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Imaging, Diagnosis, Prognosis

Clinical Cancer Research

M30 Neoepitope Expression in Epithelial Cancer: Quantification of Apoptosis in Circulating Tumor Cells by CellSearch Analysis

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Abstract

Purpose: This study aimed to detect the M30 neoepitope on circulating tumor cells (CTC) as a tool for quantifying apoptotic CTC throughout disease course and treatment.

Experimental Design: An automated sample preparation and analysis platform for computing CTC (CellSearch) was integrated with a monoclonal antibody (M30) targeting a neoepitope disclosed by caspase cleavage at cytokeratin 18 (CK18) in early apoptosis. The assay was validated using cell lines and blood samples from healthy volunteers and patients with epithelial cancer.

Results: M30-positive CTC could be detected in >70% of CTC-positive carcinoma patients, which were free for both chemotherapy and radiologic treatments. The fraction of M30-positive CTC varied from 50% to 80%, depending on the histotype. To investigate the potential application of the M30 CTC assay for the evaluation of response in early phase trials, CTC and M30-positive CTC were enumerated in a small case series of breast cancer patients during treatment. Results indicate that changes in the balance of M30-negative/positive CTC may be used as a dynamic parameter indicating an active disease, as documented by consistent radiologic findings.

Conclusions: M30 expression on CTC is detectable by immunofluorescence. The M30-integrated test has potential for monitoring dynamic changes in the quote of apoptotic CTC (in addition to CTC count) to evaluate response in clinical trials of molecularly targeted anticancer therapeutics as well as for translational research, in which there is a pressing need for informative circulating biomarkers.

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The finding of tumor cells in peripheral blood raises questions as to their metastatic potential. In fact, notwithstanding that a single tumor cell was proved to sustain metastasis *in vivo* (1), in humans the half-life of circulating tumor cells (CTC) in peripheral blood is estimated at between 1 and 2.4 hours, depending on the mathematical model of the extrapolated curve (2) and on the fact that apoptotic cells significantly contribute to the CTC fraction in breast (3) and prostate cancer (4) patients. On the other hand, a strict correlation was established between CTC count and prognosis (5), and elevated numbers of CTC at any time during therapy was reported to be an accurate indication of subsequent rapid progression and mortality (6). Nevertheless, the phenotypic and biological properties of the CTC that are necessary for the metastatic process are far from clear. Theoretically, CTC should be adapted to shed into peripheral blood and at least some of the CTC should be live cells. Moreover, although the metastatic phenotype may be later acquired as a result of selective pressure exerted at secondary sites, at least some of the CTC should be able to self-renew (7).

Addressing the role and mechanism of CTC in the development of metastasis, we investigated whether or not CTC are live cells, considering mainly when and how often the percentage of apoptotic CTC changes throughout disease course and treatment. For this purpose, we analyzed CTC in our patient cohort by the CellSearch system, an automated platform that permits serial testing with good sensitivity and reproducibility (5). CTC assay was

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Translational Relevance

The absolute number of circulating tumor cells (CTC) has proved to be a robust predictor of poor prognosis in metastatic breast, colorectal, and prostate cancer. Moreover, in the absence of tumor biopsies CTC provide a "surrogate" index for monitoring response to treatment. However, the CTC biological significance is as yet undefined: why did the median overall survival not further decrease when >5 CTC (poor-prognosis threshold, very few CTC indeed) were detected in 7.5 mL of blood?

By exploiting a M30-integrated CTC assay, we show here that CTC are a heterogeneous cell population, which includes both apoptotic and viable cells: exceedingly high numbers of live CTC were associated with radiologic recurrence of disease, and also when a switch under the threshold of poor prognosis was observed during the therapy. Our data offer a rationale to the option that a CTC subpopulation not expressing M30 may be associated with decreased chances of survival.

integrated with a monoclonal antibody (mAb), anti-M30, for recognizing (8) a neoepitope in cytokeratin 18 (CK18) that becomes available at a caspase cleavage event during apoptosis and is not detectable in vital epithelial cells; the M30 neoepitope appears early in the apoptotic cascade, with Annexin V reactivity, and it is generally regarded as a stable biomarker, specific for epithelial cell apoptosis.

The results obtained in breast, renal, and colorectal cancer patients are presented, indicating that variable numbers of apoptotic CTC can be detected in all solid tumors. The M30-integrated assay seems to be a feasible tool for monitoring apoptotic CTC.

Material and Methods

Patients

Peripheral blood was consecutively drawn from 34 breast cancer patients (33 female and 1 male, ages 39-83 years), 29 metastatic renal cancer (mRCC) patients (6 female and 23 male, ages 26-87 years), and 59 colorectal cancer patients (21 female and 38 male, ages 30-87 years) at baseline, before starting treatment. Breast and colorectal cancer patients were enrolled in this study regardless of type or line of therapy. Prior adjuvant treatment, treatment of metastatic disease, or both were permitted in the case of breast or colorectal metastatic cancer, whereas mRCC patients were consecutively enrolled in a pilot study, "Metastatic renal cancer: CTC determination in first-line Sunitinib treated patients," conducted at Istituto Oncologico Veneto (IOV-IRCCS), Padova, Italy. Whole blood was also drawn from healthy control subjects (4 female and 4 male, ages 30-60 years) who had neither known illness at the time of sampling nor history of malignant disease. All enrolled patients and healthy subjects gave their informed consent for study inclusion and were enrolled using institutional review board–approved protocols. After baseline evaluation, serially monitored (1-10 months) reevaluations of disease status in the breast cancer patients were conducted depending on the type and schedule of treatment. Tumor measurements by appropriate scans were done using Response Evaluation Criteria in Solid Tumors guidelines without independent radiology review, with no knowledge of the levels of CTC.

Cell lines

The breast cancer cell line MCF7, the prostate cancer cell line PC3, and the colon cancer cell line LoVo were purchased from the American Type Culture Collection and were grown as described (9).

Apoptosis detection

To quantitatively evaluate spontaneous and drug-induced apoptosis in the cancer cell lines, four different methodologies were compared: Annexin V apoptosis assay, anti-M30 immunostaining, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay, and Western blood analysis (WB) for poly(ADP-ribose) polymerase (PARP) cleavage, according to the manufacturer's instruction as detailed in Supplementary Materials.

CTC assay

The enumeration of CTC in whole blood was done by the CellSearch System according to manufacturer's instruction as described (5).

Phenotypic profiling of CTC

To quantify the fraction of apoptotic CTC, M30-positive CTC were detected integrating CTC assay with a specific mAb, M30 CytoDEATH Fluorescein (ALX-804-590, Alexis Biochemicals), recognizing the M30 neoepitope of CK18, analyzed with the fourth filter of the CellSearch System; results were expressed as the total number of CTC and M30-positive CTC per 7.5 mL of blood.

Statistical analysis

Data were analyzed utilizing the StatGraphics software (version 2.6), as previously reported (10). Unless otherwise indicated, all results are expressed as mean values ± 1 SD, and mean values of three experiments are shown. The nonparametric Mann-Whitney test was used to compare quantitative variables. Frequencies were compared by Fisher' exact test (two tails) or χ^2 test with Yates' correction where appropriate.

Results

Quantitative comparison of apoptosis by different methods

Several apoptosis assays devised to detect different components of the apoptosis signaling cascade or specific apoptotic features, including DNA fragmentation, caspase activity, membrane alterations, and mitochondrial changes,

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are currently available (11). Using more than one method is strongly recommended because of the limited sensitivity and specificity of current assays; moreover, choosing the most appropriate apoptotic assay should also be based on sample type (tissues or cellular effusions). Therefore, we firstly evaluated the sensitivity and specificity of M30 immunostaining, comparing spontaneous and pharmacologically induced apoptosis in cancer cell lines by flow cytometry and WB.

The MCF7, LoVo, and PC3 cell lines were cultured in the presence or the absence of cisplatin for 24 hours, raising apoptotic events in drug-treated cells, as shown (Fig. 1A) by Annexin V immunostaining (15.25% ± 4.8 MCF7-, 15.3% ± 0.3 LoVo-, and 13.6% ± 2.3 PC3-positive cells, respectively) and M30 immunostaining (15.2% ± 8 MCF7-, 17.2% ± 8.3 LoVo-, and 12.6% ± 1.3 PC3-positive cells, respectively) and confirmed by WB, showing strong PARP cleavage in MCF7 (64.2% ± 0.5), in LoVo (55.2% ± 6.6), and in PC3 (34.4% ± 9.8).

To quantitatively compare M30 immunostaining at single-cell level, double fluorescence was done with TUNEL assay in the cell lines 36 hours after apoptosis induction with cisplatin. In the drug-treated cultures double staining discriminates the initial apoptotic phase before DNA fragmentation (M30-positive TUNEL-negative cells; Fig. 1B, top left quadrant in right plot) from the apoptotic cells, which are TUNEL-positive and M30-positive (Fig. 1B, top right quadrant in right plot). Again, a fraction of cells were M30-negative and TUNEL-positive (Fig. 1B, bottom right quadrant in right plot), representing the end phase of the process, when the cells become necrotic and the M30 epitope is lost. The data are consistent with previous observations that the exposure of the M30 neoepitope occurs at the initial phase of the apoptotic cascade, before the appearance of apoptotic features in the nuclei (8).

M30 CTC assay development

The M30 mAb was integrated into the CTC assay to specifically quantify apoptosis of spreading tumor cells. The integrated test was developed using the MCF7 cell line that was maintained in culture for 24 hours in the absence or the presence of paclitaxel, raising both early and late apoptotic events in drug-treated MCF7 cells, as shown by flow cytometry (Fig. 2A). Untreated controls and drug-conditioned cells were then spiked

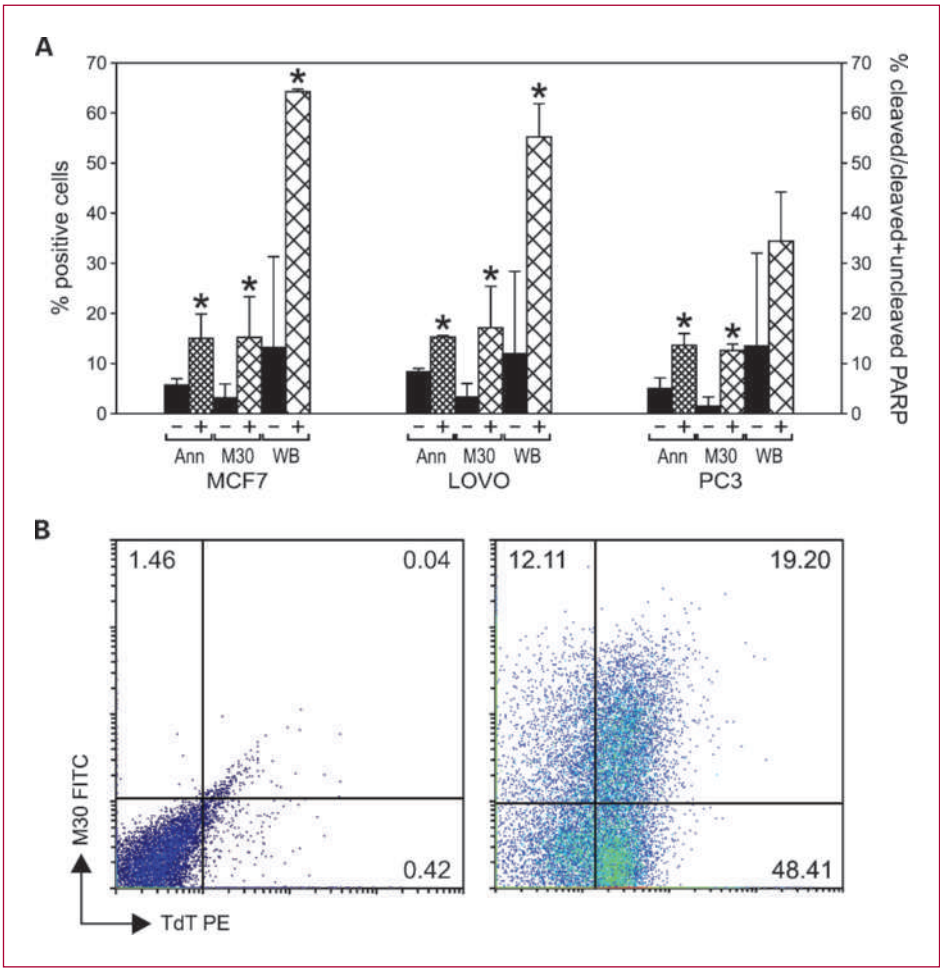

Fig. 1. Detection of epithelial apoptosis. A, comparison of different methods. Spontaneous (–) and drug-induced apoptosis (+) was evaluated by flow cytometry for Annexin V (Ann) or M30 expression or by WB to detect PARP cleavage in three cancer cell lines 24 hours after induction of apoptosis with cisplatin. *, significant increases measured in cisplatin-treated cultures (responses exceeding 2 SD the media of untreated control, $P < 0.05$ in every case). Due to the interexperimental variability of spontaneous apoptosis in bulk cultures, PARP differences were not significant in PC3 cells. B, double staining of MCF7 cells with M30 mAb and TUNEL method. Cytograms of TUNEL reactivity (*X*-axis) and M30 binding (*Y*-axis) in control (left plot) and drug-conditioned culture (right plot) 36 hours after induction of apoptosis with cisplatin. Data were acquired for 20,000 events using an EPICS-XL device. Data are representative of three experiments.

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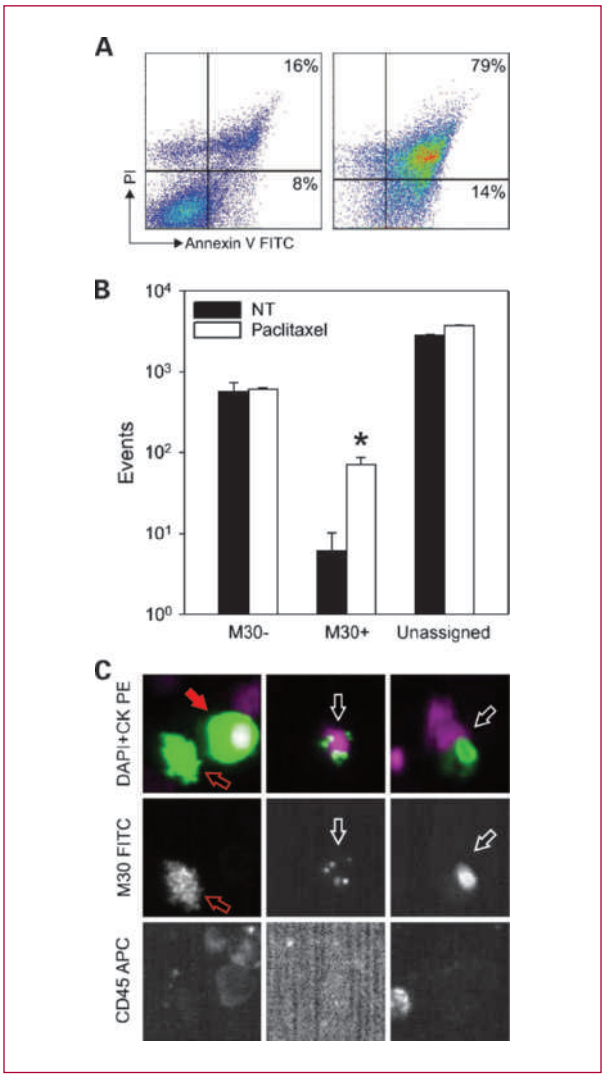

Fig. 2. Detection of tumor cells expressing the M30 neoepitope by CellSearch analysis. A, flow cytometry analysis of apoptotic MCF7 cells. Cytograms of Annexin V reactivity (*X*-axis) and propidium iodide (PI) binding (*Y*-axis) in treated culture (right dot plot) and control (left dot plot) after induction of apoptosis with paclitaxel for 24 hours. An increasing quote of early apoptotic cells (lower right quadrant in all plots) with high Annexin and low PI staining, and late apoptotic cells (upper right quadrant in all plots) with high Annexin and high PI staining was disclosed in drug-treated MCF7 cells. Data are representative of three experiments. B, quantification of apoptotic MCF7 by integrated CTC assay. MCF7 obtained from treated culture (white histograms) and control (black histograms) were spiked in blood of healthy volunteers and processed by CellSearch platform. Unassigned events are those that do not satisfy morphologic features required for a CTC or contaminating leukocytes. *, significant increase of M30-positive cells measured in paclitaxel-treated cultures (responses exceeding 2 SD the media of untreated control, $P < 0.05$). C, morphology of M30-positive and M30-negative MCF7 using an Analyzer II device. Computer-elaborated images: first row, CK and 4', 6-diamidino-2-phenylindole (DAPI) staining profile; second row, anti-M30 staining; third row, anti-CD45 specificity control. Based on CK, DAPI, and/or not-M30 staining profile, apoptotic (red open arrows) and intact cells (red closed arrow) can be clearly classified. Fragments (white open arrows) are events that cannot be assigned as a CTC (intact intermediate filament network progressively replaced by cytokeratin inclusions, cytoplasm/nuclear area overlying <50%).

into whole blood samples, at numbers similar to those observed *in vivo* in cancer patients (200-1,000 cells/7.5 mL peripheral blood) to be finally processed by the CellSearch system.

The CTC assay revealed a significant increase of M30-positive CTC (71 ± 16, 11.6%) in the drug-conditioned MCF7-spiked samples compared with untreated controls (6 ± 4, 1%; $P < 0.05$; Fig. 2B). Because the values obtained by the CTC assay and flow cytometry are generated starting from different pools of events (only nucleated cells in the CTC assay versus both cells and nude nuclei in the flow cytometry), the fraction of apoptotic CTC by the integrated test differs from the flow cytometric results obtained before the spiking. Figure 2C shows an immunofluorescence image that stresses this point: M30 immunostaining clearly discriminates intact CTC, which are M30 negative, and early apoptotic CTC, which appeared M30 positive (Fig. 2C, left photo series); both these cells satisfy the morphologic features required to be defined as CTC (clear visible nucleus, cytoplasm/nuclear area overlying >50%, uniformly immunostaining of cytoplasm). Conversely, when the intact intermediate filament network is being progressively replaced by cytokeratin inclusions (Fig. 2C, central photo series), followed by chromatin condensation and loss, all characteristic of apoptosis (Fig. 2C, right photo series), the events, which still entered in flow cytometric analysis, cannot be assigned as CTC. Remarkably, the percentage of M30-positive CTC measured in the drug-conditioned MCF7-spiked samples (11.6%) closely resembles the quote of early apoptosis (14% in Fig. 1A) as determined by flow cytometry before the spiking into the whole blood.

Because CTC are considered "fragile" cells (12), the possibility that an event assigned as M30-positive CTC could be an artifact, due to apoptotic death occurring during the procedure of enrichment and immunostaining, was addressed. To this purpose, blood samples spiked with untreated MCF7 cells were treated with paclitaxel directly into the CellSave tube, 12 to 24 hours before the CellSearch processing. Although we acknowledge that lab-adapted MCF7 cells could be less fragile than CTC, we did not disclose in this case relevant changes of the M30-positive fraction (1.5 ± 1, 0.2%).

The M30-integrated CTC assay was fully developed in blood samples obtained from healthy donors and cancer patients. To use the test in follow-up studies, we generated an on-line staining procedure that is detailed in Supplementary Materials. The obvious advantage of this approach is that antibodies of interest were added and processed simultaneously with the CK-PE and CD45-APC antibodies, minimizing cell loss or disruption (13) during permeabilization and staining steps.

Compared with the MCF7-spiked samples, analysis of patient's samples showed that the integrated assay allows discrimination of the heterogeneous staining profile of the CTC: an irregular CK staining (Fig. 3, event 519) that is frequently observed *in vivo* and may closely resemble disruption of the filamentous network can be clearly

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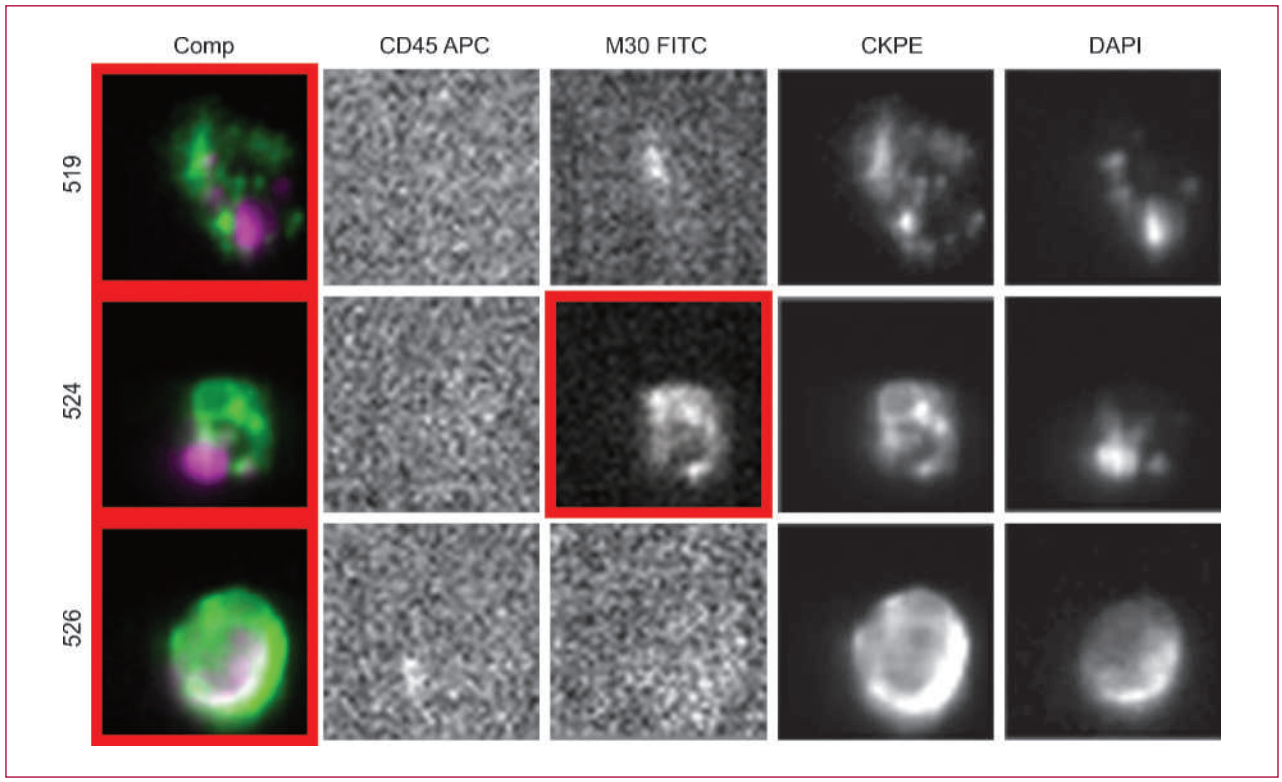

Fig. 3. M30 immunostaining of early apoptotic CTC. Analysis of three rare cells in a blood sample of a breast cancer patient using an Analyzer II device. Horizontally, the photos are of the same cell stained for the combination (Comp) of CK (green) and DAPI (violet), CD45 APC only, M30 FITC only, CK PE only, and DAPI only. Red squares, positively stained cells: events 519 and 526 are live CTC, exhibiting irregular and strong CK staining, respectively; based on M30 staining profile (sufficient signal relative to background) event 524 is classified as apoptotic CTC.

distinguished from a M30-positive CTC (Fig. 3, event 524), prospectively minimizing discretionary interpretation of morphologic features.

M30-positive CTC in solid tumors

To test whether apoptosis could be detected in CTC from patients with carcinoma, blood samples from 122 patients were tested (34 breast cancer, 59 colorectal cancer, 29 mRCC) before therapy. CTC were detected in 19 of 34 (56%) breast cancer patients; in 15 of these 19 patients (79%), M30-positive CTC were also detected. The number of CTC and M30-positive CTC ranged from 1 to 44 (median, 4) and 1 to 13 cells (median, 3), respectively. CTC were also detected in 26 of 59 (44%) colorectal cancer patients; in 24 of these 26 patients (92.3%), M30-positive CTC were detected. The number of CTC and M30-positive CTC ranged from 1 to 10 (median, 2) and 1 to 7 cells (median, 2), respectively. Finally, CTC were detected in 19 of 29 (66.5%) mRCC patients; in 17 of these 19 patients (89.5%), M30-positive CTC were detected. The number of CTC and M30-positive CTC ranged from 1 to 141 (median, 3) and 1 to 67 cells (median, 3), respectively.

The percentage of CTC-positive patients and total CTC numbers strictly resemble data previously reported in breast (5, 14, 15) and colorectal cancer patients (16). As summarized in Table 1, the presence of CTC and M30-positive CTC at diagnosis was not associated with any specific clinicopathologic feature in epithelial tumors, with remarkable exceptions. The presence of CTC at baseline seemed weakly associated with metastasis (P for trend = 0.075) in colorectal cancer (Table 1B). Moreover, the presence of CTC at baseline was associated with distant sites of metastasis (lung, mediastinal lymph node, liver, or bone, P for trend = 0.026) in mRCC; in this group, M30-positive CTC at baseline was weakly associated with clear cell tumor (P for trend = 0.08; Table 1C). In addition, M30-positive CTC were associated with elevated grading in breast cancer patients (Table 1A, P for trend = 0.018).

Serial M30-integrated CTC assay during chemotherapy

To investigate whether the integrated test may predict therapeutic response, CTC and M30-positive CTC were sequentially assessed in eight breast cancer patients. To this purpose, depending on their consensus to undergo multiple CTC tests, the patients were consecutively enrolled regardless of type or line of therapy and were monitored for a time in the range of 1 to 10 months. The results are summarized in Table 2.

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Table 1. Patients and primary tumor characteristics by CTC and M30-positive CTC count

A)

Breast cancer patients		*n*	CTC negative	CTC positive	*P**	M30+	*P**	M30+ %	*P*†
All subjects		34	15 (44%)	19 (56%)		15 (79%)		53.9	
Age at diagnosis, y	≤35	0	—	—		—		—	
	36-50	12	4	8	0.47	7	1	65.1	
	≥51	22	11	11		8		45.8	
Sex	M	1	0	1	1	0	0.21	0	
	F	33	15	18		15	56.9		
T (*n* = 19)	T_1	14	9	5‡	0.42	5‡	0.18	69.5	0.54
	T_2	4	2	2		1		100	
	T_3	1	0	1		1		100	
	T_4	0	—	—		—		—	
N (*n* = 20)	N_0	12	7	5	1	4	1	74.4	1
	N_1-N_3	8	4	4		4		68.8	
M (*n* = 17)	M_0	4	2	2	1	2	1	100	0.14
	M+	13	8	5		4		52	
Grading (*n* = 18)	G_1	5	4	1‡	0.40	1‡	0.38	50	0.018
	G_2	5	2	3		2		32.5	
	G_3	8	4	4		4		100	
Estrogen receptors (*n* = 20)	-	7	4	3	1	2	1	66.7	1
	+	13	7	6		5		49.6	
Progestin receptors (*n* = 20)	-	10	4	6	0.36	5	1	66.3	1
	+	10	7	3		2		33.3	
Her2 (*n* = 20)	-	19	11	8	0.45	7	1	52.8	1
	+	1	0	1		1		50	

B)

Colorectal cancer patients		*n*	CTC negative	CTC positive	*P**	M30+	*P**	M30+ %	*P*†
All subjects		59	33 (56%)	26 (44%)		24 (92.3%)		84	
Age at diagnosis, y	≤35	1	0	1	1	1	1	87.5	
	36-50	5	3	2		2		100	
	≥51	53	30	23		21		82	
Sex	M	38	22	16	1	16	0.138	87	
	F	21	11	10		8		79	
T (*n* = 47)	T_{is}	10	6	4‡	0.128	3‡	0.347	75	0.671
	T_0	3	3	0		—		—	
	T_1	4	2	2		2		100	
	T_2	9	4	5		5		95	
	T_3	17	11	6		6		92	
	T_4	4	0	4		4		77	
N (*n* = 47)	N_0	37	22	15‡	0.687	14‡	0.936	88	0.303
	N_1	6	2	4		4		97	
	N_2	2	1	1		1		20	
	N_x	2	1	1		1		100	
M (*n* = 47)	M_0	41	25	16	0.075	15	1	84	1
	M_1	6	1	5		5		98	

(*Continued on the following page*)

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Table 1. Patients and primary tumor characteristics by CTC and M30-positive CTC count (Cont'd)

C)

Renal cancer patients		*n*	CTC negative	CTC positive	*P**	M30+	*P**	M30+ %	*P*†
All subjects		29	10 (34.5%)	19 (65.5%)		17 (89.5%)		78	
Age at diagnosis, y	≤35	3	2	1	1	1	0.11	67	
	36-50	3	0	3		2		83	
	≥51	23	8	15		4		90	
Sex	M	23	9	14	0.84	12	1	84	
	F	6	1	5		5		96	
T (*n* = 19)	T_1	4	1	3‡	0.57	3‡	0.63	78	0.47
	T_2	3	2	1		1		80	
	T_3	11	4	7		5		67	
	T_4	1	0	1		1		100	
N (*n* = 19)	N_0	9	4	5	0.64	5	0.46	89	1
	N_1-N_3	10	3	7		5		62	
M (*n* = 11)	M_0	5	2	3‡	0.81	2‡	0.45	56	0.45
	M_1	2	1	1		1		100	
	M_x	4	1	3		3		89	
Fuhrman grading (*n* = 15)	G_1	—	—	—		—		—	
	G_2	4	1	3	1	3	1	89	*1*
	G_3	11	5	6		5		74	
Histology (*n* = 27)	CC carcinoma	22	8	14	1	13	0.33	84	0.08
	Others	5	2	3		2		38	
Sites of metastasis at blood draw (*n* = 25)	Contralateral kidney	4	4	0‡	0.026	—		—	
	Lung, mediastinal LN or liver	12	3	9		7	0.68	64	0.28
	Bone	9	3	6		6		94	

Abbreviations: T, tumor; N, node; M, metastasis; LN, lymph node; CC carcinoma, clear cell carcinoma.

*Fisher' exact test or χ^2 (‡) test were employed where appropriate.

†Median test.

‡ χ^2 test.

Overall the number of total and M30-positive CTC decreased during treatment in six and increased in two of eight patients.

In the first group, in four cases, consisting of patients 43 [progressive disease (PD)], 55 (PD), 94 (PD), and 97 [stable disease/partial response (SD/PR)], the total CTC number switched from values greater than or equal to the threshold of poor prognosis (5 CTC/7.5 mL for MBC; ref. 5) to values less than the threshold at the end of observation time, indicating a pharmacodynamic response that was related to overall disease progression only in patient 97; the M30-positive CTC were very few in these patients and the relative percentage of M30-positive CTC fluctuated, creating a pattern of peaks and troughs that were difficult to evaluate. Only one or two cells were enumerated in patients 29 (PD) and 53 (PD), being too few CTC to discriminate treatment effect.

In the second group, patient 84 (PD) showed a major shedding of CTC that went from all alive to all dead (6 CTC at the time point 3); in patient 50 (SD/PR) the total CTC number increased over the follow-up period and essentially all cells were apoptotic (10 of 11 CTC at the time point 2). The switch from values <5 to values >5 CTC was related to overall disease progression only in patient 84.

In principle, both decreased total CTC numbers to value <5 CTC and increased fraction of apoptotic CTC may represent response-related markers. However, the fact that these are both rare events may preclude the possibility to accurately assess significant differences in the M30-negative/positive CTC numbers in any patient; only follow-up studies of adequate patient cohorts monitored for an appropriate time can address the predictive relevance of apoptotic CTC.

For this purpose the observed variations were expressed by a simpler parameter: the detected numbers of M30-negative and M30-positive CTC were separately plotted in relation to time, and the area under the curve (AUC) of longitudinal graphs was calculated (Fig. 4), following a procedure commonly adopted to evaluate cumulative changes of serologic tumor markers (17). The difference

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between live and apoptotic CTC concentration-time area was calculated in all patients according to the following formula:

$$\Delta AUC = {}^{M30\text{–negative CTC}}AUC - {}^{M30\text{–positive CTC}}AUC$$

Relative numbers were obtained in the following way:

- Positive ΔAUC value is the expression of extra live CTC over the follow-up period (e.g., patients 84 and 94; Fig. 4B);
- Negative ΔAUC value is the expression of extra apoptotic CTC over the follow-up period (e.g., patients 50 and 97; Fig. 4B);
- ΔAUC = 0 derives from balanced numbers of live and apoptotic CTC.

As shown in Table 2, positive ΔAUC value was associated with radiologic recurrence of disease (P for trend = 0.036), including cases where a switch under the threshold of 5 CTC was observed during therapy (patients 43, 55, and 94 in Table 2); conversely, negative ΔAUC was associated with SD/PR also in patient 50, whose total CTC number increased to value >5 CTC.

Discussion

CTC can today be quantified in cancer patients, providing a robust predictor of treatment efficacy and survival throughout the continuum of the care (6, 18, 19). Data obtained in metastatic breast (5), colorectal (16, 20), and prostate cancer (4, 20, 21) by immunocytometric approach strongly support extending these observations to other solid tumor histotypes. Otherwise, it was recently published (22) that the threshold of 5 CTC/7.5 mL of peripheral blood, firstly set up by Cristofanilli (5), lacks prognostic significance in inflammatory metastatic breast cancer, where a value <5 CTC was not associated with better prognosis than ≥5 CTC. As suggested by the authors, the biological characterization of CTC should be addressed to discover unknown properties of these cells.

Furthermore, early clinical trials require validated pharmacodynamic biomarkers (hopefully blood-based) showing proof of mechanism (drug hits target) and/or proof of concept (tumor responds to drug). Apoptosis is often deregulated in cancer, and the induction of tumor cell death is a primary goal of many targeted therapies, directly

Table 2. Serial CTC and M30-positive CTC count during chemotherapy

Patient no.	Age/ Sex	T	N	M	Grading	ER	PGR	HER2	Test no.	Total CTC/ 7.5 mL	M30+/ 7.5 mL	M30+ %	ΔAUC*	Disease status†
29	79/M					Neg	Neg	Neg	1	2	0	0	Pos	PD
									2	1	1	100		
									3	1	0	0		
43	83/F	T_x	N+	M_1		Pos	Pos	Pos	1	8	4	50	Pos	PD
									2	1	0	0		
									3	1	1	100		
50	49/F	T_{1c}	N_0	M_0	G_3	Neg	Neg	Neg	1	3	3	100	Neg	SD/PR
									2	11	10	91		
53	64/F	T_{1b}	N_x		G_1	Pos	Neg	Neg	1	2	0	0	Pos	PD
									2	1	1	100		
55	50/F		N_0						1	8	2	25	Pos	PD
									2	Neg	Neg			
84	40/F	T_{1c}	N_0	M_0	G_3	Neg	Neg	Neg	1	Neg	Neg		Pos	PD
									2	581	18	3		
									3	6	6	100		
94	64/F	T_2	N_{1b}	M_0		Pos	Pos	Pos	1	22	0	0	Pos	PD
									2	52	6	12		
									3	6	3	50		
									4	4	0	0		
97	62/F	T_{4b}	N_2	M_0	G_3	Neg	Neg	Neg	1	5	4	80	Neg	SD/PR
									2	Neg	Neg			
									3	Neg	Neg			
									4	1	1	100		
									5	2	1	50		

Abbreviations: ER, estrogen receptor; PGR, progestin receptor; HER2, human epidermal growth factor receptor 2.

*$\Delta AUC = {}^{M30\text{-negative CTC}}AUC - {}^{M30\text{-positive CTC}}AUC$

†As determined by instrumental findings (computerized tomography or scintigrafy) simultaneously done with CTC count.

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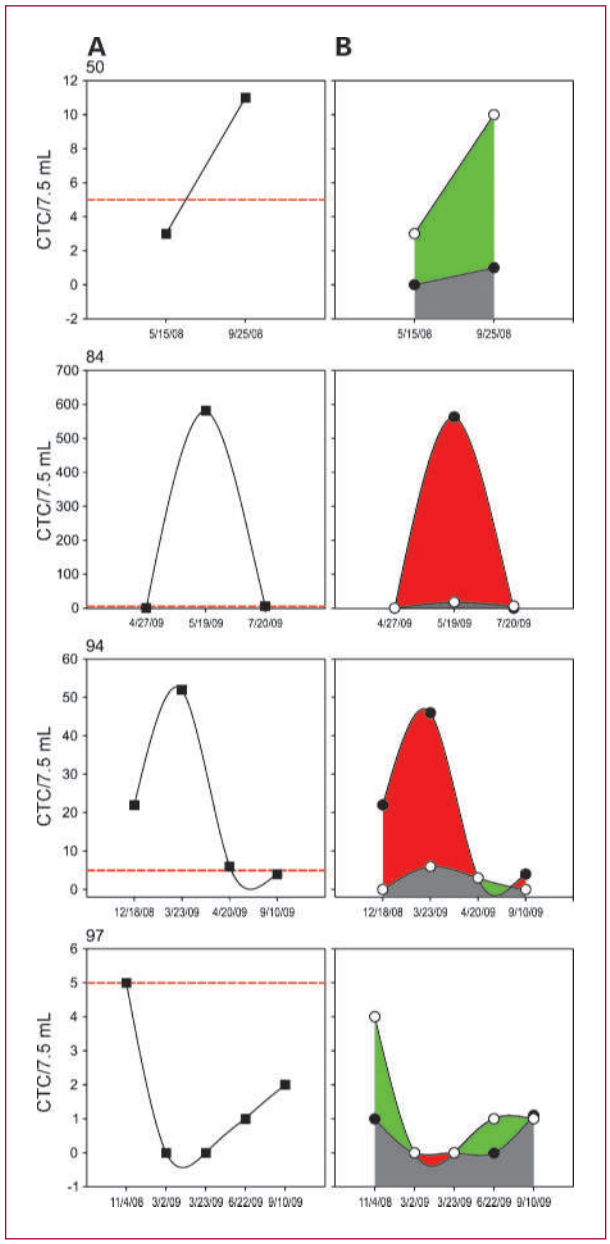

Fig. 4. Changes in the number of CTC in breast cancer patients. A, longitudinal graphs of CTC count over the indicated follow-up period for patients 50, 84, 94, and 97. Patients 94 and 97 were diagnosed with metastatic cancer at enrolling in this study; red lines, threshold of poor prognosis (5 CTC/7.5 mL for MBC; ref. 5). B, area under the blood concentration-time curve over the follow-up period of M30-negative (•, red area) and M30-positive (○, green area) CTC; gray area, overlay of the M30-positive and M30-negative longitudinal graphs. Extra live CTC over the follow-up (positive ΔAUC) were detected in patients 84 and 94, which were PD by imaging; on the contrary, extra-apoptotic CTC over the follow-up period (negative ΔAUC) were detected in patients 50 and 97, which were SD/PR by imaging.

or indirectly hinting molecular components of apoptosis regulatory pathways. Either way, apoptosis is regarded as a unique biomarker of treatment efficacy, and in the absence of tumor biopsies CTC may offer a surrogate sample.

In evaluating different components of the apoptotic cascade we focused on the M30 neoepitope for three reasons. First, the anti-M30 mAb defines an epitope of CK18 disclosing early phases of apoptosis. In this phase, despite caspase cleavage, CK18 is still retained in a filamentous network and tumor cells still satisfy the morphologic features of CTC. Second, a clinical correlate exists between serum levels of these CK fragment and tumor load and prognosis in breast (23) and colorectal cancer (24). Finally, both anti-M30 and Annexin V provided us consistent results in flow cytometry, altogether recommending including M30 in the new test.

The integrated assay was validated in 122 cancer patients before therapy at the first blood draw, disclosing that the M30 neoepitope is expressed on CTC at a very high frequency. Based on the presence of cytokeratin aggregates in their cytoplasm, apoptotic CTC in the late phase of the process were previously described in prostate (4, 25) and in lung cancer (26). M30-positive CTC were also documented in metastatic castration-resistant prostate cancer patients (27), but their enumeration was beyond the purposes of that study. To our knowledge, this is the first report on CellSearch technology applied to quantify apoptotic CTC. Larger studies are warranted to determine the prevalence of M30-positive CTC *in vivo;* however, although the detection of large numbers of these cells is counterintuitive, the data presented here are not surprising.

First, it is well known that higher grade and increased proliferation are often associated with tumor necrosis and apoptosis that may also be regarded as adverse biological features (28). Moreover, it was recently reported that both intact CTC and granular CTC (whose morphologic features strictly resemble early apoptotic cells) are inversely related to survival in castration-resistant prostate cancer (25). Indeed, in our breast cancer series, M30-positive CTC were associated with higher grading (P for trend = 0.018).

Second, apoptotic CTC were previously described in long-surviving breast cancer patients and have been considered a sign of occult niches of proliferating tumor cells, periodically shedding into the blood flow (2). Here we show that M30-positive CTC were detectable in the majority of cancer patients at different disease stages, possibly supporting that apoptotic dying is the mechanism because <0.1% of CTC released daily into the circulation successfully will settle in secondary organs (29).

Third, criticism was raised when, surprisingly, the median overall survival did not further decrease when >5 CTC were detected in 7.5 mL of blood (30). In challenging with rare cells, technical limits were evoked to account for a threshold of poor prognosis that is otherwise difficult to explain, for example increasing mistakes in assigning an event as a CTC when <5 CTC are detected or volume collected for the assay, but biological reasons cannot be excluded. Here we show that CTC frequently express a M30 neoepitope, which may offer a rationale to the argument that only the viable CTC cause the decreased chances for survival (30).

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On the other hand, the high frequency of M30-positive CTC in radio-chemo-free patients raises doubts as to whether the integrated CTC test may be a useful tool to monitor drug-induced cell death. Our data show that CTC represent a heterogeneous cell population, among which both apoptotic cells and viable cells with possible metastatic potential exist; we show that the M30-integrated assay may be used to accurately quantify them during treatment. In this setting, preliminary data obtained by tracking a small case series of breast cancer patients indicate that changes in the M30-negative/positive CTC balance as expressed by ΔAUC may be used as a "dynamic" parameter disclosing an active disease, as documented by consistent radiologic findings. As in the case of the CTC absolute number, whether such an early assessment of response to treatment may result in an improved overall outcome or quality of life needs to be prospectively assessed in clinical trials designed to investigate this question. In ongoing clinical trials at IOV-IRCCS, we are now testing whether assaying the quote of apoptotic CTC provides a more sensitive marker for rating pharmacodynamic effects in patients compared with total CTC counts.

In conclusion, although apoptosis is thought to play a major role in anticancer therapy, the clinical relevance of induction of apoptosis remains uncertain, particularly in solid tumors. The proposed test might contribute to clarify this point, and possibly provides a secondary end point other than tumor size and tumor burden for evaluation of response in early phase trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Fidler IJ, Talmadge JE. Evidence that intravenously derived murine pulmonary melanoma metastases can originate from the expansion of a single tumor cell. Cancer Res 1986;46:5167–71.
2. Meng S, Tripathy D, Frenkel EP, et al. Circulating tumor cells in patients with breast cancer dormancy. Clin Cancer Res 2004;10:8152–62.
3. Mehes G, Witt A, Kubista E, Ambros PF. Circulating breast cancer cells are frequently apoptotic. Am J Pathol 2001;159:17–20.
4. Larson CJ, Moreno JG, Pienta KJ, et al. Apoptosis of circulating tumor cells in prostate cancer patients. Cytometry A 2004;62:46–53.
5. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781–91.
6. Hayes DF, Cristofanilli M, Budd GT, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 2006;12:4218–24.
7. Amadori A, Rossi E, Zamarchi R, Carli P, Pastorelli D, Jirillo A. Circulating and disseminated tumor cells in the clinical management of breast cancer patients: unanswered questions. Oncology 2009;76:375–86.
8. Leers MP, Kolgen W, Bjorklund V, et al. Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. J Pathol 1999;187:567–72.
9. Favaro E, Nardo G, Persano L, et al. Hypoxia inducible factor-1α inactivation unveils a link between tumor cell metabolism and hypoxia-induced cell death. Am J Pathol 2008;173:1186–201.
10. Amadori A, Zamarchi R, De Silvestro G, et al. Genetic control of the CD4/CD8 T-cell ratio in humans. Nat Med 1995;1:1279–83.
11. Vermes I, Haanen C, Reutelingsperger C. Flow cytometry of apoptotic cell death. J Immunol Methods 2000;243:167–90.
12. Flores LM, Kindelberger DW, Ligon AH, et al. Improving the yield of circulating tumour cells facilitates molecular characterisation and recognition of discordant HER2 amplification in breast cancer. Br J Cancer 2010;102:1495–502.
13. Wang LH, Pfister TD, Parchment RE, et al. Monitoring drug-induced γH2AX as a pharmacodynamic biomarker in individual circulating tumor cells. Clin Cancer Res 2010;16:1073–84.
14. Krishnamurthy S, Cristofanilli M, Singh B, et al. Detection of minimal residual disease in blood and bone marrow in early stage breast cancer. Cancer 2010;116:3330–7.
15. Sandri MT, Zorzino L, Cassatella MC, et al. Changes in circulating tumor cell detection in patients with localized breast cancer before and after surgery. Ann Surg Oncol 2010;17:1539–45.
16. Sastre J, Maestro ML, Puente J, et al. Circulating tumor cells in colorectal cancer: correlation with clinical and pathological variables. Ann Oncol 2008;19:935–8.
17. de Haas EC, di Pietro A, Simpson KL, et al. Clinical evaluation of M30 and M65 ELISA cell death assays as circulating biomarkers in a drug-sensitive tumor, testicular cancer. Neoplasia 2008;10:1041–8.
18. Budd GT, Cristofanilli M, Ellis MJ, et al. Circulating tumor cells versus imaging-predicting overall survival in metastatic breast cancer. Clin Cancer Res 2006;12:6403–9.
19. Riethdorf S, Fritsche H, Muller V, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. Clin Cancer Res 2007;13:920–8.
20. Smirnov DA, Zweitzig DR, Foulk BW, et al. Global gene expression profiling of circulating tumor cells. Cancer Res 2005;65:4993–7.
21. Danila DC, Heller G, Gignac GA, et al. Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer. Clin Cancer Res 2007;13:7053–8.
22. Mego M, De Giorgi U, Hsu L, et al. Circulating tumor cells in metastatic inflammatory breast cancer. Ann Oncol 2009;20:1824–8.
23. Olofsson MH, Ueno T, Pan Y, et al. Cytokeratin-18 is a useful serum biomarker for early determination of response of breast carcinomas to chemotherapy. Clin Cancer Res 2007;13:3198–206.

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24. Ausch C, Buxhofer-Ausch V, Olszewski U, et al. Caspase-cleaved cytokeratin 18 fragment (M30) as marker of postoperative residual tumor load in colon cancer patients. Eur J Surg Oncol 2009;35: 1164–8.

25. Coumans FA, Doggen CJ, Attard G, de Bono JS, Terstappen LW. All circulating EpCAM+CK+CD45– objects predict overall survival in castration-resistant prostate cancer. Ann Oncol 2010;21:1851–7.

26. Hou JM, Greystoke A, Lancashire L, et al. Evaluation of circulating tumor cells and serological cell death biomarkers in small cell lung cancer patients undergoing chemotherapy. Am J Pathol 2009;175: 808–16.

27. Swennenhuis JF, Tibbe AG, Levink R, Sipkema RC, Terstappen LW. Characterization of circulating tumor cells by fluorescence *in situ* hybridization. Cytometry A 2009;75:520–7.

28. Rupa JD, de Bruine AP, Gerbers AJ, et al. Simultaneous detection of apoptosis and proliferation in colorectal carcinoma by multiparameter flow cytometry allows separation of high and low-turnover tumors with distinct clinical outcome. Cancer 2003;97:2404–11.

29. Dawood S, Cristofanilli M. Integrating circulating tumor cell assays into the management of breast cancer. Curr Treat Options Oncol 2007;8:89–95.

30. Tibbe AG, Miller MC, Terstappen LW. Statistical considerations for enumeration of circulating tumor cells. Cytometry A 2007;71: 154–62.

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