Clinical Chemistry 59:1
252–260 (2013)

Heterogeneity of Epidermal Growth Factor Receptor Status and Mutations of *KRAS*/*PIK3CA* in Circulating Tumor Cells of Patients with Colorectal Cancer

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BACKGROUND: Molecular characterization of circulating tumor cells (CTCs) is pivotal to increasing the diagnostic specificity of CTC assays and investigating therapeutic targets and their downstream pathways on CTCs. We focused on epidermal growth factor receptor (EGFR) and genes relevant for its inhibition in patients with colorectal cancer (CRC).

METHODS: We used the CellSearch® system for CTC detection in peripheral blood samples from 49 patients with metastatic CRC (mCRC) and 32 patients with nonmetastatic CRC (nmCRC). We assessed EGFR expression in 741 CTCs from 27 patients with mCRC and 6 patients with nmCRC using a fluorescein-conjugated antibody with the CellSearch Epithelial Cell Kit. DNA of a single CTC isolated by micromanipulation was propagated by whole-genome amplification and analyzed by quantitative PCR for *EGFR* gene amplification and sequencing for *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), *BRAF* (v-raf murine sarcoma viral oncogene homolog B1), and *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α) mutations.

RESULTS: At least 2 CTCs were detected in 24 of 49 patients with mCRC and 7 of 32 patients with nmCRC. In 7 of 33 patients, CTCs with increased EGFR expression were identified. Heterogeneity in EGFR expression was observed between CTCs from the same patient. *EGFR* gene amplification was found in 7 of 26 CTCs from 3 patients. The investigated *BRAF* gene locus was not mutated in 44 analyzed CTCs, whereas *KRAS* mutations were detected in 5 of 15 CTCs from 1 patient and *PIK3CA* mutations in 14 of 36 CTCs from 4 patients.

CONCLUSIONS: Molecular characterization of single CTCs demonstrated considerable intra- and interpatient heterogeneity of EGFR expression and genetic alterations in *EGFR*, *KRAS*, and *PIK3CA*, possibly explaining the variable response rates to EGFR inhibition in patients with CRC.

Colorectal cancer (CRC)[6] is one of the main malignancies in industrialized countries, with high mortality due to distant metastasis *(1)*. One of the most prominent therapeutic targets is epidermal growth factor receptor (EGFR) *(2, 3)*; however, prediction of responses to EGFR inhibition in patients with CRC is still challenging. The mere detection of EGFR protein expression or gene amplification on primary tumor cells has not been successful as a reliable predictor *(2–4)*. Interestingly, mutations in genes involved in the EGFR signaling pathway affect the efficacy of anti-EGFR treatment with cetuximab, panitumumab, and gefitinib. Thus, mutations in the *KRAS* locus (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog),[7] which occur in 40%–60% of patients with CRC, are relevant for responses to anti-EGFR immunotherapy *(5, 6)*. More recent studies showed an additional relevance of mutations in other downstream genes of EGFR signaling such as *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) and *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α), which are

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Received April 24, 2012; accepted October 19, 2012.
Previously published online at DOI: 10.1373/clinchem.2012.188557

[6] Nonstandard abbreviations: CRC, colorectal cancer; EGFR, epidermal growth factor receptor; CTC, circulating tumor cell; CS, CellSearch system; nmCRC, non-metastatic CRC; mCRC, metastatic CRC; WGA, whole-genome amplification; qPCR, quantitative real-time PCR; FISH, fluorescence in situ hybridization; *LINE1*, long interspersed element 1; DTC, disseminated tumor cell; CGH, comparative genomic hybridization; HER2, human epidermal growth factor receptor 2; EpCAM, epithelial cell adhesion molecule.

[7] Human genes: *KRAS*, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; *BRAF*, v-raf murine sarcoma viral oncogene homolog B1; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α; *EGFR*, epidermal growth factor receptor; *TP53*, tumor protein 53; *ERBB2*, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian).

mutated in 4%–12% and 14%–20% of primary tumors, respectively *(6–9)*.

In clinical practice, the detection of therapeutic targets is restricted to the primary tumor. However, both the expression of target molecules and the presence of therapeutically relevant mutations might differ within the tumor mass and between primary tumors and metastases *(9–12)*. Thus, the mere analysis of primary tumors for specific therapeutic targets might lead to false stratification of patients to targeted therapy.

This limitation might be overcome by the recently proposed use of circulating tumor cells (CTCs) as a "liquid biopsy" *(13–18)*. CTCs may reflect subpopulations of primary and/or metastatic tumor cells, and CTCs are easily accessible through blood collection. However, CTCs comprise only a few cells among millions of blood cells, and their detection and molecular analysis are challenging *(14)*. Currently, the automated CellSearch® system (CS) is the only FDA-cleared application for CTC detection in patients with metastatic breast, colorectal, and prostate cancer *(16–18)*.

Here, we introduce a new approach to characterize CTCs detected by CS in peripheral blood samples from patients with CRC for proteins and genes related to EGFR inhibition, including *EGFR* gene amplification and protein expression as well as mutations in the *KRAS, BRAF*, and *PIK3CA* genes.

Materials and Methods

PATIENTS

The local ethics committee approved this study, and written informed consent was obtained from all participants. Blood samples (7.5 mL) from 32 patients with nonmetastatic CRC (nmCRC) and 49 patients with metastatic CRC (mCRC), treated at the University Medical Center Hamburg-Eppendorf or at the Medical University of Graz, were collected into CellSave tubes (Veridex) and processed by CS within 96 h.

We performed further molecular analysis on CTCs from 5 patients with mCRC having advanced-stage (Dukes D) disease. Detailed patient data are provided in Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue1.

CELL LINES, CULTURE CONDITIONS, AND FLUORESCENCE IN SITU HYBRIDIZATION ANALYSIS

Method details are provided in the online Supplemental Methods.

ENUMERATION OF CTCs BY CS

We performed CTC detection by CS (Veridex) as described elsewhere *(16)*. For EGFR expression analysis, a fluorescein-conjugated antibody against EGFR (Veridex) was applied in the fourth channel of the CS. Nucleated cells showing a round to oval morphology and an epithelium-specific staining pattern (cytokeratin-positive and CD45-negative) were enumerated as CTCs. Evaluation of immunofluorescence intensity was done in a blinded fashion by 2 experienced persons independently. Questionable interpretations were evaluated again until consensus was reached.

SAMPLE PREPARATION FOR MOLECULAR ANALYSIS AFTER CellSearch DETECTION

For single-cell analysis, the scanned cartridge was removed from the MagNest and stored at 4 °C until the sample evaluation was finished. CTCs were further processed by resuspending the attached ferrofluid particles from the surface, loading the entire sample on a SuperFrost plus adhesion slide (Karl Hecht), and allowing cells to settle for 30 min.

ISOLATION OF CTCs BY MICROMANIPULATION

For isolating single CTCs, we applied a micromanipulator consisting of the microinjector CellTram vario and the micromanipulator TransferMan NK2 (both Eppendorf) supplemented with custom-made capillaries (40 μmol/L in diameter, capillary type III, Eppendorf) and connected to an Axiovert 200 inverted fluorescence microscope (Carl Zeiss). CTCs were identified by the criteria used in the CS. Single CTCs were released into 2.5 μL water in the cap of a PCR tube, spun down briefly (Galaxy Mini Microcentrifuge, VWR), and frozen at −20 °C.

WHOLE-GENOME AMPLIFICATION OF DNA FROM SINGLE TUMOR CELLS WITH THE GenomePlex KIT

We performed whole-genome amplification (WGA) with the GenomePlex Single Cell Whole Genome Amplification kit (Sigma-Aldrich) according to the manufacturer's protocol. After thawing on ice, the sample was complemented to 9 μL with nuclease-free water and 1 μL single cell lysis and fragmentation buffer, and proteinase K mix was added. The subsequent incubation for cell lysis (50 °C for 1 h) and following steps were implemented on ice as described by the manufacturer. WGA products were purified with the GenElute PCR Clean-Up kit (Sigma-Aldrich). Testing of DNA quality by multiplex PCR is described in the online Supplemental Methods.

WGA OF DNA FROM SINGLE TUMOR CELLS WITH THE GenomiPhi KIT

We performed WGA of single-cell DNA with the GenomiPhi DNA Amplification kit (GE Healthcare) as follows. CTCs were thawed on ice and lysed in 9 μL GenomPhi sample buffer by protease digestion (10.7 μAU/μL protease, 15 min at 50 °C, 15 min at 70 °C).

The released DNA was denatured (2 min, 95 °C), amplification mix (9 μL reaction buffer and 1 μL enzyme mix) was added, and amplification reaction was carried out at 30 °C for 2.5 h followed by enzyme inactivation at 65 °C for 10 min. WGA products were purified with NucleoSeq Columns (Macherey-Nagel). We analyzed the quality of WGA products by multiplex PCR (see online Supplemental Methods).

IDENTIFICATION OF *EGFR* GENE AMPLIFICATIONS BY qPCR ON WGA PRODUCTS

For quantitative real-time PCR (qPCR), 10 ng purified WGA product in a final volume of 15 μL was applied. The following primers were used: *LINE1*_for, *LINE1*_rev *(4)*, *EGFR*_exon8_F, and *EGFR*_exon8_R *(19)*. The assay was performed with Maxima™ SYBR Green qPCR Master Mix (Fermentas) as previously described *(19)*. Samples with calculated DNA concentrations <250 pg per reaction were excluded from the evaluation. Ratios ≥2 were considered amplified in the analyzed region.

SEQUENCING OF SINGLE-CELL WGA PRODUCTS

Detailed methodology and sequencing primers are described in the online Supplemental Methods.

Results

APPLICABILITY OF WGA FROM SINGLE-CELL DNA

To compare the applicability of the GenomePlex and GenomiPhi kits for WGA, we amplified single-cell DNA from 11 MDA-MB-468 cells after CS processing with both kits in parallel. DNA amounts of WGA products were higher with the GenomePlex kit (mean 8.9 μg, range 4.6–15.4 μg) than with the GenomiPhi kit (mean 1.54 μg, range 0.3–2.2 μg). Adequate DNA quality (at least 2 of 4 PCR products after multiplex PCR) (Fig. 1) *(20)* could be achieved from 8 of 11 and 11 of 11 cells amplified with GenomePlex and GenomiPhi, respectively.

The *EGFR* qPCR assay delivered PCR products with both primers (*EGFR* target and *LINE1* reference primer) in 9 of 11 reactions on GenomiPhi and 6 of 11 reactions on GenomePlex single-cell WGA products. In comparison to the mean *EGFR* gene amplification status of 14.72 (range 0.56–40.35, median 11.22) of the GenomePlex-processed cells, mean *EGFR* amplification rates of 43.89 (range 7.22–90.07, median 40.29) obtained with the GenomiPhi WGA products were more consistent with the 30- to 40-fold *EGFR* amplification measured on DNA extracted from approximately 10^7 MDA-MB-468 cells.

DETECTION OF EGFR EXPRESSION AND GENE AMPLIFICATIONS ON SINGLE CTCs

The application of an anti-EGFR antibody in the fourth channel of the CS enables the classification of EGFR expression levels on single CTCs. We first performed experiments to demonstrate that EGFR immunostaining by CS correlated with the *EGFR* gene amplification status determined by fluorescence in situ hybridization (FISH) and qPCR on WGA products from cell-line cells.

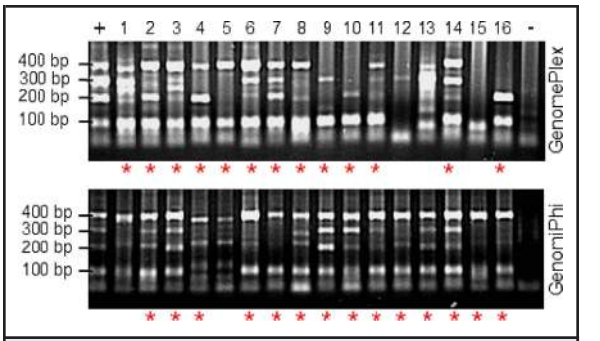

Fig. 1. Multiplex PCR to analyze the DNA quality of WGA products obtained from MDA-MB-468 single cells after CellSearch® processing.

Representative multiplex PCR results from GenomePlex and GenomiPhi WGA products are shown. Reactions with 2 to 4 amplification products of 100, 200, 300, or 400 bp indicative of adequate DNA quality are marked by asterisks. +, Positive control for WGA (10 ng DNA extract from 10^7 MDA-MB-468 cells applied to WGA procedure); –, negative control for WGA (no DNA applied).

To distinguish different levels of EGFR expression and *EGFR* gene amplification, we used the EGFR-overexpressing and/or *EGFR*-amplified cell lines MDA-MB-468, BT-20, and MDA-MB-468A *(19, 21–24)*. The *EGFR* amplicon in these cell lines has been comprehensively analyzed by high-resolution fine-tiling array analysis *(19)*. Low-level EGFR-expressing MCF-7 cells carrying a single *EGFR* gene copy served as negative control. With the help of the cell-line cells, we established an immunoscoring system for the evaluation of EGFR expression of CTCs. MCF-7 cells were either weakly positive (score 1) or negative (score 0), whereas some MDA-MB-468A cells also revealed moderate (score 2) EGFR expression (Fig. 2, A and B). In MDA-MB-468 and BT-20 cells, a moderate (score 2) to strong (score 3) intensity of EGFR-specific immunofluorescence (Fig. 2A) was observed. CTCs with a moderate to strong intensity of EGFR-specific immunofluorescence were considered EGFR-positive, whereas CTCs with negative or only weak intensity of EGFR-specific immunofluorescence were designated EGFR-negative.

EGFR expression was consistent with the mean gene amplifications determined on ≥30 cells by FISH (0.7-fold for MCF-7, 8.2-fold for BT-20, >30-fold for

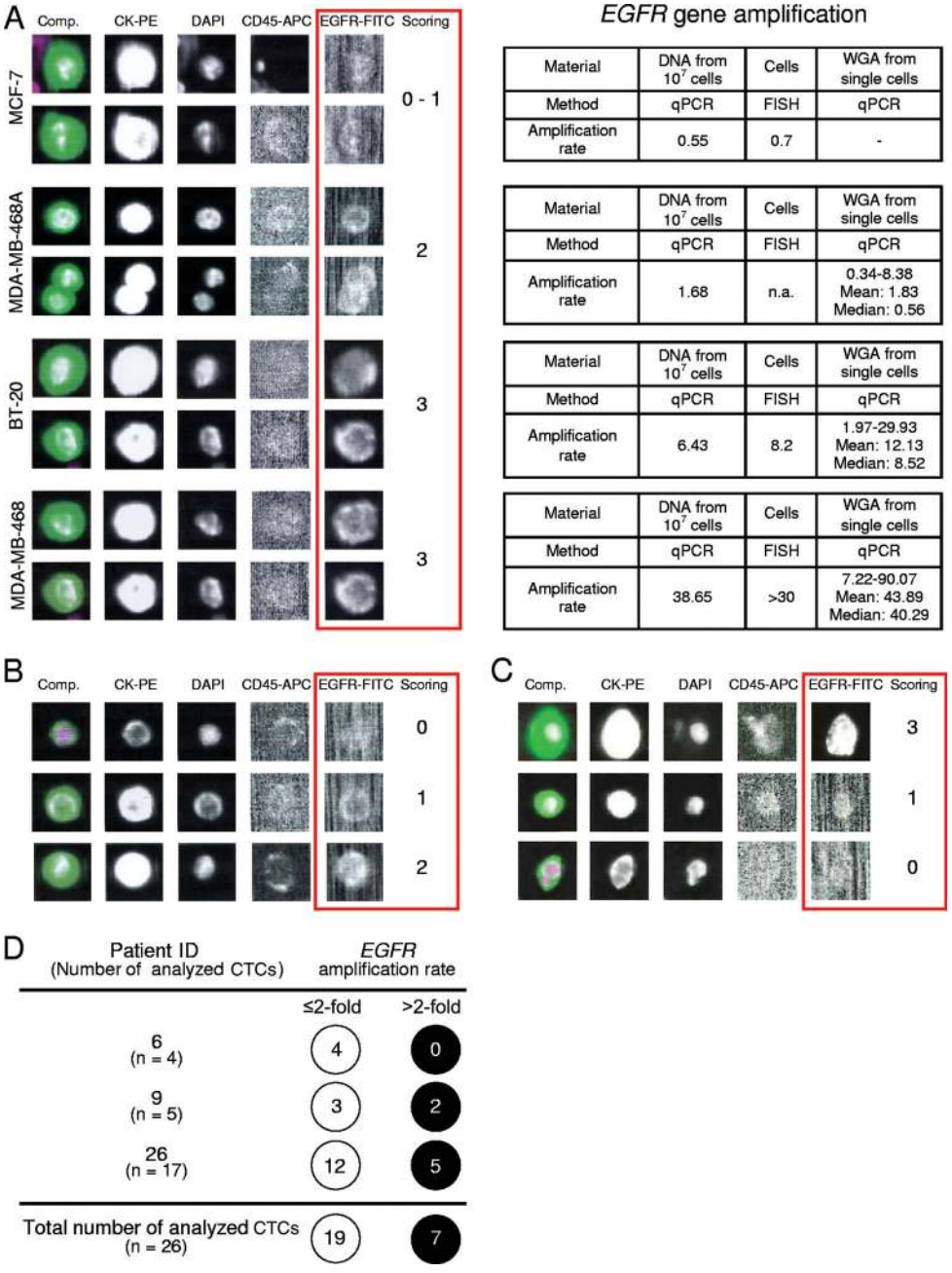

Fig. 2. EGFR immunoscoring of cells detected by CS.

(A), EGFR gene expression detected by CS (left images) correlates with *EGFR* gene amplification rates determined by qPCR and FISH (right tables) with MCF-7 (low EGFR expression = score 0–1, *EGFR* amplification rate 0.55/0.7), MDA-MB-468A (moderate EGFR expression = score 2, *EGFR* amplification rate 1.83/not analyzed), BT-20 (strong EGFR expression = score 3, *EGFR* amplification rate 6.43/8.2), and MDA-MB-468 (strong EGFR expression = score 3, *EGFR* amplification rate 38.65/>30) cells. The *EGFR* qPCR was performed on DNA extracts from approximately 10^7 cells as well as on WGA products from 10 single cells after CS. (B), Heterogeneity in EGFR expression in the MDA-MB-468A cell population. (C), Heterogeneity in EGFR expression in the CTC population of patient 9. (D), *EGFR* gene amplification rate determined by qPCR in 26 analyzed CTCs from patients 6, 9, and 26. Comp., composition; CK-PE, cytokeratin-phycoerythrin; DAPI, 4′,6-diamidino-2-phenylindole; APC, allophycocyanin; FITC, fluorescein isothiocyanate.

Table 1. EGFR immunoscoring of CTC.

Patient ID	Analyzed CTCs, n	EGFR immunoscore			
		0	1	2	3
6	181	88	65	28	0
9	202	191	9	1	1
11	2	0	0	0	2
20	9	8	0	0	1
25	7	4	2	1	0
26	100	74	22	4	0
33	6	4	1	1	0

MDA-MB-468) and qPCR on DNA extracts (approximately 10^7 cells) of the corresponding cell lines (MCF-7, 0.55-fold; MDA-MB-468 clone A, 1.83-fold; BT-20, 6.43-fold; MDA-MB-468, 38.65-fold). In line with these findings, the analysis of GenomiPhi WGA products of 8 MDA-MB-468A, 7 BT-20, and 9 MDA-MB-468 single cells by *EGFR* qPCR revealed comparable mean values of 1.83, 12.13, and 43.89, respectively. In agreement with heterogeneous EGFR expression levels observed within a cell line population (Fig. 2B), the amplification status detected by qPCR (MDA-MB-468A, median 0.56, range 0.34–8.38; BT-20, median 8.52, range 1.97–29.93; MDA-MB-468, median 40.2, range 7.22–90.07) varied among individual cell line cells.

At least 2 CTCs were detected in 24 of 49 (49%) patients with mCRC and 7 of 32 (22%) patients with nmCRC. We further assessed 741 CTCs from 33 patients with CRC (27 mCRC, 6 nmCRC) for EGFR protein expression. Altogether, ≥1 EGFR-positive CTC could be observed in 7 of 33 (21%) patients, with only 2 of 33 patients (6%) possessing strongly EGFR-positive CTCs. Whereas all CTCs detected in nmCRC patients were EGFR-negative, increased EGFR levels were observed in 7 of 27 (26%) patients with mCRC (Table 1). Furthermore, EGFR was differently expressed between CTCs from the same patients, ranging for example from EGFR-negative to strongly EGFR-positive (Fig. 2C; Table 1).

To analyze CTC heterogeneity molecularly, we focused on blood samples (n = 5) with more than 20 morphologically intact CTCs per 7.5 mL, which explains in part the low number of samples analyzed by single-cell PCR. The failure to analyze a higher number of detected CTCs is mainly due to the inability to transfer all CTCs undisturbed from the CellSearch cartridge onto slides and reidentify them for micromanipulation. Thus, from all 33 patients analyzed for EGFR expression of CTCs, only CTCs from 3 mCRC patients could also be analyzed for *EGFR* gene amplification by qPCR (Fig. 2D). Whereas patient 6 exclusively presented *EGFR*-nonamplified CTCs (fold change 0.21–1.22) but also CTCs with moderate EGFR protein expression, patients 9 and 26 carried an amplification of the *EGFR* region in ≥1 CTC. *EGFR* gene amplification could be detected in 2 of 5 CTCs from patient 9 (2.55- and 10.34-fold) and in 5 of 17 CTCs from patient 26 (2.93- to 14.48-fold). CTCs of patient 9 ranged from EGFR-negative to strongly positive. In patient 26, we found heterogeneous EGFR immunoscoring from 0 to 2.

Because it is impossible to analyze the same CTC for EGFR protein expression and *EGFR* gene amplification after CS processing, concordance on the single-cell level could not be measured with the applied methods. Immunocytochemical detection of EGFR expression in the CS, however, enables immunoscoring of each individual CTC without further loss or manipulation of these cells.

MUTATIONAL ANALYSIS OF SINGLE CTCs

For the establishment of a technique to detect mutations on WGA products from single cells, we used MDA-MB-231 cells carrying a tumor protein 53 (*TP53*) mutation. To investigate the impact of contamination of a single CTC with surrounding leukocytes during micromanipulation, we performed a mutational analysis on GenomiPhi WGA products from a MDA-MB-231 single cell supplemented with 1–2 leukocytes (Fig. 3A). An accidental contamination with >2 leukocytes during micromanipulation can generally be excluded. Although sequencing the WGA product of a single leukocyte yielded the wild-type sequence of *TP53*, the previously described *TP53* mutation R280K could be detected in all samples containing a single MDA-MB-231 cell. Neither 2 leukocytes nor 2 μL cell-free supernatant from the CS cartridge added to the sequencing reaction disturbed the detection of the expected mutation.

MUTATIONS IN DOWNSTREAM GENES OF THE EGFR SIGNALING PATHWAY

Altogether, WGA products of 69 single CTCs from 5 patients with mCRC were screened for mutations in hot-spot regions of *KRAS* (codons 12/13, 59 CTCs analyzed), *BRAF* (codon 600, 44 CTCs analyzed), and/or *PIK3CA* (codons 542–46/1047, 39 CTCs analyzed). Unlike CTCs from patient 22, CTCs from patients 6, 9, 18, and 26 showed ≥1 mutated gene (Fig. 3, B and C).

Whereas changes in the *BRAF* locus could not be detected in any analyzed CTC, the presence of a mutation in the *KRAS* or *PIK3CA* gene could be verified in CTCs from 1 of 5 (patient 6) and 4 of 5 (patients 6, 9, 18, and 26) patients, respectively.

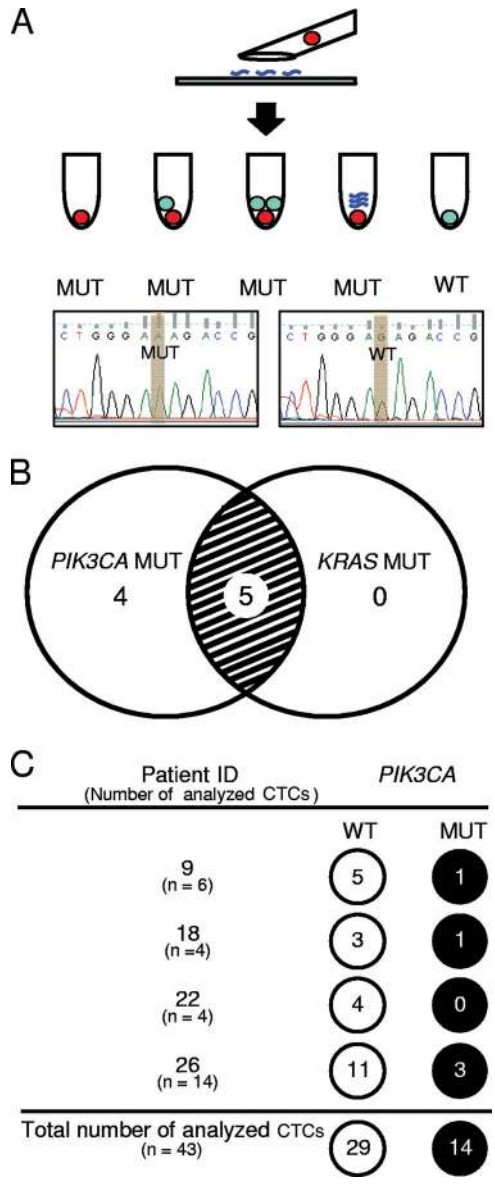

Fig. 3. Mutational analysis of single cells.

(A), Detection of the *TP53* R280K mutation in a single MDA-MB-231 cell (red). Addition of up to 2 leukocytes (green) or cell-free liquid from the CS cartridge (blue waves) to a single MDA-MB-231 cell by micromanipulation did not disturb the detection of the *TP53* mutation. (B), CTCs carrying *PIK3CA* (n = 9) and *KRAS* mutation (n = 5) obtained from patient 6 (total analyzed CTCs, n = 15; wild-type form of both genes, n = 6) illustrate the genetic heterogeneity present in a CTC population. (C), *PIK3CA* gene status in analyzed CTCs from patients 9, 18, 22, and 26. MUT, mutation; WT, wild type.

A *KRAS* (G12V) mutation was exclusively observed in 5 of 15 (33%) CTCs of patient 6. The mutation found in CTCs from this patient could also be verified on DNA extracted from the corresponding primary tumor. Moreover, in patients 9, 18, 22, and 26 with primary tumors harboring the *KRAS* wild-type gene, none of the analyzed CTCs presented a mutation in this gene locus.

The *PIK3CA* mutation E545A was frequently detected in CTCs (14 of 43 CTCs in 4 of 5 patients). Nevertheless, we observed a complex intrapatient heterogeneity. Only a subpopulation of CTCs detected in individual patients carried a *PIK3CA* mutation (9 of 15 CTCs, 60% from patient 6; 1 of 5 CTCs, 20% from patient 9; 1 of 3 CTCs, 33% from patient 18; and 3 of 11 CTCs, 27% from patient 26), whereas the other CTCs were not mutated. Furthermore, 6 of 9 *PIK3CA*-mutated CTCs from patient 6 carried the mutation E545A, while a mutation in codon 542 (E542K) was exclusively present in 3 of 9 CTCs. Moreover, 5 of 9 CTCs mutated in the *PIK3CA* gene also harbored a concomitant *KRAS* mutation in the same cells (Fig. 3B).

Discussion

We performed a comprehensive analysis of EGFR inhibition–related targets on individual CTCs of patients with CRC. Besides the assessment of EGFR expression and amplification by immunocytochemistry and qPCR, we successfully performed mutational analysis of *KRAS*, *BRAF*, and *PIK3CA* on WGA products of single CTCs. Our results show considerable intra- and interpatient heterogeneity of CTCs, which may contribute to the resistance of tumor cells to EGFR inhibition in CRC patients.

In this study, we investigated the applicability of WGA products obtained from single CTCs using two kits for PCR approaches. In comparison to GenomePlex amplification products, GenomiPhi-amplified DNA was more suitable for PCR-based approaches. This limited feasibility of GenomePlex WGAs for PCR approaches might be explained by imbalanced amplification or loss of PCR target regions during DNA fragmentation necessary for successful WGA. Despite lower DNA yield, 82% of the GenomiPhi WGA products vs 64% of the GenomePlex WGA products delivered evaluable signals in *EGFR* qPCR. The mean and median gene amplification rates (43.89- and 40.29-fold) determined on GenomiPhi WGA products are similar to those determined by qPCR on DNA extracts (approximately 10^7 cells) and by FISH analysis (30- to 40-fold) and are consistent with published data for MDA-MB-468 cell populations *(19, 22)*. Moreover, the heterogeneous gene amplification status of the

EGFR gene within the MDA-MB-468 cell population already described *(24)* was reflected by the detected variability of *EGFR* amplification among single cell-line cells ranging from 7.22- to 90.07-fold (GenomPlex 0.56- to 40.35-fold). Although the WGA from single CTCs detected by CS has not been described yet, several WGA protocols for single-cell DNA from different cell types, including cell-line cells *(19, 25–27)* and disseminated tumor cells (DTCs)/CTCs *(19, 27–29)*, have already been published. Whereas the GenomePlex technology has merely been used for comparative genomic hybridization (CGH) analysis of single-cell DNA *(26, 27)*, a linker-PCR–based approach *(28, 29)* was shown to be suitable for CGH and loss of heterozygosity analysis of single-cell DNA *(25, 28, 29)*. Moreover, a WGA protocol was previously described on single breast cancer cell-line cells with the GenomiPhi kit allowing a subsequent analysis of WGA products by fine-tiling array-CGH and qPCR to determine *EGFR* gene amplifications *(19)*.

We detected ≥2 CTCs in 49% of patients with mCRC (24 of 49) and 22% of patients with nmCRC (7 of 32). These results are similar to previous findings revealing detection rates of ≥2 CTCs in 33%–61% of patients with mCRC *(17, 30)* and 26% of patients with nmCRC *(30)*. In view of our recent work *(31)*, we applied strict morphological criteria to reduce the chance that we counted circulating nonmalignant epithelial cells. However, we cannot exclude the possibility that some of those cells classified as CTCs without detectable genomic aberrations might be nonmalignant epithelial cells.

Although 30%–90% of advanced primary CRC cases were described to be positive for EGFR expression *(32)*, CTCs with increased EGFR expression levels could be detected in only 7 of 33 (21%) CTC-positive patients. This divergence is most likely a consequence of the diversity of patient cohorts analyzed and methods and antibodies applied *(3)*. Furthermore, commonly used immunohistochemistry approaches to analyze EGFR expression on primary tumors lack a standardized scoring system *(3)*. Thus, individual interpretation of staining results and definition of EGFR positivity leads to significant discrepancies among studies concerning EGFR expression and its predictive potential for EGFR therapy response rates, which points to the need for more standardized methods to select patients for anti-EGFR immunotherapies. However, it is also possible that EGFR-overexpressing CTCs have undergone epithelial-mesenchymal transition *(33, 34)*, which might involve a downregulation of EpCAM (epithelial cell adhesion molecule) expression *(33–35)*. Thus, a proportion of EGFR-positive cells may have escaped the anti-EpCAM enrichment step of CS.

In addition to the low frequency of EGFR positivity in our patient cohort, not all CTCs of individual cases could be classified as EGFR overexpressing, revealing a substantial heterogeneity in EGFR levels among CTCs from the same patient. These varying expression levels presumably reflect intratumoral heterogeneity of EGFR expression.

Unlike the overexpression of the immunotherapy target human epidermal growth factor receptor 2 (HER2, also known as ERBB2) in breast cancer patients, which is most often connected with an amplification of the v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (*ERBB2*) gene *(36)*, the correlation of EGFR protein levels and gene amplification and their meaning for EGFR immunotherapy response is still controversial *(2–4)*. As already shown for EGFR protein expression, we also obtained a heterogeneous distribution of *EGFR* gene amplification rates between CTCs of the same patient as well as of different patients.

For a response to anti-EGFR therapies, a normal function of downstream elements of the signaling pathway (e.g., KRAS, BRAF, and PIK3CA) is essential *(5–8)*. The analysis of primary tumors as the current gold standard for therapeutic decisions might be critical owing to intratumoral genetic heterogeneity *(10, 11)* and increasing genomic instability during disease progression. This issue is further underlined by several reports showing discordant mutations (*KRAS*, *BRAF*, *PIK3CA*, *TP53*) between CRC primary tumors and corresponding metastasis in 5%–37% of patients *(9, 11, 12)*. Moreover, disagreement of HER2 protein expression between primary breast cancer and CTCs has also been reported *(37, 38)*.

To overcome these limitations, we successfully established a protocol for the mutational analysis of DNA from single CTCs detected by CS. By screening 65 CTCs isolated from 5 patients, we identified *KRAS* mutations only in CTCs of patient 6. CTCs carrying a *PIK3CA* mutation were found in 4 of 5 patients. It should be noted that all 5 patients in our study had progressive metastatic disease. Because *PIK3CA* mutations are associated with poor prognosis for patients with CRC *(39)* it is not surprising that we found a high rate of these mutations in CTCs. However, the distinct CTC populations of individual patients with mCRC revealed a similar heterogeneity as described above for EGFR expression and gene amplification. Our data are consistent with previous findings, which also showed a heterogeneous pattern of different phenotypic and genomic changes on single DTCs/CTCs obtained from breast and esophageal cancer patients *(28, 37, 38, 40)*. This divergence of CTC populations might promote the selection of tumor clones escaping from targeted

therapies and thus of potential founder cells of distant metastasis.

The main novelty of the technology described here is that it allows molecular analysis of individual CTCs after they are captured and immunostained by CS. In its current format, however, it is accessible only for a limited number of samples, mainly owing to the inability to remove all CTCs undisturbed from the CS cartridge, transfer them onto slides, and reidentify them for micromanipulation. To introduce this technology as a diagnostic tool, these problems have to be circumvented by new approaches capable of providing the captured and immunostained cells directly on slides or nonimmobilized in an appropriate buffer for further processing.

Nevertheless, we could show feasibility of several downstream applications to further characterize molecular features of single CTCs detected with CS, including immunocytochemistry, mutational analysis, and qPCR. Besides the potential utility for targeted therapies, an additional genetic characterization of CTCs will add specificity to current CTC assays *(14)*. Under certain pathological conditions such as inflammatory bowel diseases, nonmalignant epithelial cells can be released into the blood circulation, leading to false-positive CTC results unless strict morphological and molecular criteria are imposed *(31)*. However, our present work shows that CTCs are genetically heterogeneous, which points to the need for complex technologies for multiplexing single CTCs. To introduce CTC analysis to the clinical routine, there are more limitations to overcome. Furthermore, the establishment of more sensitive CTC enrichment techniques, also capturing EpCAM-negative CTCs, is an important aspect to improve CTC detection. Optimization of WGA protocols to yield highly concentrated amplification products from single CTCs suitable for a wide variation of molecular approaches should become a major focus of future experiments. The ongoing improvement of CTC enrichment and single-cell technologies may help establish a future role of CTCs for stratification of cancer patients to more individualized therapies.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: K. Pantel, Veridex.
Research Funding: K. Pantel, the European Community's 7th Framework Programme (FP7/2007–2013) under grant agreement no. 202230, acronym GENINCA, and the ERC-2010-AdG_ 20100317 DISSECT.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank Cornelia Coith and Malgorzata Stoupiec for excellent technical assistance and Professor Michael Speicher and Professor Jochen Geigl (University of Graz, Austria) for a critical reading of the manuscript.

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