

Genomic Profiling of Viable and Proliferative Micrometastatic Cells from Early-Stage Breast Cancer Patients

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ABSTRACT

Purpose: Metastases in distant organs are the major cause of death for cancer patients, and bone marrow is a prominent homing organ for early disseminated cancer cells. However, it remains still unclear which of these cells evolve into overt metastases. We therefore established a new approach based on the analysis of viable and proliferating cancer cells by single-cell comparative genomic hybridization.

Experimental Design: The bone marrow of early-stage breast tumor patients (pN₀M₀) was screened for tumor cells by immunostaining. By applying special short-term culturing, we selected for viable and proliferative tumor cells. The short-term culturing allowed us to evaluate the proliferative potential of micrometastatic cells, which we had previously shown to represent an independent prognostic marker. We assessed genomic changes in single disseminated cancer cells by single-cell comparative genomic hybridization.

Results: We found that these viable disseminated cancer cells already had a plethora of copy number changes in their genome. All of these cells showed chromosomal copy number changes with a substantial intercellular heterogeneity and differences to the matching primary tumors.

Conclusions: The established experimental strategy might pave the way for the identification of metastatic stem

cells in cancer patients. Our preliminary results support the new concept that early disseminated cancer cells evolve independently from their primary tumor.

INTRODUCTION

Currently, there are two competing views on the evolution of metastasis. The first and traditional view is that human tumors develop through a succession of genetic and epigenetic changes. Comparable with a Darwinian evolution, cells that possess advantageous characteristics will be selected to initiate the next round of clonal succession. Eventually, individual cells within the growing mass of tumor cells may acquire the capability to metastasis. This longstanding hypothesis implies that metastatic cells are rare and arise late during tumor progression (1–3). The second view arose from the finding that the clinical outcome of breast cancer patients can be predicted by a poor-prognosis gene expression signature present in the majority of primary tumor (4–6). This gave rise to the hypothesis that certain tumors may be predestined to be metastatic from their very beginning. As a consequence, the same genes that drive the development of primary tumors may also initiate metastasis. Even small primary tumors may already contain metastatic cells (7, 8).

Support for the latter scenario comes from recent whole genome analyses of single cytokeratin-positive (CK+) epithelial cells from the bone marrow of breast tumor patients. Bone marrow is a major homing site for circulating epithelial tumor cells. Isolated tumor cells in bone marrow usually escape detection if conventional histopathological techniques are used. However, disseminated tumor cells in the bone marrow as a major site of metastasis in epithelial tumors such as breast or prostate cancer can be detected with epithelial antibodies against CKs (9). The frequency of these CK+ cells in cytological bone marrow preparation from cancer patients has been estimated to be in the range of 10⁻⁵ to 10⁻⁶ (Ref. 10). In breast cancer, the very presence of occult metastatic cells is an independent prognostic indicator for the risk of death from cancer (11). Previous publications have shown that CK+ cells originate in the tumor, and their presence is associated with an increased propensity to develop metastatic disease (10, 11).

Using single-cell comparative genomic hybridization (CGH; Ref. 12) for the genomic analysis of single CK+ cells such cells from M₀ patients (*i.e.*, patients without clinical signs of overt metastases) showed little resemblance to their respective primary tumor (13). One possible explanation for this surprising finding is that the disseminated cancer cells may have separated from their primary tumor at an early stage (13) and evolve independently, driven by specific selective pressures of the particular secondary site (*e.g.*, bone marrow). However, this study did not address the viability and proliferative potential of the disseminated cells. Thus, it is unclear whether the cells analyzed are really the cells that evolve into metastases and

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Table 1 Summary of clinical data and proliferative potential of disseminated cells under special culturing conditions (for details, see text)

Bone marrow no.	Age at diagnosis in years	Tumor	Node	Metastasis	Grade	Estrogen receptor	Progesterone receptor	HER-2/neu	Ki67	Number of cells before culturing	Number of cells after culturing	Proliferative potential
1124/1125	69	1	0	0	3	p	p	3+	75%	5	105	Poor
1157/58	47	2	0	0	2	p	p	1+	5%	225	828	Poor
1186/87	41	1	0	0	2	p	p	1+	20%	0	<10	Poor
1192/93	45	1	0	0	3	neg	neg	0	70%	36	5120	Good
1153/54	54	1	0	0	2	p	p	1+	10%	150	2371	Good

whether the observed genomic alterations are of any clinical relevance (14).

We decided on a different strategy, which includes assessment of the viability and proliferative potential of disseminated cancer cells. Culturing of epithelial tumor cells is still a difficult task. We previously showed that the proliferative capacity of disseminated cells obtained from the bone marrow of patients with breast and other tumors can be assessed using special culture conditions (15). Furthermore, we showed that the proliferative potential of these cells is related to poor clinical outcome (15). Hence, we reasoned that a combination of special culture conditions and single-cell CGH may prove superior for the analysis of genetic changes associated with early metastasis. To begin exploring this strategy, we collected primary breast tumors from newly diagnosed patients at early disease stages (*i.e.*, no histopathological signs of lymph node metastases and no clinical signs of distant metastases) and tumor cells from the bone marrow of these same patients after evaluation of their proliferative potential. We performed a set of experiments, including CGH from different regions of the primary tumor and a series of single-cell CGH experiments from disseminated CK+ cells. Our results suggest that our new experimental strategy might improve the level of significance of the genomic analysis of micrometastatic cells and may pave the way for the identification of metastatic stem cells in cancer patients with important clinical implications.

MATERIALS AND METHODS

Bone Marrow Preparation. Bone marrow preparation was done as described previously (15). In brief, between 2 and 10 ml of bone marrow aspirates were perioperatively obtained from one or both sides of the upper iliac crest. We washed the bone marrow cells with Hank's medium (Biochrom, Berlin, Germany) at $170 \times g$ for 10 min. Subsequently, we obtained mononuclear cells by Ficoll density centrifugation (1.077 g/mol) at $1230 \times g$ for 30 min. The cells were washed at ambient temperature at $520 \times g$ for 10 min. For the cell culture screen, the cells were cytocentrifuged as described below.

Cell Culture Screening. The cell culture screening was done as previously published to allow a direct comparison of the results with the cutoff for good *versus* poor culture growth determined in our recent investigation (15). In brief, bone marrow cells were cultured according to our protocols (16). The number of CK+ cells was determined [at first passage] by cytocentrifugation of 3×10^4 cells onto glass slides. Immunocytochemical staining for tumor cell detection was done using monoclonal antibody A45-B/B3 (IgG1; Micromet, Munich,

Germany), which is directed to a common epitope of various CK proteins, including the heterodimers CK8/18 and CK8/19. A median number of 1.5×10^6 mononuclear cells/aspirate was examined for CK expression. The antibody reaction was developed using a Neufuchsin stain as described previously (16).

The extent of expansion for CK+ cells was calculated as the total number of cultured epithelial (*i.e.*, CK+) cells/flask. This number was calculated by multiplying the concentration of epithelial cells/cytospin, as determined by anti-CK immunostaining of an aliquot of the cultured cells, with the total number of all cultured cells (*i.e.*, epithelial cells and bone marrow stroma cells) as published previously (15).

Isolation of Cells by Laser Microdissection and Pressure Catapulting. For the isolation of cells, the Neufuchsin-stained CK+ cells could not be used because the Neufuchsin staining procedure leads to severe DNA damage. We therefore stained additional cytospins from the same cultures with a Cy3-conjugated version of monoclonal antibody A45-B/B3, resulting in a bright fluorescent labeling of CK+ cells. Two-hundred μ l of cell solution were transferred onto a microscope slide covered by a thin polyethylene membrane (PEN; PALM Microlaser Technologies AG, Bernried, Germany) by cytocentrifugation at 1000 rpm for 3 min.

Single cells were isolated by the PALM MicroBeam System (PALM Microlaser Technologies AG; Ref. 17). Isolated

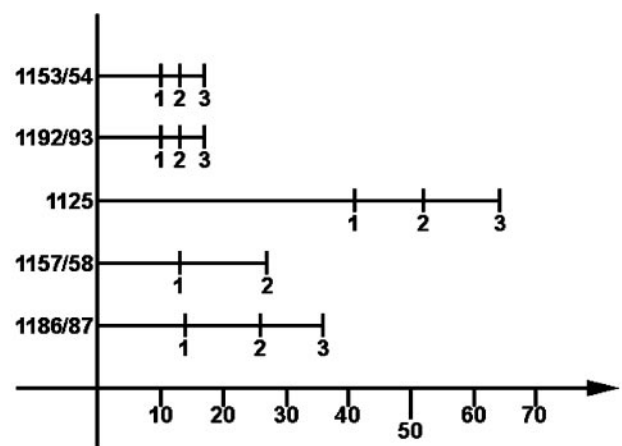


Fig. 1 Time scale for the duration of individual passages (the x axis displays the duration in days). For example, cell culture 1153/54 was split for the first time after 10 days, after 3 days for the second time, and after 4 days for the third time.

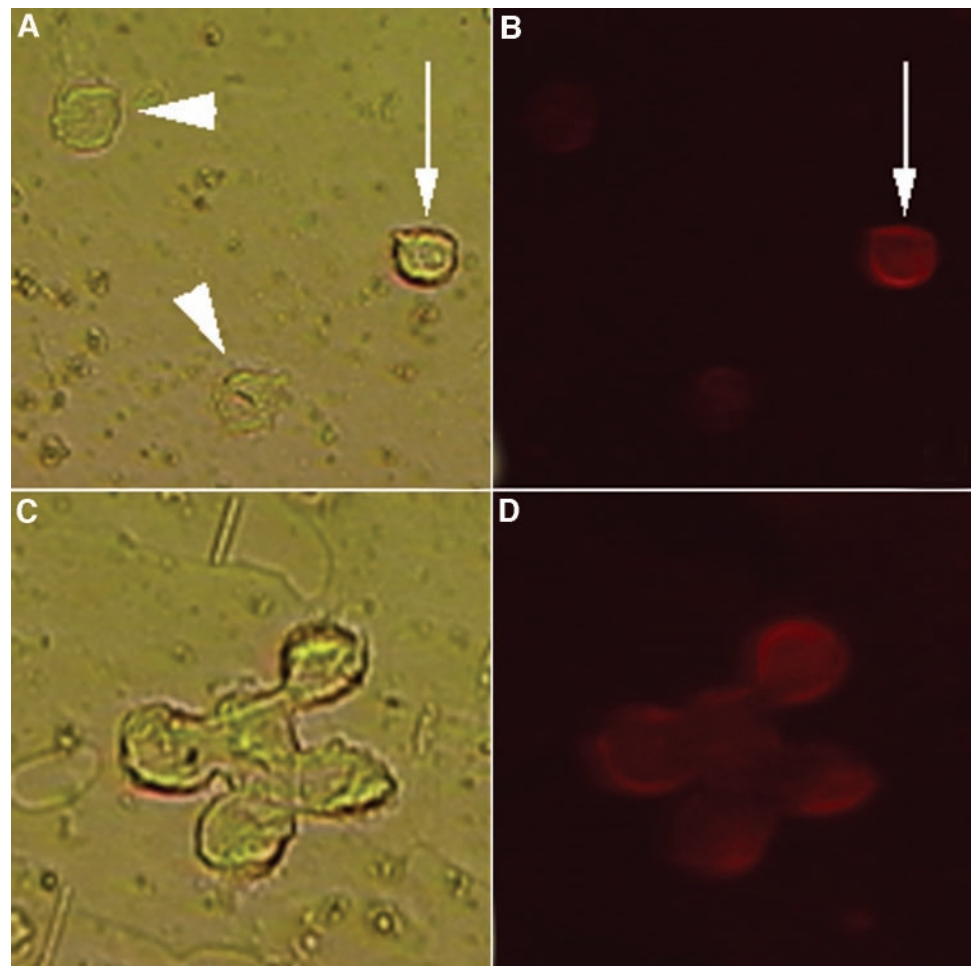


Fig. 2 A and B, example for a single cyokeratin-positive (CK+) cell. In phase contrast, three cells are visible (marked with arrowheads or an arrow; A). In the Cy3 channel, which is used to detect cells stained with the CK antibody, only the cell marked with an arrow shows the CK-specific staining pattern. C and D, example for a cluster of several CK+ cells. In phase contrast, several cells close to each other are visible (C). In the Cy3 channel, all cells show a staining pattern with the CK antibody. This cell cluster consists of five cells.

cells were lifted into a PCR cup filled up with 3 μ l 1 \times PBS/0.5% Igepal buffer. The cells were centrifuged for 10 min and digested by proteinase K at 55°C for \sim 1.5 days. Proteinase K was inactivated at 80°C for 10 min.

Single-Cell Whole Genome Amplification by PCR.

Single-cell whole genome amplification was done based on the previously published protocol (12) with a new set of primer pairs (18, 19) and with modifications which are described in detail here:

*Mse*I restriction endonuclease digest was performed by adding 0.2 μ l of 10 \times One-Phor-All-Buffer-Plus (Amersham Pharmacia Biotech), 0.2 μ l of *Mse*I (50,000 units/ml; New England Biolabs), and 1.3 μ l of H₂O for 3 h at 37°C. *Mse*I inactivation was done at 65°C for 5 min. For reference, 1 μ l of placenta DNA (225 ng/ μ l) was added to 0.5 μ l of 10 \times One-Phor-All-Buffer-Plus (Amersham Pharmacia Biotech), 0.5 μ l of *Mse*I (50,000 units/ml; New England Biolabs), and 3 μ l of H₂O and also performed for 3 h at 37°C.

Preannealing of adapters was achieved by adding LIB1 primer (5'-AGTGGGATTCTGCTGTCAGT-3') and ddMse 11 (5'-TAACTGACAGCdd-3'), 0.5 μ l each of 100 μ M stock solution, 0.5 μ l of One-Phor-All-Buffer, and 1.5 μ l of H₂O. Annealing was started at 65°C and was shift down to 15°C with

a ramp of 1°C/min. At 15°C, 1 μ l of ATP (10 mM) and 1 μ l of T4 DNA ligase (2,000,000 units/ml; New England Biolabs) were added. By adding the preannealed primers to the digested DNA, ligation was done over night at 15°C.

For primary amplification, 40 μ l consisting of 3 μ l of BM Puffer 1 (Expand long template; Roche), 2 μ l of deoxynucleoside triphosphates (10 mM), 1 μ l of Pol-Mix (3,5 units/ μ l; Roche), 1.5 μ l of deionized formamide (3%), and 32.5 μ l of H₂O were added to the ligation product.

The PCR started with 3 min at 68°C, followed by 14 cycles each with denaturation at 94°C for 40 s, annealing at 57°C for 30 s and extension at 68°C for 1 min 30 s, followed by 8 cycles of 94°C (40 s), 57°C (30 s), and 68°C (1 min, 45 s) with an increase of the annealing temperature of 1°C/cycle and a prolongation of the elongation time of 1 s/cycle, followed by 22 cycles of 94°C (40 s), 65°C (30 s), and 68°C (1 min 53 s) with a prolongation of the elongation time of 1 s/cycle, and a final cycle of 68°C (3 min 40 s). The PCR-amplification products were labeled by standard nick translation.

Chromosome-CGH. Labeled amplification product of one or several cells (in case of clusters) was mixed with reference DNA and Cot-1 DNA and salmon sperm DNA. The hybridization mix was denatured at 72°C for 7 min and hybrid-

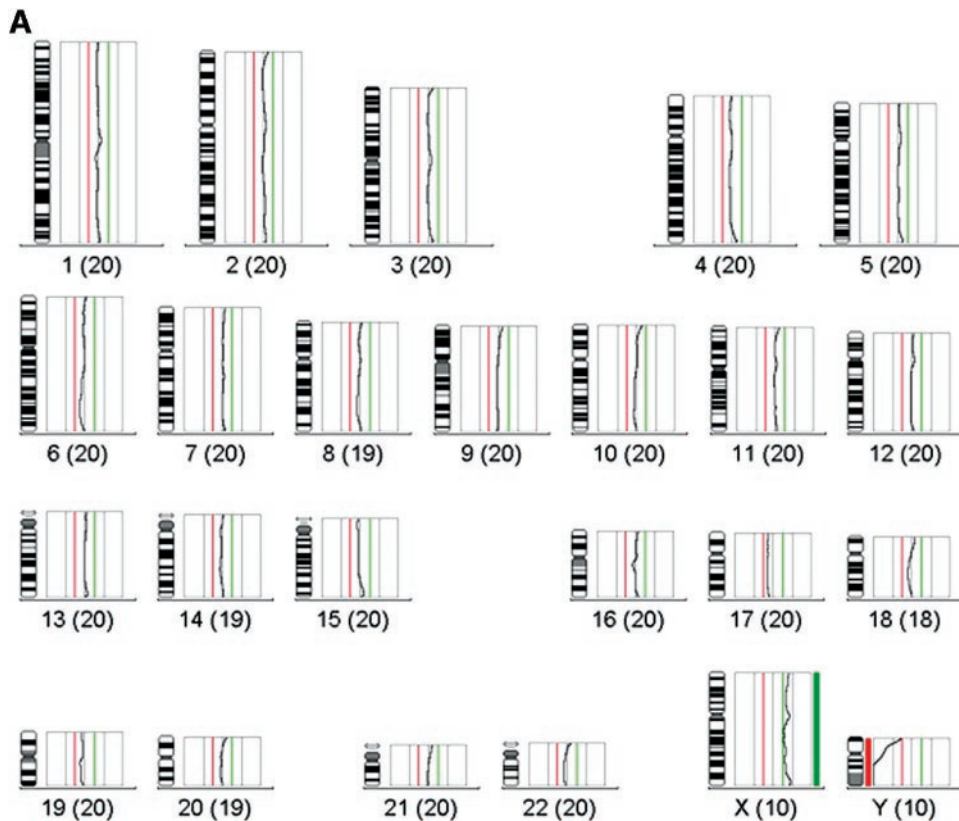


Fig. 3 Representative comparative genomic hybridization profiles derived from single cytokeratin-positive cells after short term culturing from cell culture 1125. The female sex of the patient is in each case correctly reflected in the ratio increase for the X chromosome and decrease of the ratio on the Y chromosome. A, the ratio profiles are balanced for all chromosomes. However, the ratio profile of chromosome 17 is shifted to the left (for details, see text).

ized to metaphase spreads for 2–3 days. For detection of hybridization, signals of test DNA/control DNA sheep anti-dig FITC (200 μ g; Roche)/Avidin Cy3.5 (1 mg/ml; Rockland) were used.

Image Acquisition and Evaluation. Images were captured with a Zeiss Axioplan II imaging microscope equipped with an AxioCam MRm charge-coupled device camera. The microscope and camera were controlled by the Metasystems Software (Metasystems, Altusheim, Germany). Quantitative evaluation of the ratio of test DNA and control DNA was performed using the Metasystems Isis-program or the Metasystems software.

Hierarchical Clustering Analysis. For hierarchical clustering analysis, we transformed the CGH data into a table. Each chromosome arm was designated a number for overrepresented (+1), trend for being overrepresented (+0.5), balanced (0), trend for being underrepresented (-0.5), and underrepresented (-1). For hierarchical cluster analysis, we used the programs Cluster and TreeView, both derived from the Michael Eisen Laboratory (20).⁴

RESULTS

Patient and Tumor Characteristics. All patients were newly diagnosed and staged as pN₀M₀ according to Union International Contre Cancer criteria (Table 1). Thus, they defini-

tively represent early disease stages. No neoadjuvant therapy was given.

The number of CK+ cells found before culturing did not correlate with the proliferative potential of these cells (Table 1). For example, sample 1157/58 was initially estimated to have the highest number of CK+ cells ($n = 225$). However, 1157/58 turned out to have a poor proliferative potential. In contrast, in sample 1192/93, we found only 36 cells before culturing. Yet, these cells had the best proliferative potential.

There was also no obvious relation between the proliferative potential of the primary tumor as assessed by Ki-67 staining and the proliferative potential of micrometastatic CK+ cells (Table 1). In the primary tumor 1124/25, 75% of cells showed a Ki-67 staining; however, the disseminated cells from the same tumor had a poor proliferative potential. We observed the reverse pattern in tumor 1153/54 with 10% of primary tumor cells being Ki-67 positive and a good proliferative potential of the respective disseminated cells.

Expansion of CK+ Cells. The cell cultures showed different growth patterns (Fig. 1). Cultures from patients 1192/93 and 1153/54 showed the fastest growth pattern. These two cultures also had the highest median number of expanded tumor cells with 5120 and 2371, respectively (Table 1). All other patients had cell numbers < 1000 (Table 1). We had previously shown that based on the *in vitro* expansion of CK+ cells, patients can be categorized into two groups with a cutoff point of 2105 CK+ cells/flask (15). Patients with >2105 cells/flask have a reduced overall survival rate compared with patients with

⁴ Internet address: <http://rana.lbl.gov/EisenSoftware.htm/>.

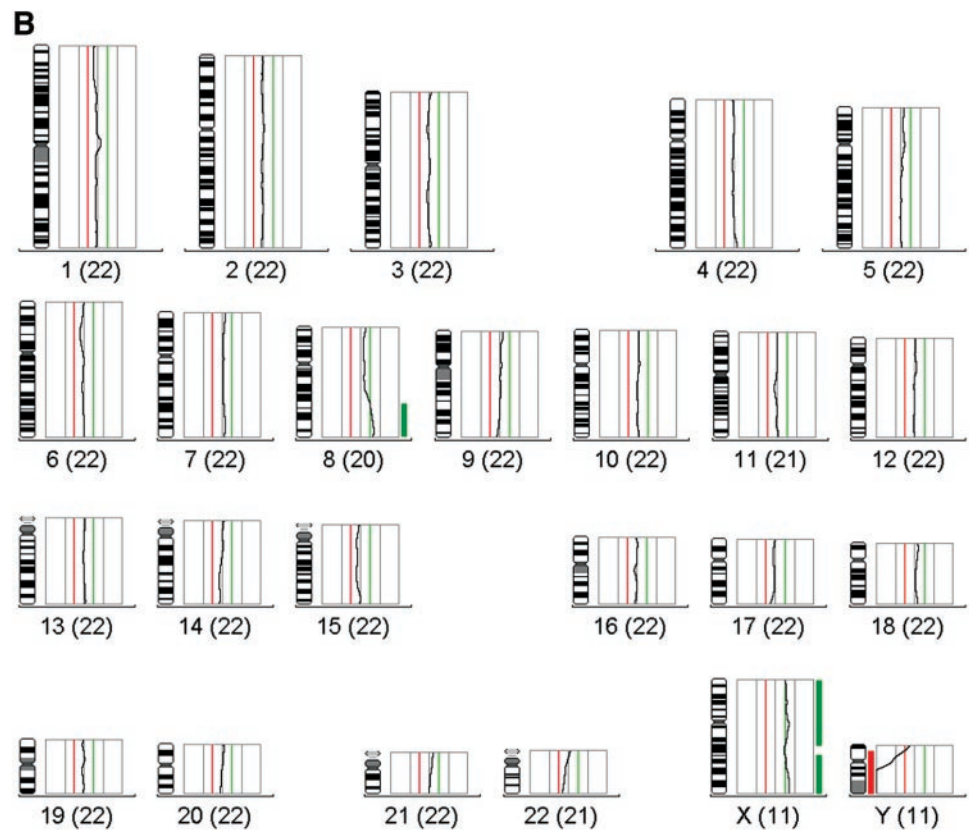


Fig. 3. B, a gain of 8q-material is the only copy number change.

<2105 cells. According to these criteria, cells from patients 1192/93 and 1157/58 showed a good expansion, which indicates a poor prognosis for these two patients.

Comprehensive Genomic Analysis. A number of different cell samples were analyzed for each patient. First, to assess copy number changes and a potential heterogeneity within the primary tumor, two different regions were isolated from the primary tumor by laser microdissection. These were separately analyzed by CGH. Second, single bone marrow cells, which were not stained with a CK antibody and should thus represent normal cells, were analyzed as controls. Third, single CK+ tumor cells were examined (Fig. 2A). Fourth, when several cells were close to each other, they were analyzed together as a cell cluster (Fig. 2B). These cell clusters typically consist of three to six cells and may represent the proliferative fraction of the cultured tumor cells.

Examples of representative CGH profiles from single disseminated cells in bone marrow cultures are depicted in Fig. 3, A and B. Fig. 3A shows an example why we decided on using classifications such as trend for over-/underrepresented. In single-cell CGH applications of disseminated cells, the ploidy level of the respective cell is not known. The ploidy level cannot be concluded from the primary tumor because there may exist considerable differences between the primary tumor and the disseminated cells impeding the selection of an appropriate threshold. In Fig. 3A, the ratio profile is balanced for all autosomes apart from chromosome 17. The ratio for chromosome 17 is shifted to the left but still within the thresholds for a diploid

chromosome complement (*i.e.*, 0.75 and 1.25). As the profile was derived from a single cell, this ratio shift cannot be explained by heterogeneity within the cell population from which the DNA was extracted. The only explanation should be that the cell is probably polyploid and that chromosome 17 is relatively underrepresented (*e.g.*, a tetraploid cell with only three copies of chromosome 17). Instead of using different thresholds, we decided to include such regions as a trend to an increased or decreased ratio.

The results of all analyzed cells are summarized in Fig. 4. In the vast majority of analyses, the X chromosome was overrepresented as indicated by the green cells. This simply reflects that we used male reference DNA in the CGH experiments so that the X chromosome overrepresentation indicates the female sex of the cell donors (*i.e.*, breast cancer patients). However, in some samples, we observed a loss of the X chromosome, which is indicated by a white instead of the green cell in Table 2.

All CK- cells showed a balanced, normal female CGH ratio profile (Table 2). This suggests that the CK- cells were indeed normal bone marrow cells. In contrast, all CK+ cells showed some or many copy number changes, which supports the tumor nature of these cells. With the exception of chromosome 2, all other chromosomes showed gains or losses in at least one analysis.

In the single CK+ cells and in the CK+ cell clusters, we observed considerable heterogeneity. The cell clusters did not show an increased or decreased number of copy number changes as compared with the single cells.

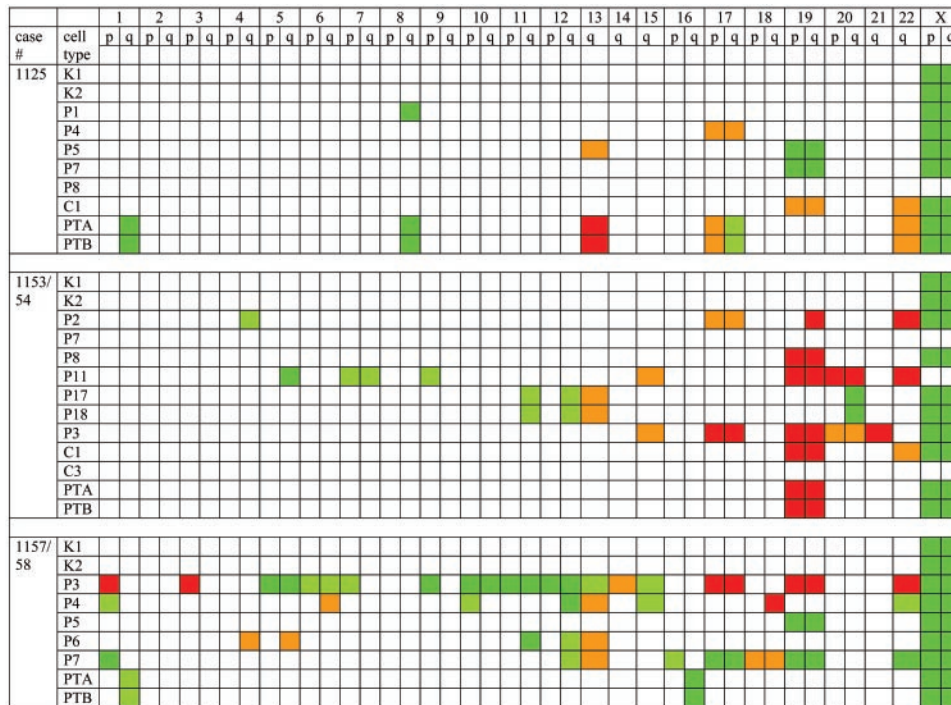


Fig. 4 Summary of gains and losses observed in different cells samples from patients with N_0M_0 breast cancer. In a cell type, the letter K indicates controls, *i.e.*, cytokeratin-negative (CK $-$) cells, P indicates a single CK $+$ cell, C a CK $+$ cell cluster, and PT primary tumor (for details, see text).

We did not observe a close correlation between the number of copy number changes in the primary tumor and its respective disseminated cells. For example, in the primary tumors 1153/54 and 1157/58, we observed only a few copy number changes. In contrast, the disseminated cells displayed a significantly higher number of such changes. A reverse pattern was found in tumor 1125 in which the disseminated cells had fewer imbalances than the respective primary tumor. Table 2 also illustrates that the two different regions from the primary tumor yielded identical (1153, 1125, and 1157) or very similar (1186 and 1192) results. Despite the limited number of samples analyzed, this finding suggests that the heterogeneity within the respective primary tumors was at best only of some degree.

The analysis did not reveal any obvious differences between good and poor proliferative capacity. Thus, we could not identify copy number changes specific for cells with good or poor proliferative capacity. Furthermore, the number of copy number changes appears not to indicate the proliferative potential. This is best exemplified with bone marrow cells from 1153/54, which proliferated well but had fewer copy number changes than 1186/87, which proliferated poorly. However, the number of cultures analyzed was only small ($n = 5$), and the study was, therefore, not designed to allow a statistical analyses.

Hierarchical Clustering Analysis. To test to what degree single disseminated cells are related or similar to each other or to their respective tumor, we performed a hierarchical clustering analysis (Fig. 5). The two different regions from the primary tumor were always clustered together, which was to be expected because of the high degree of similar or identical copy number changes (Table 2). However, there was no indication for association between the disseminated cells among each other or

to their respective primary tumor. The only exceptions were CK $+$ cells 1153-P8 and 1153-C1, which clustered together with their primary tumor.

DISCUSSION

The identification of chromosomal changes in early disseminated cells is essential for the understanding of the biology of the initial steps of the metastatic cascade. It may be important for clinical applications, including identification for therapeutic targets and improved prediction of disease course. Recently, we demonstrated that the proliferative potential of occult metastatic cells correlates highly significantly with an increased rate of cancer-related deaths and a reduced survival of patients (15). Hence, we concluded that bone marrow contains viable cells. Thus, the elucidation of genomic changes in these cells should be of particular interest because it may shed light on regions with an especial prognostic value.

However, even after *in vitro* expansion, CK $+$ cells remain rare cell events, which are not easily accessible for a detailed analysis of the entire genome. In breast cancer, the median number of CK $+$ cells is ~ 300 (Ref. 15) among thousands of normal bone marrow cells. Therefore, sophisticated methods are needed to analyze these CK $+$ cells. We have used a single-cell PCR protocol, allowing a reliable amplification of the entire genome of single cells for subsequent CGH analyses (12).

Our study provides additional evidence that the dissemination of tumor cells may be an early event during tumorigenesis. This is best reflected in the high heterogeneity of the disseminated cells and in the small resemblance of copy number changes in the disseminated cells and their respective primary

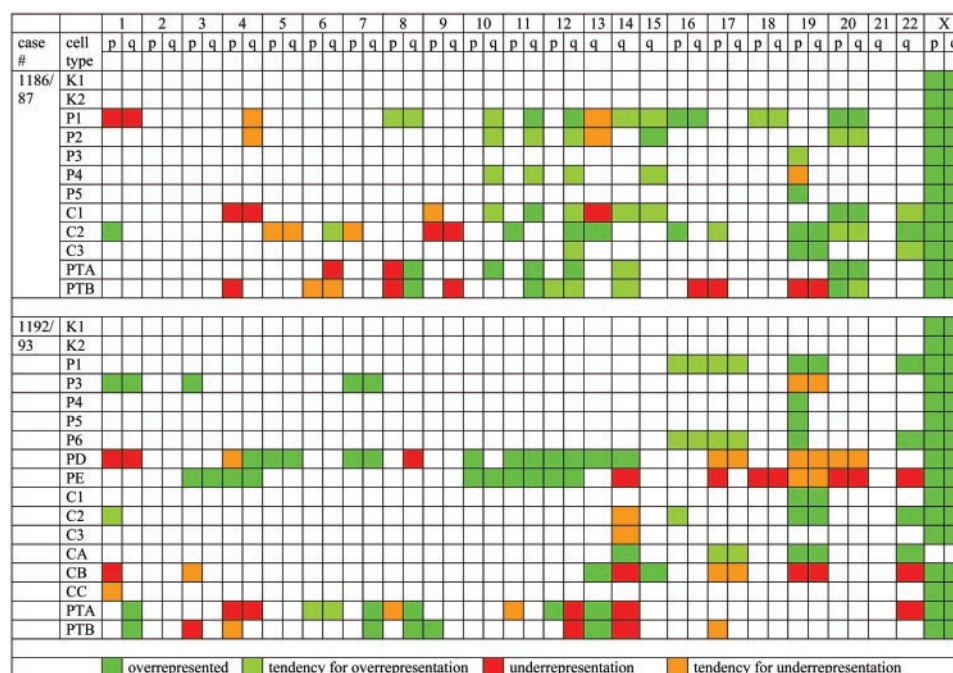


Fig. 4. Continued

tumor. It has been noted that the spectrum of chromosome aberrations seems to stabilize in advanced cancers (21). In accordance with this view, the wide range of different copy number changes observed in the analyzed disseminated cells most likely reflect a spread at an early stage.

One important limitation of previous studies focusing on the genome of micrometastatic cells in cancer patients (13, 18) is the inability to accurately distinguish between true metastatic cells and disseminated tumor cells not capable of proliferating at distant sites (14). The short-term culture probably provided a bias toward viable cells with some proliferative potential and may have increased the number of cells with metastatic potential. This is best reflected by a comparison of our data with the data derived from uncultured CK+ cells isolated from bone marrow aspirates of M_0 breast cancer patients (13). There is a striking contrast in the number of cells without copy number changes. In the study by Schmidt-Kittler *et al.* (13), 107 cells from M_0 were analyzed. In 61 of these cells (57.0%), no copy number changes were noted. In contrast, in the present study, each of the 29 individual cells and each of the 12 cell clusters, although similarly derived from M_0 patients, showed some copy number changes. This supports the notion that the genomic analyses of many cells from the former study may provide little information on the cells that do evolve into metastases (14). Some of the CK+ cells in uncultured bone marrow specimens may not even be tumor cells because of an unspecific reaction of the antibody with normal leukocytes (22). Under the special culture conditions used in this study, these cells die within the first 2 weeks of culture (16). Thus, cell culturing also leads to an additional enrichment of tumor cells through the depletion of leukocytes.

Schmidt-Kittler *et al.* (13) analyzed from M_0 breast cancer patients 46 cells with definite pathological CGH changes. Al-

though our patient number is much smaller ($n = 5$), we could analyze due to our culturing approach 29 single cells and 12 cell clusters with copy number changes. Even if we count each cell cluster as just one cell, this corresponds to data from 41 cells, which comes close to the cell number in the study by Schmidt-Kittler *et al.* (13). In this study, cells from the M_0 group had a mean number of chromosomal aberrations/cell of $5.9 (\pm 4.1 \text{ SD})$. In our study, which included only cells from patients staged as M_0 , the single cells had a mean number of chromosomal aberrations of $4.7 (\pm 4.2 \text{ SD})$ and the cell clusters of $4.3 (\pm 4.0 \text{ SD})$. Similarly to our study, Schmidt-Kittler *et al.* (13) found very few chromosomal regions frequently affected. Among the more common changes were amplifications of chromosomal regions 5cen-5q23 and 18q (13). In our study was the most common copy number change a loss of 13q (observed in five single cells), which is interesting because this region harbors the *BRCA2* gene.

All of our microdissected primary tumors displayed the CGH copy number changes typically observed in breast cancer. To determine the relationship of the disseminated cells with their primary tumor, we used unsupervised hierarchical cluster analysis. The disseminated cells did with two exceptions (CK+ cells 1153-P8 and 1153-C1; Fig. 4) not show any similarity their respective primary tumor, supporting the concept that early disseminated cancer cells evolve independently from their primary tumor (13).

A potential drawback of the culture technique may be a selection of cells. However, as we previously showed that the proliferative potential correlates with the clinical outcome (15), we argue that even if a selection occurs, it should represent a selection toward highly relevant cells. Because this article focuses on the description of our experimental strategy, the number of patients analyzed was too small to assess whether the

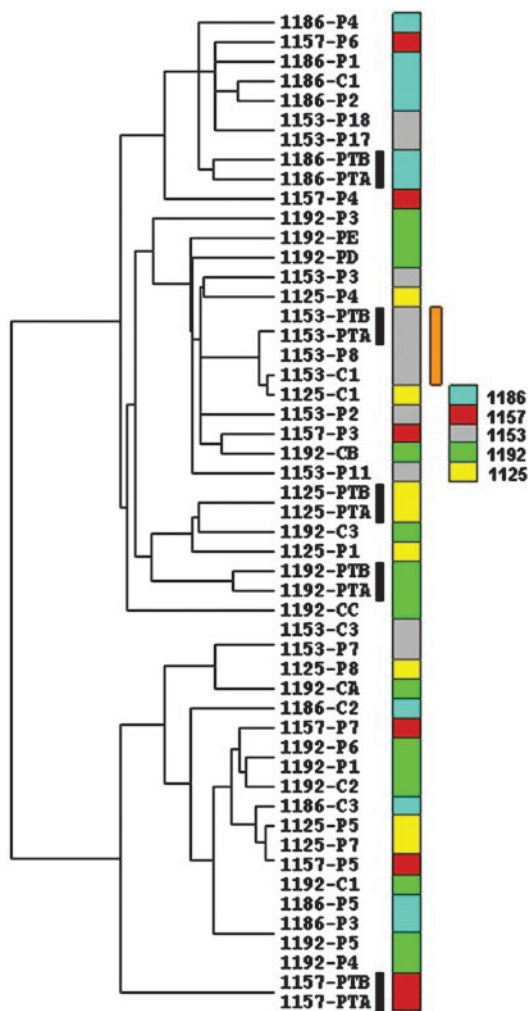


Fig. 5 Cluster analysis of comparative genomic hybridization results. Samples from the same patient are color coded. The two different areas from the primary tumor always clustered together, indicating the low differences and thus low heterogeneity observed in the primary tumors (indicated by ■). There was only one instance where cytokeratin-positive cells clustered together with the primary tumor (1153-P8 and 1153-C1; indicated by an orange bar).

proliferative potential is correlated to the type or number of copy number changes in micrometastatic cells. This analysis will be certainly an important goal of our future research.

The method presented here contributes to an improved dissection of the metastatic cascade in cancer patients. This may help to answer the key question, which of the heterogeneous genetic changes in early disseminated cancer cells are relevant for the development of overt metastasis and therefore determine the prognosis of patients with breast cancer and other solid tumors. Moreover, the example with the proto-oncogene *Her2/neu* has shown that the discovery of genetic changes can lead to new therapeutic targets for antimetastatic therapies (21). These therapies have the chance to cure patients only if applied before the occurrence of overt metastasis signal incurability. Thus, the

genomic profiling of micrometastatic cells from early-stage cancer patients has important future implications for the development of new biological therapies.

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