Enumeration and Molecular Characterization of Tumor Cells in Lung Cancer Patients Using a Novel *In Vivo* Device for Capturing Circulating Tumor Cells

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Abstract

**Purpose:** The use of circulating tumor cells (CTC) as "liquid biopsy" is limited by the very low yield of CTCs available for subsequent analyses. Most *in vitro* approaches rely on small sample volumes (5–10 mL).

**Experimental Design:** Here, we used a novel approach, the GILUPI CellCollector, which enables an *in vivo* isolation of CTCs from peripheral blood. In total, 50 lung cancer patients were screened in two subsequent device applications before and after therapy (n = 185 applications).

**Results:** By *in vivo* isolation, 58% (108/185) of the patients were positive for ≥1 CTC (median, 5 CTCs; range, 1–56 cells) as compared with 27% (23/84; range, 1–300 cells) using the FDA-cleared CellSearch system. Furthermore, we could show that treatment response during therapy was associated with significant decreases in CTC counts (P = 0.001). By dPCR, mutations in the KRAS and EGFR genes relevant for treatment decisions could be detected in CTCs captured by *in vivo* isolation and confirmed in the primary tumors of the same patients.

**Conclusions:** *In vivo* isolation of CTCs overcomes blood volume limitations of other approaches, which might help to implement CTC-based 'liquid biopsies' into clinical decision making.

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Introduction

Although tumor cells detached from primary tumors or metastases into the blood (circulating tumor cells; CTC) have been discovered almost 150 years ago, specific detection and molecular examination have only recently gained heightened attention. This may be explained by the fact that technology improvements have only recently become available to enable more consistent CTC isolation. However, despite encouraging research progress, it is still a tremendous challenge to enrich sufficient amounts of CTCs from peripheral blood of cancer patients (1, 2). Detection and molecular characterization of CTCs is challenging because CTCs are rare events, with a frequency of approximately 1 to 10 CTCs in the background of 6 × 10⁸ leukocytes, 2 × 10³ platelets and 4 × 10⁴ erythrocytes per mL of patient-derived blood sample (3).

Despite promising results demonstrating that enumeration and characterization of CTCs might provide important information on the individual risk of cancer patients, there is still an urgent demand to increase the yield of capturing CTCs.

Lung cancer is one of the most frequently occurring and deadly diseases (4). A sensitive CTC isolation method holds great promise for early detection of minimal residual disease (MRD). Furthermore, CTCs are being evaluated as predictive biomarkers to guide individual cancer treatment strategies (personalized medicine). Hence, sensitive isolation and profound molecular characterization of CTCs could serve as a "liquid biopsy" and facilitate improved individual treatment regimens for cancer patients. So far, several reports have shown that CTC counts are much higher in small cell lung cancer (SCLC) than in non–small cell lung cancer (NSCLC; refs. 4, 5). Using the FDA-cleared CellSearch, CTC values in NSCLC seem to be even lower than in other cancer entities (4, 6, 7), pointing to the need for improved CTC detection in NSCLC. Moreover, liquid biopsies would be particularly desirable in NSCLC, where an emerging number of new molecular targets have been recently discovered that would benefit from CTC-based patient selection (8).

To date, more than 40 different approaches for CTC detection have been developed (9, 10). Various approaches have also been used to improve the yield of CTCs in NSCLC patient blood samples (8); to our best knowledge, none of these assays have been sufficiently tested to support FDA approval. In the current

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study, we validated a new strategy: The CellCollector (GILUPI CellCollector, GILUPI) enables an in vivo isolation of CTCs directly from the arm vein of cancer patients. During the 30-minute incubation in the vein, the device is exposed to approximately 1 L of blood, which increases the chance to capture CTCs. Our present investigation includes (i) the analysis of a large cohort of lung cancer patients to assess the distribution of CTCs, (ii) consecutive applications of the CTC device to demonstrate reproducibility of blood sampling for CTCs (which rarely has been shown in any of the >16,000 CTC publications in PubMed), (iii) monitoring of lung cancer patients during treatment for clinical response correlated to CTC counts, (iv) comparison of CellCollector (catheter-based) results to the FDA-cleared CellSearch system (blood sample-based), and (v) molecular characterization of CTCs captured by the medical device for mutations relevant to cancer therapy and comparison to the primary tumor of the same patients. This in-depth validation will support the use of in vivo CTC capture as a new diagnostic principle for future clinical trials testing the relevance of "liquid biopsies" to direct cancer therapies.

Materials and Methods

In vitro application of the CellCollector

The GILUPI CellCollector (termed as catheter-based or wire-based enrichment of EpCAM-positive cells) is based on a sterile stainless steel medical wire, covered with 2-μm gold and a hydrogel layer at its functionalized tip. This hydrogel layer is covalently coupled with antibodies against the EpCAM protein (humanized HEA 125, GILUPI GmbH; Fig. 1A). For our in vitro studies, the wire was incubated together with unauthenticated lung cancer cells (NCI-H332, NCI-H3122, NCI-H1650, 32M1, NCI-H1975, and A549) spiked into blood (EDTA) from healthy individuals for 30 minutes on a rotator. After the incubation, the wire was carefully inserted into the vein through the catheter until the tip of the device was extended 2 cm into the vein. To assess the reproducibility of measurements with the medical wire, two subsequent applications were performed during the same day (visit) for every cancer patient participating into the study. Patients underwent two subsequent device applications both before and after 12 weeks of therapy. Fourteen patients underwent only the pretherapy but not the following applications. Exceeding the initial study design, 7 patients had an additional third visit and one of them a fourth visit, each visit with double applications.
This led to 16 additional device applications and a total of 185 analyzable applications. After the in vivo application, the wire was treated as described above. Cells captured by the device were stained and counted by different operators who were blinded to the patient's history and the CellSearch results.

**CellSearch analysis**

For method comparison, 7.5 mL of venous blood were collected into CellSave tubes immediately before the application of the wire. Blood was sent to the University Medical Center Hamburg-Eppendorf and processed within 96 hours as recommended by Janssen Diagnostics. The operator was blinded to the catheter-based results.

**Statistical analysis**

Clinical factors (T, N, M, and UICC stage) were tested for association with CTC count by multivariate analysis using ordinal logistic regression. Paired analyses between replicate measurements were tested for (i) difference in median using Wilcoxon signed rank test, (ii) difference for negative (0 CTCs) or positive (≥1 CTC) using Liddell's exact test, and (iii) correlation between ordinal values of CTCs using Kendall's tau. The difference between median number of CTCs as detected by the wire and CellSearch was assessed by the Wilcoxon signed rank test, and the agreement and correlation between the results of the two systems was assessed using Cohen's kappa and Kendall's tau, respectively. The differences in CTC count (visit 2 – visit 1) for catheter-based and
blood sample–based system results were tested for association with therapy response using the Kruskal–Wallis test.

Mutational analysis of tumor cells captured by in vivo isolation

CTC-positive wires from 2 patients (from different applications and visits) with known mutational status of the primary tumor were used for further molecular characterization of the CTCs by chip-based digital PCR (dPCR). Briefly, the wire was fragmented into small pieces into a 0.2 mL PCR-tube filled with 4.8 μL PBS (Qiagen). Whole genome amplification (WGA) was performed using the REPLI-g Single Cell Kit (Qiagen) using a 1.2 x 2 reaction volume. Amplified gDNA was quantified with the Quantiflour ONE dsDNA System (Promega) and a fluorescence reader (FluoroskanAscent; Thermo Fisher) at 485 nmEx/538 nmEm. For chip-based dPCR the ThermoFisher QuantStudio 3D system was used. Fifty nanograms of WGA DNA (2 μL) was combined with 7.5 μL of 2 x dPCR Mastermix and 0.75 μL of 2 x SNP assay for the appropriate allele. The total volume was brought to 15 μL with water, applied to the chip and sealed according to the manufacturer's instructions. The chip was placed on a flat-block PCR.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histology</th>
<th>Staging</th>
<th>CTC count: CellCollector A-1/A-2 (sum)</th>
<th>CTC count: CellSearch VI V2</th>
<th>Treatment response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T N M U</td>
<td>VI V2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>001</td>
<td>Squamous cell carcinoma</td>
<td>x 3</td>
<td>IV 2/0 (2) 2/0 μL (3) 4 0</td>
<td>SD</td>
<td></td>
</tr>
<tr>
<td>002</td>
<td>Adenocarcinoma</td>
<td>2b 1</td>
<td>IV 0/0 (0) 0/0 μL (0) n.a.</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>003</td>
<td>SCLC</td>
<td>2 3</td>
<td>IV 0/3 (3) 0/0 μL (0) 3 0</td>
<td>SD</td>
<td></td>
</tr>
<tr>
<td>004</td>
<td>Squamous cell carcinoma</td>
<td>4 2</td>
<td>IV 10/0 (0) 0/0 μL (0) 2 n.a.</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>005</td>
<td>Adenocarcinoma</td>
<td>4 3</td>
<td>O IIIIB 3/0 (3) 0/0 μL (0) 0</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>006</td>
<td>Adenocarcinoma</td>
<td>3 2</td>
<td>O IIIA 1/6 (7) 4/9 μL (15) 0</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>007</td>
<td>Adenocarcinoma</td>
<td>1b 3</td>
<td>IV 1/0 (0) 0/5 μL (5) 0 n.a.</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>008</td>
<td>Large cell carcinoma</td>
<td>4 2</td>
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<td>PD</td>
<td></td>
</tr>
<tr>
<td>009</td>
<td>Adenocarcinoma</td>
<td>3 2</td>
<td>IV 1/0 (1) 5/5 (10) 0 0</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>010</td>
<td>Adenocarcinoma</td>
<td>4 3</td>
<td>IV 0/0 (0) 1/11 μL (12) 0 0</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>011</td>
<td>Squamous cell carcinoma</td>
<td>2 3</td>
<td>IV 0/0 (0) 11/6 (17) 0 0</td>
<td>0 n.a.</td>
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<tr>
<td>012</td>
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<td>2a 0</td>
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<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>013</td>
<td>Adenocarcinoma</td>
<td>4 3</td>
<td>IV 5/5 (8) 1/2 (2) 8 24</td>
<td>PD</td>
<td></td>
</tr>
<tr>
<td>014</td>
<td>Adenocarcinoma</td>
<td>4 3</td>
<td>IV 3/11 (14) 8/18 (26) 0 0</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>015</td>
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<td>IV 0/2 (2) 4/8 μL (12) 1 n.a.</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>016</td>
<td>Adenocarcinoma</td>
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<td>PR</td>
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</tr>
<tr>
<td>017</td>
<td>Squamous cell carcinoma</td>
<td>4 3</td>
<td>IV 0/0 (0) 0/0 μL (0) n.a.</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>018</td>
<td>Squamous cell carcinoma</td>
<td>4 1</td>
<td>IV 5/13 (18) 0/0 μL (0) 0 n.a.</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>019</td>
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<td>IV 6/25 (50) 0/0 μL (0) n.a.</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>020</td>
<td>Adenocarcinoma</td>
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<td>IV 17/18 (18) 1/0 μL (1) 0 n.a.</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>021</td>
<td>Adenocarcinoma</td>
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<td>IV 20/22 (42) 0/0 μL (0) 0 n.a.</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>022</td>
<td>Adenocarcinoma</td>
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<td>IV 35/13 (48) 0/3 μL (3) 0 n.a.</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>023</td>
<td>Squamous cell carcinoma</td>
<td>3 3</td>
<td>IV 4/6 (7) 0/0 μL (0) 4 n.a.</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>024</td>
<td>Adenocarcinoma</td>
<td>x</td>
<td>IV 1/1 (1) 1/0 μL (1) 0 n.a.</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>025</td>
<td>Adenocarcinoma</td>
<td>4 1</td>
<td>IV 1/0 (1) 0/0 μL (0) 0 n.a.</td>
<td>0 n.a.</td>
<td></td>
</tr>
<tr>
<td>026</td>
<td>Adenocarcinoma</td>
<td>4 3</td>
<td>IV 2/0 (2) 0/0 μL (0) 0 n.a.</td>
<td>0 n.a.</td>
<td></td>
</tr>
</tbody>
</table>
| 027     | Adenocar...
machine and cycled: 45°C 1:00 minute, 98°C 2:30 minutes (95°C 1:00 minute, 53.5°C 0:1 minute with 30% ramp, 57.5°C 0:30 minutes) for 40 cycles, and held at either 10°C or 20°C until read. Chips were read on the QuantStudio 3D reader and analyzed by QuantStudio 3D AnalysisSuite and Microsoft Excel. SNP assays were designed using the Thermofisher Custom SNP design pipeline (Life Technologies) without modifications. PBMCs (1,000) from a healthy donor subjected to the same WGA protocol were used as a negative control to prove the specificity of the assay.

Mutational analysis of the primary tumor

Mutational analysis of the primary tumor tissue was performed in the institute of pathology at the University Medical Center Ulm. EGFR (exons 18, 19, 20, and 21) mutational analysis was done in the institute of pathology at the University Medical Center Ulm. Mutational analysis of the primary tumor metastatic lung cancer (stage III, n = 11; stage IV, n = 39) with a performance status of ECOG 0 or 1 and who were able to receive a systemic antitumor treatment (platinum-based doublet) according to current clinical guidelines (12) were enrolled into the study. Tumor morphology included squamous cell carcinoma (n = 15), adenocarcinoma (n = 30), small cell carcinoma (n = 2), and large cell carcinoma (n = 1), as well as 2 carcinoma patients with unknown morphology (Table 1). Staging for distant overt metastases was available from 49 patients; 39 (78%) patients with unknown histology (Table 1). Staging for distant overt metastases was available from 49 patients; 39 (78%) patients with unknown histology (Table 1). A schematic overview of the wire during in vivo application is presented in Fig. 1A. In total, 185 in vivo applications were performed and screened for CTCs (Fig. 1B). Briefly, the wires coated with anti-EpCAM antibodies—was placed into the arm vein of cancer patients for 30 minutes where EpCAM-positive cells can bind to the device. Afterward, the wire was placed into a special holder under the microscope and tumor cells were identified based on epithelial markers (EpCAM- and/or pan-keratin-positive) and nuclear counterstain (Hoechst33342-positive) by fluorescence microscopy. CD45 staining is necessary to discriminate possible leukocytes bound to the wire.

CTC counts and correlations to clinicopathologic characteristics

In total, 58% (108/185) of the analyzed wires were positive for ≥1 CTC (range, 1–56 cells; Fig. 2A). Most of the identified cells were single CTCs. However, in 20 of 185 analyzed wires CTC clusters (range, 2–7 cells) could also be detected. Representative image galleries of tumor cells from spiking experiments, CTCs, CTC clusters, and leukocytes (range, 0–≥1,000 per wire) are disclosed in Fig. 2B and C. No “CTC-like” events were found on the wires placed into the arm vein of healthy individuals (n = 10; data not shown).

For assessing clinical correlation to CTC counts, the sum of the CTC counts of the double applications were used in our multivariate analysis. First, an ordinal scale with four categories for CTC values was created (negative, 0 CTCs; low, 1–4 CTCs; intermediate; 5–17 CTCs, high, ≥18 CTCs) such that every category had approximately the same number of cases. The resulting regression revealed that none of the clinical factors (T, N, or M stage as well as UICC) was significantly correlated with the CTC categories, although the tumor stage was close to significant (P = 0.09).

Reproducibility of wire applications

To assess the reproducibility of measurements with the wire, the results of the two subsequent CTC analyses performed at each visit from each cancer patient (Table 1) were compared and statistically evaluated for potential differences.

At the first (pre-therapeutic) visit, CTC counts of ≥1 were detected in 78% of the study participants (combined analysis). As expected, the paired analyses resulted in some differences in CTC counts; however, these differences were not significant (P = 0.33, Wilcoxon signed rank test). Furthermore, the number of CTC-positive (≥1 CTCs) and -negative (0 CTCs) cases was also not significantly different between the two analyses (P = 0.59, relative risk 95% CI, 0.35–2.84, Liddell exact test). And finally, a positive and significant correlation could be detected between CTC values of the paired analyses of the first visit (P = 0.003, τ = 0.36, Kendall rank correlation).

At the second visit, 60% (20/33) and 56% (20/36) of the patients were identified to be CTC positive with repeated wire analyses (combined positive rate 72%). Comparing the results of the two analyses, no significant difference between the number of identified tumor cells could be detected (P = 0.42, Wilcoxon signed rank test). Furthermore, the number of CTC negative and positive patients was comparable (P = 0.64, Liddell’s exact test). Finally, the correlation between the number of CTCs detected with the two analyses was also significantly correlated (P = 0.0015, τ = 0.50, Kendall rank correlation).

Performing the same statistical analyses on the two wire analyses of visit 3, the number of CTCs and the number of CTC-positive and -negative patients were not significantly different (P = 0.88, Wilcoxon signed rank test; P = 0.25, Liddell exact test); however, the correlation between the CTC values was not significant anymore because almost all cases were CTC negative (P = 0.59, τ = 0.27, Kendall rank correlation). Visit 4 was excluded from this analysis because only one case was analyzed.

Comparison of CTC counts obtained with the wire and CellSearch

For a direct comparison of the two CTC assays, 84 analyzable blood samples (7.5 mL) were taken prior to the application of the wire and screened ex vivo for CTCs by the FDA-cleared CellSearch system. As shown in Fig. 3A, the incidence of CTC-positive lung cancer patients was higher if determined by the wire compared with the FDA-cleared technology. Direct comparison of paired patient data showed that the wire isolated a median of 3 CTCs more per patient (P < 0.0001, Wilcoxon signed rank test). Only in 11 measurements were more CTCs found with the CellSearch system than with the wire.

Taken together, these results indicated that in vivo isolation has a higher sensitivity in capturing CTCs from lung cancer patients. It should be noted that there was no correlation between the positive and negative cases as determined between in vivo
isolation and CellSearch results ($P = 0.76$, $\kappa = 0.0598$, Cohen’s kappa) or between the ordinal CTC values ($P = 0.99$, $\tau = 0$, Kendall rank correlation). A direct comparison (weighted scatter plot) between the wire and CellSearch is visualized in Fig. 3B.

**Monitoring of CTCs during systemic therapy**

To evaluate changes in CTC counts as a potential marker for monitoring treatment response, patients were grouped into responsive, stable, and progressive disease, according to current RECIST criteria, 1.1 (13). Cancer patients were screened for CTCs using two subsequent applications of the medical wire, both before and after 12 weeks of any systemic treatment. Patients were grouped according to their therapy responses assessed after initiation of therapy—responder, stable disease, or progressive disease. We could show that patients responding to the therapy [complete responders (CR) or partial responders (PR)] showed reduced CTC counts with a median decrease of 8.25 CTCs after 12 weeks initial first-line therapy (Fig. 4).

Furthermore, patients with stable disease had a median difference of 0 CTCs compared with their previous cell number investigation, whereas patients with progressive disease were diagnosed with a median increase of 4.5 CTCs ($P = 0.0013$, Kruskal–Wallis test).

Such significant differences were not seen by CellSearch (Fig. 4), where the median CTC counts between subsequent visits were similar in all response groups ($P = 0.13$, Kruskal–Wallis test).

**Mutational analysis of CTCs captured by the medical wire**

CTCs could be used for "liquid biopsy" to guide individual treatment decisions by mutational analysis of genes expressing targets or relevant pathway proteins. Therefore, we performed a proof-of-principle investigation whether CTCs captured by the wire were suitable for further molecular characterization. For this purpose, captured cells (after staining) from CTC-positive patients with known mutational status of the primary tumor were analyzed (Fig. 5A–C).
In this study, we demonstrated that in vivo isolation of CTCs is a promising approach for sensitive capture of CTCs. In vivo isolation demonstrated higher CTC capture from lung cancer patients compared with the FDA-cleared CellSearch system. Both devices capture and identify EpCAM-/keratin-positive CTCs; thus, the observed differences are most likely due to the new principle of capture and identify EpCAM-/keratin-positive CTCs; thus, the comparison with the FDA-cleared CellSearch system. Both devices, promise approaches for sensitive capture of CTCs. 

A discussion on the in vivo isolation of CTCs shows the importance of different selection methods and the potential of the ISET 80% vs. CellSearch 23% (ref. 6). CTCs exist (15) that can be lost. Nevertheless, the ISET isolation of CTCs is a difficult task (14) and smaller filter systems as well as other EpCAM-independent assays can be helpful in clarifying the relevance of “mesenchymal CTCs” with loss of epithelial markers. Experimental studies indicated that tumor cells with a mesenchymal phenotype have a higher propensity to disseminate through the bloodstream, but appear to lack the ability to form metastases at distant sites (16, 17). This would explain why CTCs expressing (at least some) epithelial markers, such as EpCAM and keratins, are excellent indicators of prognosis in cancer patients (8).

In this current investigation, we could further demonstrate that subsequent applications of the wire during the same visit are feasible and increase the capture rate of CTCs. Multiple collector wires can be used for different types of downstream analyses (e.g., immunostainings and PCR-based mutation analyses) at the same time point, which increases the applicability of the wire. Moreover, double applications provide an opportunity to analyze the reproducibility of subsequent samples performed with the wire. Although the paired in vivo analyses at the same visit resulted in differences regarding exact CTC quantification, no significant differences between repeated CTC measurements were observable, indicating that the CTC results are reproducible. This was also shown by the reproducible and specific detection of a KRAS mutation using four samples from 1 patient. Regarding the low number of CTCs detected, this aspect is of utmost importance and has been rarely addressed in real clinical specimens using other CTC assays.

It has been shown that patients who harbor clusters of CTCs have a worse clinical outcome (18). In this study, CTC clusters were detectable in 20 of 185 wires after in vivo application. Future studies are needed to clarify the clinical relevance of CTC clusters. The cellular composition of clusters can be heterogeneous and their biology is still under investigation. It can be speculated that clusters might contain CTCs from different tumor clones that are required in a cooperative manner to establish metastases at distant sites (5, 6).

Monitoring of treatment responses is one of the key applications of CTC assays (19–21). In the present study, we observed significant correlation between changes in CTC counts and
response to therapy. Decreased CTC counts after therapy initiation were frequently seen in lung cancer patients who responded to systemic treatment, whereas increased CTC numbers were observed in most patients with progressive disease. Although this finding needs to be validated in larger studies, it suggests that in vivo isolation of CTCs might be useful for monitoring therapies in NSCLC. In contrast, using the ex vivo blood sample-based approach to monitor changes in CTC counts did not correlate to treatment response in our present cohort of patients, which seems to be different from another report demonstrating that a change in CellSearch-based CTC counts after treatment was predictive of progression-free survival in NSCLC (22). This discrepancy may be explained by a different selection of patients, and larger multicenter studies should be analyzed to define the value of both assays in NSCLC.

Besides monitoring of response to therapy, the molecular characterization of CTCs can provide important information on therapeutic targets or resistance mechanisms (23-28). Thus, we evaluated whether the in vivo isolated CTCs also allow subsequent molecular characterization of genes relevant to therapy of cancer patients. We selected KRAS and EGFR as the two prominent examples already used in clinical routine for decision making (28). Although only two patients have been analyzed, we could demonstrate that the DNA from CTCs captured by the wire could be isolated, amplified, and analyzed for mutations in these genes, and—most importantly—the results matched the gene status in the primary tumor of the same patients. Control assays for mutually exclusive mutations in the same genes were negative, proving the specificity of the analysis. Although this analysis could only be done for 2 patients due to the limited clinical data set available, it clearly indicated the technical feasibility of mutational analysis on (or with) isolated CTCs. Such results may lead to treatment decisions for or against EGFR TKIs based on the presence or absence of activating or resistance mutations in EGFR or against cetuximab therapy due to the presence of a KRAS mutation. The feasibility of mutational analysis based on device-isolated CTCs is an important prerequisite for future use of the wire for "liquid biopsy" analyses in cancer patients. Taking biopsies from lung cancer patients for the assessment of molecular alterations relevant to therapy is an invasive, time-consuming, painful, and risk-associated procedure (e.g., severe bleeding events). The present investigation suggests that CTCs may offer solutions to this problem in the near future. Genomic information can also be obtained by the analysis of circulating cell-free DNA released by dying cancer cells into the blood samples, which has recently achieved great attention (29). However, CTC analysis can be performed on the DNA, RNA, protein level and capture of sufficient amounts of CTCs to also allow functional studies (25, 30, 31).

Our first validation study of the wire has produced encouraging results. However, there are still some limitations and open issues that need to be discussed. The wire application does not permit reporting of CTC counts in a given (defined) blood volume (i.e., CTCs in 10 mL of blood). Using this device, the value can only be reported as CTCs collected in 30 minutes. Because the amount of blood that flows through the vein can be variable from patient to patient, or even at different time points in the same patient, this may make it more difficult to interpret quantitative differences after treatment. However, we could show that subsequent applications of the wire during the same visit are feasible and increase the capture rates for CTCs at which time no significant differences between repeated CTC measurements were observable, indicating that the CTC results are reproducible. Besides, we had only a limited number of patients without metastases, which may also explain why no significant differences in CTC rates were observed. Larger studies should be performed including more early-stage lung cancer patients that are screened and analyzed for CTCs. Finally, the current anti-EpCAM antibodies used for capturing of CTCs have demonstrated proof-of-principle, but future generations of the device should include a mixture of different capturing antibodies to

**Figure 4.**

Reduction of CTC counts under therapy determined with the wire. Box-and-whisker plot of CTC count difference between different visits (V=visit) for patients with responsive, stable, and progressive disease.
isolate subsets of EpCAM-negative cancer cells that do not display the epithelial phenotype, e.g., cells that underwent an epithelial to mesenchymal transition (32). This development will further increase the capture rate of CTCs via the wire.

In conclusion, our present findings indicate that in vivo isolation of CTCs is a promising approach to capture sufficient amounts of CTCs amenable to downstream molecular analyses in lung cancer. This new diagnostic tool can now be tested in interventional clinical studies where the number and/or characteristics of CTCs will be used to stratify patients to particular treatments and established endpoints such as survival will be assessed. There is an increasing number of molecular targets that are only present in a small fraction of NSCLC patients (33), which requires stratification based on the molecular characteristics of tumor cells in individual patients. Furthermore, CTCs have recently been detected in patients with chronic obstructive pulmonary disease without clinically detectable lung cancer (34). The implementation of a sensitive in vivo device may now also allow early diagnosis of lung cancer. Thus, future studies are needed to explore the entire clinical potential of the wire.

**Disclosure of Potential Conflicts of Interest**

N. Brychter and O. von Ahsen report receiving commercial research grants from Bayer Pharma AG. T. Krahn is a consultant/advisory board member for Gilupi UK. K. Pantel is a consultant/advisory board member for Gilupi GmbH. No potential conflicts of interest were disclosed by the other authors.

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**Figure 5.**

Mutational analysis of the KRAS and EGFR genes in captured CTCs. A, cells isolated with the CellCollector were analyzed microscopically by means of immunofluorescence staining according to the staining procedure in the clinical setup. The wire was fragmented and subjected to WGA. Patient material examined by dPCR, CTC count, and primary tumor mutation status (NTC = none template control, WT = wild-type, and V = visit). B, amplified gDNA of patient 13 was analyzed by dPCR for the known primary tumor mutation KRAS G12D and KRAS G12C as specificity control. C, amplified gDNA of patient 47 was analyzed by dPCR for the known primary tumor mutation EGFR H773R and the other EGFR mutations L858R and T790M to control for specificity.
Other (using the CellCollectors on the patients directly (in vivo) and their further processing): N. Penkalla

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References

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