Advances in Brief

Cytogenetic Evidence That Circulating Epithelial Cells in Patients with Carcinoma Are Malignant[1]

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Abstract

Purpose: **Numerous studies of circulating epithelial cells (CECs) have been described in cancer patients, and genetic abnormalities have been well documented. However, with one exception in colorectal cancer, there has been no report of matching the genetic abnormalities in the CECs with the primary tumor. The purpose of this investigation was to determine (*a*) whether CECs in patients including those with early tumors are aneusomic and (*b*) whether their aneusomic patterns match those from the primary tumor, indicating common clonality.**

Experimental Design: **Thirty-one cancer patients had CECs identified by immunofluorescence staining using a monoclonal anti-cytokeratin antibody. Their CECs were analyzed by enumerator DNA probes for chromosomes 1, 3, 4, 7, 8, 11, or 17 by dual or tricolor fluorescence *in situ* hybridization. Touch preparations of the primary tumor tissue were available from 17 of 31 patients and hybridized with the same set of probes used to genotype the CECs.**

Results: **The number of CECs from each patient ranged from 1–92 cells/cytospin. CECs showed abnormal copy numbers for at least one of the probes in 25 of 31 patients. Touch preparations from the primary tumors of 13 patients with aneusomic CECs were available. The pattern of aneusomy matched a clone in the primary tumor in 10 patients.**

Conclusions: **We conclude that the vast majority of CECs in breast, kidney, prostate, and colon cancer patients are aneusomic and derived from the primary tumor.**

Introduction

There are numerous reports of epithelial cells in the blood (1–13) and bone marrow (14–19) of patients with carcinoma. It has been shown that the presence of micrometastases in the bone marrow is an independent prognostic indicator of an aggressive tumor with a poor outcome (20–23). In contrast to bone marrow aspirates, however, blood samples can be obtained repeatedly and easily. Longitudinal studies of CECs[3] show that their levels parallel tumor burden and response to therapy (24, 25). The blood test also has the potential of diagnosing carcinoma at an early stage, prognosticating by determining the persistence or disappearance of CECs, and the ability to follow changes in immunophenotype and genotype during tumor progression (24). The latter allows the therapeutic target to be characterized when tumor recurrence/progression occurs, for example, development of Her-2 overexpression, which calls for a specific treatment regimen (26–28). However, detection of CECs by immunophenotyping is challenging, and there have been conflicting results in evaluating the same specimen (29). Reverse transcription-PCR is a sensitive assay for detecting epithelial cell antigens, but this assay can also give false positive results (8, 9, 30–34). In addition, quantification is difficult. Most importantly, none of these findings prove that the CECs are neoplastic; they could be normal epithelial cells.

We have developed a sensitive test to detect, count, and characterize CECs (Fig. 1). However, in contrast to other studies (1–13), we find excess CECs in the majority of patients with clinically organ-confined tumor (35). They have the cytomorphology of neoplastic cells, and the number of these cells in the blood correlates with tumor burden and response to therapy (24, 25, 35). However, none of the above represents formal proof that these CECs in early carcinomas are malignant cells; proof is essential for both definitive interpretation of our data and decisions that affect treatment regimens. To obtain proof, we have searched for aneusomy in CECs and compared the pattern of aneusomy in the CECs with that of the corresponding primary tumor. If the CECs have a pattern of aneusomy that is identical to a clone in the primary tumor, then that would prove that the CECs are malignant cells derived from the primary tumor.

Changes in copy number of chromosomes or genes repre-

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[3] The abbreviations used are: CEC, circulating epithelial cell; FISH, fluorescence *in situ* hybridization; CK, cytokeratin; Mab, monoclonal antibody.

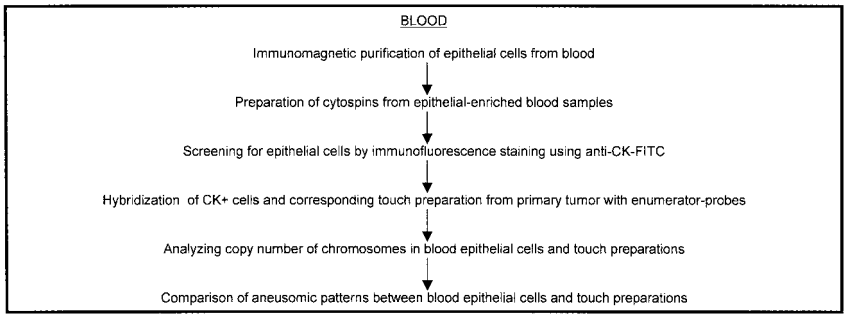

Fig. 1 Flowchart of sample processing and evaluation.

sent early events in the development of carcinoma (36–39). In the first phase of this study, CECs from patients with different types of cancer were analyzed for aneusomy involving chromosomes 1, 3, 4, 7, 8, 11, and 17 by dual color or tricolor FISH to evaluate whether the CECs are aneusomic. In the second phase of the study, we determined whether the pattern of aneusomy in the CECs was the same as that in the clones in the corresponding primary tumor using the same probes for each specimen, thereby searching for common clonality.

Patients and Methods

Patients

Thirty-one patients with histologically or clinically proven cancer and CK^+ cells in the blood were selected for this investigation from an ongoing study in which CECs were quantified in cancer patients before surgery. Peripheral blood samples (10–60 ml) were drawn into vacutainer tubes containing EDTA (Becton Dickinson) at the time of diagnosis of primary or metastatic disease and before treatment. Fresh imprints from malignant tumors were made from those cancer patients undergoing surgery. All specimens were obtained after informed consent and collected using protocols approved by the institutional review board at the University of Texas Southwestern Medical Center (Dallas, TX).

Sample Processing

Fig. 1 shows a flowchart of sample processing and individual steps of the blood test.

Touch Preparations. Touch preparations were obtained by lightly pressing the freshly cut tumor surface on precleaned microscope slides (Fischer Scientific). Slides were immediately fixed for 5 min in 95% ethanol, air dried, and then stored at –20°C until further processing.

Blood Samples. Blood samples were first enriched for epithelial cells by immunomagnetic separation. Immunomagnetic separation was performed with either (*a*) our previously published method (35) using ferrofluid coupled to antibodies against the epithelial cell adhesion molecule (Immunicon Corp.) or (*b*) the MACS system using magnetic beads labeled with MAbs against the human epithelial antigen (Miltenyi Biotec). Both procedures were performed according to the manufacturer's instructions with minor modifications (methods *a* and *b* are summarized below).

(*a*) Five ml of EDTA-anticoagulated blood were preincubated for 15 min at 20°C with 30 μg of epithelial cell adhesion molecule-ferrofluid diluted in 2 ml of dilution buffer (Immunicon Corp.) before placing the samples in a QMS 17 magnetic separator (Immunicon Corp.). After 10 min, the supernatant was carefully aspirated, and the sample was removed from the magnetic field. The enriched cell fraction was resuspended in 2 ml of cell buffer (Immunicon Corp.) and placed again in the magnetic separator to increase the purity of the cells. After a second 10-min incubation followed by aspiration of the supernatant, enriched cells were resuspended in 0.1 M DTT-PBS to reduce an excess of ferrofluid and incubated for 10 min at 20°C. To permeabilize and fix cells, 500 μl of FACS Permeabilizing solution (diluted 1:10 in distilled H_20; Becton Dickinson) were added for another 15 min at 20°C. Cells were then washed and resuspended in 200 μl of PBS.

(*b*) Mononuclear cells were isolated from 5–20 ml of EDTA-anticoagulated blood by Ficoll-Hypaque separation (Pharmacia) and washed twice in labeling buffer (PBS and 2 mM EDTA) by centrifugation at 300 × *g* for 10 min. The cell pellet was then resuspended in 300 μl of labeling buffer and incubated with 100 μl of FcR-Blocking Reagent and microbeads labeled with antibodies against human epithelial antigen (Miltenyi Biotec). After a 30-min incubation at 4°C, cells were washed and resuspended in 500 μl of separation buffer (PBS, 2 mM EDTA, and 1% BSA). The cell suspension was then applied to a MACS MS^+ separation column (Miltenyi Biotec). Nonlabeled cells were washed off the column with 1.5 ml of separation buffer. Columns were then removed from the magnetic field, and the cells retained in the column were flushed out using a plunger. Enriched cells were washed in PBS before a 10-min fixation and permeabilization step with 500 μl of FACS Permeabilizing solution. After repeating the centrifugation, cells were resuspended in 200–800 μl of PBS (100 μl for each 2.5 ml of blood).

Cytospins were prepared, independent of which separation method was used (*a* or *b*), by centrifuging 100 μl of the cell suspension onto a poly-L-lysine-coated glass slide in a cytocentrifuge (Hettich). Slides were air dried overnight and stored at –20°C until further processing.

Immunofluorescence Staining

To identify epithelial cells on slides, a FITC-conjugated MAb mouse anti-pancytokeratin, C11 (Sigma), was used. This MAb recognizes human CKs 4, 5, 6, 8, 10, 13, and 18. Cytospins were thawed and rinsed with washing buffer (PBS-0.1%

Tween 20). After blocking nonspecific binding sites with 5% BSA in PBS-0.1% Tween 20 for 30 min, slides were incubated for 40 min with 150 μl of the FITC-conjugated anti-CK MAb (diluted 1:100 in 1% BSA-PBS-0.1% Tween 20) at 37°C in a humidified chamber. Slides were then washed three times in a washing buffer for 5 min each and counterstained with 4′,6-diamidino-2-phenylindole in mounting media (Vector Laboratories). As a negative control, an isotype-matched myeloma protein conjugated to FITC was used (Sigma). SKBR3 cells (breast carcinoma cells) served as positive controls in the staining experiments.

FISH

Different pretreatments were used for (*a*) touch preparations and (*b*) blood cytospins before denaturation, as described below.

(*a*) Imprints were refixed for 45 min in methanol:acetic acid (3:1) and dehydrated in 70%, 85%, and 100% ethanol for 2 min each and then air dried.

(*b*) Blood slides with CK^+ cells were pretreated with RNase A (100 μg/ml) for 40 min, followed by a pepsin treatment in 6 μg/ml pepsin in 0.01 M HCl for 7 min. After refixing in 1% formaldehyde-PBS for 10 min, slides were dehydrated in 70%, 85%, and 100% ethanol for 2 min each and air dried.

Blood slides and touch preparations were denatured for 2 min at 80°C in 70% formamide and 2× SSC (SSC, 0.15 M NaCl and 0.015 M sodium citrate), pH 7, before dehydration in a cold ethanol series. Enumerator probes for repetitive sequence regions of chromosome 1 (satellite II/III), 3 (α satellite), 4 (α satellite), 7 (α satellite), 8 (α satellite), 11 (α satellite), and 17 (α satellite) and locus-specific probes for MYC and ERBB2 were kindly provided by Vysis, Inc. and used either in dual color (SpectrumOrange/SpectrumGreen) or tricolor combinations (SpectrumOrange/SpectrumGreen/SpectrumAqua). The FISH-DNA probe mixture was denatured in 55% formamide, 2× SSC, and 10% dextran sulfate (pH 7) for 5 min at 76°C and then added to the air-dried slide (5 μl probe mix/slide).

Cells were hybridized overnight in a humidified chamber at 37°C. Posthybridization wash was carried out at 74°C in 0.4× SSC-0.1% NP40 (pH 7; Roche Molecular Biochemicals) for 2 min followed by a 2-min wash in 2× SSC-0.1% NP40 at 25°C. Slides were counterstained and mounted with mounting media containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). Leukocytes from normal blood donors served as hybridization controls. Leukocytes from patients served as an "internal control." To ensure that the hybridization was effective, (*a*) the percentage of leukocytes with <2 signals for an individual enumerator probe had to be $\leq 10\%$, and (*b*) gains of copy numbers had to be $\leq 2\%$. Otherwise, the hybridization results were discarded. For comparison of aneusomic patterns, touch preparations were hybridized with the same combination of probes used for the corresponding blood slides.

Rehybridization (Reprobing) of Slides

Coverslips were first carefully removed from the hybridized slide. The slide was then washed in 2× SSC for 10 min and dehydrated in an increasing ethanol series. Denaturation and hybridization of the slide were performed as described above.

Selection of Enumerator Probes

Based on the literature, chromosomes 1, 3, 4, 7, 8, 11, and 17 are frequently aneusomic in breast cancer and were therefore chosen for multicolor FISH (38–46). Reports from others (47–50) led to the selection of chromosomes 3 and 11 for multicolor FISH of patients with kidney cancer. Aneusomy for the chromosomes listed above has been described for lung (51–53), colon (54–56), and prostate cancer; (57–59) therefore, these chromosomes were also used for genotyping.

Fluorescence Microscopy

Screening for Epithelial Cells. Slides were manually analyzed for the presence of CK^+ cells using a computerized fluorescence microscope (×63 or ×100 oil immersion objectives; Axiophot; Zeiss) equipped with an automated stage and cell relocation program (Applied Imaging). To screen for epithelial cells, a single-pass filter for FITC and a dual-pass filter for FITC/DAPI were used. The location of each CK^+ cell was recorded and stored. Because the immunofluorescence staining is lost during the FISH procedure, an image of each CK^+ cell and its surrounding field was also acquired to reassure the accurate relocation for the following FISH analysis.

FISH Analysis of Blood Slides. Hybridized cells were relocated with the same fluorescence microscope used for scanning. Before analyzing FISH signals, the microscopic image and previously recorded image were visually compared to ensure correct relocation. Hybridization signals in recorded cells were then enumerated separately for each enumerator probe through the appropriate single-pass filter (Spectrum Orange, Spectrum Green, or Spectrum Aqua). Criteria for analysis of hybridization signals have been outlined in detail previously (60, 61). To document the combined aneusomic pattern for a single cell, the total number of signals for each chromosome was listed, starting with the lowest chromosome number followed by each higher number *e.g.*, chromosome 1, 8, 17: cell #x: (2, 4, 1). For statistical analysis and for comparison with imprints, the signals were then classified as <2 (monosomic), 2 (disomic), and >2 [polysomic (simple classification)]. In addition, the precise number of each signal was recorded in both CECs and the corresponding tumor (precise classification).

FISH Analysis of Touch Preparations. At least 100 nonoverlapping and intact interphase nuclei were analyzed from each primary tumor. Enumeration and documentation of FISH signals were performed as described under "FISH Analysis of Blood Slides." If two or more chromosomes of the DNA probe set were aneusomic, then 5% of cells showing the same aneusomic pattern in a touch preparation was considered as the cutoff level for a cell clone. If only one chromosome displayed aneusomy, the cutoff level of monosomy (15%) and polysomy for a single chromosome (6%) and polysomy for 2 chromosomes (4%) were used to define a clone. These cutoff levels were established in a previous study (62), in which we analyzed aneusomic patterns in touch preparations from 20 patients with either benign or normal breast tissue.

Definition of Match between Primary Tumor and CECs

For comparison of aneusomic patterns between CECs and touch preparations from the primary tumor, two classifications

were used: (*a*) simplified classification of <2, 2, or >2 signals for individual chromosomes; and (*b*) the precise number of chromosome/genes in each CEC and touch preparation. The criteria for a match were as follows: (*a*) CECs had to be aneusomic for at least one of the chromosomes (therefore, disomic CECs and corresponding disomic tumors were not defined as a match; and (*b*) the classified aneusomic pattern of an individual cell(s) could be detected in a clone of the corresponding tumor touch preparation (see above for definition of a clone).

Results

Aneusomy in CECs of Patients with Carcinoma (Simple Classification). CECs from 31 patients were included in the study to be evaluated for changes in copy number of chromosome 1, 3, 4, 7, 8, 11, or 17 (Table 1). Patients were diagnosed with breast ($n = 18$), colon ($n = 4$), kidney ($n = 4$), lung ($n = 1$), or prostate cancer ($n = 4$). Additional sets of enumerator probes could be tested in four of six patients with more than one slide positive for epithelial cells (patients 9, 14, 18, and 25). The number of CK^+ cells varied between 1 and 25 cells/slide. Eleven patients had one CEC analyzed by FISH. In eight of these patients (patients 1, 2, 3, 4, 5, 6, 8, and 26), CECs were aneusomic for one or more of the enumerator probes.

As shown in Table 1 and summarized in Table 2, in 20 patients, more than one CK^+ cell could be analyzed. In three patients, CECs showed only disomy (patients 12, 15, and 22). Six patients had a population of CECs with a cytogenetically homogeneous pattern of aneusomy (patients 9, 10, 11, 13, 14, and 20). In 8 of the 10 remaining patients with more than one CK^+ cell, a cytogenetically heterogeneous pattern was observed (patients 16, 17, 21, 23, 24, 28, 29, and 30), including 6 cases (patients 16, 17, 21, 28, 29, and 30) where disomic epithelial cells were counted as a second clone. In the other two patients (patients 18 and 25), CK^+ cells showed a cytogenetically homogenous aneusomic pattern using one set of probes, but not the other.

In three patients, rehybridization was performed a second and third time (reprobing) with a different set of DNA probes (patients 9, 14, and 19). No aneusomy was detected for patient 19 using either set of enumerator probes. In patients 9 and 14, rehybridization with additional combinations of DNA probes including locus-specific probes for ERBB-2 and MYC, demonstrated a subclone of CECs with an aneusomy pattern identical to a clone in the tumor. Results of FISH from patient 25 and reprobing for patient 14 with different sets are shown in Figs. 2 and 3.

In two patients (patients 14 and 20), additional CECs that were CK^- could subsequently be identified as CECs based on the characteristic pattern of aneusomy found in CK^+ CECs. In patient 20, the number of CK^- cells identified by the pattern of aneusomy exceeded the number of CK^+ CECs. Hence, the number of CECs in our assays may represent an underestimate because of a lack of staining with anti-CK.

Aneusomy of Individual Chromosomes in CECs of Patients with Carcinoma. Changes in copy number of individual chromosomes in CECs were studied in 20 breast cancer patients (Table 3). A total of 115 CECs from eight breast cancer patients could be analyzed for aneusomy involving chromosome 1, 3, 4, 7, 8, 11, or 17. For chromosome 4, only one patient contributed CECs, and for chromosomes 3 and 11, only two patients contributed CECs; therefore, no evaluation concerning the incidence of losses and gains for these chromosomes was made in the following discussion.

Monosomy in epithelial cells could be observed for chromosomes 1, 8, and 17, but not for chromosome 7. The frequencies for monosomy were generally low and ranged between 8% and 15%. In contrast, gains of chromosomes 1, 7, 8, or 17 were detected in 44%, 64%, 75%, and 45% of CK^+ cells, respectively. Only 18 of 115 epithelial cells had normal copy numbers for the enumerator probes used to determine aneusomy (Table 1). Therefore, the majority of epithelial cells from breast cancer patients were aneusomic for at least one of the tested chromosomes.

Fourteen CK^+ cells from four patients with kidney cancer could be studied for aneusomy involving chromosome 3 and 11 (Table 4). Six CECs showed disomy. Eight CK^+ cells were aneusomic for at least one of the enumerator probes. Aneusomy of chromosome 3 could be observed in eight cells, and aneusomy of chromosome 11 could be observed in six cells. Gains were detected more often than losses for both probes.

Comparison of Aneusomic Patterns between Primary Tumor and CECs. In 17 of 31 patients, CECs could be analyzed for the pattern of aneusomy, and this pattern could be compared with that obtained from touch preparations of the primary tumor (Table 5). The results were presented in two different ways: (*a*) the copy numbers of each chromosome/gene were classified as either >2, 2, or <2 (three categories only) (simplified classification); and (b) the copy numbers of each chromosome/gene were noted for each CEC (precise classification).

Two (patients 7 and 22) of four patients with disomic CEC had tumors showing normal copy numbers for tested chromosomes. Disomic tumor cells could also be found in the primary tumors of the two other patients (patients 12 and 19), but the percentage was low, and most of the neoplastic cells in the touch preparations demonstrated aneusomy.

In six patients (patients 16, 21, 24, 29, 20, and 31), the CECs consisted of both disomic and aneusomic cells. The pattern of aneusomy and the ratio of disomic:aneusomic CECs were also reflected in the primary tumors of four patients (patients 16, 21, 29, and 30). In patient 31, it was not. In patient 24, the primary tumor was classified as disomic because the different fractions of aneusomic cells in the touch preparation were very low and did not exceed the cutoff level for a clone (see the definition for a clone in "Patients and Methods"). However, aneusomic patterns of CECs were found in the primary tumor.

Seven patients had only aneusomic CECs. In six cases, the pattern of aneusomy could be matched exactly with that of a clone in the touch preparation (patients 2, 3, 5, 14, 20, and 23). In five of these six cases, the clone was also the main subpopulation in the primary tumor. In patient 23, the combination of aneusomy of one of the four epithelial blood cells could not be found in the tumor tissue. The pattern of aneusomy could be matched for one chromosome but not for the other in one case

Table 1 Aneusomy in CECs from primary and metastatic cancer patients[a]

Cancer	ID	Stage	No. of CK$^+$ cells	Chromosomes evaluated	Aneusomic pattern of CEC	No. of CECs showing this pattern
Breast	1	M_1[b]	1	1, 7	>2, >2	1
	2	pT_2 pN_1 M_0	1	1, 17	>2, 2	1
	3	pT_2 pN_0 M_0	1	1, 17	2, >2	1
	4	M_1	1	1, 17	>2, >2	1
	5	pT_2 pN_0 M_0	1	8, 17	>2, 2	1
	6	M_1	1	8, 17	<2, 2	1
	7	pT_{1c} pN_0 M_0	1	1, 8, 17	Disomic	1
	8	M_1	1	1, 8, 17	>2, >2, >2	1
	9-1	M_1	2	1, 8, 17	>2, <2, <2	2
			2	1, 3, 11[c]	>2, 2, 2[c]	2
	9-2		1	1, 7, 8	>2, 2, <2	1
			1	1, 3, 11[c]	>2, 2, 2[c]	1
	10	pT_3 pN_0 M_0	2	1, 17	>2, 2	2
	11	pT_x pN_x M_x	2	1, 17	2, >2	2
	12	pT_{1c} pN_2 M_0	3	1, 17	Disomic	3
	13	M_1	3	8, 17	>2, <2	3
	14-1	M_1	3	1, 8, 17	>2, >2, 2	3
	14-2		5	1, 3, 11	>2, >2, >2	5
	14-3		6	8, MYC	>2, >2	8[d]
				10, 17, ERBB2	>2, 2, >2[c]	8[d]
				1, 8, 17[c]	>2, >2, 2[c]	8[d]
	15	M_1	4	1, 7	Disomic	4
	16	pT_{1c} pN_0 M_0	5	1, 7	Disomic	4
					>2, 2	1
	17	M_1	7 (3+3+1)[e]	1, 8, 17	2, >2, 2	5
					Disomic	2
					>2, >2	3
	18-1	M_1	9	1, 7	Disomic	4
					>2, 2	2
					>2, >2	3
	18-2		28 (15+13)[e]	1, 17	<2, 2	3
					<2, >2	10
					2, >2	9
					>2, 2	1
					>2, >2	5
	18-3		25	4, 7	2, >2	25
Colon	19	pT_3 pN_2 M_1	1	1, 8, 17	Disomic	1
				1, 3, 11[c]	Disomic[c]	1
	20	pT_3 pN_1 M_0	3	1, 8, 17	>2, >2, >2	7
	29	M_1	9	1, 8, 17	Disomic	3
					2, <2, 2	1
					>2, 2, 2	1
					>2, >2, >2	1
					2, 2, >2	2
					2, 2, <2	1
	31	M_1	20	1, 8, 17	Disomic	9
					>2, >2, 2	2
					2, >2, 2	3
					>2, 2, 2	1
					2, 2, >2	1
					<2, 2, <2	1
					2, 2, <2	1
					>2, 2, >2	1
					2, >2, >2	1
Kidney	21	pT_2 pN_0 M_0	2	3, 11	Disomic	1
					>2, >2	1
	22	pT_{3b} pN_0 M_0	2	3, 11	Disomic	2
	23	pT_{3a} pN_0 M_0	4 (2+2)[e]	3, 11	<2, >2	1
					>2, >2	3
	24	pT_2 pN_0 M_0	6	3, 11	Disomic	3
					<2, <2	1
					<2, 2	1
					>2, 2	1
Lung	25-1	M_1	3	1, 3, 11	>2, >2, <2	3
	25-2		3	1, 17	Disomic	1
					>2, >2	2

Table 1 Continued

Cancer	ID	Stage	No. of CK^+ cells	Chromosomes evaluated	Aneusomic pattern of CEC	No. of CECs showing this pattern
Prostate	26	M_1	1	3, 11	2, >2	1
	27	M_1	1	1, 17	Disomic	1
	28	pT_{2a} pN_0 M_0	3	1, 7	Disomic	1
					2, <2	2
	30[f]	pT_{2cp} N_0 M_0	92	1, 8, 17	Disomic	44
					2, 2, >2	5
					<2, 2, 2	4
					2, <2, 2	8
					>2, 2, 2	1
					2, >2, 2	1

[a] At the time of first diagnosis.
[b] Metastatic disease.
[c] Reprobing of the same slide.
[d] Additional CEC-CK^+ cells detected by aneusomy pattern (see last column).
[e] Several slides were hybridized with the same set of probes; () parentheses indicates the number of CK^+ cells on each side.
[f] There were many additional patterns that were left out because of space considerations.

Table 2 Patterns of aneusomy in CECs of cancer patients[a]

	Total no. of patients (%)	No. of patients with 1 CK^+ cell (%)	No. of patients with >1 CK^+ cell (%)
Disomy	6 (19)	3 (10)	3 (10)
Aneusomy	25 (81)	8 (25)	17 (55)
Cytogenetically homogeneous			4
Cytogenetically heterogeneous			13[b]
Total no.	31 (100)	11 (35)	20 (65)

[a] Simplified classification.
[b] Including two patients with identical aneusomy for one set of enumerator probes, but not for the other set.

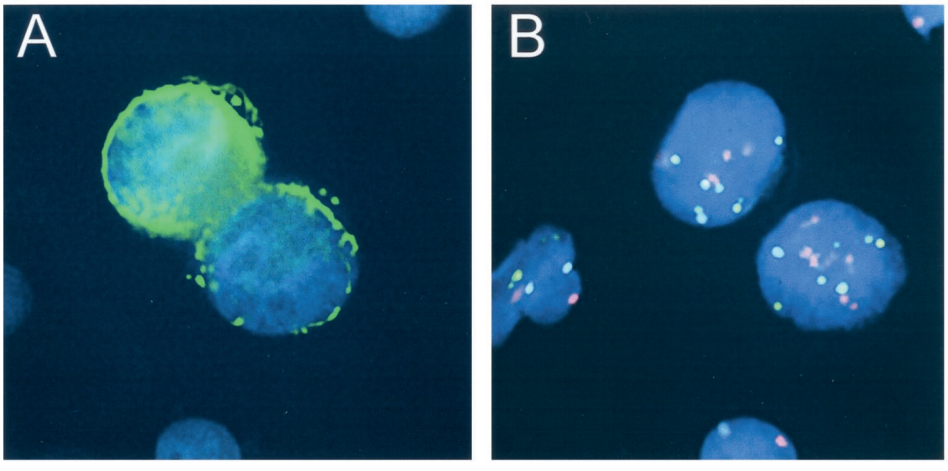

Fig. 2 Aneusomy of CECs in a patient with metastatic lung cancer (patient 25). *A*, cluster of two CK^+ cells isolated from blood. *B*, CK^+ cells hybridized with enumerator probes for chromosomes 1 (*orange*), 3 (*green*), and 11 (*aqua*); cells are polysomic for chromosomes 1 and 3 and monosomic for chromosome 11.

(patient 10). Fig. 4 demonstrates an example of a match between a CECs and primary tumor.

To summarize, excluding patients with only disomic CECs, a match between the aneusomic pattern of CECs and primary tumor could be made in 10 of 13 patients (see "Patients and Methods" for the definition of a match), regardless of whether the simplified or the precise pattern was used. We believe that the concordance between CECs and clones in the primary tumor would be greater if not for the marked heterogeneity of aneusomy in cancer. This point is illustrated in Table 6 showing the precise aneusomic pattern of 100 cells from a primary breast carcinoma.

Discussion

The major points to emerge from this study are as follows: (*a*) using FISH, the CECs isolated from breast, kidney, lung, prostate, and colon cancer patients were shown to be aneusomic

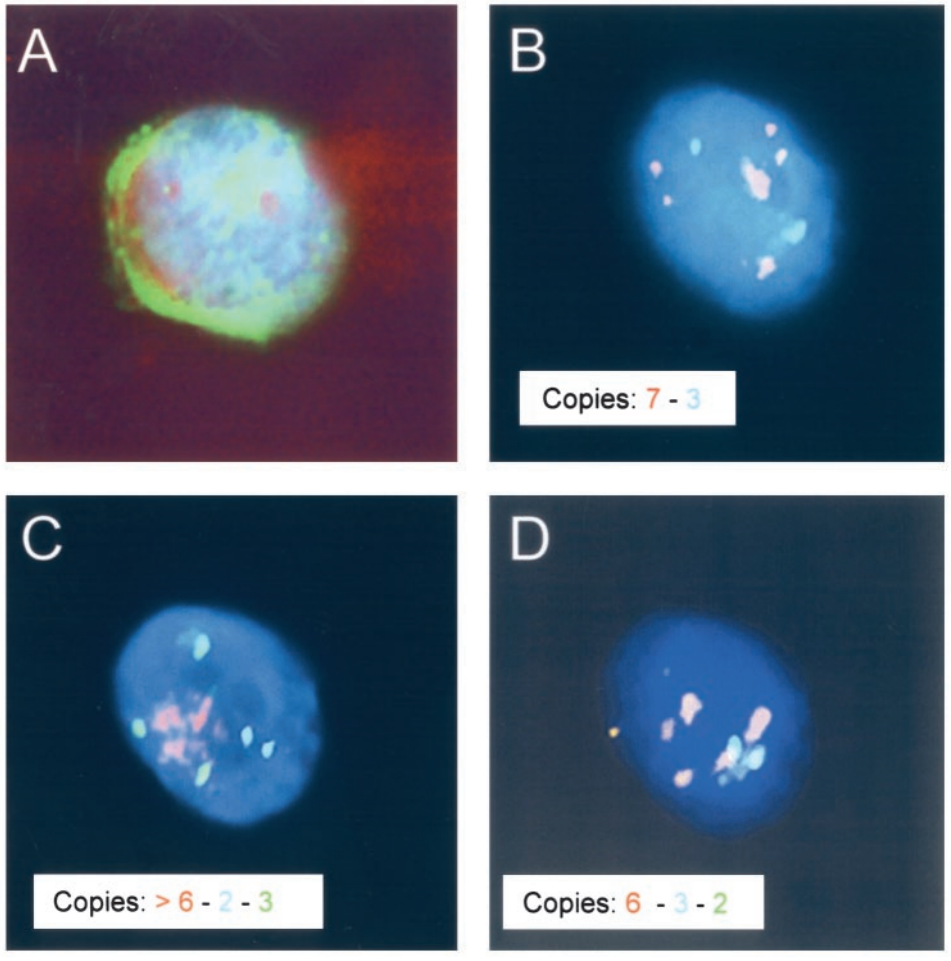

Fig. 3 Sequential phenotyping and genotyping of a CEC from a metastatic breast cancer patient (patient 14). *A*, CEC detected by an anti-CK antibody labeled with FITC; *B*, the same cell hybridized with a locus-specific probe for MYC (*orange*) and an enumerator probe for chromosome 8 (*aqua*). *C*, the same cell hybridized with a locus-specific probe for ERBB-2 (*orange*) and enumerator probes for chromosomes 10 (*green*) and 17 (*aqua*). *D*, the same cell hybridized with enumerator probes for chromosomes 1 (*orange*), 8 (*aqua*), and 17 (*green*). The copy number of the different chromosomes is shown in the pictures (*B*−*D*). Not all signals can be seen in the images because the signals are in different z planes.

Table 3 Copy number of chromosomes in CECs isolated from breast cancer patients

Copy no.	Chr 1[a] *N* (%)	Chr 3 *N* (%)	Chr 4 *N* (%)	Chr 7 *N* (%)	Chr 8 *N* (%)	Chr 11 *N* (%)	Chr 17 *N* (%)
<2 copies	13 (15)				4 (14)		5 (8)
2 copies	35 (41)	3 (38)	25 (100)	16 (36)	3 (11)	3 (38)	31 (48)
>2 copies	37 (44)	5 (63)		29 (64)	21 (75)	5 (63)	29 (45)
Total no. of CEC	85	8	25	45	28	8	65
No. of patients[b]	15	2	1	5	8	2	15

[a] Chr, chromosome. N = number of CECs; total n = 115 cells from 18 patients.
[b] Number of patients contributing cells to this group.

in the vast majority of patients; (*b*) gains of chromosomes/genes were more common than losses; (*c*) the use of tricolor FISH and, if necessary, reprobing two times with different combinations of enumerator and locus-specific probes increases the likelihood of detecting aneusomy; (*d*) in 17 patients, a comparison in the patterns of aneusomy between CECs and the primary tumor was possible, and, excluding the four disomic CECs, matches were demonstrated in 10 of 13 of these patients, regardless of whether a simplified or precise classification of signals was used. A match was defined by identity of an aneusomic CEC with a clone in the primary tumor. The latter was based on previous results (62) of analysis of 72 primary breast carcinomas and 20

Table 4 Copy number of chromosomes in CECs from kidney cancer patients[a]

No. of copies			
Chr 3[b]	Chr 11	No. of CEC	%
2	2	6	43
<2	2	1	7
<2	<2	1	7
<2	>2	1	7
>2	2	1	7
>2	>2	4	29

[a] *n* = 14 cells from four patients.
[b] Chr, chromosome.

Table 5 Comparison of patterns of aneusomy between CEC and primary tumors

Group	ID[a]	Diagnosis	No. of CECs	Chr tested	**SIMPLIFIED ANEUSOMY** pattern of CEC	No. of CECs with this pattern	% of cells in tumor matching CEC copy number pattern	**PRECISE** aneusomy pattern of CEC[b]	% of cells in tumor matching CEC **PRECISE** copy number pattern	The first three main clones in touch preparations[c]
Disomic	7	Breast Ca	1	1, 8, 17	Disomic	1	83%			**(2,2,2):83%,** (2,1,2):8%, (2,2,1):3%
	12	Breast Ca	3	1, 17	Disomic	3	33%			(3,2):48%, **(2,2):33%,** (4,2):10%
	22	Kidney Ca	2	3, 11	Disomic	2	87%			**(2,2):87%,** (2,3):4%, (4,4):4%
	19	Colon Ca	1	1, 8, 17	Disomic	1	18%			(3,2,3):23%, (3,2,4):16%, (2,2,2):18%
				1, 3, 11	Disomic[d]	1[d]	10%			(3,3,3):41%, (2,3,3):20%, (2,2,2):10%
Di-/aneusomic	16	**BREAST CA**	5	1, 7	Disomic	4	67%	4x(2,2)[b]	67%	**(2,2):67%,** (2,3):12%, (3,3):7%
					>2, 2	1	14%	(3, 2)	6%	
	21	**KIDNEY CA**	2	3, 11	Disomic	1	42%	(2, 2)	42%	**(2,2:40%, (3,4):19%,** (2,3):10%
					>2, >2	1	31%	(3, 4)	19%	
	24	Kidney Ca	6	3, 11	Disomic	3	87%	2x(2, 2)[b]	87%	**(2,2):87%,** (4,4):2%, (3,3):5%
					<2, <2	1	,	(1, 1)	0%	
					<2, 2	1	2%	(1, 2)	2%	
					>2, 2	1	2%	(3, 2)	2%	
	29	Colon	9	1, 8, 17	Disomic	3	34%	3x (2,2,2)	34%	**(2,2,2):34%,** (4,3,3):10%, (2,1,2):6%
					2, <2, 2	1	6%	(2,1,2), (3,2,2), (3,3,3)	6%, 4%, 3%	
					>2, 2,2	1	4%	2x(2,2,3), (2,2,1)	1% 1%	
					>2, >2, >2	1	3%			
					2, 2, >2	2	1%			
					2, 2, <2	1	1%			
	30	**PROSTATE**[e]	92	1, 8, 17	Disomic	44	53%	44x (2,2,2)	53%	**(2,2,2):53%,** (2,2,3):12%, (2,1,2):6%
					2, 2, >2	5	12	5x (2,2,3), 4x (1,2,2), 8x	12%, 5%, 6%	
					<2, 2, 2	4	5%	(2,1,2)	5%	
					2, <2, 2	8	6%	(3, 2,2)		
					>2, >2, 2	1	5%			
	31	**COLON**[e]	20	1, 8, 17	Disomic	9	7%	9x (2,2,2)	9%	**(4,3,2):20%** (3,3,2):17%, (3,4,2):11%
					>2, >23, 2	2	62%	(3,4,2), (3,3,2)	11% 17%	
					2, >2, 2	3	6%	(2,3,2)	6%	
					>2, 2,2	1	8%	(3,2,2)	7%	
Only aneusomic	2	**BREAST CA**	1	1, 17	>2, 2	1	40%	(4, 2)	32%	**(4,2):32%,** (2,2):11% (2,3):10%
	3	**BREAST CA**	1	1, 17	2, >2	1	11%	(2, 3)	11%	(4,3):36%, (2,4):40%, (4,3):40%
	5	**BREAST CA**	1	8, 17	>2, 2	1	47%	(3, 2)	30%	**(3,2):30%,** (4,2):13%, (4,3):12%
	10	Breast Ca	2	1, 17	>2, 2	2	0%	2x(4, 2)[b]	0%	(4,10):39%, (2,10):14%, (2,8):10%
	14-1	**BREAST CA**	3	1, 8, 17	>2, >2, 2	3	92%	(3,6,2), (4,4,2)	0%, 5%	(6,5,2):17%, (5,5,2):15%, (6,4,2):10%
	14-2		5	1, 3, 11	>2, >2, >2	5	73%	(7,3,4), (4,4,4), (5,3,3)	0%, 0%, 17%	**(6,3,3):24%,** (5,3,3):17%, (7,3,3):14%
								(6,3,3), (4,3,3)	24%, 5%	
	14-3		6	MYC, 8	>2, >2[d,f]	8[d,f]	100%	(>4,3), 2x(>4,6)[b], (>4,7),	3%, 20%, 3%	**(>4, 5):42%, (>4,4):29%, (>4,6):20%**
								3x(>4,5)[b], (>4,4)	42%, 29%	
								(3,2,>4)		
				10, 17, ERBB2	>2, 2, >2[d,f]	8[d,f]	70%	(6,3,2), (4,6,2), (5,7,2)	69%	**(3,2,>4):69%,** (2,2,>4):14%, (2,2,2):9%
								(4,5,2), (3,4,2), (3,6,2)		
				1, 8, 17	>2, >2, 2[d,f]	8[d,f]	92%	(3,5,2), (6,5,2)	1%, 1%, 2%	**(6,5,2):17%,** (5,5,2):15%, (6,4,2):10%
									1%, 0%, 0%	
									3%, 17%	
	23	**KIDNEY CA**	4	3, 11	<2, >2	1	0%	(1,3)	0%	**(4,3):80%,** (3,3):15%, (4,2):5%
					>2, >2	3	95%	(4,3), (4,4)	80%, 0%	
	20	**COLON CA**	3	1, 8, 17	>2, >2, >2	7[f]	77%	4x(4,4,4)[b], 2x(3,3,3)	11%, 1%	(4,5,4):40%, **(4,4,4):11%,** (4,5,3):10%
								(5,3,3)	5%	

[a] ID, patient identification number; Chr, chromosome; Ca, cancer.
[b] x, number of cells with this pattern (only indicated if cell number >1 cell.
[c] Clones found in CECs are bold.
[d] Reprobing of the same slide.
[e] Many additional patterns did not meet the definition of a clone and were left out for space consideration.
[f] Additional CECs were detected by unusual aneusomy patterns that were seen in other CECs in the same patient.

Table 6 Genetic heterogeneity of tumor cells in a primary breast tumor (patient 14)

Percentage of tumor cells in primary tumor with this aneusomic pattern[a]	Aneusomic pattern Chr 1, 3, and 11		
	Copy no. of Chr 1[b]	Copy no. of Chr 3	Copy no. of Chr 11
1	3	2	3
1	3	3	3
1	3	4	3
1	4	3	2
1	5	4	2
1	6	1	3
1	7	3	2
1	7	4	3
1	10	3	3
2	5	4	3
2	8	3	3
3	4	2	3
3	5	2	3
3	6	3	2
3	6	4	3
3	7	2	3
4	5	3	2
5	4	3	3
6	6	2	3
14	7	3	3
17	5	3	3
24	6	3	3

[a] Sum of percentages is less than 100% due to rounding of numbers.

[b] Chr, chromosome.

benign breast tumor/normal breast tissue and 20 leukocyte preparations that established cutoff levels for aneusomy, polysomy, and monosomy.

Twenty-five of 31 cancer patients had epithelial cells in the blood with changes in copy number of one of the enumerator probes used. Thirteen of 20 patients with more than one CK^+ cell had a cytogenetically heterogeneous cell population (simplified classification). This indicates that CECs are representative of the genetic heterogeneity associated with cancer (45, 63) and that multiple clones shed cells into the blood. However, how does one explain a pattern of aneusomy in a CEC(s) that does not represent a clone in the primary tumor or, in several cases, has no counterpart in the primary tumor using either the simplified or precise classification? We consider several possibilities: (*a*) cells that shed have undergone further genetic changes that allow them to detach from the primary tumor; *e.g.,* loss of genes involved in adherence to stroma and neighboring cells and/or amplification of genes involved in invasiveness; (*b*) overlapping signals if more than 2 copies are present; (*c*) analysis of the touch preparation for each tumor may have missed a cell(s) that matches the CEC pattern; and (*d*) in some instances, a portion of the CECs may have been derived from metastases (gross or micrometastatic) that have undergone further genetic changes. At present, we cannot exclude any of these possibilities.

Although there is a report of genetic changes in CECs (10), we could not find a study that matched the patterns as determined by bicolor or tricolor FISH between CECs in the peripheral venous blood and the primary tumor. Using mutagenic analysis of p53, Kahn *et al.* (64) compared colorectal tumors and corresponding CECs in the peripheral blood. In 41 tumors, 8 of 19 patients with CECs had a precise match. However, seven of eight of these matches involved patients with carcinomas that had spread.

To our knowledge, the present study is the first in which CECs from clinically organ-confined breast, prostate, kidney, and colon tumors have been analyzed for matches by FISH. In fact, we could not find a report on the use of tricolor FISH to analyze CECs. Without considering disomic matches, we show a general similarity of patterns between CECs and the primary tumor in 10 of 13 patients (regardless of whether the simplified or precise classification was used), and 6 of these 10 were in clinically organ-confined tumors. Two more of these 10 had only minimal lymph node involvement. This is important because we have reported excess CECs in a higher proportion of early tumors than others (1–13), and therefore proof is required that these excess CECs in early tumors represent malignant tumor cells.

Other studies have focused on detection of epithelial cells in the bone marrow (14–19). For example, Mueller *et al.* (17) investigated aneusomy for chromosomes 1, 7, and 8 separately in CK^+ cells in bone marrow of patients with prostate cancer. A total of 70–74% had aneusomy for chromosomes 7 and/or 8, and 42% had aneusomy for chromosome 1. Epithelial cells were cytogenetically heterogeneous in 3 of 10 bone marrow aspirates with multiple CK^+ cells.

It should be emphasized that because aneusomy is an early event in cancer, and hematopoietic cells are normally disomic, changes in copy numbers of chromosomes can be used to infer the malignant nature of epithelial cells. However, disomy of CK^+ cells, even when a combination of DNA probes is used, does not exclude the possibility that these cells are malignant because the malignant tumor or a clone of the tumor can be disomic for a particular number of DNA probes. Therefore, reprobing CECs with multiple sets of DNA probes including locus-specific probes (*e.g.,* ERBB-2 and MYC) should increase the sensitivity of detection of genetic changes.

In the present study, changes in copy number of chromosomes in CECs were studied in some detail for renal, colon, and breast cancer patients. Although 8 different probes were used, it was demonstrated that gains of chromosomes are more common than losses. This has previously been reported by others from cytogenetic studies on tumor tissue (40, 41, 43, 45). Whether monosomy should be considered as evidence of aneusomy in individual tumor cells has been questioned because it can be caused by hybridization inefficiency or miscounting.

Patterns of aneusomy that are detected by dual-color or tricolor FISH can be informative. For example, results from the previous analysis of patients with breast cancer, benign breast lesions, and leukocyte preparations mentioned above (62) indicate that there are several patterns of aneusomy using a combination of DNA probes for chromosomes 1, 8, and 17 that were not observed in benign breast tissues or WBCs. Thus, aneusomic patterns for chromosomes 1, 8, and 17 as (>2, >2, >2), (>2, >2, <2), (>2, >2, 2), (2, >2, >2), (>2, 2, >2), or (>2, 2, <2) are associated with malignancy by statistical analysis. Two of these patterns (>2, >2, >2) and (>2, >2, 2) were the most common aneusomic patterns represented by a cell clone in

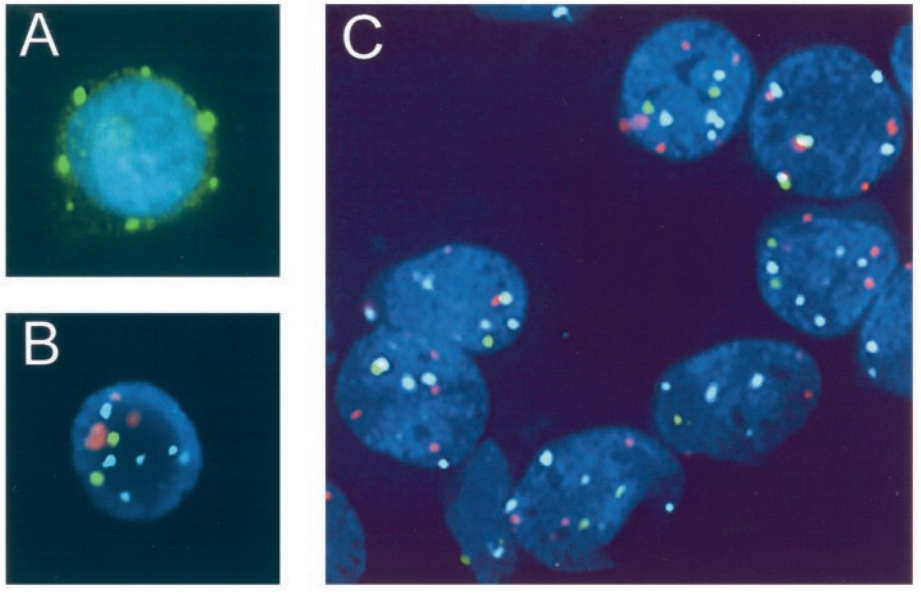

Fig. 4 Patterns of aneusomy using tricolor FISH show a match between a CEC and a clone in a breast cancer tumor (patient 14). *A*, CK^+ CEC. *B*, the same cell hybridized with enumerator probes for chromosomes 1 (*orange*), 8 (*aqua*), and 17 (*green*). *C*, primary tumor hybridized with same set of enumerator probes. Both the CECs and 92% of cells from the primary tumor have the same aneusomic pattern for chromosomes 1, 8, and 17 (>2, >2, 2).

72 primary tumors. The other four patterns were rarely represented by a cell clone. These observations led us to compare CECs and tumor cells from touch preparations with tricolor FISH in our latter comparisons.

Our results differ from those of Litle *et al*. (19), who studied numerical aneusomy involving chromosomes 1, 7, 8, and 17 in epithelial cells isolated from bone marrow of colorectal patients and their corresponding tumors. The majority of epithelial cells from the primary tumor displayed gains for these chromosomes, but only 2 of 109 $CK20^+$ cells from the bone marrow were polysomic for one of these chromosomes. It is possible that the epithelial cells in the bone marrow were not neoplastic. In any event, based on these results, common clonality could not be demonstrated. This is an important discrepancy if it indicates that there are major differences between CECs and epithelial cells in the bone marrow of the same patient. This possibility is under investigation.

Because of the small number of patients studied, the difference in the number of probes used for each specimen, the fact that different tumors were studied, and the fact that we have no baseline for the frequency of aneusomic patterns in CECs, our data do not lend themselves to conventional statistical analysis. Hence, for this study, we have defined a match operationally, depending on the extent of aneusomy in the CECs, the number of CECs, and the presence of an identical aneusomic pattern (by both the simplified and precise classification) in a clone in the primary tumor. For example, patient 14 had a total of 16 CECs representing aneusomy in five chromosomes and amplification of ERBB-2 and MYC. The various aneusomic patterns were observed in 73–100% of cells in the primary tumor by the simplified classification. When precise numbers were compared, there were CECs that matched seven clones in the primary tumor. This is a convincing match. In contrast, patient 7 is disomic for three chromosomes, and this is the same as the major clone of the primary tumor. We do not consider this a match because aneusomy was not present. We have marked patients with matches in Table 5 by setting the diagnosis in bold capital letters.

Notwithstanding the above caveats, we interpret the present data as providing evidence that CECs are aneusomic and derived from the primary tumor. We are extending the present study to a large cohort of breast cancer patients to be analyzed by tricolor FISH and reprobed, if necessary, to obtain unequivocal statistical evidence of matches so that treatment decisions can be made based on the number and characteristics of CECs.

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