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Direct Genetic Analysis of Single Disseminated Cancer Cells for Prediction of Outcome and Therapy Selection in Esophageal Cancer

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SUMMARY

The increasing use of primary tumors as surrogate markers for prognosis and therapeutic decisions neglects evolutionary aspects of cancer progression. To address this problem, we studied the precursor cells of metastases directly for the identification of prognostic and therapeutic markers and prospectively analyzed single disseminated cancer cells from lymph nodes and bone marrow of 107 consecutive esophageal cancer patients. Whole-genome screening revealed that primary tumors and lymphatically and hematogenously disseminated cancer cells diverged for most genetic aberrations. However, we identified chromosome 17q12–21, the region comprising *HER2*, as the most frequent gain in disseminated tumor cells that were isolated from both ectopic sites. Survival analysis demonstrated that *HER2* gain in a single disseminated tumor cell but not in primary tumors conferred high risk for early death.

INTRODUCTION

Despite complete surgical resection of primary cancers, a significant number of cancer patients die from metastatic spread, which manifests often years after successful initial surgery. Lacking a direct access to latent systemic cancer, routine surgical pathology and modern molecular diagnostic approaches rely on the analysis of the primary tumor to assess the risk for and the characteristics of systemic disease. Until today this approach has been justified by a concept of late metastatic spread. It holds that progression of tumor cells to so-called full malignancy mostly takes place within the primary tumor. However, recent data demonstrate that dissemination of tumor cells may occur very early after transformation with metachronous outgrowth after periods of latency (Hüsemann et al., 2008). These findings, combined with the prevailing evolutionary concept of cancer progression (Cairns, 1975), clearly discourage simple extrapolations from local to systemic disease. Rather, the evolutionary model predicts an allopatric evolution of variant tumor cells for survival and proliferation in different microenvironments and

SIGNIFICANCE

After curative resection of primary tumors, outgrowth of micrometastatic cells is the major cause for death from cancer. Therefore, therapy targets on the metastatic precursor cells are urgently needed. The finding that HER2 is a prognostic marker only when amplified in disseminated esophageal cancer cells but not when amplified in primary tumors is an example of a clinically important divergence of local disease and early systemic cancer. Since HER2 is an important molecular drug target, the results question the dominant role of primary tumors as surrogate markers for patient stratification and for therapy target research. The development of a pathology for minimal systemic cancer may therefore overcome the limitations inevitably linked to conventional diagnostic studies of primary tumors.

consequently genetic divergence (Klein, 2003). Direct genetic comparisons of primary tumors and matched clinically manifest metastases are surprisingly sparse but revealed a wide range of possible outcomes, from completely unrelated to genetically very similar (Kuukasjarvi et al., 1997). In addition, such comparative studies on the two endpoints of local and systemic disease do not reflect the evolutionary dynamics during the time of latency from dissemination of precursor cells to manifestation of metastases. Therefore, while the endpoints (i.e., the excised primary tumor and the overt metastases) might concur on some genetic alterations, it is unclear whether shared genetic aberrations reflect the general tendency of solid cancers to accumulate similar aberrations independently during outgrowth (Heim and Mitelman, 1995; Hoglund et al., 2002) or true clonal descent.

Early carcinoma spread before manifestation of distant metastasis is detected by immunostaining for epithelial cells in mesenchymal organs such as lymph nodes, bone marrow, or blood (Klein, 2003; Pantel et al., 2003). Cytokeratin antibodies for detection of disseminated tumor cells in bone marrow and EpCAM (epithelial cell adhesion molecule) antibodies in lymph nodes proved to be specific and reliable prognostic markers, with a single tumor cell among 2×10^6 bone marrow or lymph node cells sufficing to predict poor clinical outcome in breast and esophageal cancer (Braun et al., 2005; Izbicki et al., 1997). The genetic analysis of cells detected by these markers demonstrated that at least some human cancers spread early (Schardt et al., 2005; Schmidt-Kittler et al., 2003) and develop from genetically heterogeneous tumor cells to clonally expanded and selected cells at the metastatic stage of disease (Klein et al., 2002a).

Since therapeutic interventions after manifestation of metastasis are notoriously ineffective for most cancers, we tested the hypothesis that a better understanding of the actual, dynamic evolutionary processes taking place systemically before this stage provides different clinical information than analysis of the primary tumor. We investigated esophageal cancer in a prospective study, because it is one of the most aggressive human cancers with most curatively operated patients relapsing within 5 years (Enzinger and Mayer, 2003). A recent study comparing chemoradiotherapy alone with chemoradiotherapy followed by surgery for patients with esophageal cancer demonstrated that neither distant metastasis nor overall survival differed between the groups despite a lower rate of local tumor recurrence in the surgical group (Bedenne et al., 2007). It was concluded that metastatic disease determines survival and that the hope for the future is the development of more specific targeted therapies to eradicate or suppress the growth of micrometastatic disease (Ilson, 2007). To address this need we analyzed micrometastatic disease directly and isolated and compared single disseminated cancer cells from lymph nodes and from bone marrow to cover the two types of metastatic spread.

RESULTS

Detection and Whole-Genome Analysis of Single Disseminated Cancer Cells

Of 107 consecutive patients, we screened 104 bone marrow samples and 35 lymph node preparations from 18 patients with operable esophageal cancer (Table 1) for disseminated tumor cells (DTCs). As several molecular techniques were

Table 1. Clinicopathologic Data of Analyzed Patients

	Prospective Study (%)	Included in Survival Analysis[a] (%)	Extended Primary Tumor Cohort (%)
Patients	107	62	101
Male	88 (82)	50 (81)	79 (78)
Female	19 (18)	12 (19)	22 (22)
Histology			
ADC	51 (52)	32 (53)	71 (70)
SCC	56 (48)	30 (47)	30 (30)
Primary Tumor			
pT1	16 (15)	13 (21)	19 (19)
pT2	33 (31)	23 (37)	38 (37)
pT3	46 (43)	24 (39)	40 (40)
pT4	12 (11)	2 (3)	4 (4)
Lymph Node Status			
pN0	31 (29)	23 (37)	31 (31)
pN1	76 (71)	39 (63)	70 (69)
Tumor Grade			
G1	3 (3)	2 (3)	1 (1)
G2	55 (51)	31 (50)	51 (50)
G3	49 (46)	29 (47)	49 (49)
Resection Margins			
R0	87 (81)	52 (84)	97 (96)
R1	12 (11)	10 (16)	4 (4)
R2	8 (8)	0 (—)	0 (—)
AJCC Stage			
I	13 (12)	11 (18)	15 (15)
IIa	17(16)	12 (19)	16 (16)
IIb	22 (21)	17 (27)	31 (30)
III	31 (29)	14 (23)	33 (33)
IV	24 (22)	8 (13)	6 (6)
HER2 Primary Tumor			
Amplification	13 (12)	7 (17)	18 (18)
No Amplification	61 (57)	42 (83)	83 (82)
Missing	33 (31)	14 (—)	0 (—)
Synchronous Distant Organ Metastasis (M1b)			
No	94 (88)	62	101
Yes	13 (12)	0	0
DTC (Bone Marrow or Lymph Node)			
No	63 (59)	38 (61)	—
Yes	44 (41)	24 (39)	—
17q12–21 in DTC (Bone Marrow or Lymph Node)			
No	90 (84)	50 (81)	—
Yes	17 (16)	12 (19)	—
HER2 DTC (Bone Marrow or Lymph Node)			
No	98 (92)	56 (90)	—
Yes	9 (8)	6 (10)	—

[a] Includes only patients whose cells were genetically analyzed and for whom survival $\geq$ 3 months and R < 2.

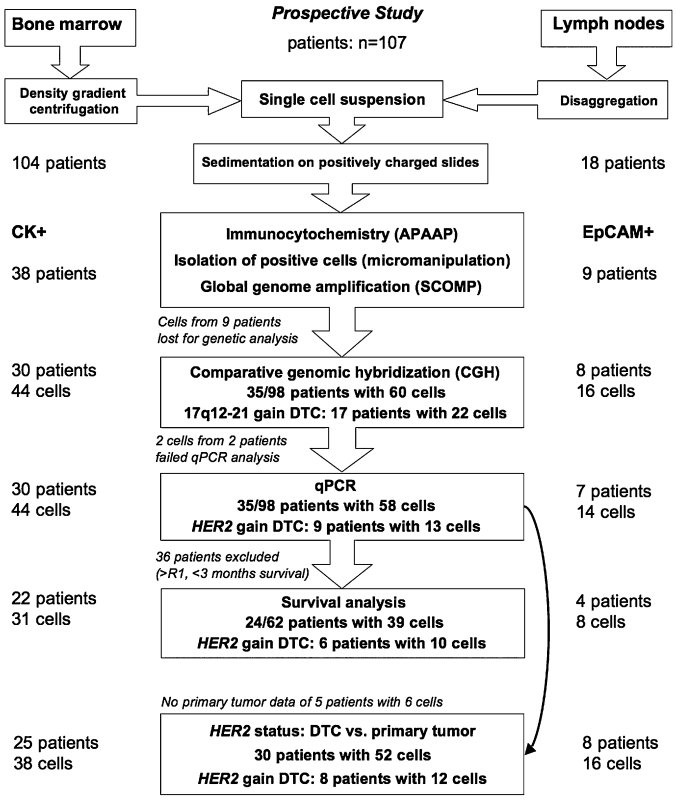


Figure 1. Experimental Outline of the Prospective Study
Disseminated tumor cells (DTCs) were detected by cytokeratin staining in bone marrow and by EpCAM staining in lymph nodes. For each experimental analysis, numbers of cells and patients are indicated.

performed on several samples of one individual patient (e.g., primary tumor, DTCs from bone marrow, and/or lymph node), which may cause confusion, Figure 1 depicts the study outline and includes numbers of patients and samples. Tumor cells that disseminated to bone marrow were detected in 38/104 (37%) cases by the mAB A45-B/B3 directed against cytokeratins 8, 18, and 19 (Figure 2A) and in 9/18 (50%) lymph node samples by an antibody against the epithelial cell adhesion molecule (EpCAM; mAb Ber-Ep4). In bone marrow and lymph node samples, 17% and 39% of patients, respectively, harbored more than one marker-positive cell (Figure S1 available online). From all positive samples, we successfully isolated 60 of the positive cells (Figure 2A, 44 cells from bone marrow and 16 cells from lymph nodes) from 35 patients and prepared their DNA for global amplification by PCR. Then, we performed single-cell comparative genomic hybridization (SCOMP) on metaphase spreads (Klein et al., 1999; Schmidt-Kittler et al., 2003), which currently allows the most reliable overview of chromosomal DNA gains and losses present in single disseminated esophageal cancer cells (Figure 2B). Among several chromosomal DNA gains and losses typical for this cancer (Figure 2C), we observed the highest number of gains for 17q12–21 (22 of 60 cells; 37%), the region of the *HER2* gene locus. This chromosomal region was significantly more often gained in cells derived from esophageal adenocarcinomas (ADC; 19/33, 58%) than in cells derived from esophageal squamous cell carcinomas (SCC; 3/27, 11%; $p < 0.001$, Fisher's exact test; Figure 2C). We then compared the genetic aberrations in cells isolated from bone marrow and lymph nodes (Figure 2D). First, in 12 cases (n = 32 cells) we had isolated more than one tumor cell displaying CGH aberrations from one of the organs and detected in three cases aberrant tumor cells in both organs of an individual patient. Here, the tumor cells from one organ (either bone marrow or lymph node) displayed mostly similar aberrations (Figure 2E), however, in all three cases with concomitantly isolated tumor cells from bone marrow and lymph nodes, the tumor cells from different organs shared almost no genomic aberrations (Figure 2E). This genetic divergence between the tumor cells isolated from the different microenvironments was also observed for all tumor cells (Figure 2D). For example, gains of chromosomes 7q and 10q and a loss at 5q were frequently found in DTCs isolated from lymph nodes while almost never observed in tumor cells from bone marrow. In contrast, the frequency of 17q12–21 gains did not differ significantly between tumor cells isolated from bone marrow or from lymph nodes (for ADC: lymph node 7/10 cells versus bone marrow 12/23 cells; for SCC: lymph node 2/6 versus bone marrow 1/21; Fisher's exact test, $p = 0.46$ and $p = 0.12$, respectively). This indicated that, while several aberrations diverged between cells disseminating to bone marrow and to lymph nodes, the region of 17q12–21 was equally gained in tumor cells disseminating via one of the two routes of systemic spread.

Assessment of *HER2* Gains in Single Disseminated Cancer Cells

Therefore, 17q12–21 aberrations might be relevant for systemic cancer spread. Because resolution of metaphase CGH ranges from 10 to 20 Mb, direct identification of amplified genes is impossible. As a potential candidate we selected the *HER2* gene for a gene-specific quantitative (q) PCR assay (Figures 3A and 3B). This assay was established and validated using whole-genomic amplification products of single metastatic breast cancer cells (Schardt et al., 2005) and can be applied to the very same genomic DNA from a single cell that had been used for CGH analysis. qPCR for the HER2 gene was successfully performed with 58 of the 60 isolated single disseminated tumor cells. Interestingly, using the qPCR assay two disseminated tumor cells were found to have a *HER2* gene gain, which had escaped detection by metaphase CGH. On the other hand, only 11/22 (50%) cells, in which CGH had uncovered chromosomal gains in the proximity of 17q12–21, had a *HER2* gene gain.

Genetic Aberrations in Disseminated Cancer Cells and Patient Survival

We then evaluated whether *HER2* or 17q12–21 gains in disseminated esophageal cancer cells put the patients at risk for death. Sixty-two patients were eligible for survival analysis following the intention-to-treat principle (Table 1 and Experimental Procedures) and were distributed into four groups: patients without

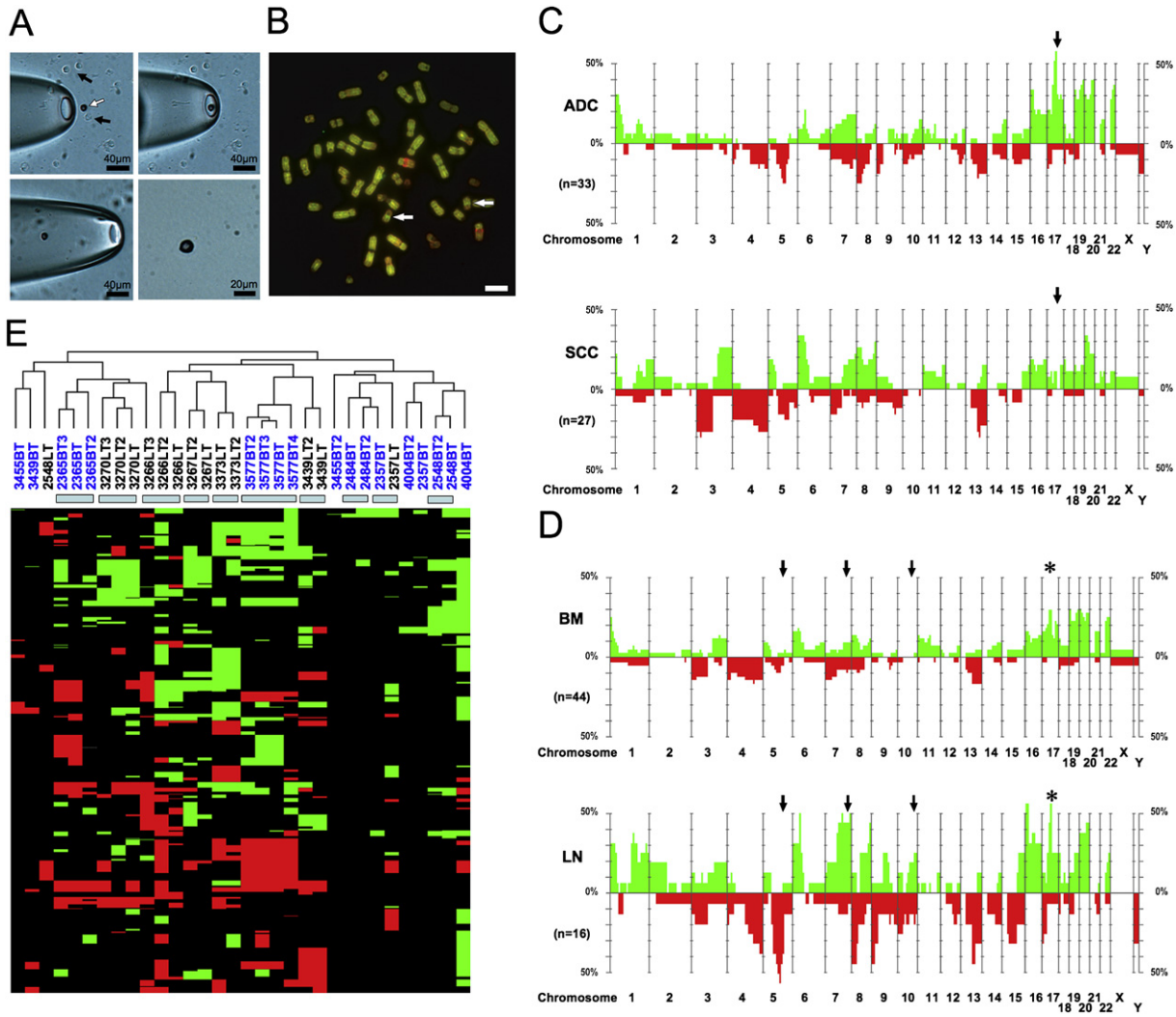


Figure 2. Whole-Genome Screen of Single Disseminated Esophageal Tumor Cells

(A) Isolation of a single cytokeratin-positive cell by micromanipulation (white arrow, tumor cell; black arrows, unstained bone marrow cells). The cell is transferred to a new slide to visually control that no other cells are coisolated (right, lower panel).

(B) Metaphase spread hybridized with the amplified genome of a single tumor cell. Arrows indicate bright hybridization signal on chromosome 17, subsequently identified as *HER2* gene gain. Scale bar, 5 μm.

(C) Horizontal histograms depicting the chromosomal gains (green) and losses (red) of disseminated tumor cells isolated from SCC and ADC esophageal cancer patients, respectively. Arrows indicate the chromosomal region 17q12–21.

(D) Horizontal histograms depicting the genomic profiles of tumor cells isolated from bone marrow (BM) and lymph nodes (LN), respectively. Note that changes at chromosome 5, 7, and 10 (arrows) frequently observed in tumor cells from lymph nodes are rare in tumor cells isolated from bone marrow. The asterisk indicates the region 17q12–21.

(E) Hierarchical cluster analysis of disseminated tumor cells isolated from bone marrow and lymph nodes. Thirty-two tumor cells from 12 patients were clustered to assess their genetic similarity. Only patients in whom we had isolated more than one tumor cell were selected. Several tumor cells were either detected in one organ (bone marrow or lymph node) or in both compartments of one individual patient. Identifiers consist of patient number, BT for bone marrow-derived tumor cell, LT for lymph node-derived tumor cell, and cell identifier. Cases 2357, 2548, and 3439 harbored DTC in both bone marrow and lymph node. Note that, even in case 2357, for which BM and LN cells are grouped in relative proximity, almost no aberrations are shared. Cells from one individual patient that cluster together are marked with a bar.

disseminated cancer cells in bone marrow or lymph nodes (n = 38); patients with disseminated cancer cells that harbored no aberration on chromosome 17q12–21 (n = 12); patients with a gain of chromosome 17q12–21 but without *HER2* gain (n = 6); and finally, patients whose cells displayed a *HER2* gain in our qPCR assay (n = 6). Among the four groups, patients with *HER2*-amplified cancer cells had an extremely poor survival (Figure 3D, log rank test, p = 0.032, df = 3) with no patient surviving longer than 23 months. Although previous studies demonstrated a significant prognostic impact of DTCs in esophageal cancer (Hosch et al., 2001; Izbicki et al., 1997; Thorban et al., 2000), the presence of DTCs alone did not confer a significant risk in our group of patients (log rank test, p = 0.134). However, when six additional patients with DTCs were added, for which

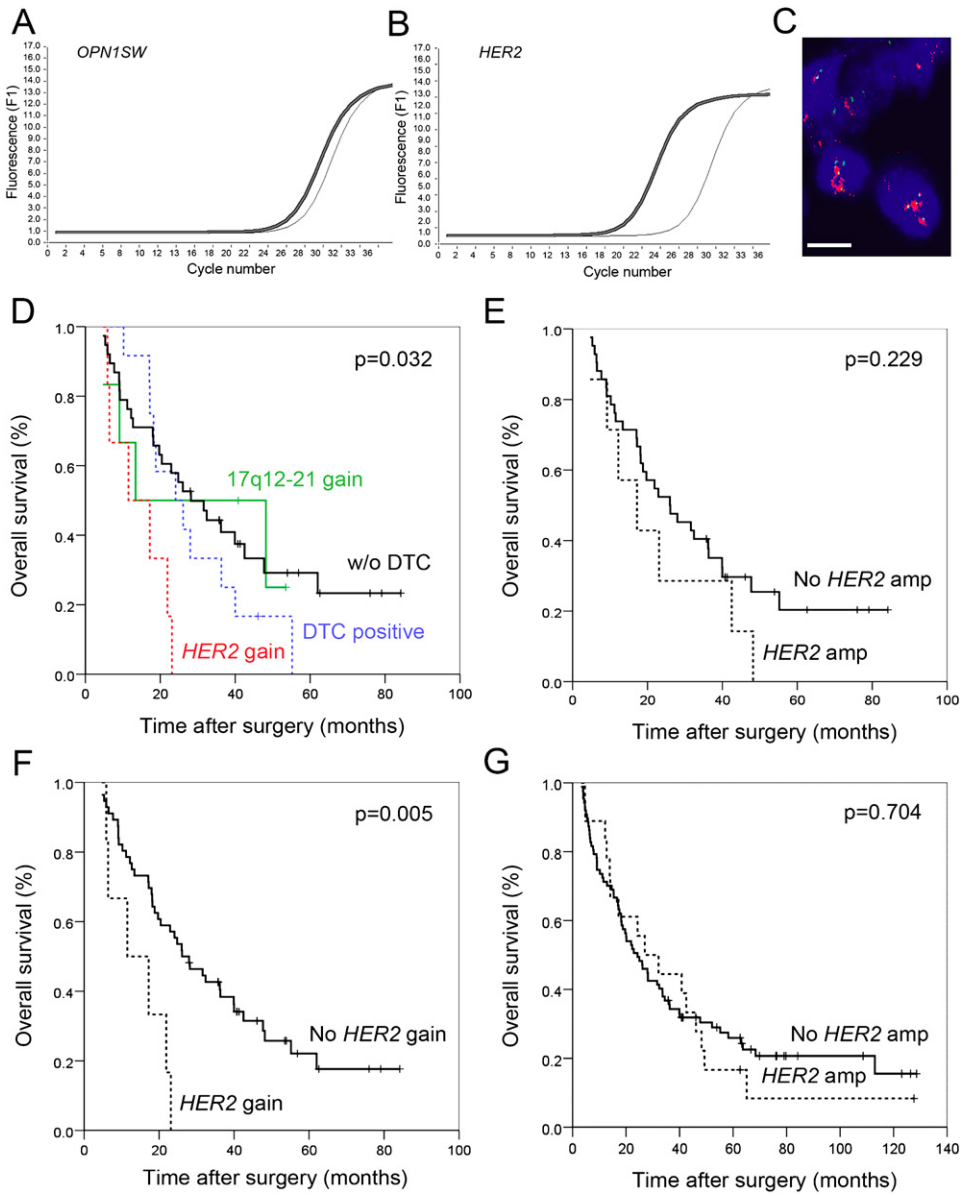


Figure 3. Identification and Prognostic Impact of *HER2* Gains in Disseminated Tumor Cells and Primary Tumors

(A and B) Quantitative PCR assay for the HER2 gene and for a reference gene of two cytokeratin-positive cells with (solid line) and without (thin line) *HER2* amplification. The HER2 gain is indicated by the significantly lower Ct value (20 versus 26) at the HER locus (B), while an almost equal Ct value (25.5 versus 26.5) was observed at the reference gene (A).

(C) FISH analysis of an ADC primary tumor with *HER2* amplification (E, red: *HER2* and green: chromosome 17 centromer). Scale bar, 10 μm.

(D) Impact of disseminated tumor cell status on overall survival (all, n = 62; DTC, disseminated tumor cells [n = 12]; 17q12–21, DTC harboring chromosomal gain of 17q12–21 but no HER2 gain [n = 6]; HER2 gain, patients with DTC that display HER2 gain [n = 6]).

(E) Impact of HER2 amplifications in matched primary tumors (all, n = 49; HER2 A, *HER2* amplification detected by FISH [n = 7]).

(F) Overall survival of patients with disseminated tumor cells that display *HER2* gain by qPCR compared to all other patients (all, n = 62; HER2 gain, patients with DTC that display HER2 gain [n = 6]).

(G) Overall survival of patients with and without *HER2* amplification analyzed by FISH in primary tumors (all, n = 101; HER2 A, *HER2* amplification detected by FISH [n = 18]).

no genetic data were available, a tendency for increased risk was observed (log rank test, p = 0.09). In the clinically relevant comparison of patients with *HER2*-amplified disseminated cancer cells against all other patients, we found a strong impact on survival upon univariate analysis (Figure 3F; log rank test, p = 0.005). Upon multivariate analysis, *HER2* amplification in disseminated cancer cells proved to be a very important risk factor. Of the other factors tested (age, lymph node status, tumor size, tumor grade, histology, and resection margin) only tumor size was also an independent risk factor and was included into the

Table 2. Multivariate Survival Analysis of Prognostic Factors in the Disseminated Tumor Cell Collective

	Univariate Analysis		Multivariate Analysis	
Risk Factor[a]	Median Survival in Months	p Value	Relative Risk (95% CI)	p Value
Age: <60 years (n = 29) versus ≥60 years (n = 33)	28.0 versus 19.7	0.510	ne[b]	—
Histology: SCC (n = 30) versus ADC (n = 32)	18.0 versus 26.1	0.058	ne	—
Lymph node status: pN1 (n = 39) versus pN0 (n = 23)	22.9 versus 36.3	0.077	ne	—
Primary tumor size: pT3–4 (n = 26) versus pT1–2 (n = 36)	19.7 versus 31.6	0.007	2.31 (1.26–4.23)	0.006
Tumor grading: G3–4 (n = 33) versus G1–2 (n = 29)	12.9 versus 26.10	0.709	ne	—
Resection margin: R0 (n = 51) versus R1 (n = 11)	26.1 versus 17.2	0.129	ne	—
Disseminated tumor cells in bone marrow or lymph nodes: present (n = 24) versus absent (n = 38)	18.8 versus 28.2	0.134	—	—
Cytokeratin-positive cells in bone marrow: present (n = 22) versus absent (n = 40)	18.2 versus 28.2	0.138	—	—
HER2 amplification in a single disseminated tumor cell: present (n = 6) versus absent (n = 56)	11.5 versus 26.1	0.005	3.57 (1.45–8.80)	0.006

Patients: n = 62.
[a] Sample numbers are given in parenthesis.
[b] ne, not entered into the final multivariate analysis model.

final model (Table 2). When we only included R0 patients (n = 52) in the multivariate analysis, HER2 gain in DTCs was the only factor included into the model (p = 0.018; relative risk: 3.321; CI = 1.226–8.997). Likewise, rescue treatment of 11 patients by chemoradiotherapy had no effect on survival (log rank test p = 0.61). *HER2* status in disseminated cancer cells was not significantly different for patients with and without rescue treatment (Fisher's exact test, p = 0.58).

HER2 Amplifications in Primary Tumors and Survival

We compared these results with the prognostic impact of *HER2* amplifications when present in primary tumors as determined by a standardized fluorescence in situ hybridization (FISH) assay (Figure 3C). Of the same patient group, for which we had analyzed the effect of DTCs, we could successfully test 49 patients for *HER2* amplification in the primary tumor. No effect of the amplification on survival was observed (Figure 3E, p = 0.229). To

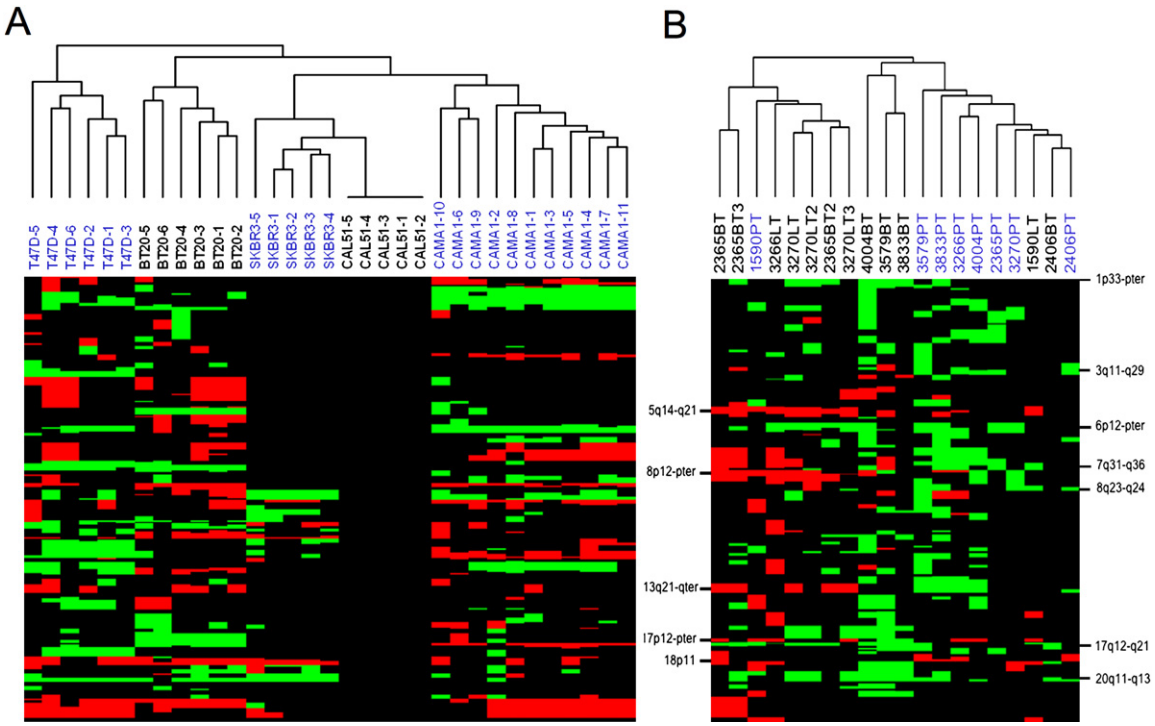


Figure 4. Whole-Genome Comparison of Single Disseminated Cancer Cells and Matched Primary Tumors

(A) Hierarchical cluster analysis of the CGH aberrations of 33 single cells isolated from five different breast cancer cell lines. Note that all single cells from each cell line are grouped according to their origin—regardless of the degree of chromosomal instability.

(B) Hierarchical cluster analysis of disseminated tumor cells that harbor *HER2* gains and their matched primary tumors. (Identifiers consist of patient number, BT for bone marrow-derived tumor cell, LT for lymph node-derived tumor cell, PT for primary tumor, and cell identifier.)

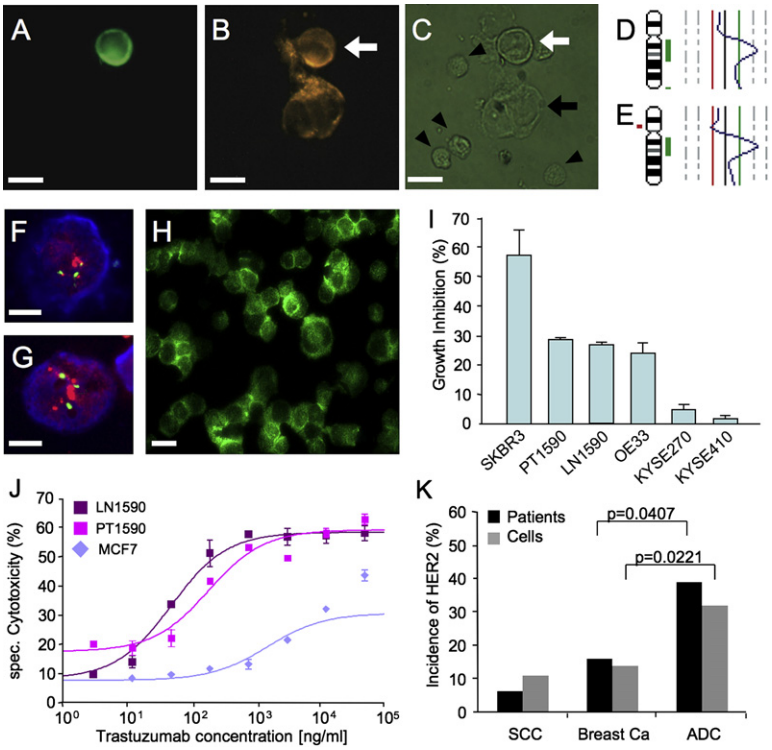


Figure 5. Evaluation of HER2 as a Potential Target for Adjuvant Treatment of Esophageal Cancer Patients

(A–C) Double staining of DTCs in lymph node samples. Scale bars, 20 μm. (A) EpCAM-positive cells in lymph node. (B) The same cells and one additional cell stained by an antibody directed against HER2. (C) Bright-field demonstrating that the surrounding lymph node cells are negative for EpCAM and HER2 (arrowheads). White arrow points at EpCAM/HER2 double-positive cell; black arrow points at HER2 single-positive cell.

(D and E) CGH profile of chromosome 17 indicating copy number gain at 17q12–21 for EpCAM/HER2 double-positive cell and for the HER2 single-positive cell.

(F and G) Dual-color FISH analysis with probes against the HER2 locus (red) and chromosome 17 centromer (green) of PT1590 (F) and LN1590 (G) showing HER2 amplification in both cell lines. Scale bars, 5 μm.

(H) Immunofluorescence using a FITC-conjugated trastuzumab revealing p185 overexpression of LN1590. Scale bar, 20 μm.

(I) Growth inhibition (mean values ± SEM, n = 3) after 72 hr of trastuzumab (10 μg/ml) treatment relative to untreated cells.

(J) Standard ADCC assay exposing target cells to trastuzumab for 4 hr. Error bars indicate ± SD from mean in triplicate determinations.

(K) Frequency of disseminated tumor cells with HER2 gain in the bone marrow of breast and esophageal cancer patients (SCC, esophageal squamous cell carcinoma, patients, n = 14; cells, n = 27; ADC, esophageal adenocarcinoma, patients, n = 18; cells, n = 27; breast, breast cancer; patients, n = 56; cells, n = 87). Patients were assigned as positive, when at least one tumor cell displayed a HER2 gain.

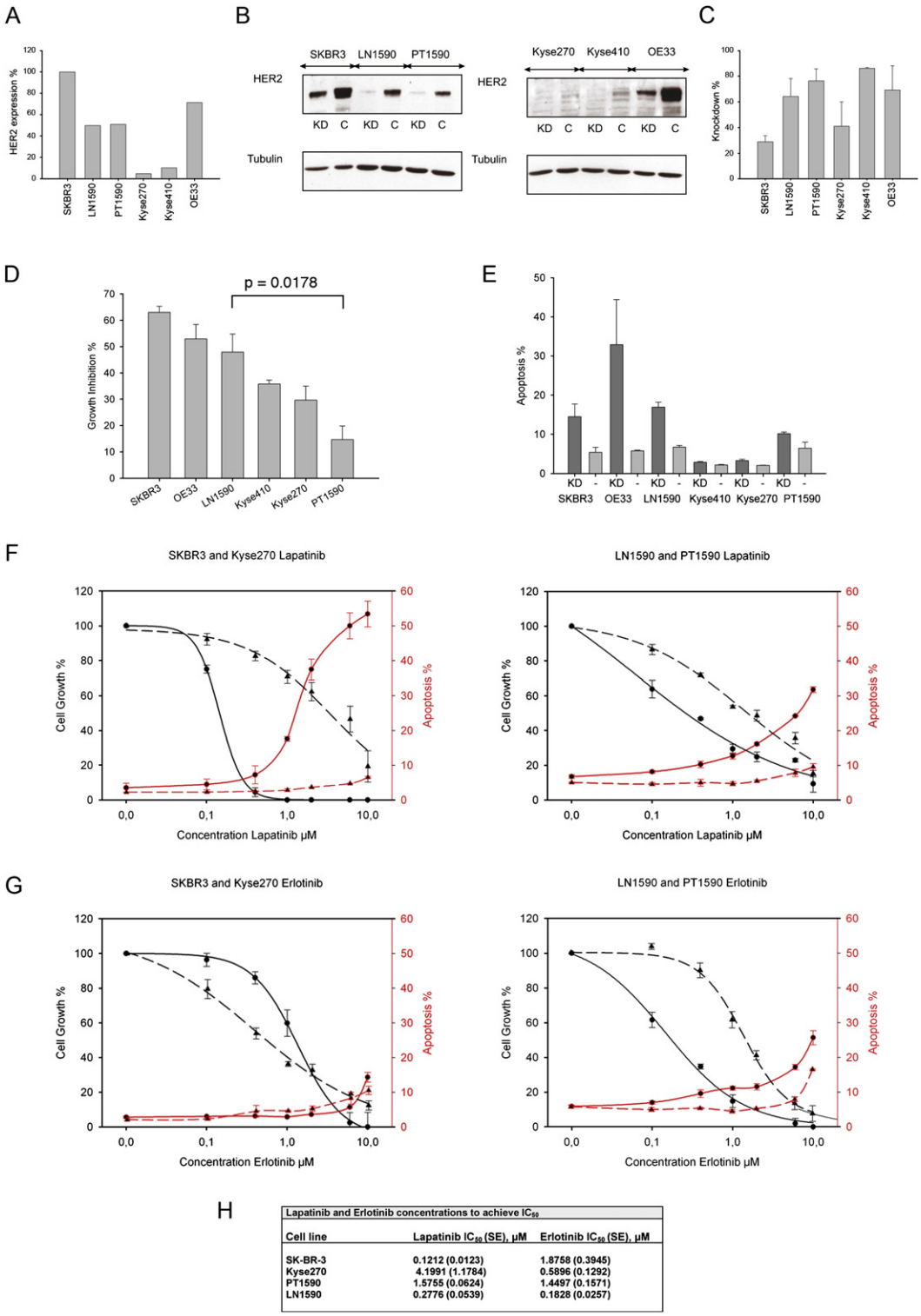
confirm this result, we added 52 unselected patients for which we could analyze the primary tumor for *HER2* gene amplification but had no data on the presence of DTCs. Also in this extended primary tumor cohort (Table 1, n = 101), *HER2* gene amplification had no effect on the survival of the patients (Figure 3G, p = 0.704). Thus, direct analysis of single DTCs had identified a genetic risk factor only relevant when present in DTCs. In 30 of the 35 cases from which we isolated marker-positive tumor cells, we were able to compare the *HER2* status of the DTCs and their matched primary tumors. The presence of *HER2* amplifications in primary tumors and DTCs was not congruent. For example, if selection for *HER2*-based therapy would rely on the analyses of primary tumors as it is currently done for breast cancer patients, four out of ten eligible cases would be missed (Table S1).

Genomic Divergence between Local and Systemic Cancer

To test whether the genetic divergence between the primary tumors and the DTCs applies only to the *HER2* gene, we compared their global patterns of genomic aberrations. We first assessed the reliability of our single-cell CGH when evaluated by hierarchical cluster analysis. We isolated 33 single cells from five different genomically well-characterized breast cancer cell lines and performed single-cell CGH analysis. All cells were correctly grouped according to their origin, although the individual cells displayed different degrees of genetic variation. Moreover, the finding that none of the single cells from the cell line CAL51—known to have a completely normal karyotype—displayed any chromosomal changes, strongly supports the validity of the analysis (Figure 4A). In striking contrast, primary tumors and matched DTCs from esophageal cancer patients were grouped remotely from each other in one of the two major branches of the dendrogram, indicating rather different and often opposing patterns of genomic changes (Figure 4B). The results demonstrate that primary tumors are inadequate surrogate markers for the genetics of early systemic esophageal cancer and suggest that direct analysis of single DTCs will uncover currently unknown genetic changes relevant for systemic progression.

HER2 as a Potential Target for Adjuvant Therapy in Esophageal Cancer

The applied whole-genome screen of DTCs and the survival analysis identified a potential therapy target, the *HER2* gene product, for treatment of micrometastases in a subgroup of esophageal cancer patients. We therefore gathered additional data that support an adjuvant therapy of esophageal cancer patients directed against HER2. First, from seven patients additional slides from disaggregated lymph nodes were available for double staining of EpCAM (the marker to detect DTCs in lymph nodes) and p185, the gene product of *HER2*. In two samples we detected three disseminated tumor cells that all expressed p185/HER2. Interestingly, one of two cells from one patient strongly expressed p185/HER2, while EpCAM could not be detected (Figures 5A–5C). Moreover, in both cells we found gains at the *HER2* region—subsequently confirmed by qPCR—among multiple chromosomal aberrations, thereby proving the malignant origin (Figure 5D). These findings suggest that p185/HER2 is expressed in DTCs with *HER2* gain, and that the antigen could be targeted by trastuzumab, an antibody directed against p185/HER2. We therefore tested whether



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Lapatinib and Erlotinib concentrations to achieve IC_{50}		
Cell line	**Lapatinib IC_{50} (SE), µM**	**Erlotinib IC_{50} (SE), µM**
SK-BR-3	0.1212 (0.0123)	1.8758 (0.3945)
Kyse270	4.1991 (1.1784)	0.5896 (0.1292)
PT1590	1.5755 (0.0624)	1.4497 (0.1571)
LN1590	0.2776 (0.0539)	0.1828 (0.0257)

Figure 6. HER2-Directed Inhibition Reveals Oncogene Dependence of LN1590 but Not PT1590 Cells

(A) HER2/p185 protein expression relative to the mammary cell line SKBR3 by densitometric evaluation of western blots.

(B) Representative western blot of the HER2 knockdown. Cells have been lysed 48 hr after siRNA treatment, and 35–50 µg of total lysate have been loaded on a 7.5% acrylamid-gel (KD = knockdown, C = siRNA control). Tubulin is shown as loading control.

(C) Mean values and standard error (n = 3) of the densitometric analysis for each HER2 knockdown performed. Histogram shows the HER2 knockdown efficiency as a percentage of the siRNA control.

esophageal cancer cells are sensitive to trastuzumab treatment in vitro and focused in particular on cells that were generated from patient 1590. So far this is the only esophageal ADC patient of whom cell lines could be generated both from the primary tumor (PT1590) and from disseminated tumor cells (LN1590) in a lymph node that had been judged macroscopically free of tumor (Hosch et al., 2000). Both DTC-derived and primary tumor-derived cell line cells harbor *HER2* amplifications and overexpress p185 (Figures 5F–5H). Upon trastuzumab treatment growth arrest was observed for LN1590 and PT1590 cells alike, as well as for the ADC cell line OE33, but not for the two esophageal SCC cell lines, Kyse270 and Kyse410 (Figure 5I). The two Kyse cell lines display a trisomy of chromosome 17, whereas OE33 displays true HER2 gene amplification upon FISH analysis (data not shown). LN1590 cells and PT1590 cells were then tested in an antibody-dependent cellular cytotoxicity assay (ADCC-assay). Since ADCC is a known effector mechanism of trastuzumab activity (Carter, 2001; Curcio et al., 2003; Prang et al., 2005), this assay allowed determining the preclinical sensitivity to trastuzumab treatment. LN1590 and PT1590 cells were lysed by effector cells with a concentration that has previously indicated clinical efficacy (Prang et al., 2005) and did not affect the p185/HER2-negative MCF7 cell line (Figure 5J). This suggests that esophageal cancer patients displaying a *HER2* gain in disseminated cancer cells would likewise benefit from adjuvant treatment with trastuzumab. Moreover, when we compared the incidence of DTCs displaying a *HER2* gain in bone marrow of nonmetastatic (stage M0) esophageal cancer patients with M0 stage breast cancer patients, for whom adjuvant therapy with trastuzumab was recently approved by the FDA, we found *HER2* gains in DTCs to be significantly more frequent in patients with esophageal adenocarcinomas than in breast cancer ($p = 0.04$ for patients and $p = 0.02$ for cells; Figure 5K).

Differential Response of PT1590 and LN 1590 Cells to HER2 Inhibition

It has been repeatedly observed that targeted therapies are only effective when the tumor cells are depending on the targeted mechanisms, a phenomenon that has been termed oncogene addiction (Weinstein and Joe, 2006) and is most often observed when the targeted mechanism is activated by genetic alterations. For example, EGFR is often overexpressed in lung and colon cancers; however, therapy responses are only observed when EGFR is mutated or amplified (Lynch et al., 2004; Moroni et al., 2005). The surprising divergence of primary tumors and DTCs for HER2-associated relapse prompted us to explore whether the DTC-derived LN1590 cells and the primary tumor-derived PT1590 cells are equally dependent on the *HER2* gene—despite the fact that both cell lines harbor HER2 amplifications. We therefore inhibited HER2 signaling by siRNA-mediated knockdown in both cell lines. Relative to the mammary cell line SKBR3, used as positive control, PT1590 and LN1590 expressed about 50% of p185/HER2 (Figure 6A), and HER2/p185 knockdown was similar for PT1590 and LN1590 (Figures 6B and 6C). However, growth inhibition and the induction of apoptosis were much stronger in LN1590 cells than in PT1590 cells (Figures 6D and 6E), indicating that LN1590 cells are more dependent on HER2 activation than PT1590 cells. Of the other cell lines, OE33 cells also responded strongly to the siRNA treatment (Figures 6D and 6E).

To extend these observations to additional clinically used inhibitors of HER2 signaling, we tested the effects of lapatinib, and erlotinib. Both inhibitors are known to affect HER2 signaling, although erlotinib was originally thought to inhibit EGFR only (Schaefer et al., 2007). SKBR3 and Kyse270 served as positive and negative controls, respectively, and responded as expected (Figure 6F). LN1590 cells but not in PT1590 cells responded strongly to lapatinib (Figure 6F), and LN1590 cells were also effectively inhibited and killed by erlotinib in contrast to PT1590 cells (Figures 6G and 6H). Thus, the low IC_{50} concentrations of both lapatinib and erlotinib further support the stronger dependence of LN1590 cells on HER2 signaling as opposed to PT1590 cells.

DISCUSSION

Our study evaluates the evolutionary model of cancer progression for potential clinical consequences and describes profound differences between local and early systemic disease. Divergent genetic aberrations were observed not only between primary tumors and DTCs from ectopic sites, but also specifically between tumor cells isolated from lymph nodes and from bone marrow. These observations most likely reflect the ongoing selection and expansion of genetically variant tumor cells and point to currently unrecognized evolutionary dynamics of disease progression, which may even exceed what we observed in our limited analysis. First, a complete description of the genomic trajectories from local to systemic disease would—in addition to DTC analysis—require inclusion of manifest lymph node and distant metastasis. Second, our analysis of genomic divergence was based on metaphase CGH. Since metaphase CGH has a limited resolution, it may miss both the true extent of genetic divergence as well as small genetic alterations (e.g., point mutations, small subchromosomal alterations) that are shared between all progeny of the initiating primary lesion. In breast cancer, we previously demonstrated the existence of such shared small

(D) Mean values ± SEM (n = 3) of the growth inhibition after the HER2 knockdown relative to the siRNA control (GL2), measured by MTT assay 72 hr after cell seeding and 96 hr after the HER2 knockdown.
(E) Percentage of apoptosis (mean values ± SEM) measured by FACS analysis (sub-G_1 content) after 72 hr (96 hr after the HER2 knockdown). KD, knockdown with HER2-directed oligo; –, control oligo GL2.
(F) Growth inhibition and induction of apoptosis by lapatinib treatment. Cell growth (black line) and apoptosis (red line) of SKBR3 (solid line) and Kyse270 (dashed line; left panel) and of LN1590 (solid line) and PT1590 (dashed line; right panel) after treatment with indicated concentrations of lapatinib for 72 hr. Mean values ± SEM.
(G) Growth inhibition and induction of apoptosis by erlotinib treatment. Cell growth (black line) and apoptosis (red line) of SKBR3 (solid line) and Kyse270 (dashed line; left panel) and of LN1590 (solid line) and PT1590 (dashed line; right panel) after treatment with indicated concentrations of erlotinib for 72 hr. Mean values ± SEM.
(H) IC_{50} concentrations for lapatinib and erlotinib.

alterations between DTCs and primary tumors despite complete divergence of chromosomal aberrations (Schardt et al., 2005). The search for genetic defects shared by all tumor cells will be particularly important because genetic activation of an oncogenic pathway often underlies oncogene dependence—a prerequisite for clinical response to targeted therapies (Weinstein and Joe, 2006). However, we cannot exclude that some of the genetic differences between DTCs from bone marrow and lymph nodes are not (only) related to the isolation from different microenvironments but also characterize epigenetically different tumor cell populations because we had to use different markers for detection. We applied the most specific markers, EpCAM antibodies for lymph node samples and cytokeratin antibodies for bone marrow, because cytokeratin-positive cells have been detected in lymph nodes (Passlick et al., 1994) and EpCAM-positive cells in bone marrow of control patients without malignancy (Klein et al., 2002b). Regardless of whether some genetic differences are associated with the specific detection method, 17q12–21 gains were the most frequent genomic aberration in both types of tumor cells, indicating that this region harbors genes that support both hematogenous and lymphatic cancer progression. Since metaphase CGH does not identify affected genes, we tested the obvious candidate mapping within this region, the *HER2* gene, by gene-specific quantitative PCR. In 50% of 17q12–21 gains, the *HER2* gene was gained both in tumor cells from lymph nodes and bone marrow. Interestingly, only gain of *HER2* but not of 17q12–21 without *HER2* gain was indicative for poor survival, suggesting that *HER2* gains are critical for systemic esophageal cancer.

Having observed the genetic divergence of cancer cells disseminated by the way of blood or lymph, we were not surprised that local disease differed from systemic cancer as well. While a gain of *HER2* in a single disseminated cancer cell was an important risk factor in multivariate analysis, *HER2* amplification in primary tumors was not associated with poor survival either in the group of patients that were analyzed for disseminated cancer cells or in a study cohort comprising twice as many patients. Our group of patients analyzed for *HER2* amplification is one of the largest of esophageal cancer patients, and the findings are in agreement with previous studies observing the lack of prognostic impact of *HER2* amplification in primary esophageal cancers (Reichelt et al., 2007). It should be noted that we applied different methods to determine the *HER2* status in single tumor cells and primary tumors. Standard FISH analysis was used for primary tumors, whereas quantitative PCR served to determine *HER2* gains in single disseminated cancer cells. All single disseminated cells identified by cytokeratin or EpCAM antibodies were isolated, and their genomic DNA was globally amplified and used for CGH and subsequently for qPCR of the *HER2* gene. A *HER2* gain was assigned to any isolated cell with significantly higher amounts of *HER2* sequence as compared to 50 single normal control cells using a robust rank sum test (Schardt et al., 2005). It will be interesting to test whether FISH analysis of single disseminated cancer cells will be equally reliable for the identification of patients with short survival.

The differential prognostic impact of HER2 gains in DTCs and primary tumors was paralleled by differential responses of the LN1590 and PT1590 cell lines to HER2 inhibition. These cell lines were isolated from DTCs (LN1590) and the primary tumor (PT1590) of a patient with esophageal cancer. While both cell lines displayed HER2 gene amplification and protein overexpression, only LN1590, the DTC-derived cell line, responded to HER2-directed siRNA and to the HER2/EGFR inhibitors lapatinib and erlotinib with increased apoptosis and growth inhibition. So far, it raises the interesting, yet puzzling and worrisome possibility not only that selection during systemic progression results in divergent genomes, as shown by the gene copy number analysis, but also that genes that are genetically altered in similar ways may play different roles in local and systemic disease. This conclusion has to be drawn with caution first, because the functional data are based on cell lines, which might have been selected in culture, and second, because PT1590 and LN1590 are currently the only available pair of cell lines derived from the primary tumor and from DTCs of the same patient.

Since genetic activation of a therapy target molecule has been demonstrated to be often essential for treatment response to various targeted therapies (Arteaga and Baselga, 2004; Weinstein and Joe, 2006), the finding of *HER2* gains in single disseminated cancer cells may provide a rationale for the stratification of esophageal cancer patients for adjuvant *HER2*-based therapies. Such targeting of *HER2* in future trials may primarily focus on esophageal adenocarcinomas, because *HER2* gains in DTCs were more frequent in patients with ADC than in SCC (39% versus 6%; Figure 5K). It is noteworthy that more M0-stage ADC patients with DTCs harbored HER2-positive cells in bone marrow than M0-stage breast cancer patients with DTCs (39% versus 16%; Figure 5K), while the incidence of HER2 amplifications in primary tumors is about 15%–17% for both tumor types (Al-Kuraya et al., 2004; Reichelt et al., 2007). While it is unknown whether the more frequent gain of HER2 in DTC of ADC compared to breast cancer patients contributes to the generally more aggressive course of esophageal cancer, another observation differentiates esophageal cancer from breast cancer. In M0-stage breast cancer patients, individual DTCs from bone marrow displayed heterogeneous chromosomal aberrations, while DTCs from breast cancer patients with manifest metastases were genetically similar (Klein et al., 2002a). Others and we interpreted this observation as a process of clonal selection and expansion taking place during a period of latency to manifest metastasis (Klein, 2003; Pantel and Brakenhoff, 2004; Weinberg, 2007). If this is correct, then the observation that individual esophageal cancer cells (taken from one of the two sites of DTC isolation; Figure 2E) share many chromosomal changes already at the stage M0 may further indicate the progressed state of minimal residual disease in esophageal cancer patients. However, the few cases of which we had isolated DTCs both from lymph nodes and bone marrow displayed heterogeneous karyotypes. This and the overall genetic divergence of cancer cells spread by the way of blood and lymph will may make it necessary to screen a large number of DTCs from both ectopic sites to identify therapy target genes. Such direct analyses of DTCs might uncover additional therapy target genes that have so far escaped identification in studies of primary tumors.

Taken together, our study exemplifies that genetic markers and therapy targets for systemic adjuvant therapies can be directly defined on disseminated cancer cells that also provide information unavailable from the primary tumor. The observed genetic divergence of local and early systemic disease emphasizes

the need for and the chances of a diagnostic pathology of minimal systemic cancer. While we do not question that primary tumors are useful to estimate prognosis, we believe that the direct analysis of metastatic precursor cells merits further exploration. With technologies at hand for comprehensive single-cell analysis (Hartmann and Klein, 2006) and automated high-throughput single-cell screening (Cristofanilli et al., 2004; Nagrath et al., 2007), we may ultimately gather therapeutically important information needed for the prevention of lethal metastasis.

EXPERIMENTAL PROCEDURES

Patients

The ethics committee of the University Hospital Hamburg-Eppendorf approved the study, and informed consent was obtained from all patients before inclusion in the study. Diagnostic procedures, inclusion criteria, and characteristics of the patients are provided in the Supplemental Data and in Table 1.

Tissue Processing

Intraoperatively, a representative sample of the primary tumor was snap frozen in liquid nitrogen immediately after removal and stored at −80°C. Five serial cryostat sections 6–8 μm thick were cut from the tissue block. Before further processing, the first slide was stained with hematoxylin and eosin to confirm the tumor within the tissue.

The procedure for bone marrow aspiration, preparation, lymph node disaggregation, and detection of single disseminated tumor cells was performed as previously described (Hosch et al., 2000, 2001; Klein et al., 2002a). Briefly, 2–10 ml of bone marrow was aspirated into a heparanized syringe from the upper iliac crest under general anesthesia before the surgical procedure. The bone marrows were washed with Hank's solution and were then subjected to Ficoll-Hypaque density-gradient centrifugation (density, 1.077 g per mole) at 900 × g for 30 min to separate mononuclear cells. Intraoperatively, during systematic lymphadenectomy, lymph nodes that were judged as tumor free by the surgeon were divided into two parts. One part was sent to routine pathology; the other part was washed twice in PBS and then minced into 1 mm pieces and diasaggregated mechanically into a single-cell suspension by rotating knives (DAKO Medimachine, Dako, Hamburg, Germany). The cell suspensions generated from bone marrow and lymph nodes, respectively, were placed in a volume of 1 ml PBS on positively charged glass slides (Menzel, Germany) at a density of 250,000 cells per 227 mm^2. After sedimentation for 45 min, the slides were dried overnight and stored at 4°C until further analysis. Immunocytological staining of 10^6 cells was carried out with the alkaline phosphatase-anti-alkaline phosphatase method (APAAP) using the monoclonal antibody A45-B/B3 (Micromet, Munich, Germany) against cytokeratin 8, 18, and 19 for bone marrow samples (Braun et al., 2000), and the EpCAM antibody BerEP4 (Dako) was used for the lymph node samples (Izbicki et al., 1997). Alkaline phosphatase was developed using BCIP/NBT (BioRad) as substrate, and slides were covered with PBS under a cover glass for evaluation with bright-field microscopy. In addition, 10^6 cells served as a control for staining with an irrelevant immunoglobulin (MOPC21, Sigma). Positive cells were isolated from the slide in PBS containing 0.5% Igepal (Sigma) using a micromanipulator after removal of the cover glass. Prior to transfer to the PCR reaction tube, all isolated single cells were placed on a fresh slide to ensure that no contaminating cell was coisolated.

FISH

For the evaluation of the HER2 gene amplification level, the commercially available dual-color FISH kit PathVision (Vysis Inc., Downers Grove, IL) was used. Cryostat sections, 5 μm thick, were cut onto silanized glass slides and were digested with 250 μg Pepsin in 50 ml 0.01 N HCl at 37°C for 10–12 min. Then the sections were fixed with 10% paraformaldehyde/50 mM $MgCl_2$/1× PBS at room temperature. Hybridization, washing, and evaluation of the signals were performed as previously described (Stoecklein et al., 2004). Data of 21 patients of the prospective study group were obtained from a previously published study to extend the number of primary tumor samples (Reichelt et al., 2007).

SCOMP

Tumor cell isolation and SCOMP were exactly performed as described by Schmidt-Kittler et al. (2003), and SCOMP of primary tumor tissue was performed as described by Stoecklein et al. (2002). The CGH aberrations were described according to the International System for Human Cytogenetic Nomenclature (ISCN 1995) guidelines. The web-based Progenetix software was used to generate histograms and to perform the hierarchical cluster analysis (860 bands) from the CGH-based reverse in situ hybridization-annotated karyotype information (Baudis and Cleary, 2001).

Quantitative PCR

Real-time PCR was performed using a LightCycler (Roche, Mannheim, Germany) and Fast Start Master SYBR Green Kits (Roche) using 1 μl of primary PCR products from the whole-genome amplification diluted 1:20 in H_2O. Analysis was done using the RelQuant software (Roche) with PCR efficiency normalization, and a reference sample was included for every run. Three primers within the HER2 locus were selected, and three primers on two chromosomes (chromosome 3 and chromosome 7) served as loading control. Primer sequences can be obtained upon request. Measurements showing unspecific products in the melting curve analysis or CGH aberrations on the control loci were discarded from further statistics. All relative expression ratios HER2/reference from duplicate measurements were compared to those from 55 normal diploid cells. Samples showing significant difference ($p < 0.05$) from the control cells in a Mann-Whitney test were classified as either "amplified" or "deleted" for HER2 depending on their mean rank value.

Double Staining

Slides were treated for 40 min with 0.1% Triton X-100 (Promega, Mannheim, Germany) in 2× HEPES buffer (140 mM NaCl, 1.5 mM Na2HPO4, 50 mM HEPES [Sigma Aldrich, Steinheim, Germany], pH 7.4) and 10% human antibody (AB) serum, then incubated with the A0485 antibody directed against p185/HER2 (5 μg/ml, DAKO) for 30 min, washed 3 × 5 min in 2× HEPES and subsequently incubated with a goat anti-rabbit-Cy3 antibody (7.5 μg/ml, Jackson ImmunoResearch Laboratories, West Grove, PA). This was followed by washing with 2× HEPES for 5 min and PBS 0.2% Tween 20 (Sigma) for 1 min and a second blocking step with 2× HEPES/10% AB serum/rabbit immunoglobulin X0903 (200 μg/ml, DAKO) for 40 min. Then biotinylated mab 3B10 (2 μg/ml) directed against EpCAM (Micromet, Munich, Germany) was added for 30 min, followed by washing with PBS for 5 min and TBS for 2 × 5 min. Subsequently, we applied an AB-complex solution (DAKO) according to the manufacturer's instructions and washed the slides in TBS for 3 × 1 min. The alkaline phosphatase activity was visualized by the ELF-97 system (Molecular Probes, Eugene, OR) according to the manufacturer's instructions (incubation time: 8 min). As negative control for the A0485 antibody we used rabbit IgG X0903 (5 μg/ml, DAKO), and as negative control for the EpCAM antibody we used biotinylated MOPC 21 (2 μg/ml, Sigma). We used as positive control PT1590 cells diluted 1:10 in human bone marrow.

Antigen-Dependent Cellular Cytotoxicity

Antigen-dependent cellular cytotoxicity (ADCC) assays were performed using breast cancer cell line MCF7 and the esophageal cancer cell lines PT1590 and LN1590 as target cells. LN1590 was generated from micrometastatic cells in a lymph node (Scheunemann et al., 1999). Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll sucrose gradient density centrifugation from fresh blood from healthy volunteer donors. Erythrocytes were removed from collected PBMC (lysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, and 100 μM ethylenediamine tetraacetic acid [EDTA]), and lysed erythrocytes and thrombocytes were removed after centrifugation of PBMC (250 g, 15 min). Target cells (5×10^6) were labeled with 50 μg calcein AM (Molecular Probes, Eugene, Oregon; C-3100MP) for 30 min at 37°C in 5 ml cell culture medium (RPMI/10% FCS). Washed PBMC were adjusted in culture medium to a density of 0.5×10^6 cells/ml, and 50,000 cells were used per replicate. Trastuzumab was diluted in culture medium to the required concentrations and added 10 min prior to PBMC at room temperature to have sufficient time for antigen binding. Washed PBMC were adjusted to 1.2×10^7 cells/ml in culture medium. A standard coincubation took 4 hr at 37°C/5% CO_2 with an effector-to-target ratio of 1:20 in a total volume of 200 μl. Subsequently, cells were collected by centrifugation and resuspended in FACS buffer containing 1 μl/ml PI (0.25 mg/ml).

Fifty thousand events were recorded by flow cytometry. Specific antibody-mediated cytotoxicity was quantified in flow cytometry and based on the number of living compared to PI-positive dead target cells in the control sample without antibody. Sigmoidal dose-response curves typically had r^2 values > 0.95 as determined by the Graphpad PRIZM4 software.

Cell Proliferation Assay

Cultured cells were seeded into 96-well plates at a density of 1000 cells/well 24 hr prior to inhibitor (Lapatinib, Erlotinib; Vichem Chemie, Hungary) treatment. The next day cells were treated with indicated concentrations of inhibitor and cultured for 72 hr in the presence of inhibitor and FBS. Cellular metabolism was assessed using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, 20 μl of MTT solution (5 mg/ml in PBS) was added to the cells. After 4 hr at 37°C the formazan crystals formed were solubilized by addition of 50 μl stop solution (10% SDS, 5% butanol, and 0.01 M HCl). Absorbance for each well was read at 570 nm using a microplate reader. Growth inhibition was calculated as a percentage of the untreated control. Experiments were done thrice or more often in triplicates for each cell line, and the inhibitory concentrations (IC_{50}) were determined using the four parameter logistic model (Sigmaplot, Systat) for curve fitting.

Flow Cytometry

Cells were seeded at a density of 2×10^4 cells/well into 12-well plates. The next day medium was changed and cells were treated with different concentrations of inhibitor. After 72 hr of cultivation in the presence of inhibitor and FBS, cells were trypsinized, collected by centrifugation, and incubated with 0.01% Triton, 0.1% sodium citrate, and 0.02 mM propidium iodide (Sigma) in the dark at 4°C. After 2 hr the cells were analyzed by flow cytometry (FACSCalibur, BD Bioscience) using the CellQuest Pro software. The sub-G_1 population was counted as the apoptotic population and represented as fraction of the total cells counted.

Western Blotting

Cells were cultivated in 6 cm plates and lysed in 100 μl lysis buffer containing 1% Triton X-100, and equal amounts of protein were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Germany), blocked for several hours in NET-gelatin and incubated at 4°C overnight with the corresponding primary antibody in NET-gelatin. The anti-HER2 (Catalog 06-562, Lot 27771) was purchased from Upstate and used in a 1:1000 dilution. Membranes were washed three times with NET-gelatin and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody diluted in Net-gelatin for 1 hr at room temperature. After additional washing (three times), detection was done using enhanced chemiluminescence (ECL; Western Lightning, Perkin Elmer) on X-ray films. Densitometric quantification was done using AIDA Image Analyzer software.

RNA Interference

Cells were seeded 24 hr prior to siRNA transfection in 6 cm plates at a density of 2×10^5 cells/plate. Transfection of 21-nucleotide siRNA duplexes (Ambion) was carried out using OligofectAMINE (Invitrogen) and OPTI-MEM media (GIBCO) without FBS.

HER2 siRNA sequence: sense, GGGAAACCUGGAACUCACCtt; antisense, GGUGAGUUCCAGGUUUCCCtg. Control siRNA (GL2; Dharmacon) sequence: sense, CGUACGCGGAAUACUUCGAtt; antisense, UCGAAGUAUUCCGCGUACGtt. After 4 hr media were changed to normal media containing FBS, and after an additional 24 hr cells were used for further experiments.

Statistical Evaluation

For statistical analysis, contingency tables were tested with Fisher's exact test and whenever appropriate with the χ^2 test. We used log rank tests for the univariate survival analysis. The primary end point was survival, as measured from a date three months after primary surgery to the time of the last follow-up or death. Data of patients who were still alive at the end of the study were censored. The joint effects with already recognized prognostically relevant variables were examined via Cox proportional hazards analysis. Histology, pT-category, pN-category, and the presence of disseminated tumor cells were entered stepwise forward into the model to test these covariables for possible prognostic joint effects with *HER2* amplification in disseminated tumor cells. The threshold for statistical significance was chosen at $p = 0.05$.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, one supplemental figure, and one supplemental table and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/5/441/DC1/.

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REFERENCES

Al-Kuraya, K., Schraml, P., Torhorst, J., Tapia, C., Zaharieva, B., Novotny, H., Spichtin, H., Maurer, R., Mirlacher, M., Kochli, O., et al. (2004). Prognostic relevance of gene amplifications and coamplifications in breast cancer. Cancer Res. *64*, 8534–8540.

Arteaga, C.L., and Baselga, J. (2004). Tyrosine kinase inhibitors: Why does the current process of clinical development not apply to them? Cancer Cell *5*, 525–531.

Baudis, M., and Cleary, M.L. (2001). Progenetix.net: An online repository for molecular cytogenetic aberration data. Bioinformatics *17*, 1228–1229.

Bedenne, L., Michel, P., Bouche, O., Milan, C., Mariette, C., Conroy, T., Pezet, D., Roullet, B., Seitz, J.F., Herr, J.P., et al. (2007). Chemoradiation followed by surgery compared with chemoradiation alone in squamous cancer of the esophagus: FFCD 9102. J. Clin. Oncol. *25*, 1160–1168.

Braun, S., Pantel, K., Muller, P., Janni, W., Hepp, F., Kentenich, C.R., Gastroph, S., Wischnik, A., Dimpfl, T., Kindermann, G., et al. (2000). Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. N. Engl. J. Med. *342*, 525–533.

Braun, S., Vogl, F.D., Naume, B., Janni, W., Osborne, M.P., Coombes, R.C., Schlimok, G., Diel, I.J., Gerber, B., Gebauer, G., et al. (2005). A pooled analysis of bone marrow micrometastasis in breast cancer. N. Engl. J. Med. *353*, 793–802.

Cairns, J. (1975). Mutation selection and the natural history of cancer. Nature *255*, 197–200.

Carter, P. (2001). Improving the efficacy of antibody-based cancer therapies. Nat. Rev. Cancer *1*, 118–129.

Cristofanilli, M., Budd, G.T., Ellis, M.J., Stopeck, A., Matera, J., Miller, M.C., Reuben, J.M., Doyle, G.V., Allard, W.J., Terstappen, L.W., et al. (2004). Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N. Engl. J. Med. *351*, 781–791.

Curcio, C., Di Carlo, E., Clynes, R., Smyth, M.J., Boggio, K., Quaglino, E., Spadaro, M., Colombo, M.P., Amici, A., Lollini, P.L., et al. (2003). Nonredundant roles of antibody, cytokines, and perforin in the eradication of established Her-2/neu carcinomas. J. Clin. Invest. *111*, 1161–1170.

Enzinger, P.C., and Mayer, R.J. (2003). Esophageal cancer. N. Engl. J. Med. *349*, 2241–2252.

Hartmann, C.H., and Klein, C.A. (2006). Gene expression profiling of single cells on large-scale oligonucleotide arrays. Nucleic Acids Res. *34*, e143.

Heim, S., and Mitelman, F. (1995). Cancer Cytogenetics, Second Edition (New York, Chichester, Brisbane, Toronto, Singapore: Wiley-Liss).

Hoglund, M., Gisselsson, D., Sall, T., and Mitelman, F. (2002). Coping with complexity. Multivariate analysis of tumor karyotypes. Cancer Genet. Cytogenet. *135*, 103–109.

Hosch, S., Kraus, J., Scheunemann, P., Izbicki, J.R., Schneider, C., Schumacher, U., Witter, K., Speicher, M.R., and Pantel, K. (2000). Malignant potential and cytogenetic characteristics of occult disseminated tumor cells in esophageal cancer. Cancer Res. *60*, 6836–6840.

Hosch, S.B., Stoecklein, N.H., Pichlmeier, U., Rehders, A., Scheunemann, P., Niendorf, A., Knoefel, W.T., and Izbicki, J.R. (2001). Esophageal cancer: The mode of lymphatic tumor cell spread and its prognostic significance. J. Clin. Oncol. *19*, 1970–1975.

Hüsemann, Y., Geigl, J.B., Schubert, F., Musiani, P., Meyer, M., Burghart, E., Forni, G., Eils, R., Fehm, T., Riethmüller, G., and Klein, C.A. (2008). Systemic spread is an early step in breast cancer. Cancer Cell *13*, 58–68.

Ilson, D.H. (2007). Surgery after primary chemoradiotherapy in squamous cancer of the esophagus: Is the photon mightier than the sword? J. Clin. Oncol. *25*, 1155–1156.

Izbicki, J.R., Hosch, S.B., Pichlmeier, U., Rehders, A., Busch, C., Niendorf, A., Passlick, B., Broelsch, C.E., and Pantel, K. (1997). Prognostic value of immunohistochemically identifiable tumor cells in lymph nodes of patients with completely resected esophageal cancer. N. Engl. J. Med. *337*, 1188–1194.

Klein, C.A. (2003). The systemic progression of human cancer: A focus on the individual disseminated cancer cell—The unit of selection. Adv. Cancer Res. *89*, 35–67.

Klein, C.A., Schmidt-Kittler, O., Schardt, J.A., Pantel, K., Speicher, M.R., and Riethmuller, G. (1999). Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells. Proc. Natl. Acad. Sci. USA *96*, 4494–4499.

Klein, C.A., Blankenstein, T.J., Schmidt-Kittler, O., Petronio, M., Polzer, B., Stoecklein, N.H., and Riethmuller, G. (2002a). Genetic heterogeneity of single disseminated tumour cells in minimal residual cancer. Lancet *360*, 683–689.

Klein, C.A., Seidl, S., Petat-Dutter, K., Offner, S., Geigl, J.B., Schmidt-Kittler, O., Wendler, N., Passlick, B., Huber, R.M., Schlimok, G., et al. (2002b). Combined transcriptome and genome analysis of single micrometastatic cells. Nat. Biotechnol. *20*, 387–392.

Kuukasjarvi, T., Karhu, R., Tanner, M., Kahkonen, M., Schaffer, A., Nupponen, N., Pennanen, S., Kallioniemi, A., Kallioniemi, O.P., and Isola, J. (1997). Genetic heterogeneity and clonal evolution underlying development of asynchronous metastasis in human breast cancer. Cancer Res. *57*, 1597–1604.

Lynch, T.J., Bell, D.W., Sordella, R., Gurubhagavatula, S., Okimoto, R.A., Brannigan, B.W., Harris, P.L., Haserlat, S.M., Supko, J.G., Haluska, F.G., et al. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N. Engl. J. Med. *350*, 2129–2139.

Moroni, M., Veronese, S., Benvenuti, S., Marrapese, G., Sartore-Bianchi, A., Di Nicolantonio, F., Gambacorta, M., Siena, S., and Bardelli, A. (2005). Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: A cohort study. Lancet Oncol. *6*, 279–286.

Nagrath, S., Sequist, L.V., Maheswaran, S., Bell, D.W., Irimia, D., Ulkus, L., Smith, M.R., Kwak, E.L., Digumarthy, S., Muzikansky, A., et al. (2007). Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature *450*, 1235–1239.

Pantel, K., Muller, V., Auer, M., Nusser, N., Harbeck, N., and Braun, S. (2003). Detection and clinical implications of early systemic tumor cell dissemination in breast cancer. Clin. Cancer Res. *9*, 6326–6334.

Pantel, K., and Brakenhoff, R.H. (2004). Dissecting the metastatic cascade. Nat. Rev. Cancer *4*, 448–456.

Passlick, B., Izbicki, J.R., Kubuschok, B., Nathrath, W., Thetter, O., Pichlmeier, U., Schweiberer, L., Riethmuller, G., and Pantel, K. (1994). Immunohistochemical assessment of individual tumor cells in lymph nodes of patients with non-small-cell lung cancer. J. Clin. Oncol. *12*, 1827–1832.

Prang, N., Preithner, S., Brischwein, K., Goster, P., Woppel, A., Muller, J., Steiger, C., Peters, M., Baeuerle, P.A., and da Silva, A.J. (2005). Cellular and complement-dependent cytotoxicity of Ep-CAM-specific monoclonal antibody MT201 against breast cancer cell lines. Br. J. Cancer *92*, 342–349.

Reichelt, U., Duesedau, P., Tsourlakis, M., Quaas, A., Link, B.C., Schurr, P.G., Kaifi, J.T., Gros, S.J., Yekebas, E.F., Marx, A., et al. (2007). Frequent homogeneous HER-2 amplification in primary and metastatic adenocarcinoma of the esophagus. Mod. Pathol. *20*, 120–129.

Schaefer, G., Shao, L., Totpal, K., and Akita, R.W. (2007). Erlotinib directly inhibits HER2 kinase activation and downstream signaling events in intact cells lacking epidermal growth factor receptor expression. Cancer Res. *67*, 1228–1238.

Schardt, J.A., Meyer, M., Hartmann, C.H., Schubert, F., Schmidt-Kittler, O., Fuhrmann, C., Polzer, B., Petronio, M., Eils, R., and Klein, C.A. (2005). Genomic analysis of single cytokeratin-positive cells from bone marrow reveals early mutational events in breast cancer. Cancer Cell *8*, 227–239.

Scheunemann, P., Izbicki, J.R., and Pantel, K. (1999). Tumorigenic potential of apparently tumor-free lymph nodes. N. Engl. J. Med. *340*, 1687.

Schmidt-Kittler, O., Ragg, T., Daskalakis, A., Granzow, M., Ahr, A., Blankenstein, T.J., Kaufmann, M., Diebold, J., Arnholdt, H., Muller, P., et al. (2003). From latent disseminated cells to overt metastasis: Genetic analysis of systemic breast cancer progression. Proc. Natl. Acad. Sci. USA *100*, 7737–7742.

Stoecklein, N.H., Erbersdobler, A., Schmidt-Kittler, O., Diebold, J., Schardt, J.A., Izbicki, J.R., and Klein, C.A. (2002). SCOMP is superior to degenerated oligonucleotide primed-polymerase chain reaction for global amplification of minute amounts of DNA from microdissected archival tissue samples. Am. J. Pathol. *161*, 43–51.

Stoecklein, N.H., Luebke, A.M., Erbersdobler, A., Knoefel, W.T., Schraut, W., Verde, P.E., Stern, F., Scheunemann, P., Peiper, M., Eisenberger, C.F., et al. (2004). Copy number of chromosome 17 but not HER2 amplification predicts clinical outcome of patients with pancreatic ductal adenocarcinoma. J. Clin. Oncol. *22*, 4737–4745.

Thorban, S., Rosenberg, R., Busch, R., and Roder, R.J. (2000). Epithelial cells in bone marrow of oesophageal cancer patients: A significant prognostic factor in multivariate analysis. Br. J. Cancer *83*, 35–39.

Weinberg, R.A. (2007). The Biology of Cancer, First Edition (New York: Garland Science).

Weinstein, I.B., and Joe, A.K. (2006). Mechanisms of disease: Oncogene addiction—A rationale for molecular targeting in cancer therapy. Nat. Clin. Pract. Oncol *3*, 448–457.