald Giemsa staining (20× magnified). Filter pores (7.5 μm).*

excluded from the statistical analysis related to cTNM. The average age was 62.5 years and most of the patients were male. The patient characteristics are presented in Table I. In terms of the clinical TNM stage, 73.9 % (n=85) of the study-population had a cT3 stage and had tumor-positive lymph nodes (cN+) on pre-therapeutic imaging. No patient had clinical signs of metastasis (cM0).

CTC categorization. Epithelioid cells with enlarged (>16 μm), irregular, hyperchromatic nuclei and increased nuclear to cytoplasmic (NC) ratio were counted as CTCs. Overall, 69.2% [n=81, 95% confidence interval (CI) (60.9%, 77.6%)] of all specimens were CTC positive.

After identification and documentation of all CTC suspected cells in all patients, we determined four categories of cells based on morphologic features: small and large single-CTCs, cluster-CTCs and circulating cancer-associated macrophage-like cells (CAML). A summary of the morphologic characteristics and exemplary pictures are shown in Figure 1. Fifty-nine percent (n=69) of the patients had small CTCs with nuclei ≥16-24 μm and 40% (n= 47) had large CTCs with nuclei ≥25 μm. Both subpopulations had an increased NC ratio with a very small rim of cytoplasm. Twelve % (n=14) of the patients were positive for cluster CTCs, which showed a very variable cell count. Furthermore, we identified the presence of CAMLs in 34.2% (n=40) of the patients. Some cells were poorly preserved and were not included in the analysis due to lacking cytomorphological features: 75.2 % (n= 88) had single CTC suspicious cells (median of 1 cells/3 ml) and 65.8 % (n=77) naked nuclei without visible cytoplasm (median of 1 cells/3 ml).

The CTC-positive patients showed relatively low counts of all types of CTC/CAML: they had a mean of 6.38 (median 3) small CTCs, a mean of 2.40 (median 1) large CTCs and a mean of 7.48 (median 4) CAML in 3 ml EDTA blood (one filter). The described CTCs, Cluster-CTC and CAML subtypes were observed not only between different patients but regularly within one specimen from the same patient (Figure 2).

CTC association with cTNM-status. The positivity for any type of CTC was not related with the pre-therapeutic T - stage (61.9%, 95%CI=41.1-82.7%, in 21 patients with cT1-cT2 and 70.2%, 95%CI=61.0-79.5% in 94 patients with cT3-cT4, p=0.46) or N-stage (73.3%, 95%CI=57.5-89.2%, in 30 patients with cN0 and 67.1%, 95%CI=57.1-77.1%, in 85 patients with cN+, p=0.52). Furthermore, the number of CTCs showed no relevant differences between the cT - stages and cN – stages (Tables II and III). Two patients were excluded from this statistical analysis, since the exact cTNM stage was not determined. Single patients with a higher cT stage 3-4 had higher maximum CTC counts compared to patients with lower cT stage 1-2, but no relevant difference in mean numbers was observed.

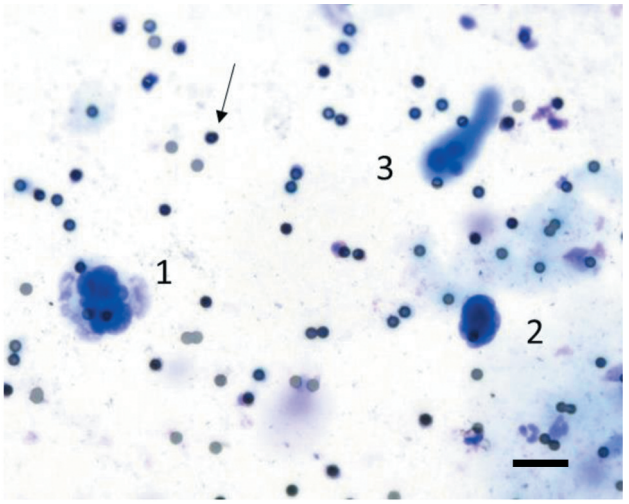


Figure 2. *Representative picture of a large CTC (1), small CTC (2) and CAML (3) in the same sample. Cells were isolated by ScreenCell®, May-Grünwald Giemsa staining (20× magnified). Filter pores (7.5 μm) marked with simple black arrow, Scale bar 30 μm.*

Single patients with clinically apparent lymph node involvement N+ had higher maximum CTC and CAML counts compared to patients with no evidence of clinically proven pre-therapeutic lymph node involvement N0 (Table III). Again, no relevant difference in mean numbers was present

Discussion

The present study shows for the first time the cytomorphology of CTCs in over one hundred patients with non-metastatic adenocarcinoma of the esophagus (EAC). After evaluating all specimens, we determined the presence of four different typical subtypes of cancer-associated cells: we defined the groups of small (16-24 μm) and large (≥25 μm) CTCs, clusters of different sizes and CAMLs (circulating cancer-associated macrophage-like cells). CAMLs were previously morphologically described and characterized by Adams *et al*. using immunofluorescence and morphology (17). Although the CAMLs are thought not to be cancer cells, we listed them next to the CTCs, considering that they originate from the tumor site and are believed to interact with the CTCs (17). The detection of CAMLs is of significance, since there is evidence of high prevalence in different cancer types even in early stages (20). Moreover, increased size of these cells and higher count of CAMLs have been found to be associated with worse progression-free and overall survival in patients with breast cancer (19, 20). In our cohort, 34.2% of the patients were positive for

Table II. *Number of CTCs and CAML in correlation with cT-stage (CTC per Filter= 3 ml blood).*

cT	n	Variable	Mean	Std Dev	Minimum	Median	Maximum
T1-T2	21	Small CTC	2.33	4.75	0	1	22
	21	Large CTC	0.85	1.59	0	0	5
	21	CAML	3.05	7.11	0	0	30
T3-T4	94	Small CTC	2.61	4.49	0	1	33
	94	Large CTC	0.99	1.92	0	0	9
	94	CAML	2.45	5.81	0	0	36

T- Stage: Size of the primary tumor; CTC: circulating tumor cell; CAML: cancer-associated macrophage-like cells.

Table III. *Number of CTCs and CAML in correlation with cN-stage (CTC per Filter= 3 ml blood).*

cN	n	Variable	Mean	Std Dev	Minimum	Median	Maximum
N0	30	Small CTC	3.20	3.85	0	2	14
	30	Large CTC	0.73	1.08	0	0	4
	30	CAML	2.27	3.91	0	0	16
N+	85	Small CTC	2.33	4.73	0	1	33
	85	Large CTC	1.04	2.06	0	0	9
	85	CAML	2.66	6.64	0	0	36

N-Stage: Degree of spread in regional lymph nodes; CTC: circulating tumor cell; CAML: cancer-associated macrophage-like cells.

CAML. The impact of the here-described CAMLs on survival or their possible use as treatment surveillance parameter remains to be determined after clinical follow-up of a minimum of 3 years.

Overall, we found quite low numbers of CTCs and CAMLs in our patient cohort. This is expected, considering the generally lower CTC-detection rate in gastrointestinal cancers (18, 28) and especially the non-metastatic state of our patients (16). The group of small CTCs was most frequently found in our patient cohort. We – as previously reported (25) - defined the cutoff of 16 μm –which is double the size of a filter pore – to ensure the most complete inclusion of possible CTCs. Since these cells are, however, relatively small compared to other CTC, it is unclear if these are true CTCs or undetermined circulating cells. Other groups describe CTCs of this size (29, 30). The relevance of the different morphologically diverse CTC subtypes for overall and progression-free survival remains to be discovered in further analysis.

Additionally, we observed some unidentified cells with damaged cytoplasm, which could be caused by the transport between the site of the blood draw and the study center. This also could influence the number of detected CTCs and could lead to false-low CTC counts across the specimens.

In this study, we described every observed CTC without the use of any cut-off for CTC positivity. Our intention was purely the cytomorphological evaluation of the CTCs. This may very well include an irrelevant fraction of circulating cells. Interestingly, the presence and the actual count of the CTCs and CAMLs showed no association to the clinical TNM stage. Regarding the connection of CTC to the cTNM stage there are contradictory reports concerning other tumor entities. In patients with small cell lung cancer, higher numbers of CTC were observed in patients with lymph node metastasis (N1-3) and III/IV stage of TNM (31). Similar results have been found in patients with colorectal and gastric cancer (28, 32, 33). A meta-analysis of 18 articles on CTCs in esophageal carcinoma has also shown a significant association with TNM staging (34). We must take into consideration, though, that these studies used the postoperative TNM classification, the pTNM stage for the analysis. However, in patients with squamous-cell carcinoma of the esophagus and colorectal carcinoma a significant correlation of the CTCs was described only with the N stage but not with the T stage (35, 36). Also, there are reports suggesting no significant correlation between TNM stage in patients with colorectal carcinoma (37). The association of CTC and cTNM and pTNM is thus yet to be determined.

The results presented here are based on the morphologic appearance of the cells using bright field microscopy and need to be evaluated with further immunofluorescence labelling. The CTC enrichment by size surely has its weaknesses due to the higher probability of false positive results in comparison to methods using cell surface antigens. At the same time this is also a strength, since it allows us a particularly good and detailed overview of the complete picture of likely circulating tumor-associated cells. Further analysis with immunofluorescence labelling and genetic singe-cell characterization of the CTCs are planned.

Most importantly, long-term prospective studies are needed to track the changes in CTCs and CAMLs over the treatment time in patients with EAC. This will hopefully show us the true potential of the separate CTC-subpopulations and CAMLs in treatment surveillance and clarify their significance as prognostic markers.

Conflicts of Interest

J.K. received travel funding from ScreenCell® to the TriCon meeting 2019. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Authors' Contributions

Conceptualization, J.K., B.K. and J.H.; methodology, B.K., T.G. and V.M.; validation, M.P. and S.T.; formal analysis, C.S.; investigation, C.B. , K.G. and J.G.; resources, B.K., S.F.F. and J.H.; data curation, C.B. and K.G..; writing – original draft preparation, J.K.; writing – review and editing, B.K., S.F.F., T.G., C.S., M.P. and J.H.; visualization, J.K.; supervision, B.K.; project administration, BK.; funding acquisition, B.K.

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