Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Characterization of Circulating Tumor Cells by Fluorescence In Situ Hybridization

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Received 15 July 2008; Revision Received 29 January 2009; Accepted 9 February 2009

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Published online 16 March 2009 in Wiley InterScience (www.interscience.wiley.com)

DOI: 10.1002/cyto.a.20718

Abstract

Tumor cells in blood of patients with metastatic carcinomas have been associated with poor survival prospects. Further characterization of these cells may provide further insights into the metastatic process. Circulating Tumor Cells (CTC) were enumerated in 7.5 mL of blood with the CellSearch™ system. After enumeration of Cytokeratin+, CD45−, nucleated cells, the cells are fixed in the cartridge while maintaining their original position. Cartridges were hybridized with FISH probes against the centromeric regions of chromosome 1, 7, 8, and 17. Next fluorescence images of the FISH probes of the previous identified CTC were acquired. Leukocytes surrounding the CTC were used as internal controls. The number of copies of chromosome 1, 7, 8, and 17 could be determined in 118 CTC containing blood samples from 59 metastatic prostate cancer patients. The samples contained a total of 21,751 CTC (mean 184, median 16, SD 650). Chromosome counts were obtained in 61% of the relocated CTC. On an average, these CTC contained 2.8 copies of chromosome 1, 2.7 copies of chromosome 7, 3.1 copies of chromosome 8, and 2.3 copies of chromosome 17. CTC in which no chromosome count was obtained most likely underwent apoptosis indicated by the expression of M30. In 6/59 patients only diploid CTC were detected these samples, however, only contained 1–5 CTC. Heterogeneity in the chromosomal abnormalities was observed between CTC of different patients as well as among CTC of the same patient. Cytogenetic composition of CTC can be reliably assessed after they have been identified by the CellSearch™ system. The majority of CTC in hormone refractory prostate cancer are aneuploid confirming that they indeed are cancer cells. An extensive heterogeneity in the copy number of each of the chromosomes was observed. © 2009 International Society for Advancement of Cytometry

Key terms

CTC; FISH; prostate cancer; aneuploidy

THE need for biomarkers to guide treatment of patients with metastatic carcinomas is increasing with the growing number of available treatment options. Tumor cells shed into the blood during metastasis have the promise to become generic biomarkers for a variety of carcinomas. In multicenter prospective studies, the presence of circulating tumor cells (CTC) have been associated with poor progression-free and overall survival in metastatic breast (MBC), colorectal (MCRC), and prostate cancer (MPC) (1–6). In these studies, a standardized method for the enumeration and characterization of CTC was utilized (7–9). The presence of specific antigens or the amplification of specific genes in these CTC may help in selecting specific therapies or families of therapies (10–15). In this study, we introduce a method that permits FISH analysis on the CTC that have previously be identified by immunofluorescent staining in a standardized and automated fashion. The method was demonstrated by assessment of chromosome 1, 7, 8, and 17 copy numbers on CTC from samples from metastatic castration resistant prostate cancer patients enrolled in a recently

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completed multicenter study that demonstrated the relation between presence of CTC and poor outcome (6).

Materials and Methods

Patient Samples

A prospective multicenter clinical trial that evaluated the utility of counting CTC for predicting response to therapy, progression-free survival, and overall survival in metastatic castration-resistant prostate cancer patients was conducted (6). A total of 65 clinical centers throughout the United States and Europe participated in this study after formal institutional review board approval. All patients were required to provide written informed consent. Blood was collected before starting a new treatment and at monthly intervals prior to the next cycle of therapy. CTC were enumerated in 7.5 mL of blood using the CellSearch™ system (Veridex Raritan, NJ) and sample cartridges were stored for later FISH analysis as described later. In this study, a total of 178 cartridges containing CTC of 70 patients from blood samples taken after initiation of therapy were used.

Enumeration of Circulating Tumor Cells

The CellTracks Autoprep (Veridex, Raritan, NJ) was used to immunomagnetically enrich epithelial cells from 7.5 mL of blood using ferrofluids coated with epithelial cell specific EpCAM antibodies and stain the CTC enriched samples with phycoerythrin conjugated antibodies directed against cytokeratins 8, 18, and 19, an allophycocyanin conjugated antibody to CD45 and the nuclear dye DAPI (7–9). After enrichment and staining the volume is reduced to 300 μL and this volume, containing all isolated CTC, is transferred to a cartridge that is present inside the MagNest™ Cell Presentation Device. Next, the cartridge is analyzed on the CellTracks Analyzer II, a four-color semiautomated fluorescence microscope (Veridex, Raritan, NJ). Image frames covering the entire surface of the cartridge for each of the four fluorescence filter cubes are captured. From the captured images, a gallery of objects meeting predetermined criteria is presented in a Web-enabled browser for interpretation by a trained operator who makes the final cell identification of the CTC. The criteria for an object to be defined as a CTC include round to oval morphology, a visible nucleus (DAPI positive), positive staining for cytokeratin and negative staining for CD45 (7–9). Results of cell enumeration are expressed as the number of cells per 7.5 mL of blood. The performance of the assay system is described in detail elsewhere (9,16).

Preservation of CTC for FISH Analysis

CTC that have been analyzed on the CellTracks Analyzer are contained in 300 μL buffer inside a cartridge and held in position against the analysis surface by magnetic forces (17,18). To preserve the location of the CTC for future interrogation the buffer inside the cartridge has to be aspirated from the cartridge without cell movement. The cartridge contained within the MagNest™ Cell Presentation Device is placed upright with the glass on which the cells reside vertically. Manual aspiration of the buffer and introduction of the fixative resulted in variable loss or movement of CTC making the need for an automated buffer aspiration and cell fixation necessary. A device was constructed using a CavroXE1000 digital syringe pump for fluid transport and transfer probes that were fabricated from 13AWG Inconel (Cavro, Sunnyvale, CA). This non-magnetic material was required to avoid movement of the probe under the influence of the magnetic field used to contain the cells in the sample cartridge in position. Aspiration and fixation steps were optimized and in the final protocol 250 μL methanol:acetic acid (3:1) is carefully injected at the bottom of the cartridge. The difference in density causes the methanol/acetic acid to swirl up along the glass to the top of the cartridge. After 2 min, 250 μL is removed from the cartridge followed by another injection of 250 μL methanol:acetic acid. After another 2 min the whole volume is aspirated followed by immediate drying using a forced air flow. Fixed and dried cartridges can be processed for FISH immediately or stored at -20°C for later use. Stability studies showed that the cartridges could be stored for at least 2 years before hybridization.

Hybridization of CTC

FISH probes specific for the centromeric regions of chromosome 1, 7, 8, and 17 labeled with Platinum*Bright*-647, -550, -505, and -415, respectively, were used in this study (Kreatech, Amsterdam, The Netherlands). Cells of the tumor cell line SKBR-3 and MCF7 were used to optimize the volume and concentrations of the probes. The optimized probe mixture constisted of 50 μL of hybridization buffer (50% Formamide / 1× SSC / 10%Dextran Sulfate) containing FISH probes against 1, 7, 8, and 17 at 2 ng/μL each. Probe mixture was added such that it covers the whole glass surface on which the cells are fixed. The cartridges were placed on a 80°C hotplate for 2 min, with the glass facing towards the hotplate, and next hybridized at 42°C for 16 h (Boekel Scientific, Feasterville, PA). After hybridization the cartridge is washed with PBS containing 4′,6-diamidino-2-phenylindole (DAPI) as a nuclear counter stain.

Fluorescent Microscope for CTC FISH Analysis

The CellTracks Analyzer II is equipped with a 10× objective (NA of 0.45). For FISH analysis the analyzer was modified and equipped with a 40× objective (NA of 0.63) (Nikon Instruments Europe, the Netherlands) to improve the resolution and light collection of the fluorescent FISH signals and was equipped with filter cubes that permitted the acquisition of the fluorescence signals from DAPI and Platinum*Bright*-647, -550, -505, and -415. The Linux based operating system was modified such that it could: (1) read stored data and the location of the previously identified CTC, (2) relocate the previous identified CTC after the FISH procedure by correlating the location of the cells in the stored data with the new location acquired on the fluorescent DAPI signal acquired with the 40× objective, (3) acquire a Z-stack of five fluorescence images of the FISH signals of CTC of interest, (4) present the acquired images to the reader for FISH analysis, (5) generate a

report of the result. CD45+ leukocytes, that are co-isolated during the magnetic isolation of the CTC and present in the cartridge, serve as internal controls for the FISH procedure and for the local signal quality for each CTC.

Results

Preservation of CTC for FISH Analysis

The protocol to aspirate the fluid from the cartridges and fix the cells in position was verified by assessment of the number of nucleated cells on the analysis surface that either moved or disappeared from the original location. For CTC identification the 10× objective is used, and 175 images need to be acquired to cover the whole glass surface. Panel A of Figure 1 displays one of the 175 frames for the DAPI signal. One DAPI stained nucleus is enlarged to illustrate the resolution that is used for the identification of CTCs. Panel B of Figure 1 displays the DAPI fluorescence image acquired with the modified instrument using a 40× objective of the area indicated by the green box in Panel A after hybridization. The field of view is 1/16 of the 10× image. The nucleus of the same cell is enlarged and illustrates the higher resolution obtained with the higher NA objective. Furthermore it can be observed that the majority of the cells remain intact and stay in position. During the fixation and FISH procedure all immunofluorescence signals used to identify the CTC disappear which permit the use of fluorescence labels for FISH that overlap the spectra of the fluorescence labels used for immunofluorescence detection. Small differences in the protocol used for aspiration can have large impact on the percentage of cells that can be retrieved and analyzed. In this study two fixation protocols were used which differed in: (1) the aspiration speed of the methanol:acetic acid in the final step of the fixation, (2) the air flow through the cartridge during drying of the cartridge after removal of the fixation reagents. Of the 2,932 CTC analyzed with one protocol, 2,707 (91%) could be relocated after FISH whereas with the other protocol 1,721 of the 3,204 CTC (53%) could be relocated. In total 4,482 of the 6,130 CTC (76%) were relocated for FISH analysis. The difference in protocol did not have an effect on the quality of the FISH signals nor did it influence the analysis.

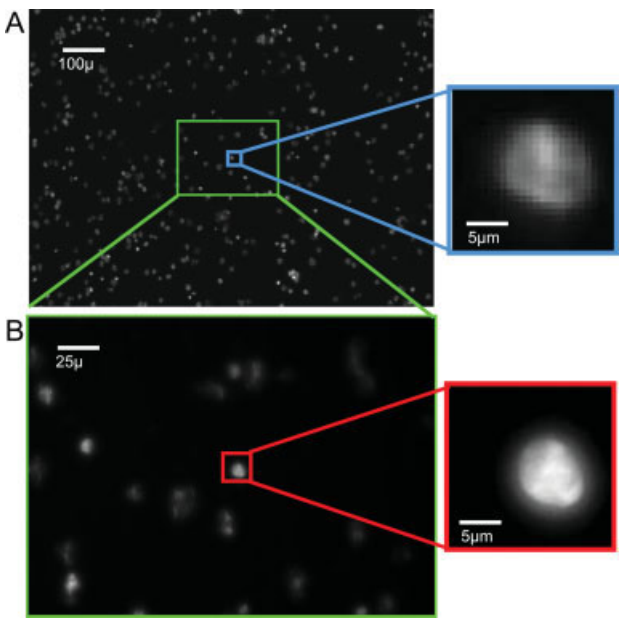

Figure 1. Fluorescence image of DAPI stained nuclei. (**A**) Fluorescence image acquired with a ×10, NA 0.45 objective. The highlighted cell in the center of the image is displayed enlarged on the right side. (**B**) Image acquired by a ×40, NA 0.63 of the area highlighted green in panel A. The field of view is 1/16th of the ×10 area. The center cell, marked by the red box is the same cell as the enlarged cell in panel A and is displayed enlarged to the right.

FISH Analysis of CTC and Leukocytes

After the FISH hybridization is completed, the cartridge is placed into the modified analyzer with the 40× objective. Fluorescence images acquired for CTC identification before FISH are imported onto the modified analyzer. To match the location of the cells before and after FISH the modified analyzer acquires DAPI images of the cartridge using the 40× objective and correlates these images with the DAPI images acquired using the 10× in the first scan of the cartridge. Next the system revisits all events that have been identified as CTC in the first scan. A Z-stack of five images with a spacing of 2 μm for each of the four FISH probes is acquired. The Z-stack images are collapsed into one image and presented to the reader. Figure 2 shows an example of DAPI, CD45-APC, and CK-PE images of nine CTC from the original identification and the corresponding DAPI, Chromosome 1, 7, 8, 17 images. Few of the images showed two copies of the chromosomes and each of the nine CTC showed at least an aberrancy of one of the chromosomes. Leukocytes surrounding the CTC serve as internal control for the FISH. The numbers of leukocytes in the cartridge vary from samples to sample. If leukocytes are present within the frame of the CTC, 1–2 leukocytes are selected as a control. In case no leukocytes are present in close proximity a larger area around the CTC is scanned. If still no controls can be selected, the quality of the FISH is judged on the leukocytes that are present at other locations. Figure 3 displays nine leukocytes that were selected as controls. The majority of the leukocytes show two copies of chromosome 1, 7, 8, and 17 in contrast with the larger heterogeneity observed in the copy numbers of chromosome 1, 7, 8, and 17 in the CTC.

One-hundred seventy eight cartridges containing CTC from 70 prostate cancer patients were analyzed for FISH analysis. To limit the analysis time, a maximum of 125 CTC were analyzed per cartridge. From the 23,341 CTC present in these cartridges 6,136 (26%) were analyzed and 4,428 (72%) could be relocated. From the 4,428 relocated CTC, 2,708 (61%) did not provide FISH signals in contrast to the surrounding leukocytes that showed good quality FISH signals. The morphologic appearance of CTC that did not provide FISH signals suggested that these cells might be disintegrating and or undergoing apoptosis. The percentage of relocated CTC per cartridge that did not provide adequate FISH signals ranged from 0 to 100% (mean 59%, median 62%, SD 35%). In 48 of the

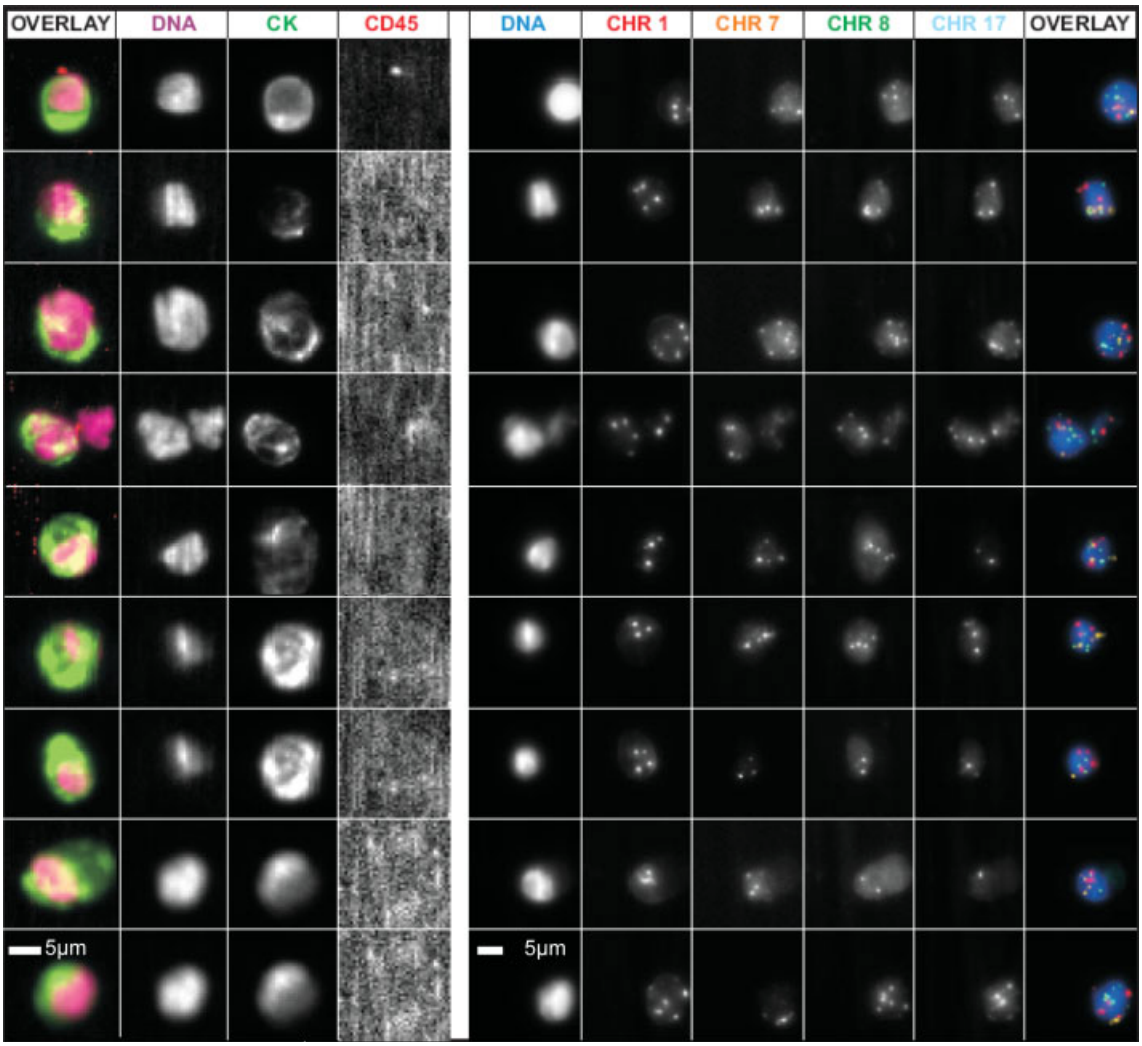

Figure 2. Fluorescence images of CTC. The four columns on the left are obtained with the ×10 objective. Column ''Overlay'' displays the overlay of the columns ''DNA'' and ''CK.'' The six columns on the right display the images of the same cells after FISH labeling using the ×40 objective. Columns show the DAPI, Chromosome 1, 7, 8, and 17 signals and an overlay of DAPI with each of the four chromosomal fluorescence signals.

178 cartridges, 100% of the CTC did not provide evaluable FISH signals and in 12 of the 178 cartridges no CTC good be relocated because of different reasons, resulting in 118 evaluable cartridges from 59 patients.

Apoptosis of CTC and FISH Analysis

To demonstrate the relation between CTC undergoing apoptosis and lack of FISH signals EpCAM enriched CTC from 7.5 mL of blood from patients with metastatic disease were stained with M30, an antibody recognizing caspase-cleaved cytokeratin 18, in addition to DAPI, Cytokeratin, and CD45. M30 was labeled with PE, Cytokeratin with FITC, and CD45 with APC. For 24 CTC positive cartridges, the cartridges were preserved and hybridized with probes identifying the centromeres of chromosome 1, 7, and 17. Panel A in Figure 4, shows an example of a CTC identified DAPI+, Cytokeratin+, M30−, CD45−, as well as the corresponding DAPI, chromosome 1, 7, and 17 images of the same CTC after hybridization and relocation. Figure 4, Panel B shows a DAPI+, Cytokeratin+, M30+, CD45− CTC from a patient but although the DAPI signal revealed that the CTC was relocated no adequate FISH signals were observed. A total of 765 CTC from the 24 samples (mean 52, median 7, SD 111) were analyzed and evaluable FISH signals were obtained for 567/590 (96%) of the M30 negative CTC but only 101/175 (58%) of the M30 positive CTC. Only CTC that were surrounded with leukocytes that contained at least one copy of each of the chromosomes were included in this analysis.

Aneuploidy of CTC in Hormone Refractory Prostate Cancer

Copies of chromosomes 1, 7, 8, and 17 could be determined in 1,720 CTC of 118 samples from 59 patients. In total, 537 leukocytes were analyzed as internal controls. Figure 5,

Panel A displays a histogram of the copy number of chromosome 1 in 1,720 CTC (red bars) and 537 leukocytes surrounding the CTC (green bars). Panel B, C and D display the copy numbers of chromosome 7, 8, and 17 plus the surrounding leukocytes respectively. On average the leukocytes shows two copies in 86% of the cases, in 10% less than two, and in 4% more than two copies. The distribution of the number of chromosome copies found in CTC is different for each chromosome. The copy number of chromosome 8 (panel C) is deviating most from normal, 8% had less than two, 38% had two copies and 54% had more than two copies. All chromosomes with more than seven copies were reported as seven copies. In 10/118 (9%) samples from six patients CTC were diploid. The number of CTC in these samples was low and ranged from 1 to 5 CTC. To arrive at a numerical value for the deviation of the chromosome copy number from normal, we defined the chromosomal composition by: ((fraction of cells with one copy * 1) + (fraction of cells with two copies * 2) + ···+ (fraction of cells with six copies * 6) + (fraction of cells with $\geq$ seven copies * 7)) − 2. For normal cells the numerical value for the deviation is thus equal to 0. In reality, two copies will not be found in all the cells and in the 537 leukocytes that were used as controls a deviation from the normal chromosomal copy number of −0.07 was found for chromosome 1, −0.02 for chromosome 7, −0.06 for chromosome 8 and −0.10 for chromosome 17. A significant larger deviation was found in CTC. For chromosome 1, the deviation ranged from −2 to 5 with an average of 0.57 (median 0.23, SD 1.04). For chromosome 7, the deviation ranged from −2 to 5 with an average of 0.66 (median 0.50, SD 1.06). For chromosome 8, the deviation ranged from −2 to 4.61 with an average of 0.85 (median 0.75, SD 1.26) and for chromosome 17, the deviation ranged from −2 to 3.33 with an average of 0.44 (median 0.09, SD 0.82). Figure 6 shows a graphical representation of the deviation of chromosome 1, 7, 8, and 17 copy number for the 118 samples from the 59 patients. In addition, the deviation of the chromosome copy number for leukocytes is provided.

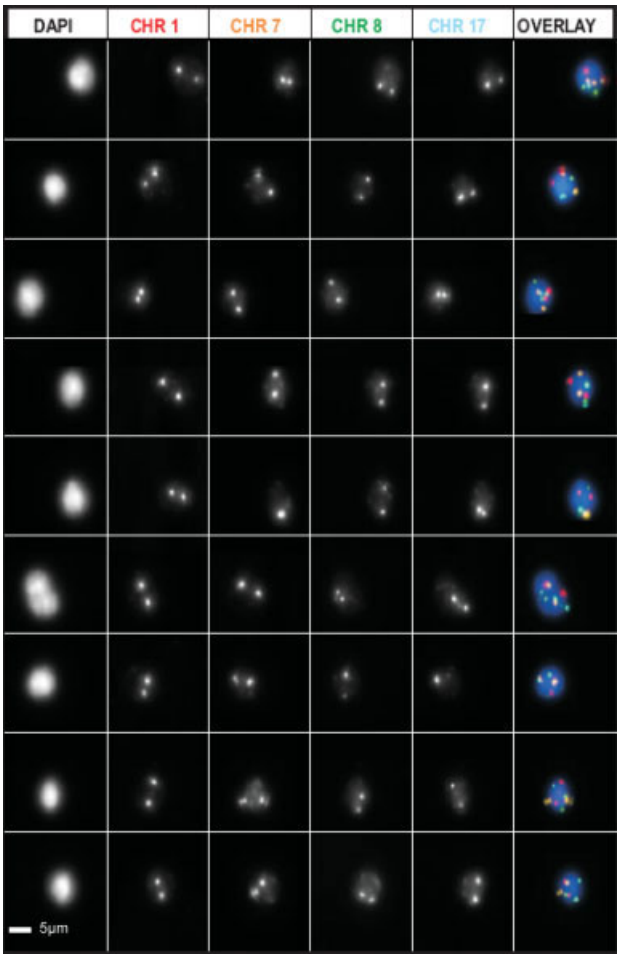

Figure 3. Fluorescence signals of leukocytes that surrounded the CTC. Column 1 displays the DAPI fluorescence signal of the nucleus. Fluorescence signals of chromosome 1, 7, 8, and 17 and an overlay of DAPI with each of the four chromosomal fluorescence signals.

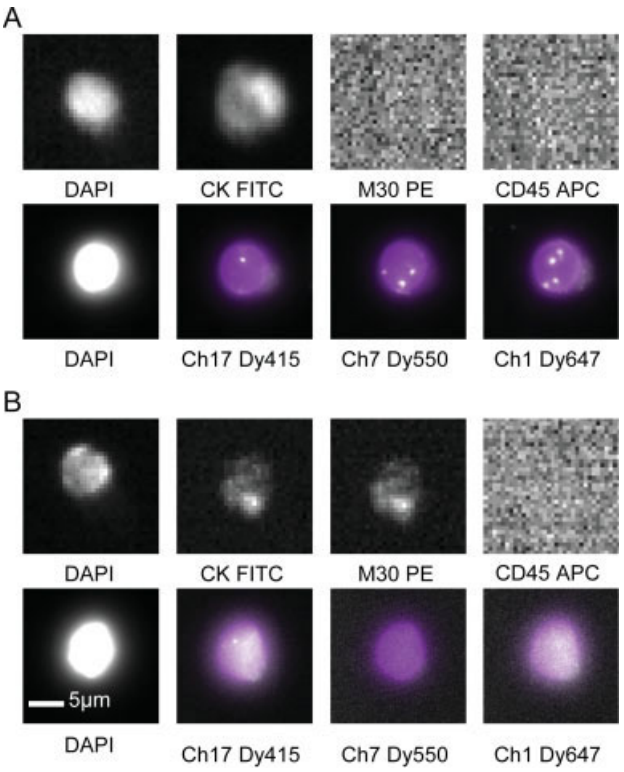

Figure 4. Two CTC before and after FISH. (**A**) Upper row of images show the different fluorescence images of a M30-negative CTC. Lower row shows the corresponding DAPI of the FISH signals of the corresponding CTC after FISH. (**B**) the same set of images but now for a M30-positive CTC.

Discussion

Although case report studies have suggested that the presence of tumor cells in blood is associated with poor outcome their low frequency has hampered exploration of their clinical utility (19–27). The recent introduction of a standardized assay has permitted the execution of prospective multicenter

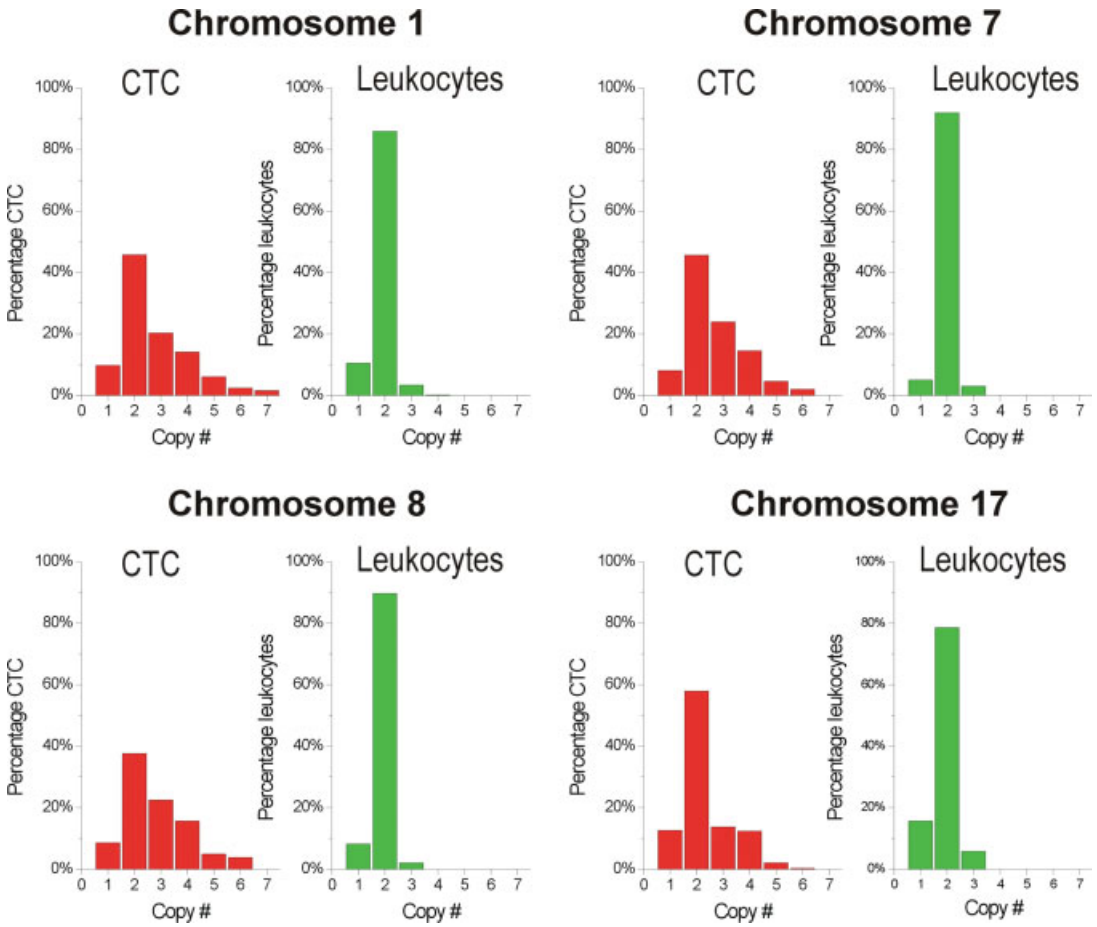

Figure 5. Four panels display the histogram of the copy number of chromosome 1, 7, 8, and 17 of 1,793 CTC (red bars) and 537 leukocytes surrounding the CTC (green bars).

clinical trials in MBC, MCR, and MPC. These studies showed that the presence of CTC indeed was associated with poor prognosis and their perseverance or occurrence after the first cycles of therapy strongly suggested that those patients were on a futile therapy (1–6). The number of therapies available to treat patients with recurrent cancer is rapidly increasing. The challenge the oncologist is facing is to determine which therapy offers the most benefit with the fewest side effects. The shift towards targeted therapies has magnified the situation. Selecting the patient most likely to respond to a target directed drug requires a more in-depth characterization of the patient. Unfortunately, use of archival primary tumor tissues to determine target status may not represent the disease at the time of a recurrence. Because of genetic instability, a percentage of tumors continue to mutate giving rise to variants that are not expressed and/or resistant to a given therapeutic regimen (28–35). Assessment of therapeutic targets on CTC constitutes may overcome this issue. The technological challenge of assessment of multiple analytes on CTC is that in most cases only few cells are available and repeated sampling or sampling of a larger blood volume are not realistic possibilities. In this study, we explored the possibility to perform FISH after the original identification of CTC. In the CellSearch assay, CTC are held by magnetic forces to an analysis surface, and in this study, we demonstrated that we were able to relocate more than 90% of the cells after hybridization. However, 61% of the CTC did not provide FISH signals that could be counted in contrast to the leukocytes that surrounded the CTC. The morphological appearance of the CTC that did not provide FISH signals suggested that these cells were disintegrating to various degrees. To prove a relation between CTC undergoing apoptosis and lack of FISH signals, we conducted a series of experiments in which CTC were characterized by the expression of caspase-cleaved cytokeratin 18 followed by FISH analysis. Although 96% of the CTC that did not undergo apoptosis provided FISH signals, only 58% of the CTC undergoing apoptosis did. It's not surprising that CTC shed into the circulation from primary or metastatic sites are undergoing apoptosis and the phenomenon has been reported earlier (36–38). Drawback of this finding is that assessment of the genetic makeup of the tumor becomes less informative as fewer CTC are available and therapeutic targets can thus be assessed in fewer patients. Recent reports using alternative technologies suggest the presence of a significant larger number of CTC in carcinoma

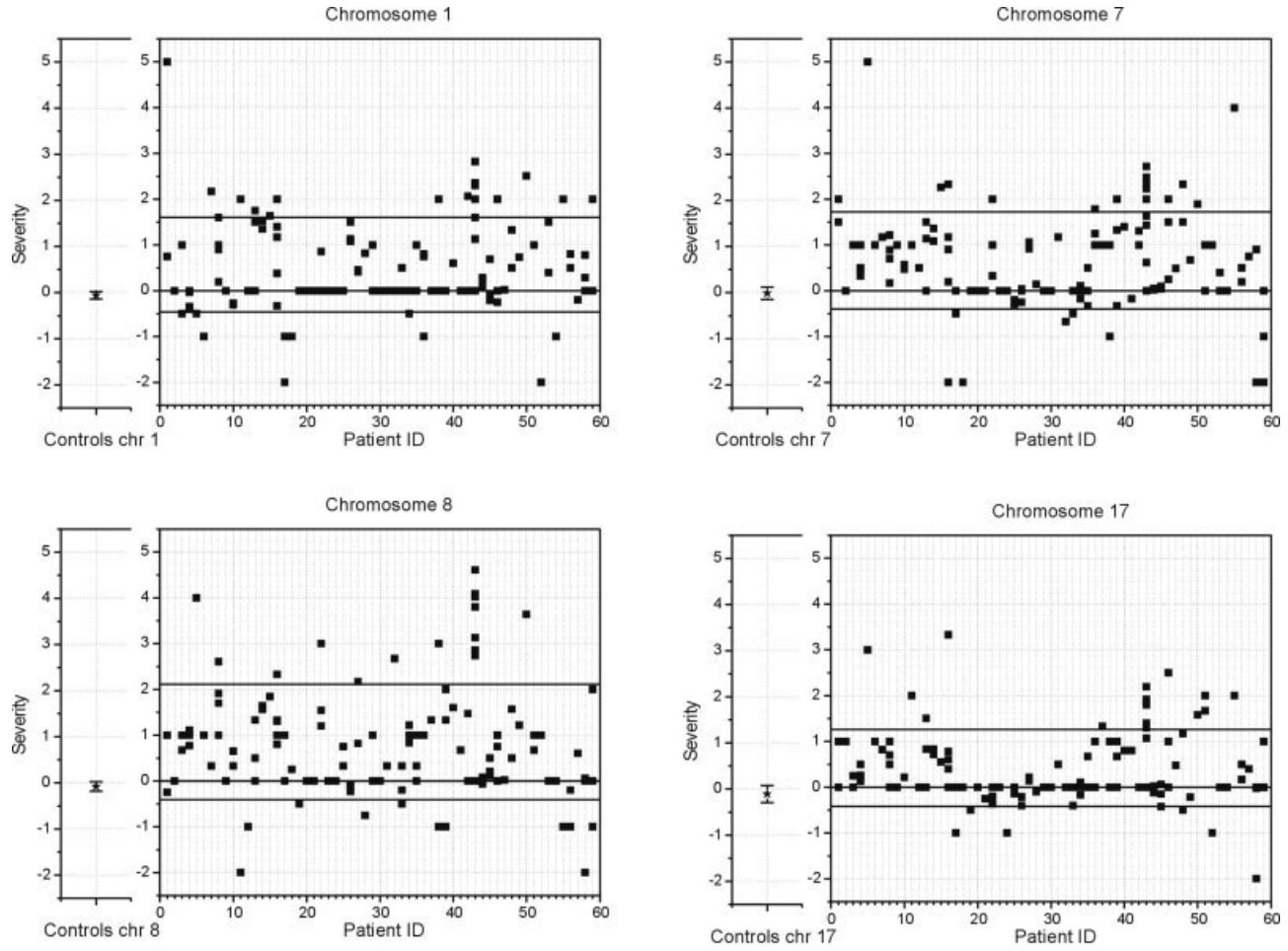

Figure 6. Four panels display the deviation from diploid cells in CTC and leukocytes for each sample in each patient of chromosome 1, 7, 8, and 17. The standard deviation for the severity factor is represented by the error bars for the controls and by the solid line in case of patients.

patients this however may well be contributed to the definition of what constitutes a CTC (39–46). Still, one would like to assess the presence or absence of therapeutic targets in as large a patient group as possible urging the need for technology that increase the yield of CTC.

The choice of the probes for the centromers of chromosome 1, 7, 8, and 17 was not to examine prostate cancer, but merely served to demonstrate the reliability of the method. This combination of four probes was also the most aberrant across carcinomas in cytogenetic databases. With this set of probes we confirmed earlier studies that CTC are indeed cancerous (14,47). Surprisingly an extreme heterogeneity was observed with respect to the aberrancy of the copy number of chromosome 1, 7, 8, and 17 between patients, but also between CTC in the individual prostate cancer patients. In the few samples with only diploid CTC less than five, CTC were detected. To quantify the deviation from normal, a numerical value was derived and determined for each sample. The value zero represents diploid cells and values obtained for leukocytes indeed were close to zero in contrast large deviations were observed for CTC. The largest deviation from normal was observed for chromosome 8 whereas the smallest deviation was observed for chromosome 1, Figure 6.

In this study, we used DAPI and the fluorochromes PlatinumBright-647, -550, -505, and -415 limiting the number of probes that can be simultaneously assessed to four. The number of treatment targets already exceeds four urging the future expansion to more probes. This may be achieved by increasing the combination of fluorochromes and or explore the feasibility of repeated FISH analysis on the same CTC.

Literature Cited

1. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Rueben JM Doyle G, Allard WJ, Terstappen LWMM, Hayes DF. Circulating tumor cells. Disease progression and survival in metastatic breast cancer. N Engl J Med 2004;351:781–791.
2. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck AT, Doyle GV, Matera J, Allard WJ, Miller MC, Rueben JM, Fritsche HA, Hortobagyi GN, Terstappen LWMM. Circulating tumor cells: A novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol 2005;23:1420–1430.
3. Budd G, Cristofanilli M, Ellis M, Stopeck A, Borden E, Miller MC, Matera J, Repollet M, Doyle G, Terstappen LWMM, Hayes D. Circulating tumor cells versus imaging—Predicting overall survival in metastatic breast cancer. Clin Can Res 2006;12:6404–6409.

4. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard JW, Doyle GV, Terstappen LWMM. Circulating tumor cells predict progression free and overall survival at each follow-up time point during therapy of metastatic breast cancer patients. Clin Cancer Res 2006;12:4218–4224.
5. Cohen SJ, Punt CJA, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse M, Mitchell E, Desch CE, Miller MC, Doyle GV, Tissing H, Terstappen LWMM, Meropol NJ. The relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:3213–3221.
6. DeBono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, Doyle GV, Terstappen LWWM, Pienta KJ, Raghavan D. Circulating Tumor Cells (CTC) predict survival benefit from treatment in metastatic castration resistant prostate cancer (CRPC). Clin Cancer Res 2008;14:6302–6309.
7. Kagan M, Howard D, Bendele T, Mayes J, Silvia J, Repollet M, Doyle J, Allard J, Tu N, Bui T, Russell T, Rao C, Hermann M, Rutner H, Terstappen LWMM. A sample preparation and analysis system for identification of circulating tumor cells. J Clin Lig Assay 2002;25:104–110.
8. Kagan M, Howard D, Bendele T, Rao C, Terstappen LWMM. Circulating tumor cells as cancer markers, a sample preparation and analysis system. In: Diamandis EP, Fritsche HA, Lilja H, Chan DW, Schwartz M, editors. Tumor Markers: Physiology, Pathobiology, Technology and Clinical Applications. Washington: AACC Press; 2002. pp 495–498.
9. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe A, Uhr JW, Terstappen LWMM. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with non-malignant diseases. Clin Can Res 2004;10:6897–6904.
10. Hayes DF, Walker TM, Singh B, Vitetta ES, Uhr JW, Gross S, Rao C, Doyle GV, Terstappen LWMM. Monitoring expression of HER-2 on circulating epithelial cells in patients with advanced breast cancer. Int J Oncol 2002;21:1111–1118.
11. Meng S, Tripathy D, Shete S, Ashfaq R, Haley B, Perkins S, Beitsch P, Khan A, Euhus D, Osborne C, Frenkel E, Hoover S, Leitch M, Clifford E, Morrison L, Herlyn D, Terstappen LWMM, Fleming T, Fehm T, Tucker T, Lane N, Wang J, Uhr J. HER-2 gene amplification can be acquired as breast cancer progresses. PNAS 2004;101:9393–9398.
12. Meng S, Tripathy D, Frenkel EP, Shete S, Naftalis EZ, Huth JF, Beitsch PD, Leitch M, Hoover S, Euhus D, Haley B, Morrison L, Fleming TP, Herylyn D, Terstappen LWMM, Fehm T, Tucker TF, Lane N, Uhr JW. Circulating tumor cells in patients with breast cancer dormancy. Clin Can Res 2004;10:8152–8162.
13. Meng S, Tripathy D, Frenkel EP, Shete S, Ashfaq R, Saboorian H, Haley B, Frenkel E, Euhus D, Leitch M, Osborne C, Clifford E, Perkins S, Beitsch P, Kahn A, Morrison L, Herylyn D, Terstappen LWMM, Lane N, Wang J, Uhr JW. uPar and Her-2 gene status in individual breast cancer cells from blood and tissues. PNAS 2006;103:17361–17365.
14. Shaffer DR, Leversha MA, Danila DC, Lin O, Gonzalez-Espinoza R, Gu B, Anand A, Han J, Smith K, Maslak P, Lilja H, Doyle G, Terstappan LWMM, Heller G, Fleisher M, Scher HI. Circulating tumor cell analysis in patients with progressive castration-resistant prostate cancer. Clin Cancer Res 2007;13:2023–2029.
15. de Bono JS, Attard G, Adjei A, Pollak MN, Fong PC, Haluska P, Roberts L, Melvin C, Repollet M, Chianese D, Connely M, Terstappen LWMM, Gualberto A. Potential applications for circulating tumor cells expressing the insulin growth factor-I receptor. Clin Can Res 2007;13:3611–3616.
16. Tibbe AGJ, Miller C, Terstappen LWMM. Statistical considerations for enumeration of circulating tumor cells. Cytometry Part A 2007;71A:154–162.
17. Terstappen LWMM, Dolan GJ. Magnetic separation apparatus and methods. U.S. Patent 6,660,159 B1, December 9, 2003
18. Tibbe AGJ, de Grooth BG, Greve J, Dolan GJ, Rao C, Terstappen LWMM. Magnetic field design for selecting and aligning immunomagnetic labeled cells. Cytometry Part A 2002;47A:163–172.
19. Recamier JCA. L'histoire de le Meme Maladie. 1829; Gabor vol: 110.
20. Ashworth TR.: A case of cancer in which cells similar to those in the tumours were seen in the blood after death. Australian Med J 1869;14:146–147.
21. Engell HC. Cancer cells in the circulating blood; a clinical study on the occurrence of cancer cells in the peripheral blood and in venous blood draining the tumour area at operation. Acta Chir Scand Suppl 1955;201:1–70.
22. Carey RW, Taft PD, Bennett JM, Kaufman S. Carcinocythemia (carcinoma cell leukemia). An acute leukemia-like picture due to metastatic carcinoma cells. Am J Med 1976;60:273–278.
23. Myerowitz RL, Edwards PA, Sartiano GP. Carcinocythemia (carcinoma cell leukemia) due to metastatic carcinoma of the breast: Report of a case. Cancer 1977;40:3107–3111.
24. Gallivan MV, Lokich JJ. Carcinocythemia (carcinoma cell leukemia). Report of two cases with English literature review. Cancer 1984;53:1100–1102.
25. Sile CC, Perry DJ, Nam L. Small cell carcinocythemia. Arch Pathol Lab Med 1999;123:426–428.
26. Seronie-Vivien S, Mery E, Delord JP, Fillola G, Tkaczuk J, Voigt JJ, Bugat R. Carcinocythemia as the single extension of breast cancer: Report of a case and review of the literature. Ann Oncol 2001;12:1019–1022.
27. Yam LT, Janckila AJ. Immunocytodiagnosis of carcinocythemia in disseminated breast cancer. Acta Cytol 1987;31:68–72.
28. Ross JS, Gray GS. Targeted therapy for cancer: The HER-2/neu and Herceptin story. Clin Leadership Manag Rev 2003;17:333–340.
29. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. N Engl J Med 1988;319:525–532.
30. Ingvarsson S. Molecular genetics of breast cancer progression. Semin Cancer Biol 1999;9:277–288.
31. Pihan GA. Purohit A. Wallace J. Malhotra R. Liotta A. Doxsey SJ. Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. Cancer Res 2001;61:2212–2219.
32. Kleer CG, Bryant BR, Glordano TJ, Sobel M, Merino MJ. Genetic changes in chromosomes 1p and 17p in thyroid cancer progression. Endocr Pathol 2000;11:137–143.
33. Alers JC, Rochat J, Krijtenburg PJ, Hop WCJ, Kranse R. Rosenberg C. Tanke HJ, Schroder FH, van Deken H. Identification of genetic markers of prostatic cancer progression. Lab Invest 2000;80:931–942.
34. Endo C, Sagawa N, Sato N, Chen Y Sakurada A, Aikawa H, Takahashi S, Usuda K, Saito Y, Fujimura S. Sequential loss of heterozygosity in the progression of squamous cell carcinoma of the lung. Br J Cancer 1998;78:612–615.
35. Simon R, Nocito A, Hubscher T, Bucher C, Torhorst J, Schraml P, Bubendorf L, Mihatsch MM, Moch H, Wilber K, Schotzau A, Kononen J, Sauter G. Patterns of her-2/neu amplification and overexpression in primary and metastatic breast cancer. J Natl Cancer Inst 2001;93:1141–1146.
36. Larson CJ, Moreno JG, Pienta KJ, Gross S, Repollet M, O'Hara SM, Russell T, Terstappen LWMM. Apoptosis of circulating tumor cells in prostate cancer patients. Cytometry Part A 2004;62A:46–53.
37. Wang ZP, Eisenberger MA, Carducci MA, Partin AW, Scher HI, Tso PO. Identification and characterization of circulating prostate carcinoma cells. Cancer 2000;88:2787–2795.
38. Racila E, Euhus D, Weiss AJ, Rao C, McConnell J, Terstappen LWMM, Uhr JW. Detection and characterization of carcinoma cells in the blood. PNAS 1998;95:4589–4594.
39. Adams AA, Okagbare PI, Feng J, Hupert ML, Patterson D, Göttert J, McCarley RL, Nikitopoulos D, Murphy MC, Soper SA. Highly efficient circulating tumor cell isolation from whole blood and label-free enumeration using polymer-based microfluidics with an integrated conductivity sensor. J Am Chem Soc 2008;130:8633–8641.
40. Campos M, Prior C, Warleta F, Zudaire I, Ruíz-Mora J, Catena R, Calvo A, Gaforio JJ. Phenotypic and genetic characterization of circulating tumor cells by combining immunomagnetic selection and fiction techniques. J Histochem Cytochem 2008; 56:667–675.
41. Pachmann K, Camara O, Kavallaris A, Krauspe S, Malarski N, Gajda M, Kroll T, Jörke C, Hammer U, Altendorf-Hofmann A, Rabenstein C, Pachmann U, Runnebaum I, Höffken K. Monitoring the response of circulating epithelial tumor cells to adjuvant chemotherapy in breast cancer allows detection of patients at risk of early relapse. J Clin Oncol 2008;10:26:1208–1215.
42. Schindlbeck C, Stellwagen J, Jeschke U, Karsten U, Rack B, Janni W, Jückstock J, Tulusan A, Sommer H, Friese K. Immunomagnetic enrichment of disseminated tumor cells in bone marrow and blood of breast cancer patients by the Thomsen-Friedenreich-Antigen. Clin Exp Metastasis. 2008;25:233–240.
43. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, Smith MR, Kwak EL, Digumarthy S, Muzikansky A, Ryan P, Balis UJ, Tompkins RG, Haber DA, Toner M. Isolation of rare circulating tumour cells in cancer patients by microchip technology 2007;450:1235–1239.
44. Roeser T, Ritzi M, Drese KS, Laureyn W, Liu C, O'Sullivan CK, Ozalp VC, Fermer C, Nilsson O, Hauch S, Albert W, Borgen E, Nygre AO. Lab-on-chip for the isolation and characterization of circulating tumor cells. Conf Proc IEEE Eng Med Biol Soc 2007;6447–6449.
45. Zheng S, Lin H, Liu JQ, Balic M, Datar R, Cote RJ, Tai YC. Membrane microfilter device for selective capture, electrolysis and genomic analysis of human circulating tumor cells. J Chromatogr A 2007;1162:154–161.
46. Tveito S, Maelandsmo GM, Hoifodt HK, Rasmussen H, Fodstad O. Specific isolation of disseminated cancer cells: a new method permitting sensitive detection of target molecules of diagnostic and therapeutic value. Clin Exp Metastasis 2007;24:317–327.
47. Fehm T, Sagalowsky A, Clifford E, Beitsch P, Saboorian H, Euhus D, Meng S, Morrison L, Tucker T, Lane N, Ghadimi BM, Heselmeyer-Haddad K, Ried T, Rao C, Uhr J. Cytogenetic evidence that circulating epithelial cells in patients with carcinoma are malignant. Clin Cancer Res 2002;8:2073–2084.