Molecular and Cellular Pathobiology

Immune Escape and Survival Mechanisms in Circulating Tumor Cells of Colorectal Cancer

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Abstract

The prognosis of colorectal cancer is closely linked to the occurrence of distant metastases. Systemic dissemination is most likely caused by circulating tumor cells (CTC). Despite the fundamental role of CTC within the metastatic cascade, technical obstacles have so far prevented detailed genomic and, in particular, phenotypic analyses of CTC, which may provide molecular targets to delay or prevent distant metastases. We show here a detailed genomic analysis of single colorectal cancer–derived CTC by array comparative genomic hybridization (aCGH), mutational profiling, and microsatellite instability (MSI) analysis. Furthermore, we report the first gene expression analysis of manually selected colorectal cancer–derived CTC by quantitative real-time PCR (qRT-PCR) to investigate transcriptional changes, enabling CTC to survive in circulation and form distant metastases. aCGH confirmed the tumor cell identity of CellSearch-isolated colorectal cancer–derived CTC. Mutational and MSI analyses revealed mutational profiles of CTC to be similar, but not identical to the corresponding tumor tissue. Several CTC exhibited mutations in key genes such as KRAS or TP53 that could not be detected in the tumor. Gene expression analyses revealed both a pronounced upregulation of CD47 as a potential immune-escape mechanism and a significant downregulation of several other pathways, suggesting a dormant state of viable CTC. Our results suggest mutational heterogeneity between tumor tissue and CTC that should be considered in future trials on targeted therapy and monitoring of response. The finding of upregulated immune-escape pathways, which may be responsible for survival of CTC in circulation, could provide a promising target to disrupt the metastatic cascade in colorectal cancer.

Introduction

Colorectal cancer ranks third among cancer-related deaths in Western countries (1). As the primary tumor can often be controlled by surgical resection, the survival of patients with colorectal cancer is closely linked to the incidence of distant metastases, mostly in the liver (2). It is widely accepted that the development of metastatic disease is mediated by circulating tumor cells (CTC), which are shed into the blood stream by the primary tumor, survive within the circulation, and home to distant organs to establish metastases (3). The prognostic relevance CTC detection in the blood of patients with colorectal cancer has been shown by several studies and a meta-analysis (4–6). Also, we showed that considerably more CTC are detectable in the mesenteric/portal venous blood as compared with central venous blood of patients with primary colon cancer, indicating that the route of hematogenous tumor cell dissemination determines, at least in part, the occurrence of metastatic lesions (7).

Despite the apparent relevance of CTC within the process of metastasis, little is known about the biology of CTC, i.e., their genotypic and phenotypic characteristics. Genomic and chromosomal profiling approaches of CTC differ widely and transcriptomic analyses are still missing mainly due to technical issues and the abundant background of blood cells (8). As a consequence, molecular markers that allow selective targeting of CTC and thus potentially interrupt the metastatic cascade have not been developed yet.

The dramatic phenotypic changes tumor cells need to undergo to become functional and tumorigenic CTC are no longer present in outgrown metastatic tumors and must therefore be reversible (9–11). This reversible phenotype is probably based on temporary differential regulation of metastasis-related genes.
As such changes may present a therapeutic target to inhibit metastatic activity, this study also aimed to evaluate the transcriptomic changes that distinguish circulating from parenchymal tumor cells and for the first time provides data about the transcriptomic characteristics in colorectal cancer–derived CTC.

The present study therefore presents both a comprehensive genomic and a transcriptional analysis of CTC derived from patients with colorectal cancer. Our findings reveal discrepancies in driving mutations between CTC and the corresponding tumor tissue. Furthermore, the results of gene expression analyses of isolated CTC provide evidence for immune-escape mechanisms involved in CTC survival in circulation, which may offer promising targets for future therapeutic approaches to delay or prevent distant metastases in colorectal cancer.

Materials and Methods

**Cell culture**

The colorectal cancer cell line HT-29 was purchased from the Leibnitz Institute DSMZ—German Collection of Microorganisms and Cell Cultures (www.dsmz.de) and maintained in RPMI (PAA) supplemented with 10% fetal calf serum (FCS; PAA), 100 U/mL penicillin (PAA) and 100 μg/mL streptomycin (PAA) in a humidified atmosphere of 5% CO₂ at 37°C. The authenticity of the cell line was regularly confirmed by DSMZ.

**Patient material**

The collection and analysis of solid and liquid patient samples as well as clinopathologic information for that study has been approved by the Medical Ethics Committee of University of Heidelberg (No. 323/2004). Written informed consent was obtained from each patient before surgery. Detailed patient characteristics are given in Supplementary Tables S5–S7.

**Blood samples**

Blood samples were obtained from patients undergoing surgical resection at the Department of Surgery, University of Heidelberg. After induction of general anesthesia, blood was drawn through a central venous catheter, or intraoperatively from the mesenteric vein, depending on the location of the tumor) or from the mesenteric (superior mesenteric vein or inferior mesenteric vein, or intraoperatively drawn through a central venous catheter, or intraoperatively from the mesenteric (superior mesenteric vein or inferior mesenteric vein, or intraoperatively drawn through a central venous catheter, or intraoperatively from the mesenteric (superior mesenteric vein or inferior mesenteric vein). After induction of general anesthesia, blood was obtained from each patient before surgery. Detailed patient characteristics are given in Supplementary Tables S5–S7.

**CellSearch CTC enumeration (Veridex)**

7.5 mL of blood, collected in CellSave Tubes (Veridex), were used for CTC analysis according to the manufacturer’s instructions. Results were evaluated by two independent and Veridex-certified operators; differences were resolved by discussion.

**Genomic DNA amplification of single cells**

We performed single-cell whole-genome amplification (WGA) using an adapter–linker PCR as originally described by Klein and colleagues (12). Single EPCAM⁺ CK(8/18/19)+ cells as detected by the CellSearch System were manually transferred into PCR tubes by a micromanipulator. One micro-liter of Proteinase K mix [1 × OnePhorAll Buffer (OPA: 100 mmol/L Tris, 100 mmol/L MgAcetate, 500 mmol/L KAcetate), 0.65% Tween, 0.65% Igepal, 1.3 mg/mL Proteinase K] was added. Cells were digested for 10 hours at 42°C in a thermocycler (Eppendorf) followed by an inactivation step of 10 minutes at 80°C. Then, 0.4 μL of Msel enzyme (Roche) was added. After incubation over 3 hours at 37°C, the enzyme was inactivated by heating to 65°C for 5 minutes. LBI1 (LBI1: 5’-AGTGGGATTCTGCTGTCAGT-3’) and ddMseI1 (ddMseI1: 5’TAACTGACAGGct-3’) primers were annealed in a reaction mix of 5 μL 10× OPA, 5 μL LBI1 100 μmol/L, 5 μL ddMseI1 100 μmol/L, and 15 μL H₂O in a water bath. For ligation, 6 μL of preannealed adapters combined with 2 μL of 10 mmol/L ATP (New England Biolabs) together with 2 μL T4 DNA ligase (Roche) were added to the digested cell. Ligation occurred overnight at 15°C. Genomic DNA was amplified with the Expand Long Template PCR System (Roche) according to the manufacturer’s instructions. Amplification was performed in a thermocycler under the following conditions: 1 × (68°C 3’); 14 × (94°C 40”, 57°C 30”, 68°C 90”+1’/cycle); 8 × (64°C 40”, 57°C 30”+1’/cycle); 68°C 1’ 45”+1’/cycle); 22 × (94°C 40”, 65°C 30”, 68°C 1’53”+1’/cycle); 1 × (68°C 3’40”). The PCR products were subsequently purified with the GenElute PCR Clean-up Kit (Sigma) according to the manufacturer’s instructions.

**Sequencing**

Amplified PCR products generated by sequence-specific primers (Supplementary Table s12) were primarily preamplified by AmpliTaq Gold DNA Polymerase (Applied Biosystems) in a thermocycler under the following conditions: 1 × (95°C 10’); 38 × (95°C 45”, 58°C 10”, 72°C 40’); 1 × (72°C 10’). Sequence analysis of preamplified PCR products was performed by a sequencing laboratory (GATC Biotech). For that purpose, newly designed primers are shown in Supplementary Table S13.

**Tissue preparation and DNA extraction**

Fresh frozen tumor tissue samples were used for sectioning. Sections of 10 μm were cut in a cryostat at −20°C and adhered to object slides. One section of each sample was stained with hematoxylin and eosin and examined by light microscopy to discriminate tumor tissue from stroma and healthy tissue. Between 10 and 20 sections, depending on the amount of tumor tissue, were collected for genomic DNA (gDNA) extraction with the Qiagen DNeasy Tissue Kit according to the manufacturer’s instructions. The estimated amount of tumor tissue within the dissected area was, in every case, at least 70%.

**Microsatellite instability analysis**

Amplified gDNA of CTC and tumor tissue samples was tested for microsatellite instability (MSI) using the markers NR21, NR24, and BAT 25 from the standard NCI/ICG-HNPCC marker panel and analyzed as previously described (13–15). These markers were selected because respective amplicons do not contain Msel restriction sites (Supplementary Table S14). In case of defining a CTC as MSI+(+), a multiplex PCR was subsequently conducted as previously described, targeting the most prominent gene candidates for MSI in their coding sequences (15).
Single-cell array comparative genomic hybridization

Array comparative genomic hybridization (aCGH) for single cells was performed following the protocol developed by Möhlenbeck and colleagues (16). In case of single CTC analysis, the SurePrint G3 human CGH 4 × 180K Oligo Microarray Kit (Agilent) was used according to the manufacturer’s instructions. Regarding the control experiment to prove the sensitivity of aberration detection, male and female leukocyte gDNA was hybridized against each other. Single CTC analysis was conducted with matched leukocytes. aCGH analysis of microdissected snap-frozen tumor tissue probed against matched healthy tissue was carried out with the SurePrint G3 human CGH 8 × 60K, Oligo Microarray Kit (Agilent). Data preprocessing was performed with Scan Control (V 7.0.3; Feature Extraction (V 10.1.1.1: Agilent). Data analysis and visualisation were performed by Genomic Workbench (V 5.0.14: Agilent; parameters: Genome build: hg18; Evaluation algorithm ADM-2, threshold 6; aberration filter: Minimal number of probes in area = 3, minimal mean log2 ratio = 0.25) and subsequently by the R-based package “GLAD” (17) with its defaults (except breaksFdrQ = 0.01) and using the haarsseg (18) algorithm and “aCGH” (ref. 19; aberration filter: Minimal number of probes in area = 3, minimal mean log2 ratio = 0.2).

Mononuclear cell enrichment from blood samples

25 to 30 mL of blood was layered over LSM 1077 lymphocyte gradient (PAA) and the peripheral blood mononuclear cell (PBMC) fraction was enriched according to the manufacturer’s instructions.

CTC enrichment and staining

PBMC were resuspended in 1.5 mL of beads buffer (PBS, 2 mmol/L EDTA, 0.1% AB serum). Then, 450 μl anti-CD45 magnetic beads (Dynal) were added to the mononuclear cells and incubated in an overhead shaker for 20 minutes at 4°C, followed by magnetic cell separation. The cell pellet was stained with Alexa-Fluor488-anti-EpCAM antibody (Biolegend). EpCAM-positive cells were identified under a fluorescence microscope (Leica, please see Supplementary Fig. S5), manually isolated with a micromanipulator (Eppendorf) and immediately transferred into RNA lysis buffer (PicoPure RNA isolation kit; Invitrogen). To obtain more valid expression data, immediately transferred into RNA lysis buffer (PicoPure RNA isolation kit; Invitrogen). To obtain more valid expression data, immediately transferred into RNA lysis buffer. Then, 450 μL anti-CD45 magnetic beads (Dynal) were added to the mononuclear cells and incubated in an overhead shaker for 20 minutes at 4°C, followed by magnetic cell separation. The cell pellet was stained with Alexa-Fluor488-anti-EpCAM antibody (Biolegend). EpCAM-positive cells were identified under a fluorescence microscope (Leica, please see Supplementary Fig. S5), manually isolated with a micromanipulator (Eppendorf) and immediately transferred into RNA lysis buffer (PicoPure RNA isolation kit; Invitrogen). To obtain more valid expression data, immediately transferred into RNA lysis buffer.

RNA extraction and amplification

Total RNA was extracted by the Arcturus PicoPure RNA Isolation Kit (Invitrogen) according to the manufacturer’s instructions. Amplification of total RNA was with the WT-Ovation RNA Amplification Kit (Nugen) according to the manufacturer’s instructions.

RT-PCR and statistical methods

Before CTC transcriptome analysis, we confirmed the technical feasibility of single-cell qPCR by a pilot study performed with decreasing numbers of HT-29 colorectal cancer tumor cells (Supplementary Figs. S5 and S6). SYBR Green RT-PCR was performed on a LightCycler qPCR cycler (Roche) according to the manual with an annealing temperature of 60°C and 55 amplification cycles. Amplification plots were analyzed using the second derivative maximum method. All Primers (Supplementary Table S11) were designed with Primer3Plus software and span large intronic regions to exclude amplification of gDNA. Ct values > 35 were considered unspecific. CTC expression data were normalized to the corresponding tumor’s gene expression by calculating the ΔΔCt values with the following formula: ΔΔCt = ΔCt tumor − ΔCt CTC = [Ct gene of interest (tumor) − Ct housekeeper (tumor)] − [Ct gene of interest (CTC) − Ct housekeeper (CTC)]. Statistical analysis of CTC expression profiles was achieved by Wilcoxon matched-pairs signed rank test, comparing the ΔΔCt values of CTC to the ΔΔCt values of their corresponding tumor tissue. P < 0.05 was considered statistically significant. Statistical outliers were identified and excluded with the ROUT method. (20) Only genes that were expressed in either tumor or CTC in at least 30% of the samples were analyzed. GraphPad Prism 6.0.1 (GraphPad Software Inc.) was used for statistical analyses and data plotting.

Expression results from a few genes [CK18 (KRT18), CK20 (KRT20), CXCR4, Ki-67 (MKI67), β-catenin (CTNNB1)] in a subgroup of CTC samples (4 of 11 samples) with metastatic disease to the liver have been used in a different publication (Rahbari and colleagues, submitted for publication) to demonstrate the metastatic potential of CTC derived from colorectal liver metastases.

Results

Single-cell aCGH confirms the genomic tumor cell identity of CTC detected by the CellSearch System

To genomically confirm the tumor cell identity of CTC detected by the CellSearch System [CD45 / EpCAM+/CK (8,18, 19)] we performed single-cell aCGH analysis (cf. Fig. 1). Eight single CTC from 8 individual patients and the corresponding microdissected tumor tissue were analyzed (Supplementary Table S1), revealing almost identical chromosomal aberrations in CTC and matched tumor tissue along the genome. The penetrance plot diagram showed a typical colorectal cancer pattern of recurrent chromosomal aberrations (gains at 1q, 7, 8q, 13, and 20 and losses at 1p, 4, 8p, 17p, and...
18; Fig. 2A; ref. 11). By dissecting the pooled data set into individual ratio plots, cases with rather similar or punctually different profiles could be identified (Supplementary Fig. S1). Two cases are representatively shown in Fig. 2B. An alternative analytic algorithm (haarseq, see Materials and Methods) confirmed the identified alterations of the single CTC (Supplementary Fig. S3).

These data underline that CTC detected by the CellSearch System are genomically true colorectal tumor cells, which, in turn, validates the following in-depth analyses of single CTC.

**Genetic characterization of colorectal cancer–derived single CTC reveals disparities in key mutations to the primary tumor**

We next performed a comprehensive genetic characterization of single CTC via genomic sequencing (cf, Fig. 1).

Initially, CTC were screened for common colorectal cancer–related mutations (KRAS, BRAF, and TP53). In total, we examined 126 CTC isolated from 31 individual patients (Supplementary Tables S2 and S3). Mutations in the KRAS gene in either CTC or tumor tissue were detected in 11 (35.5%), the BRAF V600E mutation in 3 (9.7%), and TP53 mutations in 12 patients (38.7%), which is in the range reported for colorectal cancer mutation rates (21).

MSI at one or more markers (Supplementary Table S2) was detected in CTC from 2 patients (of 25 with complete MSI data sets; 7.7%, Fig. 2C). In 1 patient, two of 11 tested CTC were MSI despite a microsatellite stable (MSS) tumor (Table 1).

Of note, 17 of 31 (54.8%) patients exhibited mutational disparity between CTC and primary tumor in any of the tested mutations; in 3 of these 17 patients (17.6%) at least one of the CTC was mutated, whereas the tumor was wild-type (1 × MSI, 1 × KRAS, 1 × TP53 E8); in 13 of 17 patients (76.5%) the CTC were wild-type despite a mutated tumor. In case of the patient with an MSS tumor with MSI CTC (patient HD-2215), we performed immunohistochemical expression analysis of DNA mismatch repair proteins MLH1, MSH2, MSH6, and PMS2 in sections obtained from the patient’s primary tumor and metastatic lesions to rule out contaminating stromal cells as the cause of the MSS genotype. In none of the sections, lack or reduction of nuclear MMR protein expression was observed.

Furthermore, to make sure that contaminating normal cells did not obscure MSI, we reanalyzed the MSI status of HD-2215 in all sections that were also stained for MMR protein expression (one primary tumor, two metastatic lesions). To achieve maximal sensitivity, microdissection was performed from three or more regions of each specimen, and MSI was analyzed using a
A set of four highly sensitive mononucleotide microsatellite markers (BAT25, BAT26, CAT25, and BAT40). None of the 10 analyzed regions (with estimated tumor cell content of >85%) displayed any alleles indicative of MMR deficiency or MSI.

In 18 of 31 patients (58.1%), we were able to analyze more than one CTC, of which 6 (33.3%) showed cytogenetically heterogeneous CTC populations. One patient is representatively shown in Table 1. The heterogeneity of CTC in terms

Figure 2. Genomic analysis of single colorectal cancer–derived CTC. A, aCGH data of eight CTC and the matched tumor tissue are illustrated as a penetrance plot mapping the frequency of detected aberrations along the human set of chromosomes (1–22, X, Y). Gains are shown in red and losses in green. B, two CTC/tumor tissue ratio profile pairs are representatively displayed. Fairly similar profiles can be observed for patient HD-2095, punctual differences for patient HD-2116. C, amplified length polymorphism analyses exemplified shown for CTC and control leukocytes of patients HD-2215 and HD-2341 for NR21 (blue) and NR24 (green). Asterisks indicate the unchanged microsatellite length as detected in control leukocytes. Arrows point out an altered microsatellite length in single CTC or tumor specimen.
of their microsatellite stability is representatively shown in Table 2.

In 11 of the 18 patients (61.1%) from which more than one CTC was analysed, we found mutational disparity between tumor and CTC; in contrast, we detected such disparity in only 3 of 13 patients (23.3%) with only one analyzed CTC. This indicates that increasing numbers of analysed CTC consequently increase the sensitivity of CTC genotyping in detecting new mutations not detected during tumor genotyping.

In summary, genomic analysis of single colorectal cancer–derived CTC demonstrated typical key mutations of colorectal cancer in expected frequencies. Interestingly, clinically relevant mutations were found in a subgroup of CTC that were not detected in the primary tumor.

Transcriptome analysis of colorectal cancer–derived CTC reveals a downregulation of epithelial markers, CTC dormancy, and an immune-escape phenotype.

To perform qPCR on CTC, we isolated viable CD45⁻/EPCAM⁺ CTC from blood samples of patients with colorectal cancer by manual isolation with a micromanipulator (cf. Fig. 1; ref. 22). To increase the likelihood of CTC detection, we isolated CTC from blood drawn from the tumor-draining vein (mesenteric venous blood in stage III tumors, hepatic venous blood in stage IV disease with hepatic metastases) and compared the isolated CTC with the respective tumor tissue (PT or LM; ref. 7). Blood samples from 72 patients with colorectal cancer were enrolled (Supplementary Tables S4 and S5). To independently control the applied CTC enrichment and isolation protocol, we also analyzed corresponding blood samples on the CellSearch System (Supplementary Table S6).

We succeeded in isolating CTC in nine of 72 cases (14%). Five samples from 3 patients were isolated from the mesenteric vein and compared with tissue from the primary tumor; 6 samples from 5 patients were isolated from the hepatic vein and compared with tissue from liver metastases. A panel of 48 genes (Supplementary Table S8) covering biomarkers for leukocytes, colorectal cancer, EMT (epithelial–mesenchymal transition), cancer, and stemness was chosen.

Table 1. Genomic analysis demonstrated heterogeneity between tissue and CTC and between individual CTC, representatively shown for patient HD-2215

<table>
<thead>
<tr>
<th>Sample</th>
<th>Microsatellite status</th>
<th>KRAS C12/13</th>
<th>BRAF V600E</th>
<th>TP53 E5/6</th>
<th>TP53 E7</th>
<th>TP53 E8</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT</td>
<td>MSS</td>
<td>G12S</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>LM</td>
<td>MSS</td>
<td>G12S</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>HV CTC 1</td>
<td>MSS</td>
<td>G12S</td>
<td>WT</td>
<td>n.d.</td>
<td>n.d.</td>
<td>WT</td>
</tr>
<tr>
<td>HV CTC 2</td>
<td>MSS</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>WT</td>
</tr>
<tr>
<td>MV CTC 1</td>
<td>MSS</td>
<td>G12S</td>
<td>WT</td>
<td>n.d.</td>
<td>n.d.</td>
<td>WT</td>
</tr>
<tr>
<td>MV CTC 2</td>
<td>MSS</td>
<td>G12S</td>
<td>WT</td>
<td>n.d.</td>
<td>n.d.</td>
<td>WT</td>
</tr>
<tr>
<td>MV CTC 4</td>
<td>MSS</td>
<td>G12S</td>
<td>WT</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>MV CTC 5</td>
<td>MSS</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>MV CTC 7</td>
<td>MSI</td>
<td>G12S</td>
<td>WT</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

NOTE: Numbers e.g., CTC 1, CTC 2) indicate different single CTC from the same patient. Pathologic results are indicated by bold text to show that the CTCs are heterogeneous and differ from the PT and the LM. Abbreviations: HV, hepatic venous; MV, mesenteric venous; WT, wild-type; PT, primary tumor; LM, liver metastasis; n.d., not detected.

Table 2. CTC with signs of MSI were also examined for MSI within coding regions (cMSI)

<table>
<thead>
<tr>
<th>Sample</th>
<th>NR-21</th>
<th>NR-24</th>
<th>BAX</th>
<th>DD5</th>
<th>FLT3LG</th>
<th>ELAVL</th>
<th>MSI classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV CTC 1</td>
<td>MSI</td>
<td>—</td>
<td>—</td>
<td>MSS</td>
<td>MSS</td>
<td>MSI</td>
<td>H</td>
</tr>
<tr>
<td>MV CTC 2</td>
<td>MSI</td>
<td>MSI</td>
<td>MSS</td>
<td>MSS</td>
<td>MSS</td>
<td>MSI</td>
<td>H</td>
</tr>
<tr>
<td>MV CTC 3</td>
<td>MSI</td>
<td>MSS</td>
<td>MSS</td>
<td>MSS</td>
<td>MSS</td>
<td>MSI</td>
<td>H</td>
</tr>
<tr>
<td>MV PBMC</td>
<td>MSS</td>
<td>MSS</td>
<td>—</td>
<td>MSS</td>
<td>MSS</td>
<td>MSS</td>
<td>—</td>
</tr>
</tbody>
</table>

NOTE: Results of patient HD-2341 are representatively depicted. Three single CTC were classified as MSI-high level (MSI-H) and showed a mutation in the coding region of the ELAVL gene. A corresponding PBMC from patient HD-2341 did not show any signs of MSI. MSI events are shown in bold text to stress the fact that different markers have shown different results in these CTC. Abbreviation: MV, mesenteric venous.
CD45 expression in CTC did not differ significantly from the corresponding tumor tissue. The epithelial origin of the isolated CTC was confirmed by the expression of EPCAM, CK18 (KRT18), CK19 (KRT19), and CEACAM7 and the absence of those markers in control leukocyte samples. Generally, gene expression in the CTC samples was on a lower level compared with the corresponding tumor tissue (Fig. 3A and B). Together with the relative downregulation of Ki-67 (MKI67), which was expressed in only 3 of 11 CTC samples (n.s.), and c-Myc (MYC, P = 0.008) as compared with the corresponding tumor tissue, these data indicate a reduced proliferative activity of CTC, which may indicate a state of dormancy in these cells. Interestingly, β-catenin (CTNNB1) is also downregulated (n.s.), indicating reduced activity in the Wnt signaling cascade.

As expected, most epithelial markers were strongly expressed in the tumor tissue and downregulated in CTC, indicating a potential loss of epithelial phenotype in CTC; the epithelial markers EPCAM (P = 0.027), CK19 (P = 0.012) and CEACAM7 (P = 0.002) were significantly downregulated in CTC. However, we did not observe increased expression of the mesenchymal marker vimentin (VIM, P = 0.765).

CTC do not seem to overexpress markers generally associated with "stemness" such as CD166 (ALCAM), CD26 (DPP4), or CD44s, CD133 (PROM1) was not expressed in any of the CTC examined.

Interestingly, the only gene that was overexpressed in CTC compared with the corresponding tumor tissue was CD47 (P = 0.039). CD47 is generally considered as an anti-phagocytic "don't eat me" signal expressed on cancer cells to prevent macrophages and dendritic cells from attacking tumor cells (23). The counterpart of this anti-phagocytic mechanism, the pro-phagocytic calreticulin (CALR), was significantly downregulated in CTC (P = 0.001).

Migration- and motility-associated genes were generally downregulated, some of which reached or were close to statistical significance [fibronectin (FN1), P = 0.047; CD44v6, P = 0.027; CD151, P = 0.062; TSPAN8, P = 0.064].

In summary, we succeeded in identifying a CTC-specific expression profile as well as disparities to the matched tumor tissue. Tumor cells circulating in the blood stream seem to be in a state of dormancy, indicated by the downregulation of most major signaling pathways, including those responsible for cell migration and motility. The only gene we found significantly upregulated was CD47, which is associated with tumor immune escape.

Discussion

The prognosis of colorectal cancer is closely linked to the occurrence of distant metastases (2). Although CTC play a pivotal role in the metastatic cascade (3) and their prognostic impact has been repeatedly demonstrated (4, 5, 24–27), little is known about their genomic and transcriptomic hallmarks due to the technical obstacles involved in single-cell analysis. The identification of CTC-specific traits would facilitate the development of compounds directly targeting CTC, which could dramatically improve the prognosis of localized disease by preventing metachronous metastases. The aim of the present work was therefore to further characterize CTC and identify genetic and phenotypic hallmarks of CTC, enabling them to survive in the blood stream and ultimately form tumors distant from the primary lesion.

On the basis of the initial proof of genomic tumor cell identity of CTC, we performed a broader mutational characterization of single colorectal cancer–derived CTC. To this end, we aimed to isolate CTC and compare them to the tumor bulk they most likely originated from. We therefore isolated CTC intraoperatively from the appropriate tumor draining vein (e.g., the hepatic vein in case of hepatic metastases, or the mesenteric vein in case of a primary tumor) and compared the isolated CTC with tumor cells isolated from the corresponding tumor tissue. As all blood samples were obtained intraoperatively during tumor resection, we were able to obtain complete sets of blood samples and corresponding tissue samples.

For mutational profiling, we used a robust adapter–linker PCR for WGA that allows comprehensive genomic profiling of fixed and unfixed single cells (12, 16, 28–32). As expected, our data show that CTC are a profoundly heterogeneous cell population. We were able to identify several CTC with mutations that were not present or detected in the matched tumor tissue. This may represent newly acquired mutations in CTC; however, it is more likely that the tumor cell subclone responsible for shedding the mutated CTC into the blood stream was not contained in the biopsy used for genomic analysis of the tumor tissue. Other groups recently observed a similar phenomenon and were subsequently able to detect the mutations found in the CTC at subclonal levels by deep sequencing of the primary tumor tissue (33). As our data strongly support the recent findings of Heitzer and colleagues and other groups (34, 35), especially in the case of KRAS mutations, it is conceivable that many patients receive anti-EGFR treatment although the metastatic subclone within the tumor has already a constitutively active EGF receptor (EGFR) signaling cascade. Testing CTC for KRAS mutations ("liquid biopsy") may identify such patients and could therefore be a valuable addendum to traditional tumor genotyping; it may detect genetic changes of small regions or CTC-dispatching tumor cell subpopulations in the primary tumor that are not necessarily contained in the tissue sample used for tumor genotyping. Also, as tumor tissue genotyping can only provide a current snapshot and the tumor genotype is subject to constant change, sequential genotyping of CTC may be reasonable to early detect new mutations that may have further therapeutic implications. This hypothesis is well in line with recent findings that circulating KRAS-mutant DNA can be an early predictor of future resistance to anti-EGFR therapy (36). In summary, the current approach using the solid tumor as the sole source of information for therapeutic decision may be substantially improved by additional analysis of CTC or circulating tumor DNA.

Most distant metastases genotypically and phenotypically resemble their primary tumor (11). This implicates that the changes enabling CTC to perform the necessary steps to cause distant metastases must be reversible and, thus, on a transcriptomic rather than a genomic level. Because of the
volatile nature of RNA and the miniscule amounts available for analysis, transcriptomic profiling of CTC is technically challenging. We succeeded in isolating viable CTC by manual isolation from the PBMC fraction and subsequently analyzed the transcriptome by qPCR. We demonstrated reduced expression of most tested genes, indicating a state of dormancy of CTC. The downregulation of c-Myc and Ki-67 supports this assumption and is well in line with other reports (37).

Surprisingly, most genes related to cell migration and invasion such as TSPAN8 (38, 39) and CD44v6 were significantly downregulated as well. This, again, may indicate the short-lived nature of the phenotypic changes in CTC; once in circulation, CTC do not require migratory capabilities anymore and may have already lost the mesenchymal phenotype at the time of analysis. Consequently, the tetraspanin CD151, which is involved in cell–cell contacts and which has been shown to be downregulated in metastatic colorectal cancer as compared
with nonmetastatic colorectal cancer (40, 41), was significantly downregulated, indicating reduced cell–cell adhesion capabilities of CTC.

The blood stream is a hostile environment for tumor cells; although a tumor presumably sheds millions of tumor cells into the blood stream every day (42), only few can be detected at any given time. This may be due to the rapid clearance of CTC from the blood stream, most likely mediated by natural killer (NK) cells, monocytes/macrophages, and neutrophils. CTC are therefore under evolutionary pressure to develop a phenotype that can protect or hide them from the immune system. In line with this hypothesis, we found CTC to exhibit a distinct nonimmunogenic phenotype by overexpressing CD47, a protein inhibiting the cytotoxic and phagocytic activity of activated immune cells (43–45). CD47 binds to SRPα (46), an inhibitory receptor on macrophages and T cells (47–49) and is notably the only gene we found to be overexpressed in otherwise mostly dormant CTC. Previous studies on patients with breast cancer indicated the presence of CD47-positive CTC in the bone marrow, which may be responsible for tumor relapse (50). Other groups have shown that constitutive CD47 upregulation is a pivotal requirement for non-Hodgkin lymphoma immunotolerance and dissemination (51, 52). In concordance with the upregulation of CD47 in CTC is the marked downregulation of calreticulin, a chaperone protein acting as a homing beacon, inducing a strong cytotoxic and phagocytic reaction in NK cells and other immune effector cells (53, 54).

The presented data provide new insight into the biology of CTC as it becomes clear that colorectal cancer–derived CTC are mostly dormant and disguised from the immune system. To our knowledge, this is the first study demonstrating both a comprehensive genomic and transcriptomic profiling of colorectal cancer–derived CTC. The data also demonstrate the distinct heterogeneity among CTC, which may explain the relatively low number of metastases compared with the number of CTC present in the blood stream (42), and suggest that only a small fraction of CTC is actually able to form new lesions. A recent work by Baccelli and colleagues showed that the number of CTC present in the blood stream (42), and suggest that relatively low number of metastases compared with the number of CTC. Our expression data show a significant downregulation of CD44 and CD47+, a distinct heterogeneity among CTC, which may explain the biological relevance of EPCAM+ CTC. This question needs to be addressed in the future once standardized and reliable markers or physical properties allowing EPCAM-independent detection of CTC have been established. To date, neither the existence of EPCAM-negative colorectal cancer–derived CTC nor their biologic relevance have been reported and the biologic relevance of EPCAM+ CTC in colorectal cancer is well established. Hence, EPCAM still remains the best marker for CTC detection and isolation available today.

In conclusion, our study confirms the genetic heterogeneity of colorectal cancer–derived CTC and demonstrates mutational disparities to the primary tumor. Transcriptome analyses suggest a dormant state of CTC and, for the first time, provide evidence of immune-escape mechanisms of CTC in humans. These data further support the concept of CTC as predictive biomarkers in colorectal cancer. Furthermore, they identified immune-escape mechanisms as potential targets to disrupt the metastatic cascade in colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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