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Establishment of immunocytochemical and molecular methods for characterization of circulating tumor cells in breast cancer patients

Dissertation

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Abbreviations

APC	Allophycocyanin
APS	Ammonium persulfate
AR	Antigen retrieval
BSA	Bovine serum albumin
CK	Cytokeratin
CTCs	Circulating tumor cells
DAPI	4’,6-Diamidino-2-phenylindole
DCIS	Ductal carcinoma in situ
DEME	Dulbecco’s modified eagle medium
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DOP	Degenerate oligonucleotide primed
DTCs	Disseminated tumor cells
DTT	Dithiothreitol
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
FCS	Fetal calf serum
FFPE	Formalin-fixed paraffin- embedded
FISH	Fluorescence in situ hybridization
FITC	Fluorescein
GITC	Guanidine isothiocyanate
ICC	Immunocytochemistry
IF	Immunofluorescence
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LCM	Laser capture microdissection
MDA	Multiple displacement amplification
NBT	Nitro blue tetrazolium chloride
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEP	Primer extension preamplification
PR	Progesterone receptor
RT-PCR	Reverse transcriptase polymerase chain reaction
SNP	Single nucleotide polymorphism
SSC	Saline sodium citrate
TB	Terrific broth
TBE	Tris-borate electrophoresis
TEMED	N,N,N’,N’-Tetramethylethylenediamine,1,2-bis(dimethylamino)-ethane
VEGF	Vascular endothelial growth factor
WGA	Whole genome amplification

Aims of the study

Breast cancer is the most prevalent cancer in the world and one of the leading causes of cancer mortality among women, and metastasis is the main cause of cancer-related death. However, early spread of single tumor cells usually remains undetected by conventional staging. These single tumor cells in regional lymph nodes, peripheral blood and in distant organs, such as bone marrow are referred to as 'micrometastases', which now can be identified by sensitive immunocytochemical and molecular assays. Furthermore, tumor cells detected in peripheral blood are defined as circulating tumor cells (CTCs). The presence of CTCs in patients with metastatic breast cancer is significantly associated with worse outcome. A standardized method to detect CTCs yielding reliable results could provide benefits for breast cancer patients from designing target-directed therapies, monitoring therapeutic efficacies, and long term follow-up. The CellSearch^{TM} system is an automated and standardized system for the detection and enumeration of CTCs in peripheral blood and has been cleared by the FDA for monitoring patients with metastatic breast cancer.

Major aims for the present study are:

1. To detect CTCs in peripheral blood from breast cancer patients with the help of the CellSearch^{TM} system as precondition for the further phenotypical and molecular characterization of these cells. Although the clinical significance of CTCs is being investigated in various studies, the biological characteristics of CTCs are largely unknown thus far.

2. To validate the detection of HER2 expression on CTCs with the help of the CellSearch^{TM} system. HER2 is currently the most important biological target in clinical breast cancer management. The introduction of trastuzumab, a humanized monoclonal antibody directed against the extracellular domain of HER2, into adjuvant breast cancer treatment led to significantly improved clinical outcome for breast cancer patients. However, discrepancies between the HER2 status in primary tumors and CTCs in peripheral blood have been recently reported. Therefore, analysis of

HER2 expression on CTCs in the blood of breast cancer patients may contribute to identifying additional molecular markers for targeted therapy.

3. To evaluate the expression of some other important biomarkers for breast cancer, such as estrogen receptor (ER), progesterone receptor (PR), Ki-67 and apoptosis marker M30.

4. To establish and optimize a set of methods including immunofluorescence (IF), FISH, combined FISH and IF, and real time PCR to further characterize CTCs after processing blood samples from breast cancer patients with the CellSearch^{TM} system.

5. To establish the method of whole genome amplification (WGA) on DNA derived from processed cells and to test the suitability of generated amplification products for real time PCR in a small pilot study for paving the path to the oncoming genetic analysis of CTCs at the single-cell level.

1. Introduction

1.1 Breast cancer staging and therapy

Breast cancer is the most prevalent cancer in the world and one of the leading causes of cancer mortality among women, accounting for about 14% of female cancer deaths (Parkin et al. 2005; Jemal et al. 2007). An estimated number of 178,480 newly diagnosed breast cancer cases are expected to occur and the expected number of deaths from breast caner for 2007 in the United States is 40,460 among women (Jemal et al. 2007). Relapse and metastasis are the main causes of cancer-related death.

Breast cancer can be categorized as noninvasive and invasive carcinomas on the basis of pathologic features. Ductal carcinoma in situ, or DCIS, is the most common type of noninvasive breast cancer, which is defined by the proliferation of carcinoma cells within the milk ducts and the absence of protrusion through the basement membranes (Figure 1.1). Approximately 85% to 95% of invasive carcinomas are ductal in origin. The invasive ductal carcinomas include unusual variants of breast cancer, such as colloid or mucinous, adenoid cystic and tubular carcinomas, which have an especially favorable outlook (National Comprehensive Cancer Network 2007).

The staging of cancer depends on the primary tumor (T), metastasis in regional lymph nodes (N) and distant metastasis (M). The American Joint Committee on Cancer (AJCC) TNM staging system for breast cancer (AJCC 2002) is described below:

T0 No evidence of primary tumor

Tis Carcinoma in situ

T1 Tumor 2cm or less in greatest dimension

T2 Tumor more than 2cm but not more than 5cm in greatest dimension

T3 Tumor more than 5cm in greatest dimension

T4 Tumor of any size with direct extension to chest wall or skin.

N0 No regional lymph node metastasis

N1 Metastasis to movable ipsilateral axillary lymph node(s)

N2 Metastases in ipsilateral axillary lymph nodes fixed or matted, or in clinically apparent ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastasis

N3 Metastasis in ipsilateral infraclavicular lymph node(s), or in ipsilateral internal mammary lymph node(s) and axillary lymph node(s), or in ipsilateral supraclavicular lymph node(s)

M0 No distant metastasis

M1 Distant metastasis

Stage grouping:

Stage 0	Tis N0 M0
Stage I	T1 N0 M0
Stage IIA	T0 N1 M0, T1 N1 M0, T2 N0 M0
Stage IIB	T2 N1 M0, T3 N0 M0
Stage IIIA	T0-3 N2 M0, T3 N1 M0
Stage IIIB	T4 N0-2 M0
Stage IIIC	Any T N3 M0
Stage IV	Any T Any N M1

The treatment of breast cancer patients involves the local managements such as surgery and radiation therapy (Russell et al. 2004), and the adjuvant systemic therapies including cytotoxic chemotherapy, endocrine therapy and biologic targeted therapy (Zielinski et al. 2005; Robert et al. 2006; Coates et al. 2007). The selection of treatment is based on a number of prognostic and predictive factors such as tumor stage, hormone receptor content, and HER2 status. With the emergence of HER2-targeted therapy, trastuzumab, a humanized monoclonal antibody directed against the extracellular domain of HER2, the clinical response to chemotherapy and outcomes in patients with primary and metastatic breast cancer have been significantly improved (Piccart-Gebhart et al. 2005; Romond et al. 2005). The

recommendation of the incorporation of trastuzumab into adjuvant treatment of breast cancer patients with HER2-positive tumors has been one of the biggest changes in the National Comprehensive Cancer Network (NCCN) guidelines in the past few years (NCCN, 2007). Recently, several ongoing clinical trials of targeted therapy involving lapatinib, a selective inhibitor of both epidermal growth factor receptor (EGFR) and HER2 tyrosine kinase; cetuximab, an antibody directed against EGFR; and bevacizumab, an antibody directed against vascular endothelial growth factor (VEGF) are promising perspectives for patients with hormone receptor and HER2 negative breast cancer (Ramaswamy et al. 2006; Cleator et al. 2007).

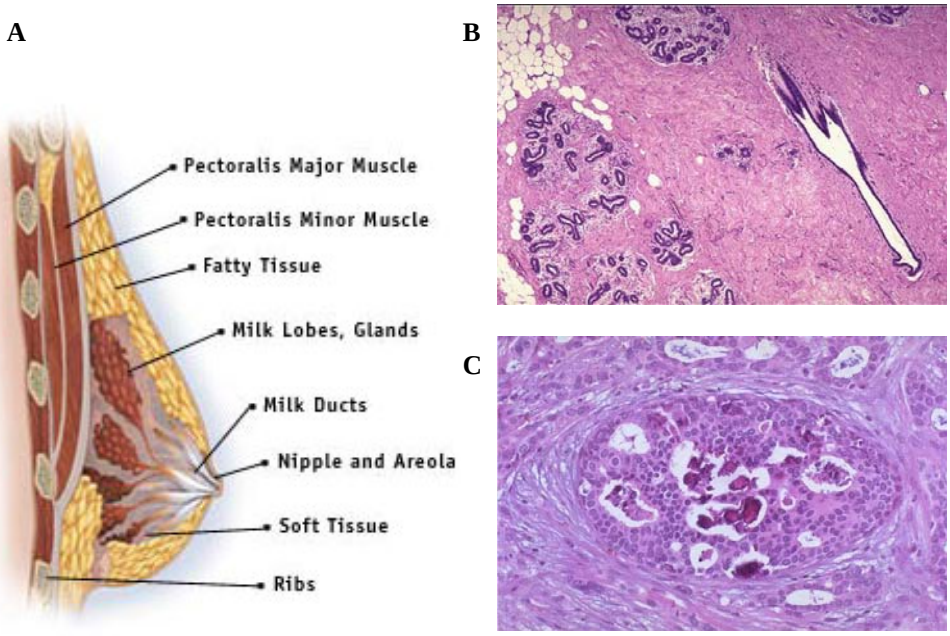


Figure 1.1 Anatomy of breast (A) and H&E staining of normal breast tissue (B) and breast ductal carcinoma (C) with DCIS in the center surrounded by infiltrating tumor cells.

(http://www.mentorcorp.com/global/breast-reconstruction/mastectomy-anatomy.htm and http://www.binglixue.com/image/bre/bre1.asp 29 Mar 2007).

1.2 Metastatic cascade of breast cancer

Metastasis is an exceedingly complex multistep process, during which the disseminated cancer cells from the primary sites spread through the circulatory system, arrest in the capillary bed, resume proliferation and establish secondary tumors in distant organs (Yang et al. 2004; Mehlen et al. 2006). Approximately 10-15% of breast cancer patients have an aggressive disease and develop distant metastases within 3 years after the initial detection of the primary tumor (Weigelt et al. 2005). Once cells are shed from primary breast carcinomas, metastases can be formed in various organs. The most common metastasis sites of breast cancer are bone, lung and liver (Lee et al. 1983).

The prevailing model of metastasis suggests that metastatic capacity of tumor cells is acquired relatively late in tumorgenesis and only occurres in rare subpopulations with advantageous potential (Fidler 2003; van't Veer et al. 2003). Recent findings obtained by gene-expression profiling from human breast carcinoma tissues have challenged this traditional model (van't Veer et al. 2002; Ma et al. 2003; Schmidt-Kittler et al. 2003; Weigeit et al. 2003). The newly understanding of breast cancer is that it is a systemic disease, not just a local disease and the metastatic capacity is an early and inherent property of the breast carcinoma (Weigelt et al. 2005).

The routes of metastasis can be categorized as lymphatic and haematogenous route according to the transportation system via which cancer cells disseminate to the remote organs (Pantel and Brakenhoff 2004; Figure 1.2). These two routes are complementary in the metastasis of breast cancer. In the lymphatic route, cancer cells disseminated from the primary tumor to the lymph nodes through lymphatic vessels initially, and then to distal organs through blood vessels (Cao 2005). The disseminated tumor cells survive in lymph nodes and grow to form a detectable metastatic lesion, whereas tumor cells spreading to distant sites through blood die or keep dormant during the early stages of breast cancer disease (Meng et al. 2004a; Pantel and

Brakenhoff 2004). At later stages, metastasis to distant organs might also result from the cancer cells disseminated from the established lymph-node metastasis.

In the haematogenous route, cancer cells spread from the primary tumor to distant organs through the blood, forming overt metastases by bypassing previous passage through the lymph nodes (Pantel and Brakenhoff 2004). The fact that about 20-30% of breast cancer patients with absence of lymph-node metastasis develop metastatic lesions at distant sites might support this view (Braun et al. 2000a). In bone marrow and peripheral blood, single tumor cells can also be detected in breast cancer patients without manifest metastasis by epithelial-specific anti-cytokeratin (CK) antibodies (Braun et al. 2005; Muller et al. 2005). Results from recent gene-expression profiling studies suggest as well that the lymphatic and the haematogenous route might be governed by different molecular determinants (Woelfle et al. 2003).

The secondary tumor can further metastasize to other organs and corresponding regional lymph nodes through haematogenous dissemination, resulting in metastases at multiple sites (Pantel and Brakenhoff 2004). Breast cancer patients frequently develop multi-organ metastases, which perhaps are due to either primary or secondary distant metastasis. In Minn and colleagues' study, the authors suppose that the breast cancer cells from primary tumors with metastatic capacity have a distinct organ-specific metastatic potential which is controlled by a particular gene-expression profile (Minn et al. 2005).

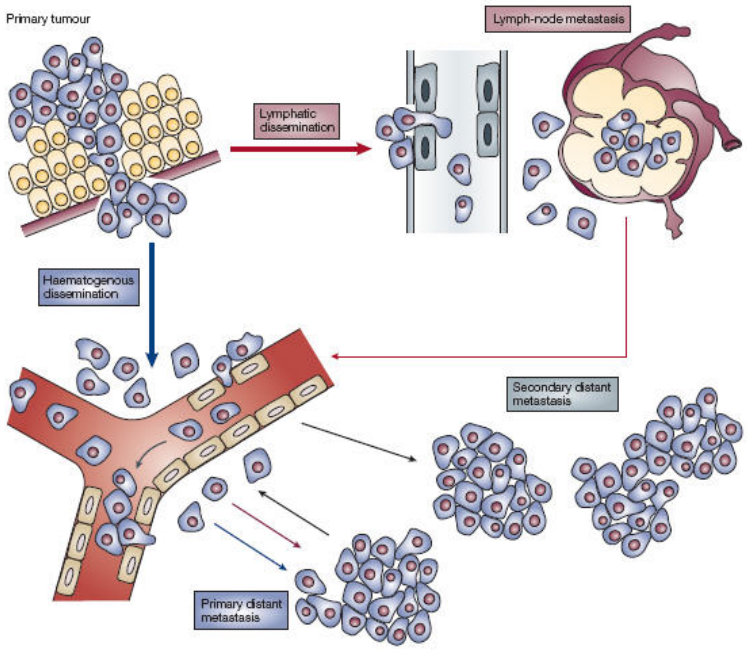


Figure 1.2 Model of the metastatic cascade. Cancer cells can disseminate from the primary tumor to distant organs and tissues via lymphatic routes (red arrows) and haematogenous routes (blue arrows). Secondary tumors can further metastasize to other distant organs through haematogenous dissemination (black arrows) (from Pantel and Brakenhoff 2004).

1.3 Micrometastasis in breast cancer patients

Single tumor cells that spread to regional lymph nodes, peripheral blood and distant organs, such as bone marrow at early-stage disease but can not be detected by conventional staging have been referred to as micromeatastasis (Pantel and Brakenhoff 2004). Peripheral blood represents the haematogenous route for tumor cell dissemination and might therefore become an obvious tissue to study micrometastasis (Balic et al. 2005; Pantel and Panabieres 2007). Alternatively, bone marrow is a prominent metastatic site in breast cancer, and is a major harbor for occult metastatic cells. To date, bone marrow and peripheral blood have been extensively investigated for micrometastasis of breast cancer (Slade and Coombes 2007). Immunocytochemistry (ICC) and reverse transcriptase polymerase chain reaction (RT-PCR) are the two main approaches that are used to detect tumor cells by their

specific protein or marker gene expression, respectively (Zach et al 2006). The presence of circulating tumor cells (CTCs) in peripheral blood or disseminated tumor cells (DTCs) in bone marrow of patients with breast cancer has been proven to be related to the worse clinical outcomes (Braun et al. 2000b, 2005; Wiedswang et al. 2003; Giatromanolaki et al. 2004). The detection of CTCs/DTCs can also be used to monitor the response to the adjuvant therapy (Kasinir-Bauer et al. 2002; Becker et al. 2006).

1.3.1 Methods of tumor cell enrichment

Regardless of the method used for the detection of CTCs/DTCs, the initial step of sample preparation is the typical separation of mononuclear cells and isolated tumor cells from the other cells in the sample. Ficoll density gradient centrifugation is the most common method for tumor cell enrichment (Pfitzenmaier et al. 2003). The tumor cell recovery rate of this enrichment method ranges from 34% to 84% (Rosenberg et al. 2002; Gertler et al. 2003; Choesmel et al. 2004a). Other media similar to Ficoll that are used for the enrichment of tumor cells by density gradient centrifugation are OncoQuick, Biocoll, PolymorphPrep and NycoPrep (Berteau et al. 1998; Baker et al. 2003; Becker et al. 2006).

To improve the recovery rate of tumor cells, immunomagnetic enrichment has been developed (Witzig et al. 2002; Fonseca et al. 2003; Zieglschmid et al. 2005). This method is based on the employment of immunomagnetic beads or ferrofluids conjugated with monoclonal antibodies directed against tumor-associated antigens, such as epithelial cell adhesion molecule (EpCAM), HER2, or EGFR (Chroesmel et al. 2004b; Meng et al. 2004b; Woelfle et al. 2005a; Hager et al. 2005). Due to the heterogeneous nature of CTCs/DTCs, immunomagnetic enrichment methods using a combination of antibodies have a higher recovery rate than those concentrating only to a single antibody (Hager et al.2005). A recently published gene-expression profiling study (Woelfle et al. 2005b) indicated that the immunomagnetic enrichment

using a special antibody coupled beads does not considerably change the gene expression pattern of tumor cells.

1.3.2 Detection of circulating/disseminated tumor cells

To detect rare tumor cells in peripheral blood or bone marrow, methods with a high sensitivity and specificity are required. As stated above, ICC and RT-PCR are the two main approaches that are used to detect tumor cells by their specific protein or marker gene, respectively (Zach et al 2006). For epithelial tumors, such as breast cancer, cytokeratins (CKs) have become the best marker for detection of disseminated or circulating tumor cells (Pantel and Brakenhoff 2004). Cytokeratins belong to the intermediate filament protein family and are ubiquitously expressed by epithelial cells. The A45-B/B3 antibody that predominantly recognizes CK8, CK18 and CK19 has been most commonly used for the detection of CTCs/DTCs by ICC with high sensitivity and specificity (Braun et al. 2000a; Kasimir-Bauer et al. 2002; Muller et al. 2005; Becker et al. 2006). CK has also proven to be the most robust marker for the detection of micrometastasis by RT-PCR, and which is in particular true for CK19 (Slade and Coombes 2007).

Although RT-PCR seems to be more sensitive than ICC, the latter still remains the standard method for the detection of tumor cells in blood or bone marrow (Kruger et al. 2000). One advantage of ICC is that further characterization of CTCs/DTCs can be performed by additional ICC or fluorescence in situ hybridization (FISH) or PCR, which can help to confirm the biological characteristics of CTCs/DTCs (Goeminne et al. 2000), whereas the divergence of sensitivity and specificity between laboratories may be the critical limitation of RT-PCR for the detection of CTCs/DTCs (Goeminne et al. 2000; Silva et al. 2001; Altaras et al. 2002). The other problem associated with RT-PCR is that the unusual expression of tumor-associated transcripts by normal cells could increase the risk of false positives (van Houten et al. 2000). If one considers that this illegitimate expression is very weak, the development of quantitative

RT-PCR assays should help to solve this problem (Pantel and Brakenhoff 2004).

A major step forward in the detection of circulating tumor cells was the development of an automated system (CellSearch™ system, Veridex, Warren, NJ, USA), which uses ferrofluids coated with anti-EpCAM antibodies to enrich EpCAM-positive cells from whole blood. In a second step, these cells are categorized into CK-positive and CK-negative ones by staining with fluorescently labeled monoclonal antibodies against CK. Leukocytes are excluded by staining with an anti-CD45 antibody (a pan-leukocyte marker). Cells are classified as tumor cells when they are nucleated, have a diameter of at least 4μm and are characterized by positivity for CK and negativity for CD45 (Check 2005). The CellSearch™ system has been cleared by the FDA for monitoring patients with metastatic breast cancer. A multicenter validation study by Riethdorf and colleagues (2007) confirmed the reliability of the CellSearch™ system for the measurement of CTCs in metastatic breast cancer patients suggesting that the system might be a suitable tool for routine assessment of therapeutic response in these patients.

1.3.3 Clinical significance of circulating/disseminated tumor cells

The presence of DTCs in bone marrow of breast cancer patients has demonstrated to be an independent prognostic factor for primary breast cancer patients. In a meta-survey analysis involving 4703 patients with stage I-III breast cancer, DTCs were detected in 30.6% of the patients by ICC. The presence of DTCs was a significant prognostic factor with respect to poor overall survival, disease free survival and distant-disease-free survival (Braun et al. 2005). The study of Braun et al. (2005) also revealed that the presence of DTCs correlated with tumor size, lymph node involvement, histological grade and hormone receptor status, which is partly contradictory to the results of a previous study by Fehm and colleagues (2004). The other application of DTCs is the possibility to monitor the therapeutic efficacy of a particular anti-tumor treatment (Braun et al. 2000b). This might help the clinicians to

change the therapeutic regimes for individual patients. However, whether this change will bring benefits to patients is still under investigation.

Although the clinical significance of CTCs in the blood of patients with early-stage disease without overt metastasis is still under investigation, the presence of CTCs in peripheral blood of patients with metastatic breast cancer has been proven to be an independent prognostic factor (Pantel and Panabieres 2007). In the study of Cristofanilli and colleagues (2004), CTC detection with the CellSearch^{TM} system was reported to be a prognostic factor in patients with metastatic breast cancer, and 5 CTCs per 7.5ml blood was determined as a cutoff level of CTC with a prognostic significance. Patients with ≥5 CTCs at baseline (before treatment initiation) and at first follow-up (4 weeks) have a shorter progression-free survival and overall survival than patients without or with less than 5 CTCs (Cristofanilli et al. 2005). The continued follow-up (3 to 5, 6 to 8, 9 to 14, and 15 to 20 weeks) and CTC levels at each follow-up time point were evaluated by Hayes and colleagues (2006). The results of this study indicated that the assessment of CTC levels at "any" subsequent follow-up time points accurately and reproducibly predicted the clinical outcome. The comparison of CTC detection to radiological imaging methods showed that the enumeration of CTCs is an earlier, more reproducible indication of disease status than current serial radiographic imaging evaluation (Budd et al. 2006).

1.4 Characterization of circulating/disseminated tumor cells

Although the clinical significance of CTCs/DTCs has been demonstrated by various studies, the biological characteristics of CTCs/DTCs are largely unknown. In our study, we will characterize CTCs/DTCs with several known prognostic factors of breast cancer including HER2, Ki-67, and hormone receptor. Caspase-cleaved CK18 as an apoptotic marker will be also investigated.

1.4.1 HER2

The HER2 proto-oncogene is located on chromosome 17q21 and encodes an 185kD transmembrane tyrosine kinase receptor, which belongs to subclass I of the receptor tyrosine kinase superfamily comprising four members: HER1 (EGFR), HER2, HER3 and HER4 (Hynes et al. 2005). The overexpression of HER2 has been observed in approximately 18% to 20% of breast cancer cases (Slamon et al. 1987; Wolff et al. 2007) and is associated with poor clinical outcome in node-negative and node-positive breast cancer patients (Press et al.1997; Ross et al. 1999). The HER2 positivity also predicts the resistance to tamoxifen antiestrogen therapy and the response to anthracycline and paclitaxel chemotherapy (Konecny et al. 2004; De Laurentiis et al. 2005; Pritchard et al. 2006). Currently, the most important factor is that the HER2 status of primary tumor can predict the benefit from the therapy with trastuzumab, a humanized monoclonal antibody directed against the extracellular domain of HER2 receptor. Trastuzumab has been demonstrated to improve the clinical outcome when used alone or combined with chemotherapy in metastatic breast cancer patients (Slamon et al. 2001).

The HER2 status can be assessed by immunohistochemistry (IHC) for evaluation of protein overexpression and by FISH for evaluation of gene amplification. In the HER2 testing guideline from the American Society of Clinical Oncology/College of American Pathologists (Wolff et al. 2007), an algorithm defining positive, equivocal, and negative values for HER2 status is recommended (Figure 1.3): a positive HER2 result has been given to IHC staining of 3+ (uniform, intense membrane staining of more than 30% of invasive tumor cells), or a FISH result of more than six HER2 gene copies per nucleus or a FISH ratio (HER2 gene signals to chromosome 17 signals) of more than 2.2. On the other hand, a negative result is an IHC staining of 0 or 1+, a FISH result of less than four HER2 gene copies per nucleus, or a FISH ratio of less than 1.8. Equivocal results require additional action for final determination. Although it is more time consuming, expensive and requires special equipment, FISH remains

the current “gold standard” for the assessment of HER2 gene amplification (Gokhale et al. 2004). Recently, a rapid, easy handling technique, real time PCR, has been proposed for the detection of HER2 gene amplification in breast cancer. The results of HER2 amplification determined by real time PCR are comparable to results obtained by FISH (Gjerdrum et al. 2004; Nistor et al. 2006).

The HER2 status in CTCs/DTCs derived from breast cancer has been analyzed by immunostaining and FISH. The results of these studies indicate that the presence of HER2 positive CTCs in blood or HER2 positive DTCs in bone marrow is associated with poor clinical outcomes in stage I-III breast cancer patients (Braun et al. 2001; Wulfing et al. 2006). Interestingly, the conversion of the HER2 status in CTCs, from HER2 negativity in the primary tumor to HER2 positivity in CTCs has been also reported, supporting the possibility that HER2 gene amplification can be acquired during the progression of breast cancer (Hayes et al. 2002; Meng et al. 2004b; Wulfing et al. 2006). Another possibility is that the HER2-positive CTCs/DTCs are due to heterogeneity in the primary tumor being also HER2-negative when less than 10% of HER2-positive cells are present. However, it is unknown whether this conversion can be turned to clinical benefit.

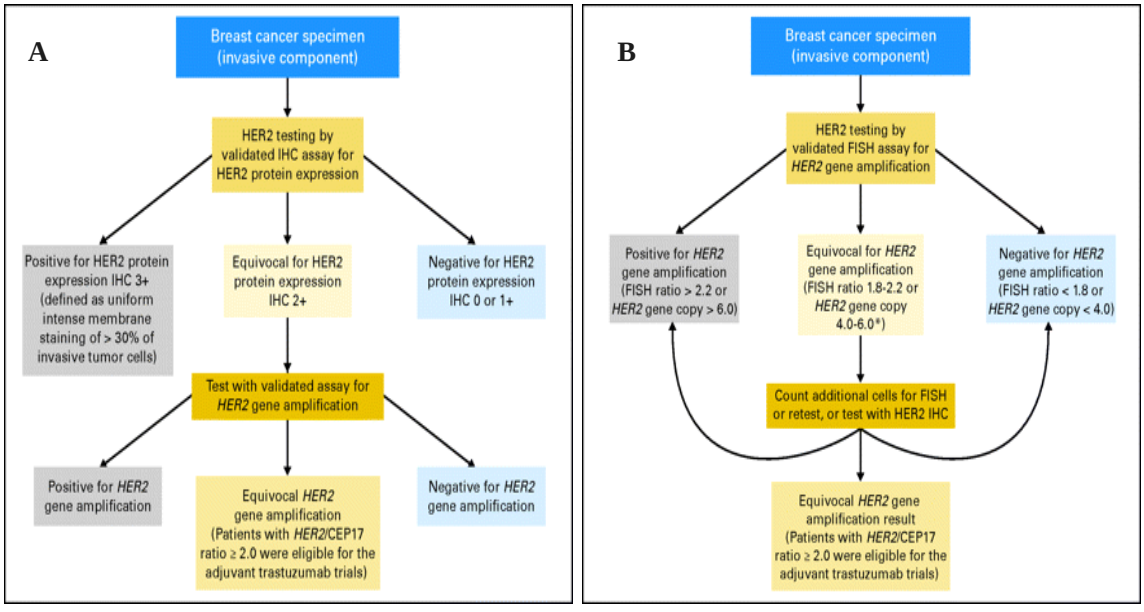


Figure 1.3 Algorithm for HER2 testing. A, algorithm for immunohistochemistry. B, algorithm for FISH (from Wolff et al. 2007).

1.4.2 Proliferation marker: Ki-67

Ki-67 protein, a proliferation marker, is present in the nuclei of cells in all active phases of the cell cycle (G1, S, G2, and mitosis), but absent in the quiescent or resting cells in the G0 phase (Colozza et al. 2005). This antigen can be detected by immunostaining and the different staining patterns reflect the different cell cycle phases. The Ki-67 staining is localized principally in nucleoli in G1 phase, and the nucleoplasmic staining increases with cell cycle progression as well as a strong perichromosomal staining is present during mitosis (Verheijen et al. 1989; du Manoir et al. 1991).

Among several proliferation markers, Ki-67 revealed to be the most frequently used protein in breast cancer analysis (Schindlbeck et al. 2005). Many studies have shown that the evaluation of Ki-67 in primary breast cancer is an independent prognostic factor for disease-free survival and overall survival (van Diest et al. 2004; Colozza et al. 2005). Rare detection of Ki-67 expression in CTCs/DTCs of breast cancer patients indicates that most of these disseminated tumor cells remain in a dormant state in peripheral blood and bone marrow (Pantel et al. 1993; Muller et al. 2005).

1.4.3 Apoptotic marker: caspase-cleaved cytokeratin 18

The programmed cell death is called apoptosis, which is usually committed by the activation of a specific family of cysteine proteases called caspases (Salvesen et al. 1997). As showed in Figure 1.4, during apoptosis, CK18 cleavage at the ^{393}DALD/S site by caspases 3, 7, and 9 is an early event, which generates a fragment of approximately 45kD. A second cleavage at the L1-2 linker region by caspase 6 is responsible for the final collapse of the keratin skeleton into large aggregates, generating a fragment of 20 kD (Schutte et al. 2004). The monoclonal antibody M30 can identify these newly generated ~45kD and 20kD fragments by detecting a 10-residue epitope of CK18 on the liberated C terminus as a cleavage at ^{393}DALD/S

site (Leers et al. 1999; Schutte et al. 2004). Consequently, detection of caspases-cleaved CK18 by M30 can show a wide spectrum of apoptotic processes from early stage to late stage. With double immunofluorescence, Grassi and colleagues (2004) reported that the appearance of the M30 neoepitope corresponds to the gradual disappearance of cytoskeleton cytokeratins and to the presence of nuclear DNA fragmentation.

Circulating tumor cells in peripheral blood have a short lifespan measured in hours (Meng et al. 2004a). Their death is correlated to apoptosis. In fact, most of CTCs exhibit apoptotic morphologic changes (Marrinucci et al. 2007). Larson and colleagues (2004) further confirmed this phenomenon by characterizing CTCs with an M30 antibody. In their study, 74% of CTCs were positive for M30. The identification of apoptotic CTCs/DTCs may provide relevant information regarding the therapeutic response in cancer patients. In the study of Fehm and colleagues (2006), apoptotic DTCs determined by the M30 antibody could be detected in 23% (36/157) of breast cancer patients after neoadjuvant chemotherapy. The presence of only apoptotic DTCs was higher in responding patients than in non-responding patients, whereas no apoptotic DTC could be detected in patients with progressive diseases.

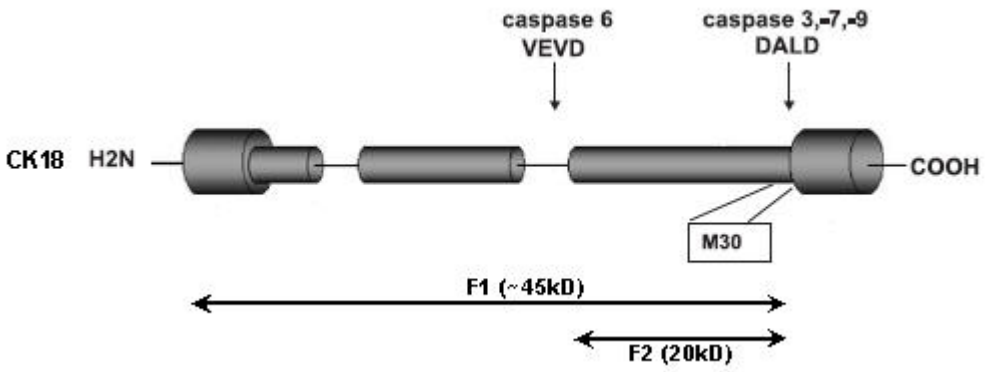


Figure 1.4 Schematic presentation of the CK18 structure, caspases cleavage sites and M30 neoepitope location (modified from Bantel et al. 2000; Schutte et al. 2004).

1.4.4 Hormone receptor

The importance of the hormone receptors (estrogen receptor [ER] and progesterone receptor [PR]) in breast cancer has been clearly shown for many years. Approximately 75% of breast cancers express ER, and more than half of these cancers also express PR (McGuire 1978). Patients with ER-positive/PR-positive tumors have a much better prognosis than patients with ER-negative/PR-negative tumors (Collett et al. 1996). However, the prognostic value of both ER and PR might be lost after long-term follow-up (5 years) (Bardou et al. 2003). Therefore, the hormone receptor status is just a weak prognostic factor in breast cancer (Hayes et al. 2001).

The hormone receptor status is a strong predictive factor for response to endocrine therapy. Adjuvant tamoxifen therapy can significantly decrease the recurrence and death in breast cancer patients with ER-positive tumors (Early Breast Cancer Trialists' Collaborative Group 2005). Other trials prospectively designed to test the efficacy of tamoxifen in ER-negative tumors further confirm the role of ER as a predictor of response to endocrine therapy (Hutchins et al. 1998; Fisher et al. 2001). Similarly, PR is also a powerful predictor of response to endocrine therapy (Cui et al. 2005). Furthermore, PR-negativity predicts *de novo* (intrinsic) resistance to tamoxifen in ER-positive patients (Tovey et al. 2005).

The evaluation of the hormone receptor status of primary breast carcinoma has been a routine work for providing information on selection for endocrine therapy. However, the expression of hormone receptor in circulating/disseminated tumor cells is unknown thus far.

The major aim of the present study was to establish and optimize a set of methods including immunofluorescence, FISH and real time PCR to further characterize CTCs for HER2, ER, PR, Ki-67 and M30 expression after processing blood samples from breast cancer patients with the CellSearch^{TM} system. The second aim of the study was

to evaluate the HER2 expression in CTCs with the help of the CellSearch™ system, and to document the correlation between the HER2 status and the staining patterns obtained by the CellSearch™ system. Additionally, whole genome amplification (WGA) on DNA derived from processed cells and the suitability of generated amplification products for real time PCR were determined. WGA from single cells might provide sufficient DNA materials for further experiments such as real time PCR and comparative genomic hybridization to discern genetic aberrations in CTCs.

2. Materials and methods

2.1 Materials

2.1.1 Reagents

2× qPCR Master Mix for Probe Assay	Eurogentec, Kologne, Germany
2× SYBR Green PCR Master Mix	Qiagen, Hilden, Germany
Acetic Acid	Merck, Hohenbrunn, Germany
Acetone	J.T.Baker, Deventer, Holland
Acrylamide/Bisacrylamide (19:1, 40%)	Appligene Oncor, Illkirch, France
Agar-Agar	Carl Roth, Karlsruhe, Germany
Ammonium Persulfate	Serva, Heidelberg, Germany
Ampicillin	Carl Roth
Antibiotic/Antimycotic	PAA Laboratories, Pasching, Austria
Antibody Diluent	Dako, Carpinteria, CA, USA
Antibody Diluent with Background Reducing Components	Dako
Bacto tryptone	BD, Sparks, MD, USA
Boric Acid	Carl Roth
Bovine Serum Albumin	Sigma-Aldrich, Steinheim, Germany
Calcium Chloride ($CaCl_2$)	Merck

Cot Human DNA	Roche, Mannheim, Germany
DEME	Gibco, Grand Island, NY, USA
DEME/Ham's F-12	PAA laboratories
Demecolcine	Sigma-Aldrich
Dextran Blue	Fluka, Steinheim, Germany
Digoxigenin-11-dUTP	Roche
Dimethyl sulfoxide (DMSO)	Serva, Heidelberg, Germany
di-Potassium Hydrogen Phosphate (K_2HPO_4)	Merck
Dithiothreitol (DTT)	Merck
DNA Molecular Weight Marker VIII,VII,IV	Roche
Ethanol	J.T.Baker
Fetal Calf Serum	PAA Laboratories
Ficoll-Paque Plus	Amersham Biosciences, Uppsala, Sweden
Fixation Solution B (37% Formaldehyde)	Micromet AG, Munich, Germany
Fixogum Rubber Cement	Marabuwerke GmbH, Tamm, Germany
Formamide	Fluka
GelStar Gel stain	Cambrex Bio Science, Rockland, ME, USA
Glycerol	Carl Roth
Guanidine Isothiocyanate (GITC)	Fluka
Hank's Salt Soulition	Biochrom AG, Berlin, Germany
Human Erythrocyte Lyse Buffer	R&D Systems, Minneapolis, MN,USA
Human Placental DNA	Sigma-Aldrich
Hydrochloric Acid (HCl)	Merck
Insulin-Transferrin-Selenium A	Gibco
IPTG	Carl Roth

L-Glutamine 200mM	Gibco
Magnesium Chloride ($MgCl_2$)	Sigma-Aldrich
Magnesium Sulfate ($MgSO_4$)	Merck
Manganese Chloride ($MnCl_2$)	Merck
MetaPhor® Agarose	FMC Bioproducts, Rockland, ME, USA
Methanol	Carl Roth
NBT/BCIP Stock Solution	Roche
PBS, Dulbecco's	Gibco
Peptone	Merck
PIPES	Carl Roth
Potassium Chloride (KCl)	Sigma-Aldrich
Potassium Chloride Solution 0.075M	Sigma-Aldrich
Potassium Dihydrogen Phosphate (KH_2PO_4)	Merck
Protein Block Serum-Free Ready-To-Use	Dako
Protein Block Serum-Free Ready-TO-Use	Dako
REAL™ Target Retrieval Solution (10×, pH6,0)	Dako
RPMI 1640	Gibco
Seakem® LE Agarose	Cambrex Bio Science
Sodium Acetate	Merck
Sodium Chloride (NaCl)	Carl Roth
Sodium Dihydrogen Phosphate Dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	Merck
Sodium Hydroxide (NaOH)	Merck
Spectrum Green-dUTP	Vysis, Downers Groves, IL, USA
Spectrum Orange-dUTP	Vysis
tri-Sodium Citrate Dihydrate	Merck
Triton X-100	Sigma-Aldrich

TRIZMA® base (Tris)	Sigma-Aldrich
Trypan Blue 0.4% Solution	Sigma-Aldrich
Trypsin-EDTA	Gibco
Tween 20	Promega, Modison, WI, USA
Vectabond® Reagent	Vector Laboratories, Burlingame, CA, USA
Vectashield® Mounting Medium with DAPI	Vector Laboratories
X-gal	Carl Roth
Xylene	Fluka
Yeast Extract	Carl Roth
β-Mercapto-Ethanol	Sigma-Aldrich

2.1.2 Antibodies

Antibody	Working dilution	Source
Alexa 488, goat anti-mouse IgG (H+L)	1:200	Molecular Probes, Eugene, OR, USA
Alexa 546, goat anti-mouse IgG (H+L)	1:200	Molecular Probes
Anti-DIG-AP Fab fragment	1:5000	Roche, Mannheim, Germany
Anti-DIG-Fluoresin	1:200	Roche
Anti-DIG-Rhodamine	1:200	Roche
CK 18 (Clone M30, FITC)	1:250	Roche
ER (Clone 6F11)	1:50	Novocastra, Newcastle, UK
HER2/neu (Clone CB11)	1:50	Novocastra
Ki-67 (Clone MIB-1)	1:50	Dako, Carpinteria, CA, USA
Ki-67 (Clone Ki67, FITC)	1:10	Dako
Mouse IgGκ (MOPC-21)	1:2000	Sigma-Aldrich,Steinheim,Germany
Pan-cytokeratin (Clone A45-B/B3,Cy3)	1:300	Micromet AG, Munich, Germany

PR (Clone 1A6)	1:50	Novocastra

2.1.3 Enzymes

DNA Polymerase I	Roche, Mannheim, Germany
DNase I	Roche
Pepsin ready-to-use	Zymed Laboratories, San Francisco, CA, USA
Proteinase K	Roche
Restriction Endonuleases	New England Biolabs, Beverly, MA, USA
RNase A	Roche

2.1.4 Kits

CellSearch™ Epithelial Cell Kit	Veridex LLC, Warren, NJ, USA
CellSearch™ Tumor Phenotyping Reagent HER2/neu	Veridex LLC
CEP® Chromosome Enumeration DNA FISH Probes	Vysis, Des Plaines, IL, USA
DNA Blood Mini Kit	Qiagen, Hilden, Germany
Large Construct Kit	Qiagen
HER2 FISH pharmDx™ Kit	Dako, Carpinteria, CA, USA
Bioprime® DNA Labeling System	Invitrogen, Carlsbad, CA, USA
Silver Stain Plus Kit	Bio-Rad, Hercules, CA, USA
GenomiPhi DNA Amplification Kit	GE Healthcare, Freiburg, Germany

2.1.5 Real time polymerase chain reaction (PCR) primers and probes

HER2 (product size: 93bp)

Forward primer: 5-CTG CTG GTC GTG GTC TTG G-3

Reverse primer: 5-CTG CAG CAG TCT CCG CAT C-3

Probe: 5-FAM-CTC ATC AAG CGA CGG CAG CAG AAG AT-BHQ1-3

In this study, we designed a pair of primers for 17q11.2 region, which was so-called Contr17.

Contr17 (product size: 99bp)

Forward primer: 5-TGG CCA GTG GCA CAA TCC-3

Reverse primer: 5-ACC AAG TGC CGT GCT GAT G-3

Probe: 5-Yakima Yellow-ACA GAG CTC CAC CTC AAC CAC TTG GCA-BHQ1-3

All these oligonucleotides were designed by Primer Express 2.0 (Applied Biosystem, Foster City, CA, USA) and purchased from Operon (Cologne, Germany) and Eurogentec (Seraing, Belgium).

2.1.6 Equipments and accessories

Centrifuges

Rotofix 32	Hettich, Tuttlingen, Germany
Centrifuge 5417R	Eppendorf, Hamburg, Germany
Heraeus Biofuge pico	Thermo Scientific, Langenselbold, Germany
Heraeus Multifuge 3S-R	Thermo Scientific
Sorvall RC5C plus	Thermo Scientific

Microscopes

Axiostar plus	Carl Zeiss, Gottingen, Germany
Axioplan 2 imaging	Carl Zeiss
Leica DM LB	Leica, Wetzlar, Germany
Inverted Microscope	Hund Wetzlar, Wetzlar, Germany

Others

Neubauer improved counting chamber	Marienfeld, Lauda-Konigshofen, Germany
Hybond^{TM}- N^+ Membrane	Amersham Biosciences, Little Chalfon, UK
Bio-Spin® 30 Tris Column	Bio-Rad Laboratories, Hercules, CA, USA
NucleoSEQ Column	Macherey-Nagel, Duren, Germany
Peltier Thermal Cycler PTC-200	Biozyme, Hessisch Oldendorf, Germany
Decloaking Chamber	Biocare Medical, Walnut Creek, CA, USA
Thermomixer comfort	Eppendorf, Hamburg, Germany
Spectrophotometer ND 1000	NanoDrop,Wilmington, DE, USA
Microcon Centrifugal Filter YM-30	Millipore, Billerica, MA, USA
CellSearch^{TM} system	Veridex LLC, Warren, NJ, USA
Mastercycler ep realplex 4.0	Eppendorf

2.1.7 Cell lines

Breast cancer cell lines included SK-BR-3(ATCC HTB-30), MDA-MB-468(ATCC HTB-132), MDA-MB-231(ATCC HTB-26), MDA-MB-435s (ATCC HTB-129), T47D (ATCC HTB-133), MCF7 (ATCC HTB-22) and BT474 (ATCC HTB20), provided by the Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf.

2.1.8 BAC DNA

The human genomic clone RP11-94L15(GenBank number: AC079199) was chosen as it encompassed the HER2 gene. It was purchased from RZPD German Resource Center for Genome Research (Berlin, Germany).

2.1.9 Tumor specimens

One formalin-fixed paraffin-embedded pleural effusion specimen from a breast cancer

patient and slides with 2µm sections from eleven formalin-fixed paraffin-embedded breast tumor specimens with known HER2 FISH status were obtained from the Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf.

The examination of blood samples was approved by the local ethics review and all enrolled patients gave their informed consent.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Culture medium

For cultivation of the cell lines SK-BR-3, MCF7 and MDA-MB-231 DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% (v%v) fetal calf serum (FCS), 2mM L-Glutamine and 1% Antibiotic/Antimycotic solution was used.

For cultivation of the cell line T47D RPMI-1640 supplemented with 10% FCS, 2mM L-Glutamine and10µg/ml Insulin was used.

For cultivation of the cell line MDA-MB-468 DMEM supplemented with 5% FCS, 2mM L-Glutamine and 1% Antibiotic/Antimycotic was used.

For cultivation of the cell line MDA-MB-435S DMEM supplemented with 10% FCS and 2mM L-Glutamine was used.

For cultivation of the cell line BT474 DMEM-Ham's F12 supplemented with 10% FCS was used.

2.2.1.2 Cell culture

All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. When confluency had reached 70%~80%, cells were sub-cultured. Culture medium was removed and discarded. Cells were rinsed by pre-warmed DPBS once and then incubated with Trypsin/EDTA for 3~5min at 37°C. When nearly all cells were detached, medium containing FCS was added to neutralize Trypsin/EDTA. Cells were collected after a centrifugation at 1200rpm (240×g) for 5min and resuspended in the medium. Aliquots of the cell suspension were added to new culture flasks.

Cells were preserved in the appropriate medium containing 10% DMSO. The cryotubes labeled with the cell line name, passage number and date were placed into a -80°C freezer overnight and then transferred to the vapor phase of a liquid nitrogen storage tank.

2.2.2 Enrichment of blood by Ficoll

Ten to twenty milliliter peripheral blood were filled up to 50ml with Hank's solution and centrifuged at 1400rpm (400×g) for 10min. Then the supernatant with lipid phase was carefully removed and the sediment was filled up with cold DPBS to 30ml. 20ml Ficoll-Paque Plus were added into a new Falcon tube and carefully overlaid with the diluted sample. After centrifugation at 1400rpm (400×g), 4°C for 30min, the upper layer and the interface (lymphocyte layer) were immediately carefully transferred into a new Falcon tube and then filled up to 50ml with DPBS. After careful mixing, samples were centrifuged at 1400rpm (400×g), 4°C for 10min. Supernatant was removed and cells were resuspended in 5 to 10ml DPBS. However, if there were too many erythrocytes in the pellet, an erythrocyte lysis should be performed. In this case, cells were resuspended in 1ml H-lyse buffer and incubated for 3min under gentle shaking. Then 30ml DPBS were added and samples were spun at 1400rpm (400×g), 4°C for 10min again. Supernatant was removed and cells were resuspended in 5 to

10ml DPBS.

Ten microliter of cell suspension were mixed with 10µl of trypan blue solution and counted in a Neubauer improved counting chamber. Subsequently, cytospins were prepared with 700,000 cells per slide by a spin for 5min at 1200rpm (180×g). The slides were air-dried overnight at room temperature and stored at -80°C.

2.2.3 Immunomagetic enrichment of epithelial cells from blood by the CellSearch™ system

The CellSearch™ system (Veridex) consists of CellSave sample tubes (Immunicon) for preserving and transporting blood samples, the CellSearch™ epithelial cell kit (Veridex) containing all of the reagents and consumables for conducting the test, CellSearch™ control cells (Veridex) for assuring proper performance on a daily or run-to-run basis, an automated instrument for adding reagents and washing cells (CellTracks® AutoPrep system, Veridex), and a semi-automated microscope for scanning and reading results (CellSpotter® Analyzer, Veridex).

Peripheral blood (7.5ml) is drawn into a CellSave sample tube and transferred to a 15ml conical tube (provided with the CellSearch™ kit). Dilution Buffer (6.5ml) is added to the blood sample and samples are mixed well by inversion 5 times. The sample is processed by the CellTracks® AutoPrep System within 1 hour after a centrifugation at 800×g for 10min. During the AutoPrep System procedure, firstly, plasma is aspirated and ferrofluids conjugated with anti-EpCAM are added. Secondly, immunomagnetic labeled cells are collected in a magnetic field and unlabeled cells are aspirated when magnets engaged. Thirdly, magnets are removed and cells are resuspended. Subsequently, staining reagents including 4',6-diamidino-2- phenylindole (DAPI), anti-CK-phycoerythrin (PE) and anti-CD45- allophycocyanin (APC) are added. At the end, the enriched sample is dispensed into a cartridge that is inserted into a MagNest®, a fixture of two magnets held together by steel. The strong magnetic

field of the MagNest® causes the magnetically labeled epithelial cells to move to the surface of the cartridge. The trapped cells are scanned by the CellSpotter® Analyzer.

The circulating tumor cells are classified based on morphology and correct phenotype. A cell is considered as a CTC when it is CK-PE positive, DAPI positive and CD45-APC negative. The cells are usually round or oval, but may sometimes be polygonal or elongated. The nuclear area should be smaller than the cytoplasmic area and more than 50% of the nucleus has to be surrounded by the cytoplasm. CTC must have a diameter of at least 4μm.

2.2.4 Vectabond-coated slide preparation

Firstly, Vectabond™ Reagent treatment solution was prepared by adding the entire content (7ml) of the bottle to 350ml of acetone and stirring both well. Subsequently, SuperFrost/Plus slides in slide racks were immersed in acetone for 5min. After being removed from acetone, slides were immediately placed in Vectabond™ Reagent solution for 5min. The racks were then gently dipped several times over 30 seconds in distilled water by changing the water for every 5 racks in order to eliminate excess of reagent. Slides were removed from the rack and air dried thoroughly at 37°C. At this point, gentle agitation or tapping of the rack before allowing drying will decrease water droplets and their resulting spots. Slides were stored at room temperature in boxes, labeled with VC.

2.2.5 Immunofluorescence (IF)

In this study, breast cancer cell line cells obtained after the CellSearch™ run are called *processed cells*, while breast cancer cell line cells directly taken from the cell culture flasks are called *native cells*.

2.2.5.1 Membranous antigen staining

2.2.5.1.1 IF staining for native cells

Prior to fixation with 0.5% formaldehyde (Fixation solution B, dilution 1:74) areas on the slides where the cells were located were circled with Dako Pen, followed by blocking with Protein Block Serum-Free Ready-To-Use (DAKO Cytomation) for 20min. Subsequently, cells were incubated with the first primary antibody (HER2) for 45min at room temperature. The antibody was visualized by a goat anti-mouse IgG conjugated with the fluorochrome Alexa 488. For double staining, another blocking step was required. The second primary antibody- A45-B/B3 directly conjugated with Cy3- was incubated for 45min at room temperature. The nuclei were stained with DAPI. Washing of slides by TBST buffer 3×3min was conducted between each step except that between blocking and application of the primary antibody. The primary antibody was diluted with Antibody Diluent (DAKO Cytomation), and the secondary antibody was diluted with Antibody Diluent with Background Reducing Components (DAKO Cytomation).

2.2.5.1.2 IF staining for processed cells

After the CellSearch^{TM} system run and the CellSpotter$^{®}$ analysis, cells were removed from the cartridge by using a 21 gauge needle to scrap the wall of the cartridge. The cell suspension was directly placed onto a slide, and the slide was dried completely at 37°C for at least two hours.

The directly labeled primary antibodies, A45-B/B3 conjugated with Cy3 or HER2 conjugated with FITC, were incubated for 45min at room temperature after rinsing the slides with TBST 2×3min. Subsequently, slides were washed with TBST buffer for 3×3min and stained with DAPI.

2.2.5.2 Nuclear antigen staining

2.2.5.2.1 IF staining for native cells

Cells were fixed with acetone at room temperature for 5min, followed by blocking with Protein Block Serum-Free Ready-To-Use for 20min. Cells were then incubated with the first primary antibody (ER, PR, or Ki-67) for 45min at room temperature. These immunoreactions were visualized by goat anti-mouse IgG conjugated with Alexa 488. For double staining, repeated blocking was required. The second primary antibody- A45-B/B3 directly conjugated with Cy3- was incubated for 45min at room temperature. The nuclei were stained with DAPI. Washing of slides by TBST buffer for 3×3min was conducted between each step except that between blocking and application of the primary antibody. The primary antibody was diluted with Antibody Diluent, and the secondary antibody was diluted with Antibody Diluent with Background Reducing Components.

2.2.5.2.2 IF staining for processed cells

A heat induced antigen retrieval technique was adopted for the detection of nuclear antigens in processed cells.

After fixation with -20°C acetone for 5 min and rinsing with PBS briefly, slides were put into the boiled citrate buffer of pH 6.0 (Dako REAL Target Retrieval Solution, 1×), and simmered for 10min in a microwave oven by setting the power to140-150W. Slides remained in buffer for 20min for cooling down, followed by a brief wash step with distilled water. Before incubation with the antibody Ki-67 directly conjugated with FITC for 45min at room temperature, blocking was conducted with Protein Block Serum-Free Ready-To-Use for 20min. Subsequently, slides were washed twice with TBST for 3min each and once with PBS for 3min. Counterstaining of nuclei was performed with anti-fading DAPI Vectashield.

10×PBS-buffer pH 7.4

NaCl	90g
$Na_2HPO_4 \times 2H_2O$	14.33 g
KH_2PO_4	2.67g
Distilled H_2O	to 1000ml

1×PBS-buffer pH 7.4 dilute 10×PBS-buffer pH 7.4 1:10 with distilled water

10×TBST pH 7.6

Tris	181.65g
NaCl	262.98g
Tween-20	15g
25%HCl	170ml
Distilled H_2O	to 3000ml

1×TBST pH 7.6 dilute 10×TBST pH 7.6 1:10 with distilled water

2.2.6 Fluorescence in situ Hybridization (FISH)

2.2.6.1 BAC DNA isolation

BAC DNA was isolated with the help of the Large-Construct Kit (Qiagen) according to the manufacturers' instructions as described below.

1) A single colony from a freshly streaked selective plate was isolated and a starter culture of 5ml TB medium containing 25µg/ml chloramphenicol was inoculated, followed by incubation for at least 8h at 37°C with vigorous shaking (~300rpm).
2) 0.5-1.0ml of the starter culture were diluted into 500ml selective TB medium (1/500 to 1/1000 dilution) and bacteria were propagated at 37°C for 12-16h with vigorous shaking (~300rpm).

3) The bacterial cells were harvested by centrifugation at 6000 ×g for 15min at 4°C. All traces of supernatant were removed by inverting the open bottle until all medium had been drained.

4) The bacterial pellet was resuspended in 20ml Buffer P1. Buffer P1 and Buffer P3 should be placed on ice previously.

5) Twenty milliliter of Buffer P2 were added into the solution. Subsequently samples were mixed gently but thoroughly by rotating and inverting 4~6 times at room temperature for no more than 5min.

6) When solution became clean, 20ml chilled Buffer P3 were added to stop the lysis reaction. Samples were mixed immediately but gently by inverting 4~6 times, and incubated on ice for 10min.

7) Samples were centrifuged at ≥ 20,000× g for 30min at 4°C.

8) Supernatant containing BAC DNA was removed and filtered through a folded filter prewetted with distilled water.

9) DNA was precipitated by adding 0.6 volumes room-temperature isopropanol to the cleared lysate followed by mixing and centrifugation immediately at ≥ 15,000× g for 30min at 4°C. The supernatant was carefully decanted.

10) DNA pellet was washed with 5ml room-temperature 70% ethanol, and centrifuged at ≥ 15,000× g for 15min at 4°C. The suspernatant was carefully decanted without disturbing the pellet.

11) The tube containing the DNA pellet was placed upside down on a paper towel for allowing the DNA to air-dry for 2-3min. After any additional liquid visible on the tube opening was carefully removed, the DNA was carefully redissolved in 9.5ml Buffer EX by very gentle shaking.

12) 200µl ATP-Dependent exonuclease and 300µl ATP solution were added to the dissolved DNA, and mixed gently but thoroughly, and incubated in a water bath or heating block at 37°C for 60min.

13) Equilibration of a QIAGEN-tip 500 was carried out by applying 10ml Buffer QBT, and the column was empty by gravity flow.

14) 10ml Buffer QS were added to the DNA sample from step 12, then the whole

sample was applied to the QIAGEN-tip, and allowed to enter the resin by gravity flow.

15) The QIAGEN-tip was washed with 2 × 30ml Buffer QC.

16) DNA was eluted with 15ml Buffer QF, prewarmed to 65°C. The eluate was collected in a 50ml Falcon polypropylene tube with 10.5ml (0.7 volumes) room-temperature isopropanol.

17) The tube was placed on ice for 1h in order to increase precipitation. The eluted DNA was aliquoted in 1.5ml Eppendorf tubes and centrifuged at ≥ 15,000×g for 30min at 4°C. The supernatant should be carefully decanted.

18) DNA pellet was washed with 0.5-0.7ml room temperature 70% ethanol in each tube, and centrifuged at ≥ 15,000× g for 15min at 4°C. The supernatant was carefully decanted without disturbing the pellet.

19) DNA pellet was dried in a speedvac by virtue of a vacuum-desiccator. The pellet should not be overdried, otherwise DNA will be difficult to redissolve.

20) The DNA was redissolved in a suitable volume (10 or 20µl) of buffer (normally, TE buffer, pH 8.0).

21) DNA concentration was determined by the Spectrophotometer ND 1000 (NanoDrop).

22) DNA was stored at -20°C.

LB Agar

Peptone	10g
Yeast Extract	5g
NaCl	10g
Agar-Agar	15g
Distilled H_2O	to1000ml

Autoclave and cool to 45°C, then add following reagents into solution and mix well.

Ampicillin (100mg/ml)	1000µl	end concentration: 100µg/ml
IPTG (200mg/ml)	200µl	40µg/ml
X-Gal (20mg/ml)	2000µl	40µg/ml

Terrific Broth (TB) medium

Solution 1

Tryptone (or Peptone) 12g

Yeast Extract 6 g

Glycerol 5 g

Distilled H_2O to 900ml

Solution 2 pH 7.2

KH_2PO_4 2.31g

K_2HPO_4 12.54g

DistilledH_2O to 100ml

Solution1 and Solution 2 are prepared and autoclaved separately and combined afterwards.

Buffers

Buffer P1 (resuspension buffer)	50mM Tris·Cl, pH 8.0 10mM EDTA 100μg/ml RNase A
Buffer P2 (lysis buffer)	200mM NaOH 1% SDS
Buffer P3 (neutralization buffer)	3.0M potassium acetate pH 5.5
Buffer QBT (equilibration buffer)	750mM NaCl 50mM MOPS 15% isopropanol 0.15% Triton X-100 pH 7.0
Buffer QC (wash buffer)	1.0M NaCl 50mM MOPS 15% isopropanol pH 7.0

Buffer QF (elution buffer)	1.25M NaCl 50mM Tris·Cl 15% isopropanol pH 8.5
Exonuclease Solvent	20mM KCl 20mM KPO_4 pH 7.5
TE buffer	10mM Tris·Cl 1mM EDTA pH 8.0

2.2.6.2 Digestion by restriction endonucleases

DNA (2µg) was mixed with 2µl *Eco*RI or *Xho*I respectively and 2.5µl enzyme buffer (10×) in a 0.5ml reaction tube, BSA addition was optional according to the enzyme used. The total volume of the reaction mixture was 25µl. The mixture was incubated at 37°C overnight. After incubation, the mixture was heated to 65°C for 20min to stop the reaction. The sample was cleaned by NucleoSEQ column for late real time PCR application. Alternatively, 6× DNA loading buffer was added directly to the sample and a digestion map for BAC DNA was generated by agarose gel electrophoresis.

2.2.6.3 Probe labeling by nick translation

Solutions

10× NT-Buffer	0.5M Tris·HCl 50mM $MgCl_2$ 0.5mg/ml BSA pH 7.5

0.1M β-Mercaptoethanol	0.1ml β-Mercaptoethanol 14.4ml distilled H_2O
DNase Ⅰ	1mg DNase Ⅰ 0.5ml NaCl (0.3M) 0.5ml Glycerin
dNTP-Mix (ACG-Mix)	0.5Mm dATP 0.5Mm dCTP 0.5Mm dGTP 0.1Mm dTTP

Nick-Translation-Mix

10× NT-Buffer	6µl
0.1M β-Mercaptoethanol	6µl
ACG- Nucleotide-Mix	6µl
Biotin-16-dUTP(1mM) **or**	1.2µl
Digoxigenin-11-dUTP(1mM)	
DNA-Polymerase I	1.2µl
DNase I (1:75)	1.2µl

DNA-Solution

DNA	2µg
Distilled H_2O	to 32µl

1) 32µl DNA were mixed with 18µl nick- translation mix and incubated at 15°C for 45 min.

2) Sample was placed on ice and analyzed by separating a 1.5µl aliquot on a 1.2% high melting agarose gel.

3) If the mean fragment size was below 200bp a new sample should be prepared using a lower concentration of DNase I.

4) If the mean fragment size was higher than 500bp, 1µl DNase I should be added and the sample should be incubated for another 15min before analyzing a 1.5µl sample on a gel.

5) If the mean fragment size was 200-500bp 0.05M EDTA could be added to the sample, which was subsequently incubated for 20min at 70°C to stop the enzyme reaction. Subsequently, the sample was cleaned up.

6) The sample was cleaned up with Microcon centrifugal filter device YM-30 according to the manual. Briefly, the sample was pipetted into the reservoir and centrifuged for 15min at 9000rpm (Biofuge Pico, Heraeus). Subsequently reservoir was washed with 100-200µl water followed by centrifugation again for 5min. 200µl water was added to reservoir after centrifugation. Reservoir was placed upside down in a collection tube, and then spun 1min at 9000rpm (Biofuge Pico). The eluted DNA was stored at -20°C for later use.

2.2.6.4 Probe labeling by random priming

1) 1µg DNA was dissolved in 18µl water.
2) DNA was boiled for 5min and kept immediately on ice for 5min.
3) The following components were added:

5µl	10× dNTP
5µl	0.5mM Spectrum Orange (or Green) - dNTP
20µl	2.5× random primer
1µl	Klenow fragment

4) Sample was mixed gently and centrifuged briefly.
5) Incubation was carried out at 37°C for at least 2 hours or longer (overnight).
6) Sample was purified by using Bio-Spin® 30 Tris Column.

   -The column was inverted sharply several times to resuspend the settled gel and remove any bubbles. The tip was snapped off and the column was placed in a 2ml microcentrifuge tube.

   -Centrifugation was performed for 2min at 1000×g (or 2200 rpm) to remove the

packing buffer. The tube containing the filtrate was discarded.

-The column was placed in a clean 2ml microcentrifuge tube and sample (20~100µl) was carefully applied directly to the center of the column.

-Centrifugation was performed for 4min at 1000×g (or 2200 rpm).

-Following centrifugation, the purified sample was then in Tris buffer.

7) 5µl 3M sodium acetate (NaAc) and 150µl ethanol were added to the purified sample to precipitate DNA, and sample was incubated at 4°C for 1h (or overnight).

8) Centrifugation was performed for 30min at 14,000 rpm (Centrifuge 5417R, Eppendorf), 4°C.

9) The supernatant was carefully removed and the pellet was dried in a speedvac.

10) The pellet was dissolved in 25µl TE buffer and stored at -20°C.

2.2.6.5 Dot blot assay

Solutions

Buffer AP1	0.1M Tris·Cl 0.1M NaCl 20mM $MgCl_2$ 0.05%(v/v) Triton X-100 pH 7.5
Buffer AP2	30mg/ml BSA in buffer AP1 pH 7.5
Buffer AP3	0.1M Tris·Cl 0.1M NaCl 50mM $MgCl_2$ pH 9.5
NBT/BCIP staining solution	NBT/BCIP Stock solution 200µl in 10ml Buffer AP3

1) 1µl of the 1:10, 1:100, 1:1000 and 1:10000 diluted probes were spotted on Hybond-N^+ membrane.

2) 1µl of the 1:10, 1:100, 1:1000 and 1:10000 diluted positive controls (any probe which has been identified to be labeled with DIG) were spotted on the same membrane.

3) The membrane was incubated with probes and controls in the microwave oven (>600W) for 2min.

4) The membrane was equilibrated with 10ml Buffer AP1 for 1min.

5) The membrane was incubated with 10ml Buffer AP2 for 10min at room temperature.

6) The membrane was incubated with anti-DIG AP antibody (1:5000 in AP1) for 10min at room temperature.

7) The membrane was washed for 2×3min with Buffer AP1.

8) The membrane was washed for 3×3min with Buffer AP3.

9) Incubation with NBT/BCIP staining solution (5-10ml) until signals were strong enough. The membrane was kept in the dark during incubation.

2.2.6.6 Metaphase preparation

Breast cancer cell line cells were cultured in DMEM or RPMI medium as described under 2.1.7 and harvested using Trypsin/EDTA. Demecolcine (0.02µg/ml) was added and the cells were incubated for 2h before harvest in order to increase the number of chromosome being in the metaphase. The cells were washed with DPBS by centrifugation at 300×g for 10min and resuspended in the residual solution. After dropwise addition of 7ml potassium chloride (75mM) to the tube cells were incubated for 15min at 37°C for hypotonic treatment. After the incubation seven drops of fixative (2:1 methanol/glacial acetic acid) were added to the solution and a centrifugation at 300 ×g, 10min was carried out. Cells were then incubated with 7ml of fixative for at least 1h at -20°C, followed by washing with fixative once. Finally, cells were resuspended in several drops of fixative. Cell suspension was dropped from

a place about 1m high to slides coated with water to obtain optimal chromosome spreading. One or two slides were stained with DAPI to proof chromosome spreading. The other slides were air-dried overnight at room temperature and stored at -80°C in aluminum foil until use.

2.2.6.7 Fluorescence in situ hybridization

2.2.6.7.1 FISH on native cells

2.2.6.7.1.1 FISH with HER2 probe labeled with Spectrum Orange and commercial CEP17 probe

Slides were pretreated with proteinase K (0.1µg/ml) for 7min at 37°C before dehydration in 70%, 85%, and 100% ethanol for 2min each and then air dried. DNA was denatured for 5min at 73°C in denaturation solution followed by dehydration in ice-cold 70% and 80% ethanol for 2min each. Slides were then kept in 100% ethanol at room temperature until all slides were ready to be dried.

Probe mixture for each slide was prepared as follows: HER2 in-house probe labeled with Spectrum Orange (about 30ng/µl) 1µl, human Cot-1 DNA 30µg, human placental DNA 10µg, dextran blue 20µg, 1/7th volume of 1M sodium acetate and 2.5 volumes of cold (-20°C) 100% ethanol. Probe mixture was incubated for 1h at -80°C and then centrifuged at 13,000rpm (Centrifuge 5417R), 4°C for 30min. The blue pellet was redissolved in 9µl of CEP Hybridization Buffer (Vysis) and mixed with 1µl CEP17 Spectrum Green DNA probe (Vysis). The probe mixture was denatured at 75°C for 5min and 10µl were applied to each air-dried slide immediately. Coverslip was applied and sealed with rubber cement. Hybridization was carried out at 42°C overnight in the Peltier Thermal Cycler PTC-200.

Posthybridization wash was conducted in solutions consisting of 50% formamide/2×SSC for 3×10min, 2×SSC for 10min, and 2×SSC/0.1%Tween20 for

5min. All washing steps were performed at 45°C. Nuclei were counterstained and slides were mounted with anti-fading DAPI Vectashield.

2.2.6.7.1.2 FISH with HER2 probe labeled with digoxigenin

Slides were pretreated with proteinase K (0.1µg/ml) for 7min at 37°C before dehydration in 70%, 85%, and 100% ethanol for 2min each and then air dried. DNA was denatured for 5min at 73°C in denaturation solution followed by dehydration in ice-cold 70% and 80% ethanol for 2min each. Slides were then kept in 100% ethanol at room temperature until all slides were ready to be dried.

Probe mixture for each slide was prepared as follows: HER2 in-house probe labeled with digoxigenin (about 30ng/µl) 1µl, human Cot-1 DNA 30µg, human placental DNA 10µg, dextran blue 20µg, $1/7^{th}$ volume of 1M sodium acetate and 2.5 volumes of cold (-20°C) 100% ethanol. Probe mixture was incubated for 1 hour at -80°C and then centrifuged at 13,000rpm (Centrifuge 5417R), 4°C for 30min. The blue pellet was redissolved in hybridization buffer (50% formamide, 2×SSC, and 10% dextran sulfate, pH 7.0). The probe mixture was denatured at 70°C for 5min, followed by annealing of repetitive elements to Cot-1 DNA at 41°C for 20min, and chilled on ice for 5min. 10µl of probe mixture were applied to each air-dried slide immediately. Coverslip was applied and sealed with rubber cement. Hybridization was carried out at 37°C overnight in the Peltier Thermal Cycler PTC-200.

After hybridization slides were washed with 50% formamide/2×SSC at 45°C for 4×5min, 0.1×SSC at 60°C for 3×5min, and 4×SSC/0.1%Tween20 at 45°C briefly. Slides were incubated with anti-DIG-Fluorescein or anti-DIG-Rhodamine (diluted with 1% BSA in 4×SSC/0.1%Tween20) at room temperature in a moist chamber after blocking with 3%BSA in 4×SSC/0.1%Tween20 for 30min at 37°C. Slides were then washed three times with 4×SSC/0.1%Tween20 for 5min each at 45°C and nuclei were counterstained with anti-fading DAPI Vectashield.

20×SSC pH 7.0	NaCl 175.3g Na_3-Citrat·$2H_2O$ 88.2g Distilled H_2O to 1000ml autoclave, store at room temperature
2×SSC pH 7.0	20×SSC 50ml Distilled H_2O to 500ml store at room temperature
2×SSC/0.1%Tween 20 pH 7.0	20×SSC 50ml Tween 20 500µl Distilled H_2O to 500ml store at room temperature
50%Formamide/2×SSC pH 7.2	Formamide 105ml 20×SSC 21ml Distilled H_2O to 210ml
4×SSC/0.1%Tween 20 pH 7.0	20×SSC 72ml Tween 20 360µl Distilled H_2O to 360ml
0.1×SSC pH 7.0	20×SSC 1050µl Distilled H_2O to 210ml
Denaturation Solution pH 7.4 (70% formamide/2×SSC)	Formamide 35ml 20×SSC 5ml Distilled H_2O to 50ml store at -20°C
Proteinase K-Solution pH 7.5 (0.1µg/ml)	1M TRIS-HCl 1ml 1M $CaCl_2×H_2O$ 100µl Distilled H_2O to 50ml. pre-warm to 37°C Proteinase K stock solution (1mg/ml) 5µl add just prior to use

Hybridization Buffer pH 7.0 (50% formamide, 2×SSC, 10% dextran sulfate)	Deionized formamide 5ml 20×SSC 1ml Dextran sulfate 1g Distilled H_2O to 10ml heat to 70°C for 1h to dissolve the dextran sulfate, aliquot and store at -20°C
3%BSA (blocking solution)	BSA 150mg 4×SSC/0.1%Tween20 5ml Aliquot store at -20°C
1%BSA (antibody dilution buffer)	3% BSA 1:3 diluted with 4×SSC/0.1%Tween20

2.2.6.7.1.3 FISH with HER2 FISH pharmDx™ kit

The HER2 FISH pharmDx™ kit (DAKO)contained Texas Red labeled HER2 DNA probe and fluorescein labeled CEN17 PNA probe.

Slides were prepared as described above. 10µl of HER2/CEN17 Probe Mix were applied to each slide. Coverslip was applied and the edges of the coverslip were sealed with rubber cement. Co-denaturation at 82°C for 5min and hybridization at 45°C overnight were carried out in the thermocycler described above. After hybridization, slides were washed in Stringent Wash Buffer at 65°C for 10min, followed by washing in Wash Buffer for 2×3min at room temperature. Nuclei were counterstained and slides were mounted with anti-fading DAPI Vectashield.

2.2.6.7.2 FISH protocol for processed cells

Slides were pretreated with RNase A (100µg/ml) for 40min, followed by a pepsin pretreatment for 7min at 37°C. Slides were refixed in 1% formaldehyde for 10min,

before dehydration in 70%, 85%, and 100% ethanol for 2min each and then air dried. DNA was denatured for 2min at 80°C in denaturation solution followed by dehydration in ice-cold 70% and 80% ethanol for 2min each. Slides were then kept in 100% ethanol at room temperature until all slides were ready for drying and probe application.

Probe mixture for each slide was prepared as follows: HER2 in-house probe labeled with Spectrum Green or Spectrum Orange (about 30ng/µl) 1µl, human Cot-1 DNA 30µg, human placental DNA 10µg, dextran blue 20µg, 1/7th volume of 1M sodium acetate and 2.5 volumes of cold (-20°C) 100% ethanol. Probe mixture was incubated for 1h at -80°C and centrifuged at 13,000rpm (Centrifuge 5417R), 4°C for 30min. The blue pellet was redissolved in 10µl of hybridization buffer. The probe mixture was denatured at 75°C for 5min. Then 10µl of probe mixture were applied to each air-dried slide immediately. Coverslip was applied and sealed with rubber cement. Hybridization was carried out at 42°C overnight in the thermocycler as described above.

Posthybridization wash was conducted in solutions consisting of 50% formamide/2×SSC for 3×10min, 2×SSC for 10min, and 2×SSC/0.1%Tween20 for 5min. All washing steps were performed at 45°C. Nuclei were counterstained and slides were mounted with anti-fading DAPI Vectashield.

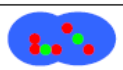
2.2.6.7.3 FISH assessment

Slides were evaluated using an Axioplan2 fluorescence microscope equipped with a CCD camera. Results were documented using the MetaSystems Isis imaging system. At least 60 non-overlapping and intact nuclei were analyzed. For single color FISH, only cells with at least one gene signal were counted. For dual color FISH, only cells with one or more gene signals of each color were counted. In FISH analysis, signals are often in different planes of focus, necessitating focusing up and down through the

section to find all the signals present in any given cell (Hicks and Tubbs, 2005). Split signals- two smaller signals in very close proximity- were counted as one signal (Table 2.1). In cells with high level HER2 amplification, the HER2 signals may form a cluster. In these cases the number of HER2 signals could be counted, and was therefore given as "cluster" meaning high level of amplification.

The assessment of HER2 amplification was performed according to the guideline recommended by the American Society of Clinical Oncology/ College of American Pathologist (Wolff et al. 2007). HER2 gene copy numbers of more than 6 per nucleus or HER2/CEP17 ratios of more than 2.2 were defined as HER2 amplification. In contrast, HER2 nonamplication was a result of HER2 gene copy numbers of less than 4 per nucleus or a HER2/CEP17 ratio of less than 1.8. A result of HER2 gene copy number 4-6 per nucleus or HER2/CEP17 ratio of 1.8-2.2 was defined as HER2 equivocal.

Table 2.1 Signal counting guidelines for dual color FISH

1		Do not count. Nuclei are overlapping, not all areas of nuclei are visible
2		Two green signals, do not score nuclei with signals of only one color
3		Count as 3 green and 12 red signals (cluster estimation)
4		Count as 1 green and 1 red signal. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one
5		Do not count (over-digested nuclei)
6		Count as 2 green and 3 red signals. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one
7		Count as 1 green and 5 red signals
8		Count as 3 green (1 green out of focus) and 3 red signals
9		Cluster of red signals hiding green signals, check the green signals with a specific FITC filter, or do not count

(from HER2 FISH pharmDx^{TM} kit package insert, www.dakousa.com)

2.2.7 Combined FISH and IF staining

2.2.7.1 Pretreatment of samples

In this study, different pretreatments were used for samples from native cells, processed cells and formalin-fixed paraffin-embedded pleural effusion before heat treatment. To prevent the detachment of cells, all kinds of samples were baked at 60°C for at least one hour. Then samples of native cells were treated with the antigen retrieval technique as described below. For samples of processed cells, slides were pretreated with RNase A (100μg/ml) for 40min before heat treatment. The formalin-fixed paraffin-embedded pleural effusions were deparaffinized through two changes of xylene for 10min each, two changes of 100% ethanol for 5min each, and 80% ethanol briefly and air-dried followed by heat treatment.

2.2.7.2 Heat treatment

In this study, REAL^{TM} Target Retrieval Solution (citrate buffer of pH 6.0) was used. A plastic jar filled with the citrate buffer was placed into the center of the pressure cooker. The slides were immersed into the buffer, and the pressure cooker was sealed after the temperature rose to 100°C. In our study, we tested different temperatures (100°C, 120°C, 125°C) and different heating periods (1min, 2.5min, 5min, 10min). When the pressure dropped to 0, the jar was removed from the pressure cooker. The slides were kept in the buffer for 20min to cool down, subsequently washed in distilled water and DPBS, before proceeding with further experiments.

2.2.7.3 One color FISH combined with IF staining

After heat treatment, slides were fixed with 1% formaldehyde for 10min, followed by dehydration in 70%, 85% and 100%ethanol for 2min each and air drying. Slides were denatured for 2min at 80C in 70% formamide/2× SSC before dehydration in ice-cold

70% and 80% ethanol for 2min each. Slides were then kept in 100% ethanol at room temperature until all slides were ready to be dried.

Probe mixture for each slide was prepared as follows: HER2 in-house probe labeled with Spectrum Orange or Spectrum Green (about 30ng/µl) 1µl, human Cot-1 DNA 30µg, human placental DNA 10µg, dextran blue 20µg, $1/7^{th}$ volume of 1M sodium acetate and 2.5 volumes of cold (-20°C) 100% ethanol. Probe mixture was incubated for 1h at -80°C and then centrifuged at 13,000rpm (Centrifuge 5417R), 4°C for 30min. The blue pellet was redissolved in hybridization buffer and denatured at 75°C for 5min. The probe (10µl) was applied to each air-dried slide immediately. Coverslip was applied and sealed with rubber cement. Hybridization was performed at 37°C overnight in the thermocycler as described above.

Posthybridization wash was carried out in 50% formamide/2×SSC for 3×10min, 2×SSC for 10min, and 2×SSC/0.1%Tween20 for 5min. The whole wash procedure was performed at 45°C.

For additional immunocytochemistry, slides were incubated with primary antibody (CB11 or A45-B/B3 conjugated with Cy3) for 45min at room temperature after blocking for 20min in ready-to-use protein block (serum-free). When the directly fluorescently labeled antibody A45-B/B3 was used, slides were treated with the anti-fading DAPI Vectashield after a rinse in TBST to counterstain the nuclei. When the unlabeled primary antibody CB11 for the detection of HER2 expression was used, immunoreaction was detected with a secondary goat anti-mouse IgG antibody conjugated with Alexa488 or Alexa555. The choice of the Alexa fluorochrome depended on the HER2 probe labeled either with Spectrum Green or Spectrum Orange. Slides were washed in TBST after the incubation with secondary antibody for 45min at room temperature. Subsequently, nuclei were counterstained with anti-fading DAPI Vectashield. The samples were evaluated immidiately after staining.

2.2.7.4 Dual color FISH combined with IF staining

After heat treatment, slides were fixed with 1% formaldehyde for 10min, followed by dehydration in 70%, 85% and 100% ethanol for 2min each and air drying. Ten microliter HER2/CEN17 Probe Mix (HER2 FISH pharmDx^{TM} kit) were applied to each slide. Coverslips were placed and the edges of coverslips were sealed with rubber cement. Co-denaturation at 82°C for 5min and hybridization at 45°C overnight were carried out in the thermocycler. After hybridization, slides were washed in Stringent Wash Buffer (HER2 FISH pharmDx^{TM} kit) at 65°C for 10min, followed by washing in Wash Buffer (HER2 FISH pharmDx^{TM} kit) for 2×3min at room temperature. Subsequently, immunofluorescence staining was performed as described above. In this case, CEN17 probe was labeled with fluorescein, whereby only Alexa 555 conjugated secondary antibodies could be used.

2.2.8 Gel Electrophoresis

2.2.8.1 Agarose gel electrophoresis

Agarose gels (0.75% to 2.5%) were used to analyze results of enzyme digestion and real time PCR. SeaKem LE Agarose, a standard high melting agarose for routine nucleic acid electrophoresis of fragments between 500bp-23,000bp, was chosen to analyze the results of enzyme digestion. MetaPhor Agarose, an intermediate melting agarose with high resolution separation of 20bp-800bp DNA fragments, was chosen for analysis of monoplex real time PCR. The optimal gel concentration depended on the size of the DNA fragments to be resolved (Table 2.2).

Agarose gels were prepared by heating the mixture of 1×TBE buffer and agarose powder at a desired concentration until the agarose was completely melted. Once the agarose solution cooled to about 60°C, GelStar Gel Stain was added by diluting the stock 1:10,000 into the gel solution prior to pouring the gel (1µl per 10ml). After the

gel had solidified in a horizontal gel casting tray, samples containing DNA mixed with loading buffer were loaded and the gel was run in 1×TBE buffer.

Table 2.2 Sieving properties of different percentage agarose gel

	Concentration of agarose (%, in 1×TBE buffer)	Size range (bp)
SeaKem LE Agarose	0.50	1,000-23,000
	0.70	800-10,000
	0.85	400-8,000
	1.00	300-7,000
	1.25	200-4,000
	1.75	100-3,000
MetaPhor Agarose	1.80	150-800
	2.00	100-600
	3.00	50-250
	4.00	20-130
	5.00	<80

(The sourcebook a handbook for gel electrophoresis, www.cambrex.com)

10× TBE

Tris	108g
Boric acid	55g
0.5M EDTA (pH8.0)	40ml
Distilled H_2O	to 1000ml

1× TBE dilute 10× TBE 1:10 with distilled water

0.5M EDTA pH 8.0

EDTA	93g

NaOH	11g
Distilled H_2O	to 500ml

6× Loading Buffer

Glycerol	30%
Bromophenol Blue	0.25%
Xylene Cyanol	0.25%

2.2.8.2 Native polyacrylamide gel electrophoresis

An 8% native polyacrylamide gel was used for analysis of multiplex real time PCR. Polyacrylamide gels have a higher resolution capability than agarose gels and can therefore separate molecules of DNA whose lengths differ by as little as 0.2%. Native polyacrylamide gels can separate DNA fragments of 20bp-2000bp. Table 2.3 indicates the suggested polyacrylamide concentration.

Table 2.3 Sieving properties of different percentage native polyacrylamide gel

Concentration of acrylamide (%)	Size rang (bp)
3.5	1,000-2,000
5.0	80-500
8.0	60-400
12.0	40-200
15.0	25-150
20.0	5-100

(The sourcebook a handbook for gel electrophoresis, www.cambrex.com)

The polyacrylamide gel solution was prepared as described below. After the acrylamide had completely polymerized, the comb and the tape of the bottom of the chamber were removed. Each well was flushed with 1×TBE buffer to remove debris.

Samples containing DNA were mixed with loading buffer and loaded onto the gel. The gel was run in 1×TBE buffer. Nucleic acids were visualized by staining with ethidium bromide or Silver Stain Plus.

For DNA staining with ethidium bromide, the polyacrylamide gel was submerged for 20min in ethidium bromide solution (0.5μg/ml in gel buffer) and subsequently destained with distilled water twice for 20min each.

For silver staining of DNA, the gel was fixed with Fixative Enhancer Solution for 20min, then washed with distilled water for 2×10min, and subsequently stained with Staining Solution for 20min. Then the gel was incubated with 5% acetic acid for 15min to stop the staining reaction and finally the gel was rinsed in distilled water for 5min.

8% Polyacrylamide gel solution

10× TBE	1ml	
Acrylamide/Bisacrylamide (19:1, 40%)	2ml	
Distilled H_2O	to 10ml	
TEMED	5μl	
10% APS	90μl	add prior to use

10% APS

APS	0.1g
Distilled H_2O	1ml

Fixative Enhancer Solution

Methanol	200ml
Acetic Acid	40ml
Fixative Enhancer Concentrate	40ml
Distilled H_2O	to 400ml

Staining Solution

Distilled H_2O	35ml	
Silver Complex Solution	5ml	
(contains NH_4NO_3 and Ag NO_3)		
Reduction Moderator Solution	5ml	
(contains tungstosilicic acid)		
Image Development Reagent	5ml	
(contains formaldehyde)		
Development Accelerator Solution	50ml	add prior to use
(contains Na_2CO_3)		

2.2.9 DNA isolation from cell lines

DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen). Cell suspensions (200µl) containing up to 5×10^6 cells were incubated with 20µl proteinase K and 200µl lysis buffer overnight at 56°C, followed by adding 200µl absolute ethanol to the sample. After lysis DNA was purified on QIAamp Spin Columns and eluted in 200µl Buffer AE. The concentration of DNA was measured with the help of the Spectrophotometer ND 1000 (NanoDrop). DNA was stored at -20°C.

2.2.10 DNA isolation from paraffin-embedded tissues

The formalin-fixed paraffin-embedded tissue sections (2µm thick) were deparaffinized through three changes of xylene for 5min each, hydrated in serial ethanol (100%, 96%, 80%) for 1min each, and rinsed in distilled water. One of four serial sections was stained with hematoxylin and eosin (H&E) for evaluation of tumor cells, and other three sections were stained with 0.1% toluidine blue O/ 0.02% methylene blue for manual dissection of tumor cells, so that the interference of normal cells and stromal cells was minimized. For H&E staining, tissue sections were stained with hematoxylin for 5min, subsequently decolorized in acid ethanol (2/3 volume of

hydrochloric acid, 1/3 volume of 95% ethanol) briefly and under running tap water for 10min, and counterstained with eosin for 20 seconds. Slides were rinsed in distilled water and dehydrated in 80%, 96%, and absolute ethanol briefly followed by placing into xylene for 2min and air-drying. For other serial sections, slides were stained with 0.1% toluidine blue O/ 0.02% methylene blue for 30 seconds, rinsed in distilled water for 1min, and dehydrated in 75% and 100% ethanol briefly.

Tumor cells stained with toluidine blue O/methylene blue were manually dissected by needles and collected into a tube containing 180µl Buffer ATL and 20µl proteinase K (Qiagen). DNA was extracted using QIAamp DNA Micro Kit (Qiagen). The samples were incubated at 56°C for 48h, followed by incubation with 200µl Buffer AL and 200µl absolute ethanol for 5min at room temperature. Entire lysates were purified on QIAamp miniElute Columns and eluted in 30µl distilled water. The concentration of DNA was measured with the help of the Spectrophotometer ND 1000. DNA was stored at -20°C.

2.2.11 Determination of DNA concentration

DNA concentration was measured using the Spectrophotometer ND 1000 according to the manual. An A260/A280 ratio of 1.7-1.9 was accepted as “pure” for DNA.

2.2.12 Whole genome amplification for single cells

2.2.12.1 Preparation of hydrophobic slides

The clean slides were immersed in sulfuric acid (H_2SO_4) for 1h. After washing with distilled water, slides were dried at 110°C for 30min. Slides were immersed in 0.5% octadecyltrichlorosilane for 2h or longer with a gentle shaking, followed by cleaning with ethanol and distilled water sequentially. Slides were kept at room temperature from dust.

2.2.12.2 DNA extraction

Lysis buffer was prepared as described by Hartshorn et al. (2005) with slight modifications. Dry droplets of lysis buffer were prepared by delivering 0.2µl lysis buffer onto hydrophobic slides.

Target cells were aspirated into a glass capillary with an internal diameter of 30µm, and then expelled directly onto the lysis dot in a minimum volume of distilled water. After drying at room temperature for 4-5min, 1µl proteinase K (Qiagen) and 9µl Sample Buffer (GenomiPhi^{TM} DNA Amplification Kit, GE Healthcare) were added. These 10µl solutions were transferred into a PCR reaction tube for whole genome amplification.

2.2.12.3 Whole genome amplification

Whole genome amplification was performed using the GenomiPhi DNA Amplification Kit (GE Healthcare) according to the manual. The sample (10µl) containing 9µl Sample Buffer was denatured at 95°C for 10min and kept on ice for 3min. After combination with 9µl Reaction Buffer and 1µl Enzyme Mix, sample was incubated at 30°C for 16h. Subsequently the enzyme was inactivated by heating for 20min at 65°C.

2.2.12.4 Purification of amplification products

Post-amplification cleanup was conducted by NucleoSEQ columns (Macherey-Nagel). The gel matrix was hydrated with 600µl water by incubation at least for 1h or overnight at room temperature or 4°C. The hydrated gel resin was spun down at 750× g for 2min. After loading the sample to the center of the column, a centrifugation at 750× g for 5min was carried out to recover the purified sample. Afterwards, this DNA could be used directly for real time PCR, or stored at -20°C.

2.2.13 Real time PCR

Monoplex real time PCR was performed using SYBR Green chemistry. HER2 and Contr17 amplifications were performed in separate wells. For each reaction, 2µl DNA was added to a final volume of 10µl with a final concentration of 1× SYBR Green PCR Master Mix and 200nM of each primer.

Multiplex real time PCR was carried out using a TaqMan assay. HER2 and Contr17 amplifications were performed in the same wells. The total reaction volume was 25µl containing 0.8× PCR Master Mix for probe assay, 4.5mM $MgCl_2$, 80nM HER2 primers, 60nM HER2 probe, 320nM Contr17 primers, 240nM Contr17 probe and 2µl DNA.

All PCR reactions were conducted in duplicates. The cycling program of the monoplex PCR was: Uracil-N Glycosylase activation at 50°C for 2min, initial denaturation at 95°C for 15min, followed by 40-50 cycles that consisted of a denaturation step at 95°C for 15 seconds, and an annealing and elongation step at 60°C for 1min. The program of the multiplex PCR was slightly modified: Uracil-N Glycosylase activation at 50°C for 2min, initial denaturation at 95°C for 15min, followed by 40-50 cycles that consisted of a denaturation step at 95°C for 15 seconds, an annealing step at 55°C for 30 seconds and an elongation step at 65°C for 30 seconds.

Genomic DNA was serially diluted at dilution factor of 10 to generate standard curves for quantification. HER2 and Contr17 standard curves were obtained respectively. The amount of HER2 and Contr17 in an unknown sample was determined by comparison with the corresponding standard curve. A sample was considered as HER2 gene amplified when the ratio of HER2/Contr17 was no less than 2.0 (≥2.0).

3. Results

3.1 Detection of CTCs

Detection and enumeration of CTCs in peripheral blood from breast cancer patients were conducted with the CellSearch™ system. For the further characterization of CTCs, 65 samples with at least one detectable CTC were collected. Ten of 65 cases (15.4%) had ≥5 CTCs /7.5ml of blood, which is the cutoff level of CTCs with prognostic relevance for metastatic breast cancer patients determined by the studies of Cristofanilli and colleagues (2004, 2005) using the same analysis system. In two samples more than 100 CTCs could be detected (Table 3.1).

In Figure3.1A, two CTCs (marked in composite image) are shown in a part of a gallery of images generated by the CellSpotter® Analyzer. These CTCs are positive in the DAPI and CK-PE channels but negative in CD45-APC and FITC channels. Figure 3.1B and 3.1C show pictures that were directly taken from a cartridge after sample processing. Among ten nucleated cells captured by EpCAM-coated ferrofluids (Figure 3.1B), only three are CTCs (CK-positive) (Figure3.1C) whereas the others are CK-negative.

In order to establish methods for further characterization of CTCs, cells from the breast cancer cell lines SK-BR-3 and MCF7 were spiked into the dilution buffer and processed by the CellSearch™ system. Recovered cells were then called *processed cells*. After this processing, the cells are labeled with EpCAM-coated ferrofluids and CK-PE and the nuclei are stained with DAPI.

In order to prepare slides for further characterization of CTCs, all cells in a cartridge regardless whether from patients or cell lines were directly transferred onto Vectabond-coated slides and dried for two hours at 37°C.

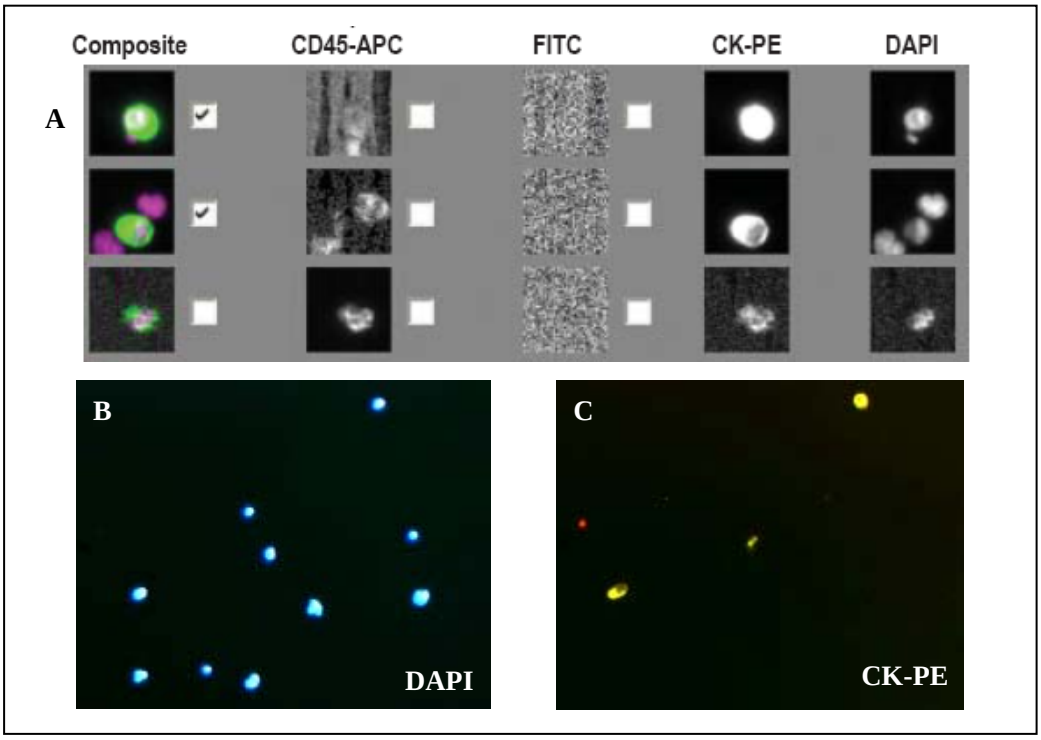


Figure 3.1 Detection of CTCs in peripheral blood from breast cancer patients. A, gallery of images that has to be screened for CTCs. Two CTCs are marked in the composite image. B and C, pictures taken from a cartridge in DAPI channel and CK-PE channel, respectively.

Table 3.1 Number of CTCs/7.5ml blood from 65 CTC-positive breast cancer patients

No. CTCs in 7.5ml blood	No. patients	% patients
1-4	55	84.6
≥5	10	15.4

3.2 Evaluation of HER2 expression in CTCs

3.2.1 Spiking experiments

To find out whether HER2 immunostaining performed in the CellSearch™ run represents the known HER2 status of different breast cancer cell lines, we performed

spiking experiments with SK-BR-3 and MCF7 cells. HER2 expression was analyzed using a FITC-labeled anti-HER2 antibody (Veridex, Warren, NJ) in the CellSearch™ system. As shown in Figure 3.2, SK-BR-3 cells were strongly positive for HER2, whereas MCF7 cells were only weakly stained with this antibody. Therefore, according to the DAKO score we scored these cells as 3+ and 1+, respectively. The results of immunostaining were concordant with FISH results as described later (see part 3.7).

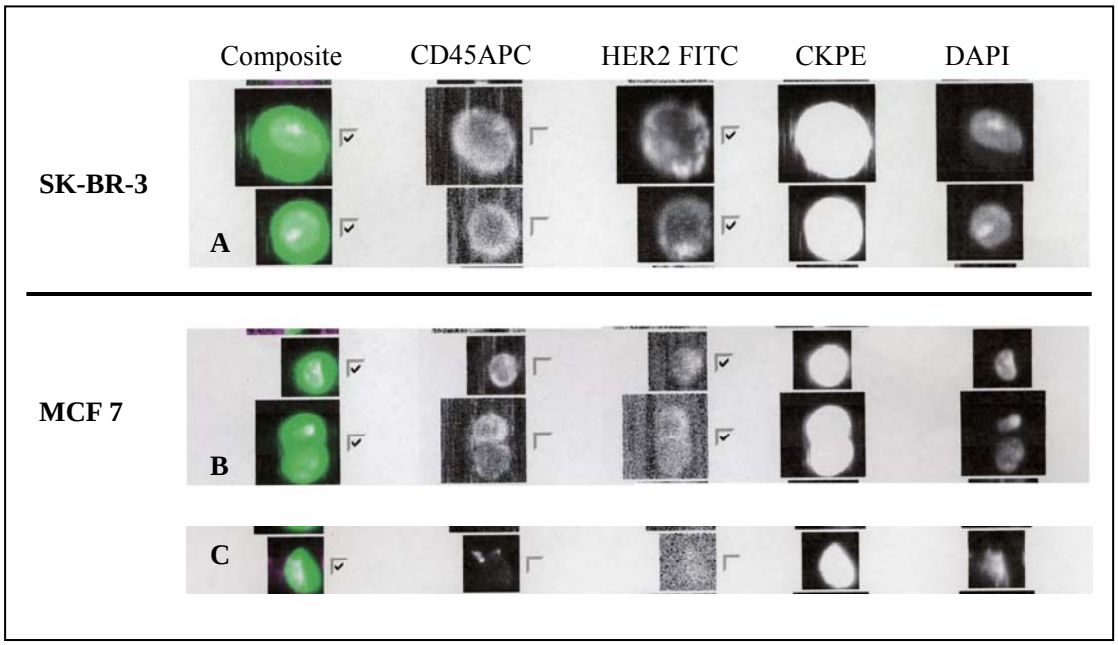


Figure 3.2 HER2 staining patterns of SK-BR-3 and MCF7 cells after the CellSearch™ run. Three patterns are presented: strongly positive (A), dim positive (B), and negative (C).

3.2.2 Clinical samples

The evaluation of HER2 expression in CTCs was performed in 51 samples with the help of the CellSearch™ system using the FITC-labeled anti-HER2 antibody described above. Since MCF7 is weakly positive for HER2 but clearly non-amplified, cells with dim positive patterns are defined as HER2-negative cells in the analysis of clinical samples. In contrast, only CTCs with strongly positive patterns are defined as

HER2-positive cells. Figure 3.3 shows three HER2 stained CTCs (marked in composite image, two CTCs are strongly HER2-positive and one CTC is weakly positive) in a part of a gallery of images, which are positive in DAPI, PE (CK-PE) and FITC (HER2-FITC) channels, but negative for CD45-APC.

HER2-positive CTCs were detected in 15 of 51 patients (29.4%). In some cases discrepancies between the HER2 status in primary tumors and CTCs in blood were observed. Five cases with HER2-negative primary tumors had strongly HER2 positive CTCs in peripheral blood. Furthermore, the expression level of HER2 in CTCs was heterogeneous, even among CTCs from the same patient. Table 3.2 shows all samples with HER2-positive CTCs. Figure 3.4 presents heterogeneity of HER2 expression in CTCs.

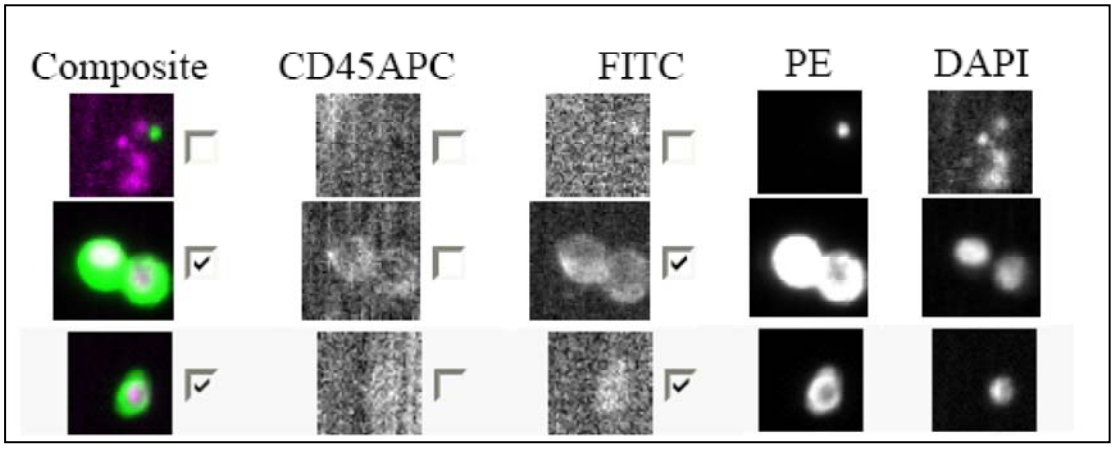


Figure 3.3 Detection of HER2 expression in CTCs with CellSearch™ system. Three of HER2 stained CTCs are marked in composite and FITC image. Here two cells are strongly positive which are classified as HER2-positive CTCs and the other one is weakly positive which is classified as HER2-negative CTC.

Table 3.2 Number of HER2-positive CTCs in selected samples (CellSearch^{TM} system)

Patient number	HER2 status of primary tumor	No. CTCs	No. HER2 positive cells
1	negative	3	3
2	---	37	8
3	---	22	10
4	positive	1	1
5	---	1	1
6	negative	1	1
7	---	4	1
8	positive	113	12
9	negative	1	1
10	negative	1	1
11	positive	4	2
12	positive	8	7
13	negative	1	1
14	positive	183	24
15	---	2	1

--- data unavailable

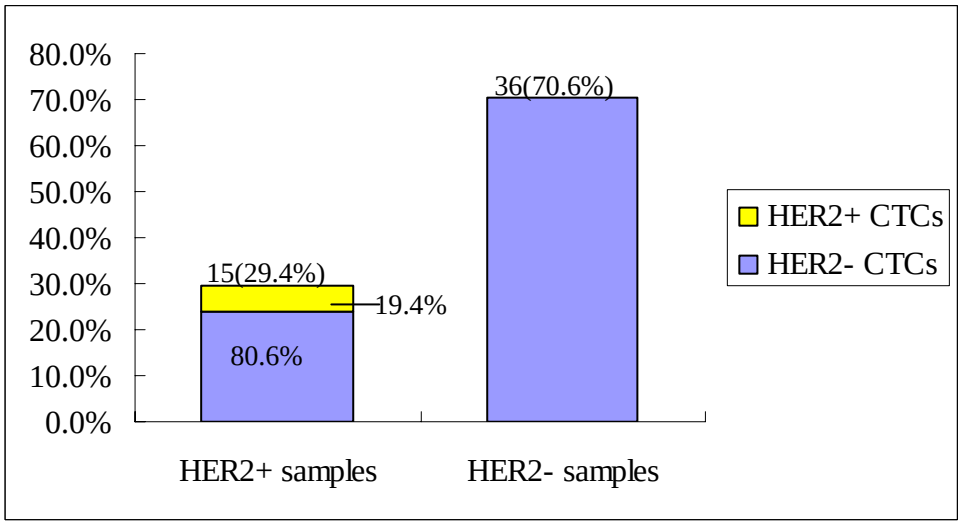


Figure 3.4 Heterogeneity of HER2 expression in CTCs. From 15 HER2 positive samples, only 19.4% of CTCs were strongly positive for HER2 while 80.6% were HER2- negative or only weakly positive.

3.3 Immunofluorescence (IF) of native cells

In contrast to *processed cells* obtained after the CellSearch™ run, breast cancer cell line cells directly taken from the cell culture flasks are called *native cells* in this study.

3.3.1 HER2 and cytokeratin

HER2 and cytokeratin expression were detected in the following breast cancer cell lines: BT474, SK-BR-3, T47D, MCF7, MDA-MB-231, MDA-MB-435s and MDA-MB-468 by double immunofluorescence staining. All cell lines except for MDA-MB-435s were positive for cytokeratin. BT474 and SK-BR-3 had a very strong expression of HER2, consequently scored as 3+. T47D was also positive for HER2, but compared to BT474 and SK-BR-3 its HER2 expression was weaker, therefore scored as 2+. MCF7, MDA-MB-231, MDA-MB-435s and MDA-MB-468 were negative for HER2 (Figure 3.5, Table 3.3).

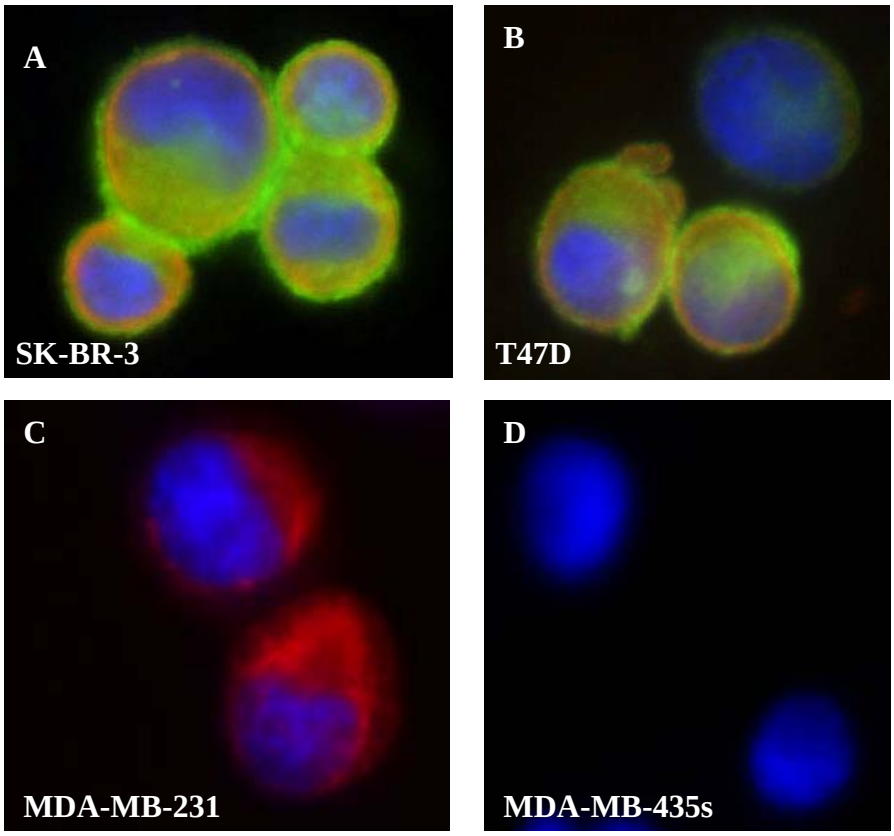


Figure 3.5 HER2 (green) and cytokeratin (red) double immunofluorescence of

breast cancer cell lines. SK-BR-3 (A) and T47D (B) are positive for both HER2 and cytokeratin, so overlay of red and green turns to yellow. MDA-MB-231 (C) is only positive for cytokeratin and MDA-MB-435s (D) is negative for both HER2 and cytokeratin.

3.3.2 Detection of hormone receptor expression

BT474, MCF7 and T47D are estrogen receptor (ER)- and progesterone receptor (PR)-positive cell lines. In contrast, MDA-MB-231 is negative for ER and PR as well as SK-BR-3, MDA-MB-435s and MDA-MB-468 are negative for ER but positive for PR (Figure 3.6, Table 3.3). The difference in PR expression of SK-BR-3, MDA-MB-435s and MDA-MB-468 compared to published data in the literatures (Maemura et al. 1995; Tong et al. 1999; Fu et al. 2003) could possibly be due to heterogeneity between the cell lines cultivated in different laboratories and different numbers of passages the cell lines went through.

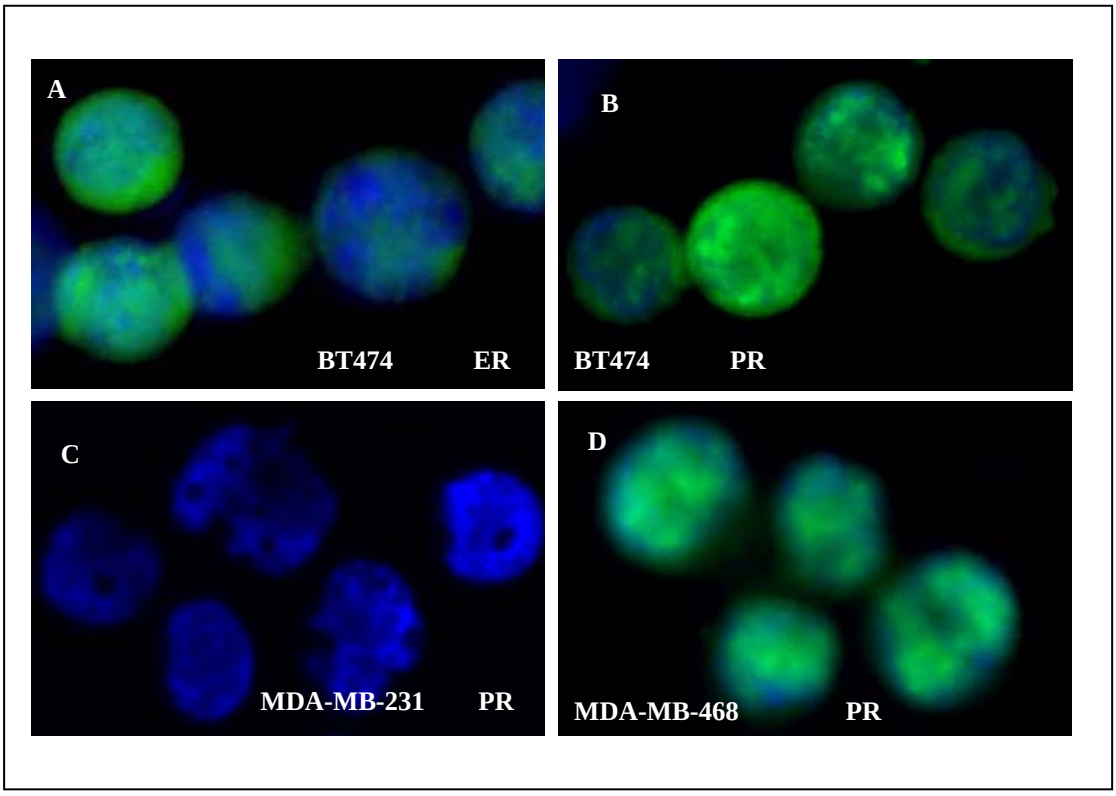


Figure 3.6 ER and PR expression in breast cancer cell lines. BT474 (A, B) is

ER+/PR+, and MDA-MB-231 (C) is negative for both ER and PR. In this study, we found that MDA-MB-468 (D) is positive for PR.

Table 3.3 Characterization of breast cancer cell lines concerning HER2, ER and PR expression

Cell line	Cytokeratin	HER2	ER	PR
BT474	+	3+	+	+
SK-BR-3	+	3+	-	+
T47D	+	2+	+	+
MCF7	+	1+	+	+
MDA-MB-231	+	0	-	-
MDA-MB-468	+	0	-	+
MDA-MB-435s	-	0	-	+

+ positive, - negative

3.3.3 Expression of the proliferation marker Ki-67

In this study, we compared two methods of fixation for the native cells for subsequent analysis of nuclear Ki-67 IF staining. Both formaldehyde fixation and cold acetone fixation worked well, and no permeabilization reagent was applied following the fixation step. In Figure 3.7, MCF7 cells present Ki-67 positivity, pretreated with either acetone fixation (left) or formaldehyde fixation (right).

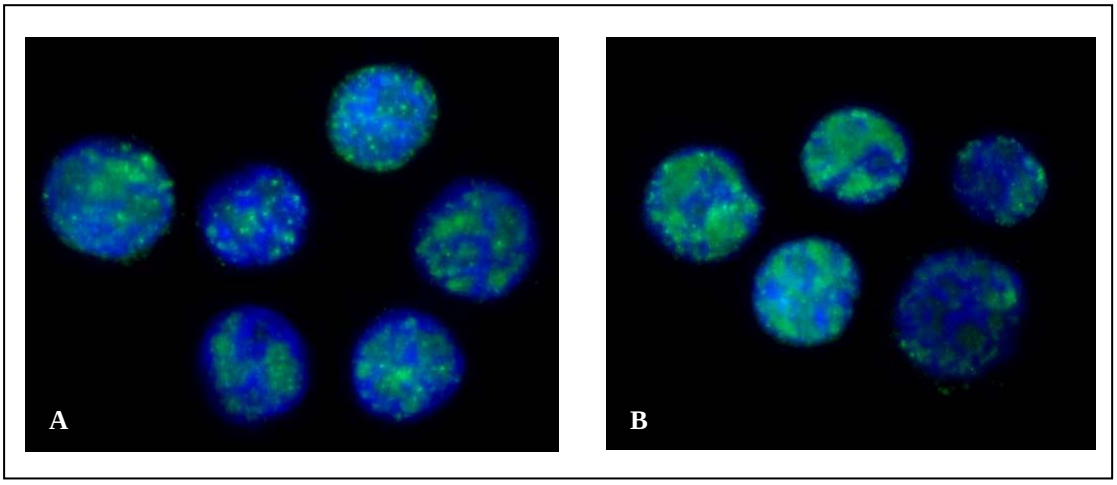


Figure 3.7 Ki-67 immunofluorescence of breast cancer cell line MCF7. A, cells were fixed with acetone. B, cells were fixed with formaldehyde.

3.4 Establishment of IF methods for processed cells

3.4.1 Immunodetection of membranous antigens

The processed cells had been fixed and permeabilized during the CellSearch^{TM} system processing, thus there was no need for a further fixation step for membranous antigen staining. We detected HER2 antigens in these cells without any further fixation or permeabilization step. Figure 3.8A demonstrates HER2 staining with strong intensity.

3.4.2 Immunodetection of nuclear antigens

Firstly, several fixation and permeabilization methods were tested which were all not successful for Ki-67 detection in the processed cells (Table 3.4). These included two methods, formaldehyde fixation and cold acetone fixation without permeabilization reagent, which worked well for nuclear antigen staining of native cells. The other three protocols included only methanol fixation, paraformaldehyde fixation followed by Tween20 or Triton X100 permeabilization. Cold acetone fixation followed by antigen retrieval technique (citrate buffer of pH 6.0, heating for 10min by microwave oven) was eventually adopted for nuclear antigen staining of the processed cells resulting in very good nuclear staining (Figure 3.8B).

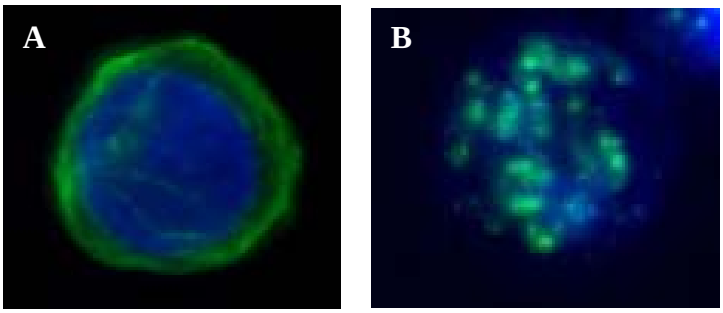


Figure 3.8 HER2 (A) and Ki-67 (B) immunofluorescence of processed SK-BR-3 cells.

Table 3.4 Summary of tested methods to detect Ki-67 by IF
in the processed SK-BR-3 cells

Method	Fixative	Time	Permeabilizing reagent	Time
1	0.5% Formaldehyde	15min	No	---
2	Acetone (-20°C)	5min	No	---
3	Methanol	5min	No	---
4	4% Paraformaldehyde	20min	0.5%Tween20	20min
5	4% Paraformaldehyde	20min	0.5% Triton X100	20min
6	Acetone (-20°C)	5min	Antigen retrieval	10min

3.5 Immunofluorescence of processed cells

3.5.1 Ki-67

Different Ki-67 staining patterns were observed in the processed MCF7 cells. Consistent with published data (du Manoiret al. 1991; Suurmeijer et al. 1999) these different staining patterns possibly reflect the various phases of the cell cycle. The upper row of Figure 3.9 shows results from Suurmeijer et al. (1999), and the lower row demonstrates results from our study.

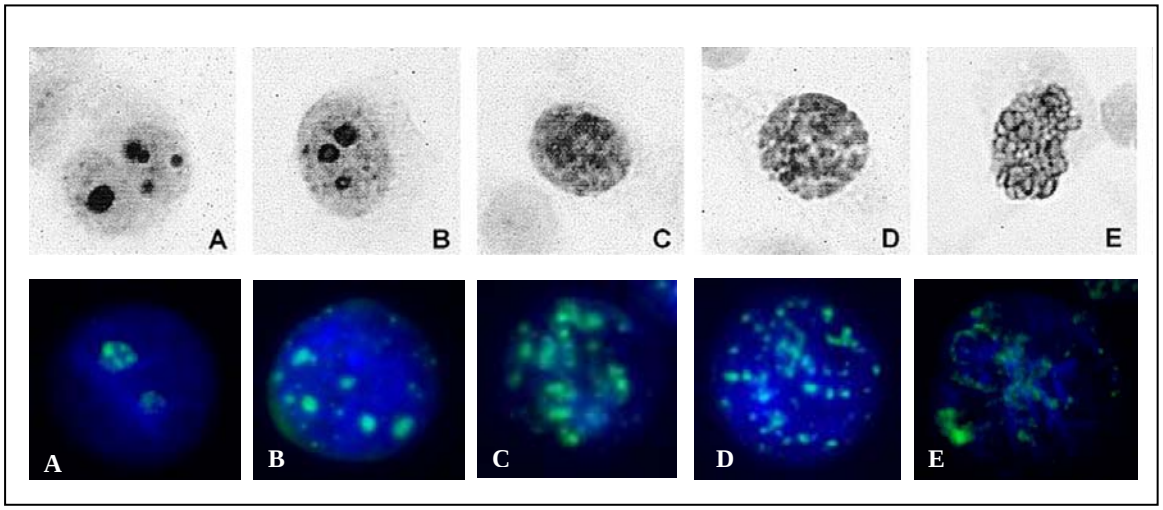


Figure 3.9 Different Ki-67 staining patterns of MCF7 cells corresponding to various phases of the cell cycle. A, later G1 phase; B, S phase; C, G2 phase; D,

prophase; E, metaphase.

3.5.2 M30

Double immunofluorescence of A45-B/B3-Cy3 and M30-FITC on SK-BR-3 cells allowed us to detect apoptosis. The intact cells with strong cytokeratin positivity were negative for M30 (Figure 3.10A). Conversely, in the apoptotic cells, the expression of cytokeratin became weaker or negative, meanwhile, the nuclear fragmentation took place and M30 staining became strong (Figure 3.10B,C,D,E,F). The apoptotic body indicated the final stage of apoptosis (Figure 3.10F).

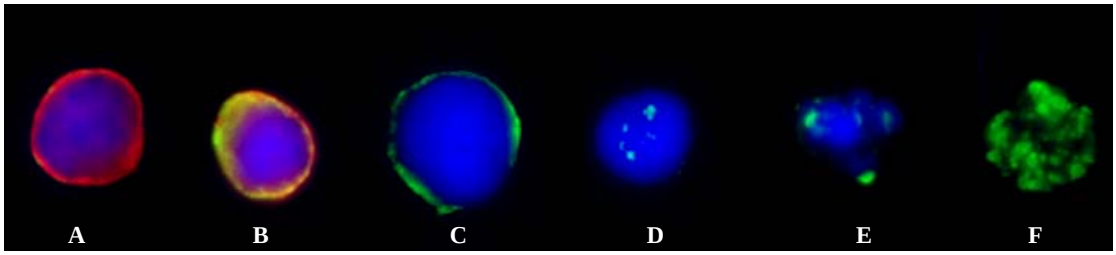


Figure 3.10 Cytokeratin (red) and M30 (green) double IF of the processed SK-BR-3 cells. Cytokeratin positive cells were negative or moderate positive for M30 (A, B). Cells with strong positivity for M30 and nuclear fragmentation were cytokeratin negative (C, D, E, F). F shows an apoptotic body without detectable nuclear content.

3.6 Generation and labeling of the HER2 FISH probe

3.6.1 Confirmation of BAC clone identity

For the generation of a HER2 FISH probe, the BAC clone RP11-94L15 was chosen as it encompassed the HER2 gene. In order to confirm the BAC clone identity, restriction endonucleases *Eco*RI and *Xho*I were used to construct a restriction map (Figure 3.11, 3.12). By comparing the obtained fragments lengths with those expected, the identity of the BAC clone could be ensured.

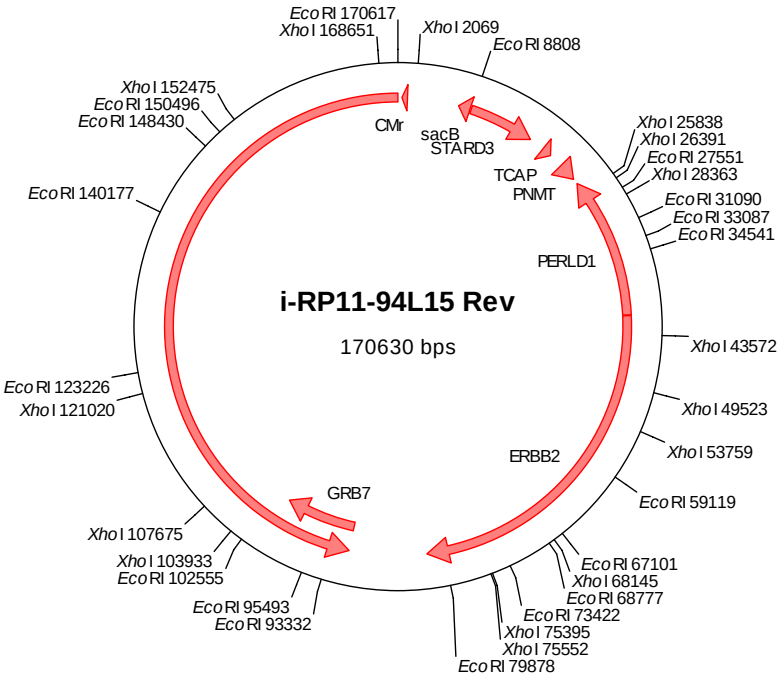


Figure 3.11 Restriction map of RP11-94L15.

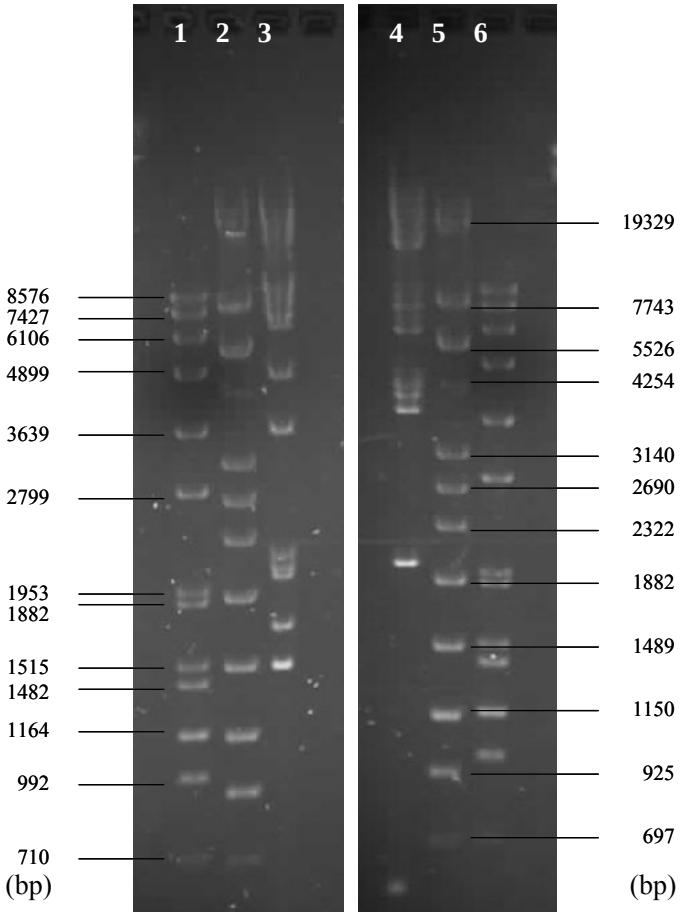


Figure 3.12 RP11-94L15 DNA digested with *Eco*RI (lane 3) and *Xho*I (lane 4).

Fragments were separated in a 0.75% agarose gel and stained with ethidium bromide. Lane 1 and 6 show DNA Molecular Weight Marker VII (Roche, Mannheim, Germany); lane 2 and 5 show DNA Molecular Weight Marker IV.

Fragment lengths after *Xho*I digestion (bp): 31455, 28381, 23769, 16176, 15209, 14386, 13345, 7250, 5951, 4236, 4048, 3742, 1972, 553, 157.

Fragment lengths after *Eco*RI digestion (bp): 24578, 20671, 20121, 18743, 16951, 13454, 8821, 8253, 7982, 7062, 6456, 4645, 3539, 2161, 2066, 1997, 1676, 1454.

3.6.2 Labeling of the HER2 FISH probe with haptens or fluorochromes

The BAC DNA was either indirectly labeled with a hapten such as biotin or digoxigenin by nick translation or directly with a fluorochrome such as Spectrum Green or Spectrum Red by random priming. The labeled probes were verified by their fragment sizes by gel electrophoresis. The final fragment sizes should be between 200bp and 500bp. In the example shown below (Figure 3.13), the probes in lane 1, 3 and 5 had been nicked to the appropriate sizes.

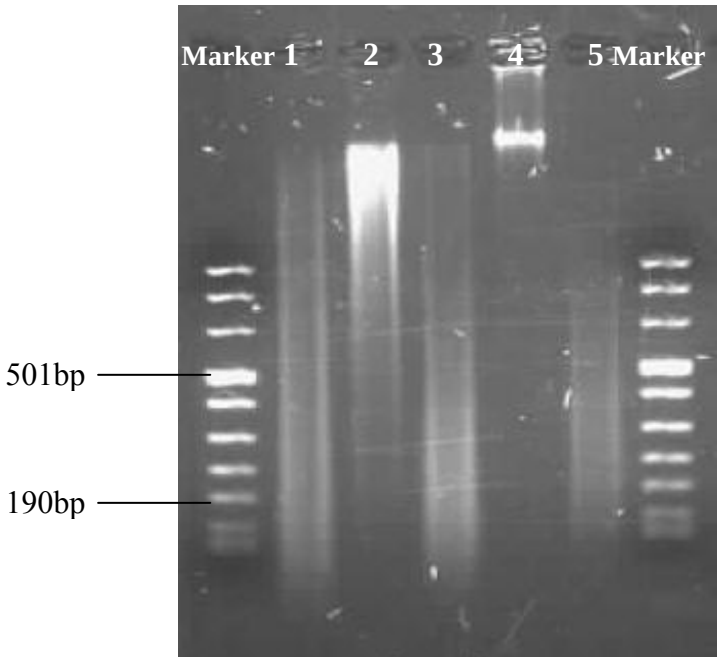


Figure 3.13 Nick translated DNA fragments separated in an 1.2% agarose gel.

3.6.3 Dot blot assay

The degree of hapten incorporation into the BAC DNA was determined by a dot blot assay. This method is useful to ensure that a lack of FISH signals after hybridization is not due to poorly labeled probes. In the example shown in Figure 3.14, compared to the labeled control probe, the labeled probe also had incorporated digoxigenin very well.

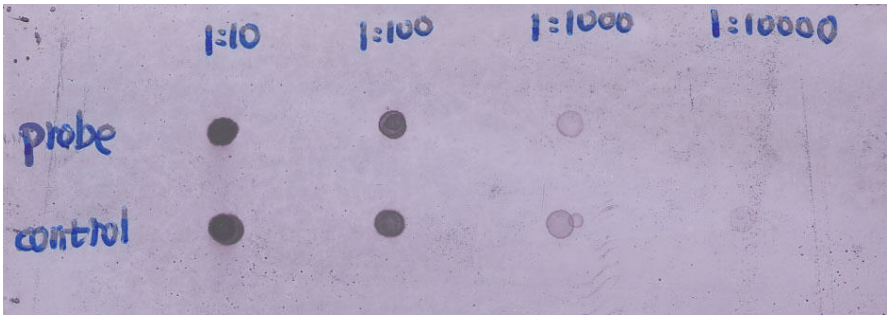


Figure 3.14 Dot blot assay of a probe labeled with digoxigenin. Both probes were 10-fold serially diluted.

3.6.4 Determination of specificity of the probe

After the probe had been labeled, the FISH experiments were carried out on normal lymphocytes and metaphase spreads from two HER2 non-amplified tumor cell lines, MCF7 and MDA-MB-468. Only two strong HER2 signals in each nucleus or metaphase and low background were observed (Figure 3.15). The results showed that the probe hybridized specifically only to one chromosome (two copies) and had been labeled efficiently. The results of dual color metaphase FISH containing a commercial centromere probe CEP17 indicated that the HER2 probe mapped to the correct chromosome.

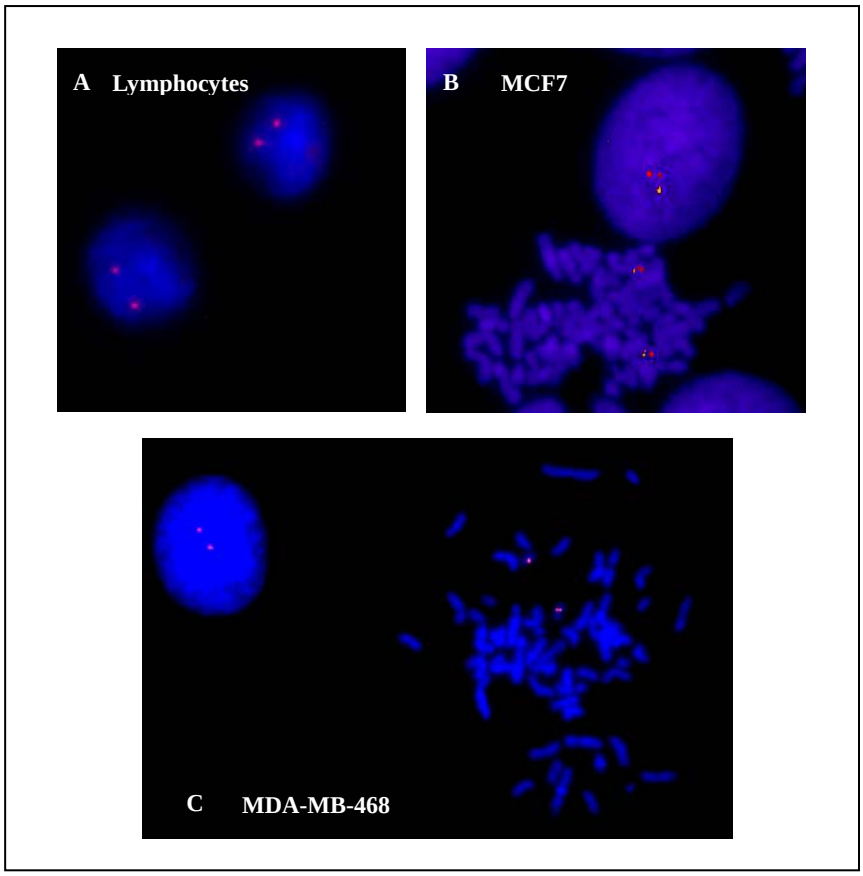


Figure 3.15 Determination of specificity of the labeled HER2 probe. In normal lymphocytes (A), MCF7 (B) and MDA-MB-468 (C) cells, only two HER2 signals in each nucleus or metaphase were detected. In MCF7 metaphase FISH (B) the yellow signal is generated from CEP17 probe labeled with Spectrum Aqua by a false color program of the MetaSystem Isis system (magnification ×63). The HER2 gene mapped to chromosome17.

3.7 FISH of native cells

HER2 gene copy number was detected in seven breast cancer cell lines. HER2 gene amplification is defined as FISH ratio (HER2 gene signals to chromosome 17 signals) of ≥ 2.2. In contrast, HER2 gene non-amplification is given when the FISH ratio is less than 1.8. The gene status and protein expression were consistent for SK-BR-3, BT474, MCF7, MDA-MB-231, MDA-MB-435s and MDA-MB-468 cells, but not for T47D cells (Table 3.5). SK-BR-3 and BT474 showed HER2 amplification, while the other cell lines as expected did not amplify the HER2 gene. T47D with weak (2+)

HER2 positivity and HER2 gene nonamplification (HER2/CEP17=1.1) was found to have on average five copies of chromosome 17 (Figure 3.16).

Table 3.5 HER2 status of breast cancer cell lines

Cell line	IF	Ratio HER2/CEP17	Mean HER2 signal number	HER2 amplification
BT474	3+	9.2	48.9	Positive
SK-BR-3	3+	5.5	36.6	Positive
T47D	2+	1.1	5.6	Negative
MCF7	1+	1.0	2.4	Negative
MDA-MB-231	0	0.9	2.6	Negative
MDA-MB-435s	0	1.0	2.1	Negative
MDA-MB-468	0	1.0	2.0	Negative

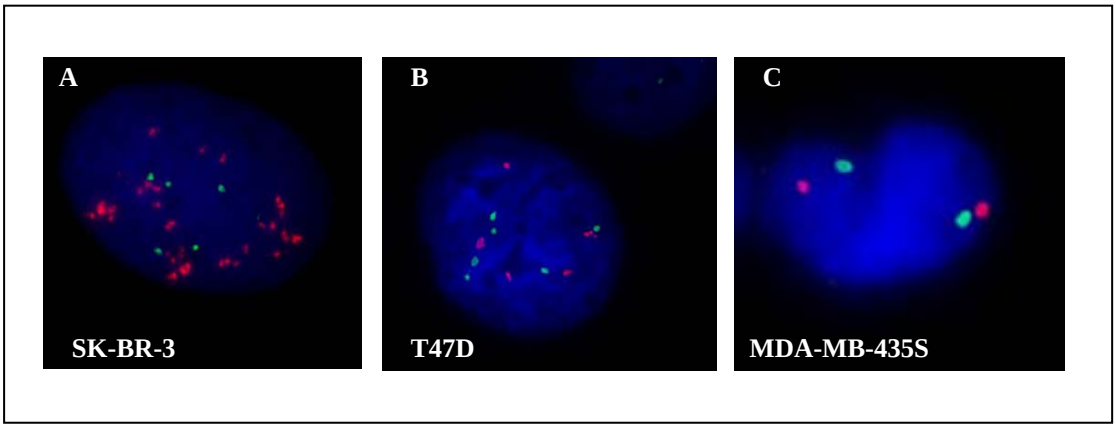


Figure 3.16 HER2 status of different cell lines. A, SK-BR-3 cell with HER2 (red) amplification and polysomy of chromosome 17 (green). B, T47D cell with polysomy of chromosome 17, but without HER2 gene amplification. C, MDA-MB-435s cell without HER2 amplification and with a normal diploid chromosome 17 status.

3.8 FISH of processed cells

As to be seen in Figure 3.17A, the protocol established for native cells did not work for processed cells. Figure 3.17A indicates a high background throughout the staining area and no FISH signal was found in the nuclei. This could be due to the CellSearch^{TM} system processing including a fixation step avoiding probe penetration

into the nucleus. Subsequently, we modified the protocol and changed the pretreatment and the stringency of the hybridization. RNase A and pepsin treatment replaced the proteinase K and the hybridization temperature was increased from 37°C to 42°C. Figure 3.17B shows a good FISH result with clear FISH signals and low background by using the modified FISH protocol.

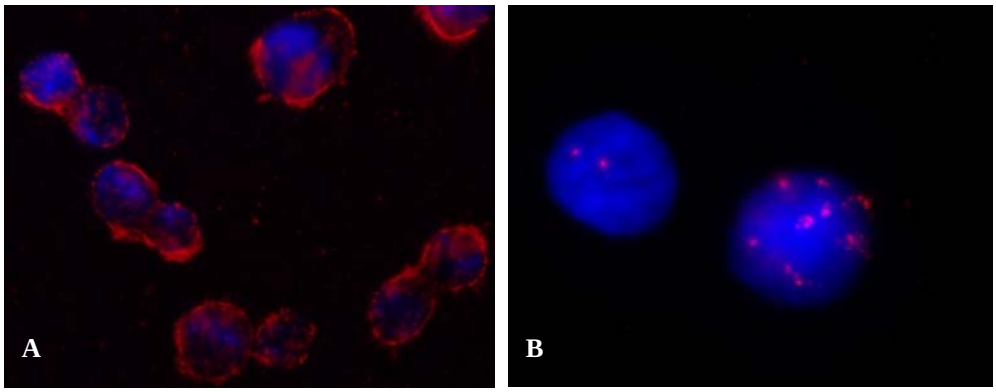


Figure 3.17 HER2 FISH of processed cells. A, result obtained by applying the protocol established for native cells. B, result obtained by the modified protocol (MCF7 cell, left; SK-BR-3 cell, right).

3.9 Combined FISH and immunofluorescence of native cells

The antigen retrieval technique applied in this study comprised citrate buffer of pH 6.0 as antigen retrieval buffer, and the pressure cooker as heating source. Heating temperature and heating time had to be optimized. Table 3.6 shows the effects of different temperatures (100°C and 120°C) and heating times (2.5min, 5min, 10min) on simultaneous FISH and IF staining for native cells. In this study, we conducted combined FISH and IF on SK-BR-3 cells, which are positive for HER2 and cytokeratin. At 120°C, heating for 2.5min or 5min can acquire the same staining results where both FISH signal and IF staining intensity are very good. But there was an influence of the heating time on the nuclear morphology, which was better conserved using shorter heating periods. However, at 120°C, prolonging heating duration did not result in an increase of the IF staining intensity. On the contrary,

FISH signals were significantly decreased and the background was high when applying a temperature of 120°C for 10min.

At 100°C, all three tested times did not change nuclear morphology and resulted in satisfactory FISH results, however, IF intensity was lower than that after 120°C pretreatment. Especially, at 100°C for 2.5min, there was a much higher background than at the other conditions.

For routine use, the following condition revealed to be optimal: 120°C, 2.5min in citrate buffer of pH 6.0 using a pressure cooker. The following probes were tested: HER2 in-house probe labeled with Spectrum Orange, HER2 in-house probe labeled with Spectrum Green, and the HER2 FISH pharmDx kit probe. The quality of the FISH signals generated from the in-house probes was comparable to that obtained using the commercially available probes (Figure 3.18).

Application of the combined FISH and immunofluorescence enables distinguishing cell populations with different cytokeratin expression and different HER2 levels. As Figure 3.18 B and C shows, SK-BR-3 can be detected distinctively from blood cells and MCF7 by cytokeratin and HER2 immunostaining, since HER2 gene amplification and polysomy of chromosome 17 can be evaluated in individual cells.

Table 3.6 Effects of temperature and heating time on the results of combined FISH and immunofluorescence of native cells

	120°C			100°C		
	2.5min	5min	10min	2.5min	5min	10min
Nuclear morphology	++	+	±	++	++	+
FISH intensity	++	++	+	++	++	++
IF intensity	++	++	+	±	+	+
Background	no	no	yes	yes	yes	yes

± not good, + sufficient, ++ good.

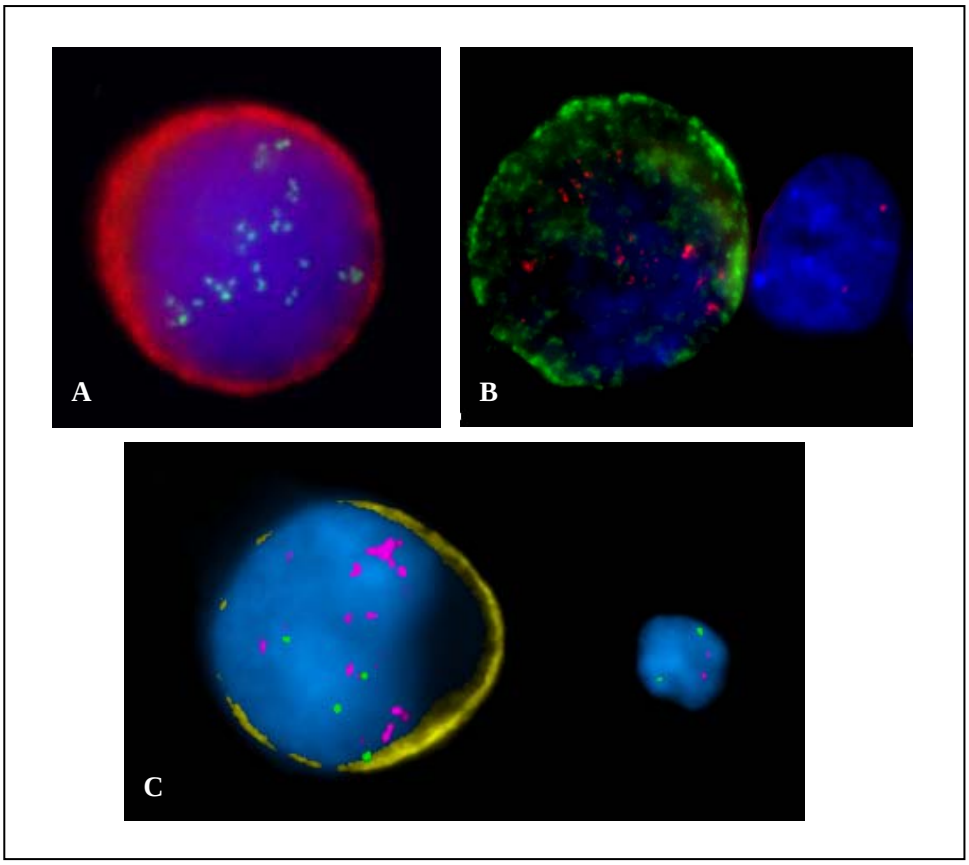


Figure 3.18 Combined FISH and immunofluorescence of native cells. A, SK-BR-3 cells with HER2 amplification (green: HER2 signals) and cytokeratin (red) positivity. B, SK-BR-3 cell (left) with HER2 overexpression (green: HER2 immunofluorescence) and HER2 amplification (red: HER2 signals), MCF7 cell (right) with two copies of the HER2 gene. C, SK-BR-3 cell (left) with cytokeratin positivity (yellow), HER2 amplification (red: HER2 signals) and polysomy of chromosome 17 (green: CEP17 signals), leukocyte (right) with two copies of each the HER2 gene and chromosome 17.

3.10 Combined FISH and immunofluorescence of processed cells

As mentioned above, the situation of processed cells was different from that of native cells. Thus, the heating conditions might be different from that for native cells. The results are summarized in Table 3.7. Increasing the heating temperature from 120°C to 125°C did not improve the results. Heating for 2.5min or for 5min at 125°C even resulted in the complete absence of the FISH results. By heating to 125°C for 1min,

FISH signal could be found in some cells, but the intensity in general was weak. Because the processed cells had been labeled with CK-PE, the background existed under all heating conditions. Nevertheless, the optimal condition of heat retrieval of processed cells is 120°C for 2.5min. Under this condition the FISH signals were clear, the intensity of immunofluorescence was strong and the background was low (Figure 3.19A).

Table 3.7 Effect of temperature and heating time on FISH results of processed cells

	120°C		125°C		
	2.5min	5min	1min	2.5min	5min
Nuclear morphology	++	+	+	±	±
FISH intensity	+	-	±	-	-
Background	yes	yes	yes	yes	yes

± not good, + sufficient, ++ good, - no FISH signal.

3.11 Combined FISH and immunofluorescence of paraffin-embedded cells from pleural effusion

For one sample of formalin-fixed paraffin-embedded pleural effusion cells from a breast cancer patient, only the optimal antigen retrieval condition was adopted (120°C, 2.5min). Besides cytokeratin immunofluorescence, EpCAM immunofluorescence staining (to distinguish tumor cells from mesothelial pleural cells) was also conducted combined with HER2 FISH. In this case, many cytokeratin or EpCAM positive cells were detected in the pleural effusion, but all without HER2 amplification. Most of these cells only had one copy of the HER2 gene (Figure 3.19B).

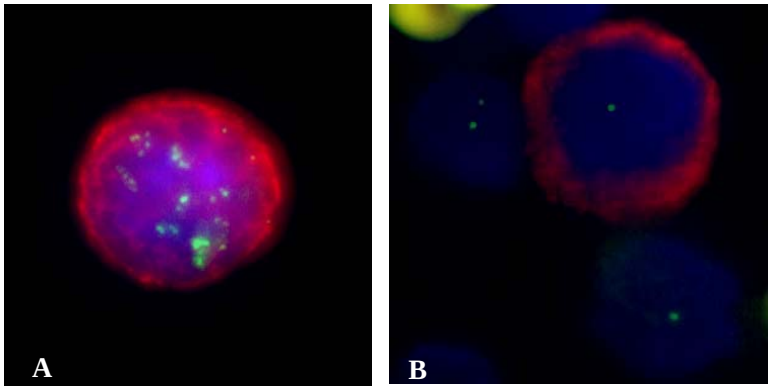


Figure 3.19 Combined FISH and immunofluorescence of a processed SK-BR-3 cell (A) and formalin-fixed paraffin-embedded cells from pleural effusion (B). Cytokeratin expression (red) and HER2 gene (green) amplification were detected.

3.12 Detection and characterization of CTCs from breast cancer patients using combined FISH and immunofluorescence

To further confirm the feasibility of the combined FISH and immunofluorescence procedure, three clinical samples with high number of CTCs detected with the CellSearch^{TM} system were analyzed using this method. In this study, only the HER2 probe labeled with Spectrum Green without a CEP17 probe was used. Therefore the HER2 positivity was defined as HER2 gene copy number >6.0 per nucleus according to the ASCO/CAP HER2 testing guideline (2007).

175 CTCs were recovered after the procedure, and no HER2-FISH-positive cells were found among these 175 CTCs. The majority of CTCs (137/175, 78.3%) had two HER2 signals and 17.1% (30/175) had one HER2 signal. An elevated number of HER2 gene copies was detected in seven CTCs (7/175, 4%) with three HER2 signals, and only one cell (1/175, 0.6%) had six copies of the HER2 gene, which was defined as equivocal in the ASCO/CAP HER2 testing guidelines. Figure 3.20 shows CTCs with different numbers of the HER2 gene.

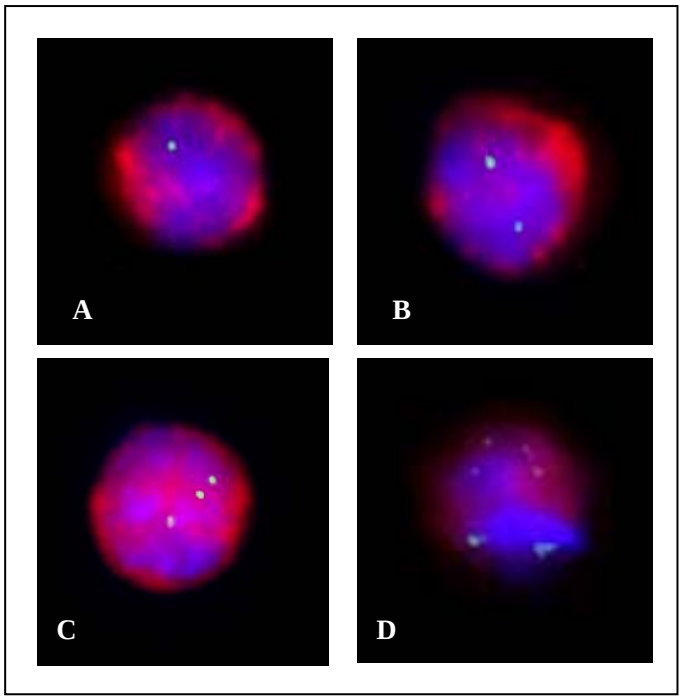


Figure 3.20 CTCs from breast cancer patients were detected and characterized by combined HER2 FISH (green) and CK immunofluorescence (red). A-D were CTCs with one, two, three and six copies of the HER2 gene, respectively.

3.13 Optimization of multiplex real time PCR

The conditions for a particular PCR reaction must be optimized for specific primers, probes, chemistry as well as for the specific PCR machine. In this study, the conditions were optimized for data obtained on the Mastercycler ep realplex 4.0 (Eppendorf) and the qPCR Master Mix for Probe Assay (Eurogentec).

In this study, the multiplex real time PCR conditions were optimized to obtain reliable, sensitive, stable and reproducible results. Some important PCR parameters such as the concentration of the primers, probes, Mg^{2+}, and PCR buffer were adjusted under the same cycling program: Uracil-N Glycosylase activation at 50°C for 2min, initial denaturation at 95°C for 15min, followed by 40-50 cycles that consisted of a denaturation step at 95°C for 15 seconds, an annealing step at 55°C for 30 seconds and an elongation step at 65°C for 30 seconds.

Firstly, the ratio of primers and probes of HER2 (17q21.1) and Contr17 (17q11.2) was optimized. Initially, equimolar primer and probe concentrations (200nM and 150nM respectively) of the two regions were tested. Subsequently, the ratio of primers of HER2 to Contr17 was adjusted while the ratio of the primers to the corresponding probe was kept. Although the efficiency of the PCR reaction was not optimal (Figure 3.21) when the ratio of HER2/Contr17 was 1:4, the signal curves were least scattering(Figure 3.22) comparing to those generated from other ratios of HER2/Contr17 (2:3, 3:2, 1:1, 4:1).

Secondly, the concentration of the PCR buffer was optimized. The results showed that the efficiency obtained by using a 0.8× PCR buffer was higher than 95%, being comparable to that obtained by higher buffer concentrations. Therefore, this PCR master mix concentration was chosen for the following experiments.

Finally, the concentration of $MgCl_2$ was adjusted. The tested concentrations were 4mM, 4.5mM, 5mM, 7mM and 9mM. Increasing the concentration of Mg^{2+} improved the efficiency of the multiplex reaction, especially for Contr17. At 4.5mM of $MgCl_2$, the efficiencies of HER2 and Contr17 were 93% and 96% respectively and the signal curves were least scattering.

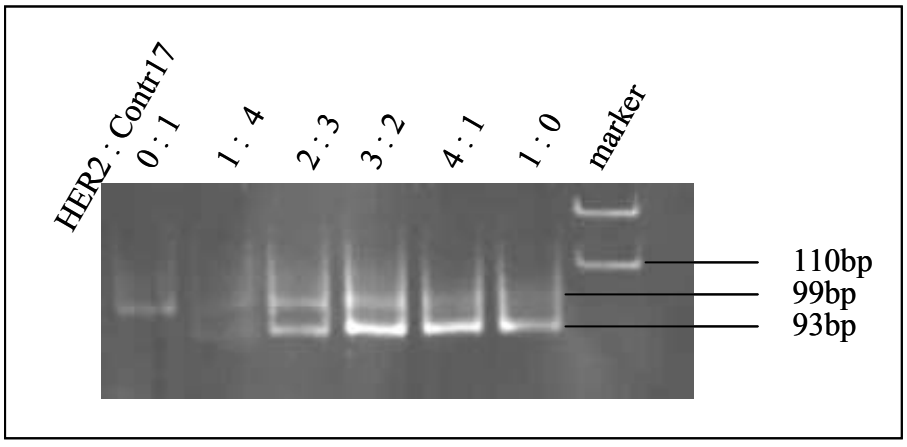


Figure 3.21 Native polyacrylamide gel electrophoresis of multiplex real time PCR. The amplicon sizes of HER2 and Contr17 are 93bp and 99bp, respectively.

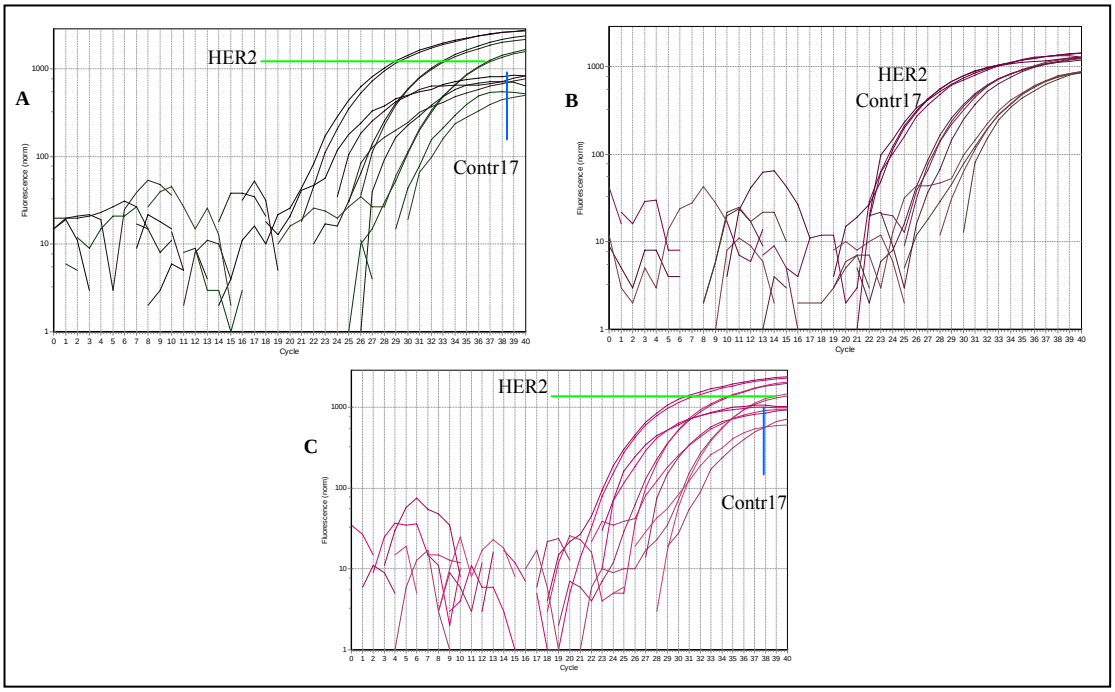


Figure 3.22 Real time PCR of human genomic DNA with different concentrations of primers and probes. A, HER2: Contr17 is 1:1. B, HER2: Contr17 is 1:4. C, HER2: Contr17 is 2:3.

3.14 Real time PCR analysis of HER2 gene amplification in breast cancer cell lines

The ratio of the HER2 gene to the internal control Contr17 was quantified in the following breast cancer cell lines with known, but different HER2 amplification values by monoplex real time PCR (SYBR Green): BT474, SK-BR-3, T47D, MCF7, MDA-MB-231, MDA-MB-435S and MDA-MB-468. Figure 3.23A shows an example of the signal curves of HER2 amplification and Figure 3.23B shows typical nonamplification signal curves, which were generated from SK-BR-3 and MCF7 cell lines, respectively. Figure 3.23C shows the standard curves generated by plotting the log concentration of the standards versus the Ct value. The slopes of the standard curves are not equal, indicating a small efficiency difference between the two reactions. But this efficiency difference has no effect on the quantification because

each target gene is compared to its own standard curve in every reaction. The ratio was calculated using the mean Ct values of samples run in duplicates. The results are summarized in Table 3.8. As expected, BT474 and SK-BR-3 showed HER2 amplification with ratios above the cutoff value of 2.0 whereas the ratios of the other cell lines were lower than the cutoff value.

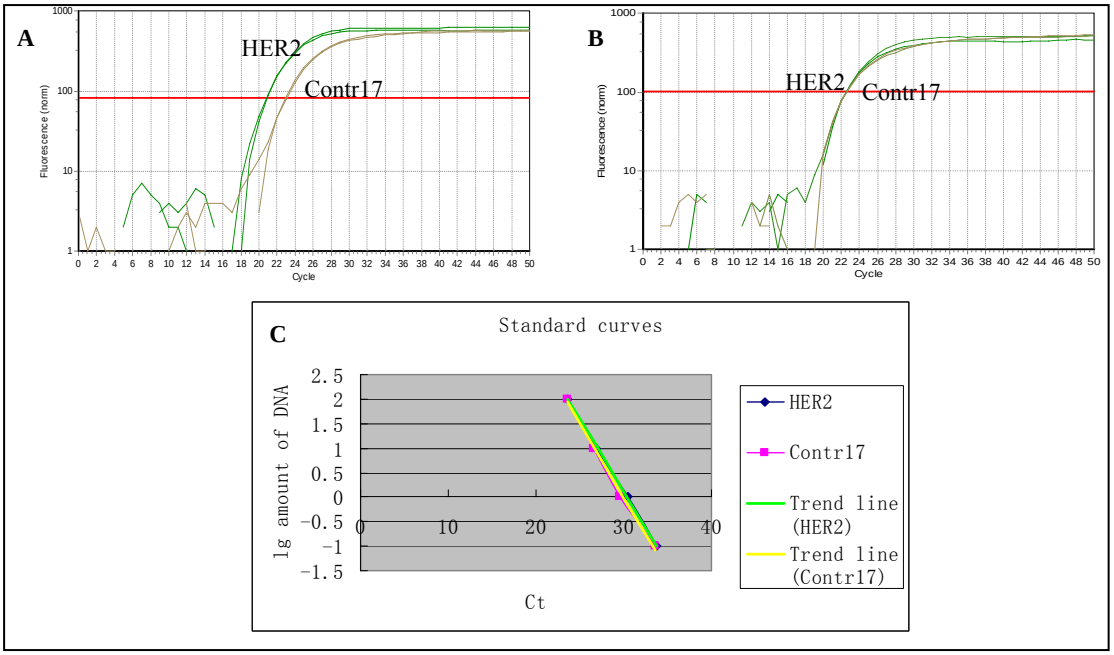


Figure 3.23 HER2 gene detection in breast cancer cell lines by real time PCR. A, signal curves of SK-BR-3. B, signal curves of MCF7. C, standard curves for quantification.

Table 3.8 HER2 status in breast cancer cell lines

Cell line	FISH ratio	PCR ratio
BT474	9.2	14.0
SK-BR-3	5.5	5.0
T47D	1.1	1.1
MCF7	1.0	1.0
MDA-MB-231	0.9	1.3
MDA-MB-435s	1.0	1.1
MDA-MB-468	1.0	1.5

3.15 Real time PCR analysis of HER2 gene amplification in paraffin-embedded breast cancer tissues

To see whether the primers used for the determination of the HER2 status in breast

cancer cell lines are also applicable for clinical specimens, a real time PCR protocol was established for DNA extracted from paraffin-embedded breast cancer tissues. Therefore, DNA samples from 11 breast cancer patients with known HER2 status determined by FISH were analyzed for HER2/Contr17 ratio by monoplex and multiplex real time PCR. Afterwards, the results obtained by PCR were compared with those obtained by FISH to determine the sensitivity and specificity of the PCR method.

3.15.1 Monoplex real time PCR

In ten of eleven samples interpretable signal curves by monoplex SYBR Green real time PCR were obtained. The results are shown in Table 3.9. In one sample, there was no adequate DNA amplification. Thus, no interpretable signal curves could be generated. In eight samples HER2 amplification was detected with ratios of HER2/Contr17 higher than 2.0. The other two samples did not indicate HER2 amplification since ratios of HER2/Contr17 were lower than 2.0. Typical FISH results and signal curves for significant HER2 gene amplification (from sample 7) and HER2 non-amplification (from sample 11) are shown in Figure 3.24 and Figure 3.25, respectively.

Table 3.9 Results of HER2 monoplex real time PCR in breast cancer patients

	PCR		FISH	
Sample	HER2/Contr17	Status	HER2/CEP17	Status
1	29.1	amplified	cluster	amplified
2	8.0	amplified	cluster	amplified
3	12.5	amplified	cluster	amplified
4	16.3	amplified	2.8	amplified
5	15.1	amplified	cluster	amplified
6	10.6	amplified	2.8	amplified
7	23.0	amplified	cluster	amplified
8	7.0	amplified	cluster	amplified
9	---		1.9	nonamplified
10	1.9	nonamplified	1.0	nonamplified
11	1.3	nonamplified	1.6	nonamplified

--- no amplificable DNA available

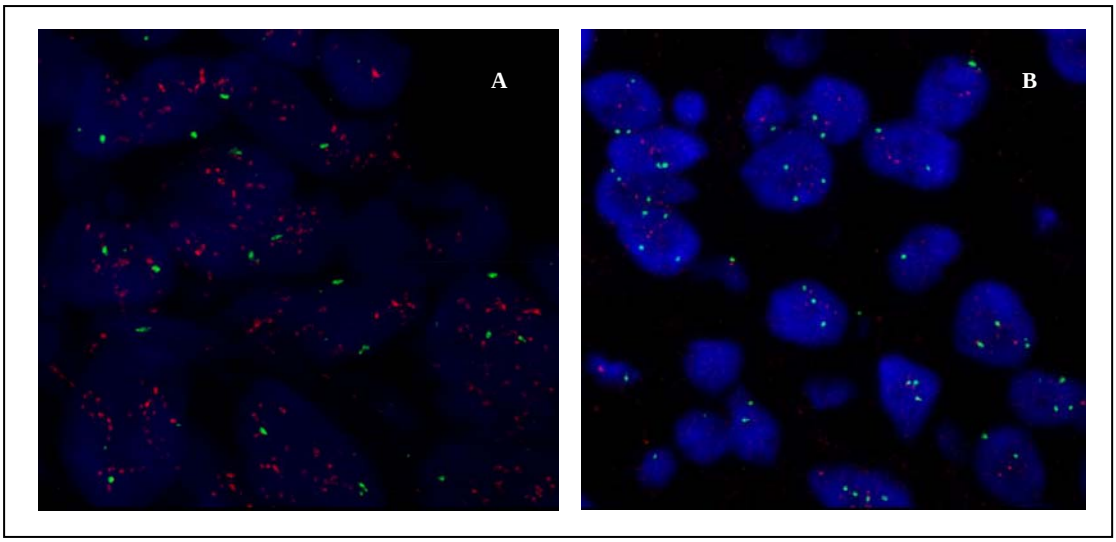


Figure 3.24 HER2 status determined by FISH in tissue samples from breast cancer patients. A, HER2 amplification with HER2 signal clusters (red) in sample 7. B, HER2 non-amplification with HER2/CEP17=1.6 in sample 11. The green signals are from CEP17.

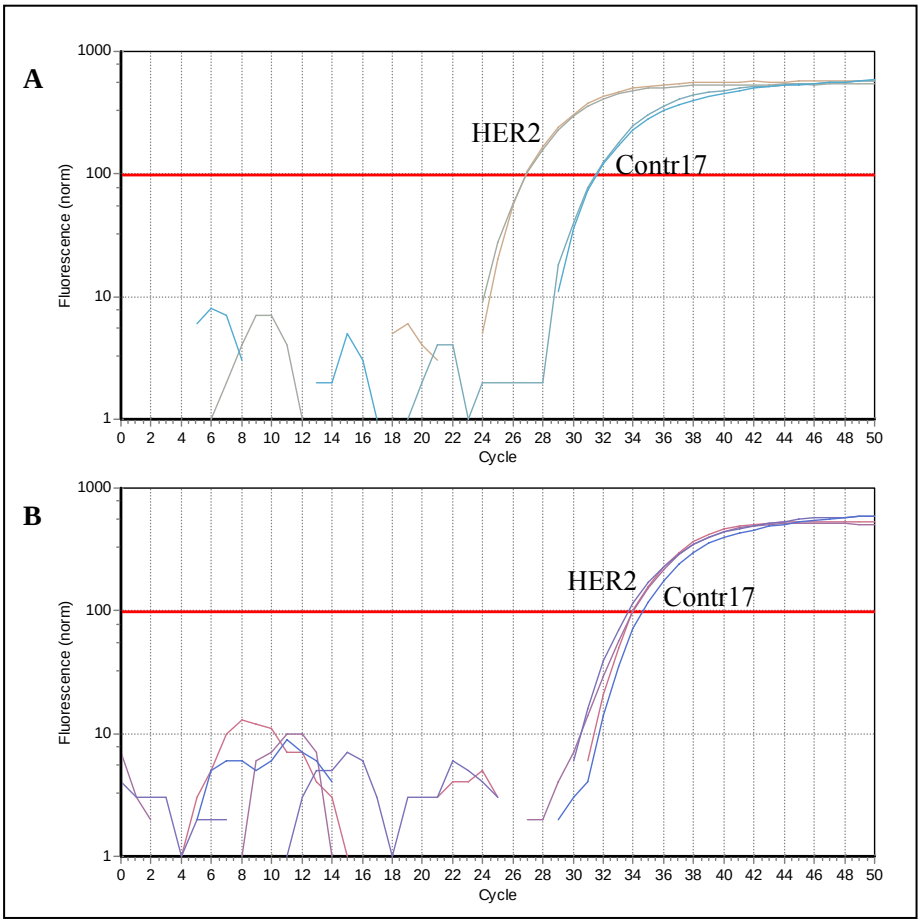


Figure 3.25 Real time PCR analysis of HER2 gene amplification in tissue samples from breast cancer patients. A, the HER2 gene is significantly amplified with

HER2/Contr17= 23 from sample 7. B, the HER2 gene is not amplified with HER2/Contr17= 1.3 from sample 11.

3.15.2 Multiplex real time PCR

In all of eleven analyzed samples interpretable signal curves by multiplex real time PCR (TaqMan assay) were obtained. The results are summarized in Table 3.10. In nine samples HER2 amplification was detected with ratios of HER2/Contr17 higher than 2.0. The other two samples did not indicate HER2 amplification since the ratios of HER2/Contr17 were lower than 2.0.

Table 3.10 Results of HER2 multiplex real time PCR in breast cancer patients

	PCR		FISH	
Sample	HER2/Contr17	Status	HER2/CEP17	Status
1	19.7	amplified	cluster	amplified
2	8.8	amplified	cluster	amplified
3	11.5	amplified	cluster	amplified
4	6.0	amplified	2.8	amplified
5	13.2	amplified	cluster	amplified
6	4.8	amplified	2.8	amplified
7	21.0	amplified	cluster	amplified
8	8.1	amplified	cluster	amplified
9	1.7	nonamplified	1.9	nonamplified
10	1.2	nonamplified	1.0	nonamplified
11	2.2	amplified	1.6	nonamplified

3.16 Comparison of FISH and real time PCR analysis of HER2 status

3.16.1 Cell lines

As shown in Table 3.8, there is a high concordance between FISH and real time PCR analysis concerning the HER2 status. The cell lines with HER2 amplification detected by FISH (BT 474 and SK-BR-3) were found to be amplified using real time PCR as well. None of other cell lines that were HER2 non-amplified by FISH presented

HER2 amplification by real time PCR.

3.16.2 Clinical samples

Results of both monoplex and multiplex real time PCR are highly concordant with FISH analysis from formalin-fixed paraffin-embedded tissue sections of breast cancer patients (Table 3.11).

In monoplex PCR, HER2 status of ten patients with interpretable signal curves completely (10/10,100%) agreed with that determined by FISH. In multiplex PCR, only one sample showed HER2 amplification determined by PCR while non-amplification was determined by FISH. In the other ten samples (10/11, 90.9%), the results obtained by multiplex PCR were similar to those obtained by FISH.

Table 3.11 Comparison of the HER2 status in clinical samples by FISH and real time PCR analysis

	Monoplex		Multiplex	
FISH/PCR	+	-	+	-
+	8	0	8	0
-	0	2	1	2

+ HER2 amplification; - HER2 non-amplification.

3.17 Real time PCR analysis of HER2 gene amplification in processed cells at the single-cell level

To eliminate the contamination of non-neoplastic cells and improve the accuracy of HER2 evaluation by real time PCR, analysis at single CTC level could be needed. The recently developed technique of whole genome amplification (WGA) can overcome the limit of the scarce DNA to a PCR at single-cell level.

To establish the methods involving DNA extraction from single cell, WGA and real time PCR for DNA amplified by WGA, a small number of cells were primarily

analyzed. In the present study, two to five hundred processed and native SK-BR-3 cells were lysed by lysis buffer on a hydrophobic slide, subsequently the lysate was transferred into a new tube to preserve or continually conduct WGA. Normal human genomic DNA was also amplified by WGA. HER2 gene quantification was performed by real time PCR after WGA.

Initial experiments were performed to screen six samples of WGA amplified SK-BR-3 DNA using SYBR Green chemistry and a TaqMan assay. We found that there was no interpretable signal curve obtained in SYBR Green real time PCR using amplified DNA as template, but PCR products could be observed by agarose gel electrophoresis, with a wide range of high molecular weight DNA producing high background (Figure3.26). Whereas in TaqMan assay, among six screened cases, four yielded both HER2 and Contr17 amplification curves, one case only had HER2 curve, and another one had no signal curve for either HER2 or Contr17. The subsequent agarose gel electrophoresis presented the same results as the TaqMan assay indicated. The absence of amplicons might be due to the loss of the target region during the transfer from hydrophobic slides to reaction tubes.

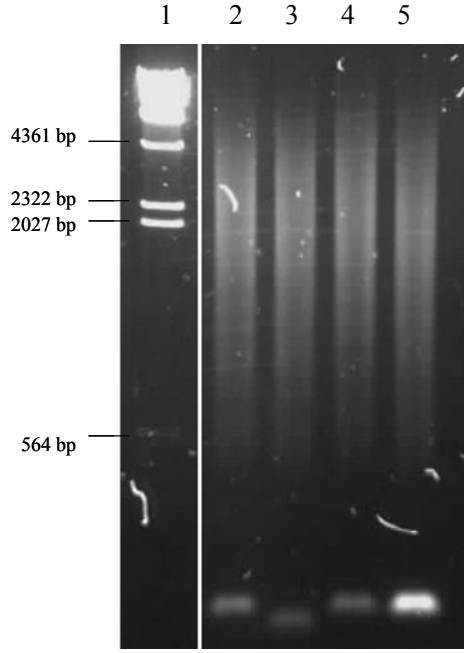


Figure 3.26 Products of SYBR Green real time PCR visualized by agarose gel electrophoresis. Lane 1, molecular weight DNA marker II. Lane 2-5, amplified SK-BR-3 DNA with WGA.

In a next step we digested amplified DNA and corresponding original DNA with *Eco*RI, not cleaving the target region, before SYBR Green real time PCR. Subsequently interpretable signal curves were obtained in samples. The reason for this result is that the digestion results in smaller fragments and increasing the reaction efficiency as well as signal-to-noise ratio. Therefore we employed SYBR Green chemistry combined with prior *Eco*RI digestion to analyze HER2 gene amplification.

To answer the question, whether the CellSearch™ system processing has an interference with the accuracy of the evaluation of the HER2 status by real time PCR, we quantified the HER2 gene in DNA from native and processed SK-BR-3 cells. The results showed that the HER2 quantification in processed SK-BR-3 cells (HER2/Contr17=5.0±0.8) are quite similar to that obtained with native SK-BR-3 cells (HER2/Contr17=3.9±0.1), indicating that the CellSearch™ system processing has no significant impact on HER2 evaluation by real time PCR.

Whether the WGA procedure affects the downstream real time PCR is another question we should discern. In this pilot study, SYBR Green PCR was initially conducted in amplified healthy human genomic DNA in two replicates. The ratios of HER2/Contr17 in the amplified DNA varied from 1.2 to 6.0 relative to the original control DNA, while an ideal ratio of HER2/Contr17 in human genomic DNA should be 1.0. However, the reaction efficiency of both target regions in amplified DNA varied from 147% to 196%, which was significantly higher than the efficiency of around 90%-100% in original DNA. The efficiency of much higher than 100% definitely means the generation of unspecific products. Thus, the results cannot be trusted. Subsequently, we employed the TaqMan assays, a more specific method, to repeat the experiment. Although the reaction efficiency was acceptably around 90% in both amplified and original DNA, the ratios of HER2/Contr17 in the amplified DNA varied from 4.7 to 5.3 relative to the original control DNA. Therefore, to achieve reliable and accurate results, the methods must be further optimized.

4. Discussion

In the present study, CTCs were detected by the CellSearch™ system. The aim of this study was to establish and optimize methods for further phenotypical and molecular characterization of these cells.

The CellSearch™ system is an automated and standardized system for the detection and enumeration of CTCs in peripheral blood (Balic et al. 2005). It has been cleared by the FDA for monitoring patients with metastatic breast cancer. By utilizing a cutoff of ≥5 CTCs per 7.5ml blood, detection of CTCs with the help of the CellSearch™ system has been shown to be a reliable independent prognostic factor and a predictive factor for patients with metastatic breast cancer (Cristofanilli et al. 2004; Cristofanilli et al. 2005; Hayes et al. 2006). The presence of ≥5 CTC per 7.5ml blood predicts a decreased progression-free survival and overall survival, and the increase of CTC levels at follow-up strongly suggests resistance to treatment (Cristofanilli et al. 2004). The validation of the analytic performance of the CellSearch™ system in routine setting has been proved by our institute previously (Riethdorf et al. 2007). Within this multicenter validation study it was shown that the CellSearch™ system has a high sensitivity and specificity, and using special preservation tubes for blood drawing and transport at room temperature, the enumeration of CTC is stable for at least 72h.

The CellSearch™ system provides four channels to distinguish different fluorescently-labeled cells yielded by using differently labeled antibodies. In general, a cell is classified as CTC when it is DAPI positive/CK-PE postive/CD45-APC negative with nearly round or oval morphology, carries a visible nucleus within the cytoplasm, and has a cell size of at least 4μm. Therefore the FITC channel is left for an additional marker of interest, such as HER2. Nevertheless, to our best knowledge, there is no publication available about HER2 testing with the help of the CellSearch™ system until now. Thus, there are no data available yet about a correlation of HER2 evaluation between the CellSearch™ system and the gold standard-FISH. In addition,

we were interested in the evaluation of some other important biomarkers for breast cancer, such as ER, PR, Ki-67 and M30 on CTCs. Therefore, the major aim of this study was to establish and optimize a set of methods including immunofluorescence, FISH, combined FISH and immunofluorescence, and real time PCR to further characterize CTCs after processing blood samples from breast cancer patients with the CellSearch^{TM} system.

4.1 HER2 expression in circulating tumor cells

With the introduction of trastuzumab, a humanized monoclonal antibody directed against the extracellular domain of the HER2 receptor, into adjuvant treatment regimens of breast cancer patients, the clinical outcome could be significantly improved (Slamon et al. 2001). Therefore, HER2 testing in formalin-fixed paraffin-embedded tissue sections from the primary tumor after operation has become a routine test in pathologic labs. Furthermore, more and more studies (Hayes et al. 2002; Wulfing et al. 2006) are paying attention to HER2 evaluation in CTCs, which might provide a new therapeutic target for patients with HER2-negative primary tumors. The conversion of the HER2 status in CTCs, from HER2 negativity in the primary tumor to HER2 positivity in CTCs, supports the possibility that HER2 gene amplification can be acquired during the progression of breast cancer (Hayes et al. 2002; Meng et al. 2004b; Wulfing et al. 2006). In the study of Meng et al. (2004b), four patients with this HER2 conversion received trastuzumab-containing therapy. Three of them had response to the therapy: one showed a complete response, and the other two presented a partial response. Although the results give inspiring information for the patients with HER2-negative primary tumors, prospective randomized clinical trials must be done to clarify the benefit of an additional trastuzumab treatment for those patients.

The conventional pathologic analysis of the HER2 status comprises immunohistochemistry (IHC) and FISH. For evaluation of HER2 expression by IHC,

results are scored as 0, 1+, 2+, and 3+ depending on the intensity of membrane staining and the percentage of detectable staining (Jacobs et al. 1999). In the FISH assay, at least 40 nuclei have to be counted from nonoverlapping invasive tumor cells, and the average number of HER2 gene signals per nucleus or the average ratio of HER2/CEP17 is calculated (Hicks et al. 2005). Both methods determine the average gene and expression status of a population of tumor cells, not necessarily reflecting the HER2 status of an individual tumor cell. Data presented at the 43rd American Society of Clinical Oncology annual meeting demonstrated that patients with HER2 FISH-negative and IHC score of 0-2+ tumors can also benefit from trastuzumab treatment (Paik et al. 2007). This phenomenon might be due to the intratumoral heterogeneity of the HER2 status in breast cancer tissues (Shin et al. 2006). In a recent study, uPAR and HER2 were detected in individual CTCs from 52 samples (Meng et al. 2006). The results showed that the distribution of uPAR and HER2 is not homogeneous between the cells in a given sample. Our study confirmed the findings of heterogeneity in that HER2-postive CTCs were detected in 15 of 51 patients (29.4%) by the CellSearch^{TM} system. However, from these 15 samples, only 19.4% (74/382 CTCs) of CTCs were positive for HER2 while 80.6% (308/382 CTCs) were HER2-negative. Thus, the HER2 status of one cell is not necessarily representative for that of all cells in a given sample.

As mentioned above, the HER2 status of primary tumors determined by IHC or FISH strongly predicting the benefit of trastuzumab treatment is based on the evaluation of at least 40 cells. Similarly, a prognostically relevant cutoff level of ≥5 CTCs per 7.5ml blood is also determined for the clinical significance of CTC detection. However, there is no information about the number of HER2-positive CTCs which can reflect the gene status of patients and predict the benefit of trastuzumab treatment for breast cancer patients with HER2-negative primary tumors. So the question came up, whether trastuzumab treatment can be administrated to a patient with one HER2-positive CTC. Meng et al. (2004b, 2006) used 10 consecutive CTCs (a bin) to determine the HER2 gene status and classified the entire bin as HER2-positive or

–negative if five or more cells revealed to be of the same phenotype or genotype. The results indicated a high concordance between CTCs and overall HER2 gene status of patients. In contrast, another study enumerated HER2-positive cells individually and observed a discrepancy between the detection of HER2-positive CTCs and the HER2 status of corresponding primary tumors (Wulfing et al. 2006). In their study, 17 patients contained HER2-positive CTCs, and twelve of them just had 1-4 HER2-positive cells. Although the authors did not describe the frequency of HER2-positive CTCs in 12 patients with HER2-negative primary tumors, we can deduce that the concordance between CTCs and primary tumors might be increased by using the criteria described by Meng et al. to evaluate the HER2 status of CTCs. Nevertheless a significant correlation between the presence of HER2-positive CTCs even as few as 1-4 cells and poor clinical outcome was observed in Wulfing's study. Taking together, it is difficult to answer the question raised above. In our study, firstly more attention is paid to the analytic performance of the CellSearch^{TM} system than to the clinical significance of HER2-positive CTCs. The number of HER2-positive CTCs which is sufficient for diagnosis should be determined in future experiments, and the appropriate cutoff must be selected considering high costs and potential cardiotoxicity of trastuzumab.

In our study, HER2 expression in CTCs detected by the CellSearch^{TM} system was divided into three categories: negative, dim positive, and strongly positive. The breast cancer cell lines SK-BR-3 and MCF7 with known HER2 expression and gene amplification were spiked into blood to perform a calibration. SK-BR-3 is strongly positive for HER2 and as such scored immunocytochemically 3+ and the average ratio of HER2/CEP17 signals determined by FISH was 5.5. MCF7 cells are weakly positive in immunocytochemistry, therefore scored as 1+ and FISH negative with a HER2/CEP17 ratio of 1.0. After CellSearch^{TM} processing SK-BR-3 cells presented strongly positive for HER2, and the majority of MCF7 cells were only weakly positive or negative for HER2. In CTCs from breast cancer patients we also found these different expression levels of the HER2 protein. However, so far, there are no

data published to demonstrate whether these staining patterns captured by the CellSpotter® Analyzer reflect the "true" HER2 status of CTCs, especially for those dim positive cells, lacking comparison with results of FISH. To validate the specificity and accuracy of determination of the HER2 status in CTCs by the CellSearch™ system, more clinical samples should be collected and reinvestigated by FISH. To our knowledge, Immunicon Corporation is developing a technique to detect cytogenetic aberrations in CTCs with FISH by the CellSearch™ system. This technique will give a promising perspective for an accurate evaluation of the HER2 status in CTCs by the CellSearch™ system.

We developed a combined FISH and immunofluorescence procedure to redetect CTCs concerning their HER2 gene status simultaneously following CellSearch™ system processing. Most of 175 CTCs from 3 cases analyzed with this technique were HER2 non-amplified and only one CTC had an equivocal result with six copies of HER2 gene. Unfortunately HER2 testing in these three samples was not performed by the CellSearch™ system, thus the assessment of HER2 status between the CellSearch™ system and the following FISH assay could not be compared. Furthermore the results obtained from such a small number of clinical samples cannot represent the trend of the presence of HER2-positive CTCs from breast cancer patients. Recent studies (Meng et al. 2004b; Hayes et al. 2002) suggested that the presence and frequency of HER2-positive CTCs at initial diagnosis might be related to the status of the primary tumor, but a conversion of HER2 could be observed during tumor progression. Interestingly, it was reported that the HER2 gene copy numbers in HER2-positive CTCs were 2~3 fold lower than those in corresponding HER2 amplified primary tumors (Meng et al. 2004b). The comparison of the HER2 status between primary tumors and CTCs in peripheral blood or DTCs in bone marrow from breast cancer patients was also performed by recent studies (Braun et al. 2001; Solomayer et al. 2006; Wulfing et al. 2006; Vincent-Salomon et al. 2007). These results revealed that although in the majority of breast cancer patients the HER2 status remained stable between the primary tumor and the micrometastasis (Vincent-Salomon et al. 2007),

discrepancies between HER2 status in the primary tumors and CTCs/DTCs were commonly observed (Braun et al. 2001; Solomayer et al. 2006; Cristofanilli et al. 2006; Wulfing et al. 2006). These discrepancies might be due to either heterogeneity of the primary tumor or gain of HER2 gene amplification during tumor progression. Our result that five patients with HER2-negative primary tumors had strongly HER2 positive CTCs in peripheral blood is in line with these published data. These results imply that the HER2 overexpression of micrometastasis might provide a new target for breast cancer therapy in patients with HER2-negative primary tumors. Thus the accuracy of the HER2 evaluation in CTCs/DTCs appears extraordinarily important. Our combined FISH and immunofluorescence method could be a robust tool for this purpose due to its potential to combine phenotyping and genotyping target cells simultaneously.

4.2 Pretreatment for immunofluorescence staining of nuclear antigens

Estrogen receptor (ER), progesterone receptor (PR), and proliferation marker Ki-67 are very important prognostic and predictive factors for breast cancer (Hayes et al. 2001). The most common method to detect these nuclear antigens is immunostaining. In immunocytochemical techniques preliminary cell fixation and permeabilization, the two crucial steps that are mandatory for the detection of nuclear antigens, can be performed in various ways (Millard et al. 1998). Fixation methods are generally categorized into two classes: organic solvents and cross-linking reagents. Acetone and methanol belonging to organic solvents can remove phospholipids from cell membranes resulting in the access of antibody to the intracellular antigen (DiDunato et al. 2003). Formalin and paraformaldehyde belonging to cross-linking reagents react predominantly with the protein amino groups and form a network of linked antigens (Namimatsu et al. 2005). Cross-linkers preserve cell structures better than organic solvents, but may require the addition of a permeabilization step for the detection of some intracellular antigens. Detergents such as Tween 20, Triton X-100, or saponin are often used to permeabilize the membrane (Jacob et al. 1991; Landberg et al. 1992;

Glasova et al. 1995). Indeed, the correct choice of a method depends on the cell type examined and the characteristics of the antigens to be detected (Millard et al. 1998). Our findings underline this issue. For native cells, fixation with formaldehyde or cold acetone resulted in comparable results of immunofluorescence staining for the detection of nuclear antigens, whereas for cells resulting from CellSearch™ processing, five fixation-permeabilization methods, including fixation with formaldehyde, cold acetone, methanol, or paraformaldehyde, and permeabilization with Tween 20 or Triton X100, failed to detect Ki-67 expression. These results confirm that optimization experiments of fixation and permeabilization have to be performed prior to the conduction of particular immunocytochemical analysis.

Antigen retrieval (AR), a new approach for restoration of immunoreactivity using heating tissues or cells in solutions containing various metals, has been widely applied for the demonstration of ER, PR, Ki-67, HER2, etc. expression in formalin-fixed paraffin- embedded (FFPE) tissues (MacGrogan et al. 1996; Pertschuk et al. 2000). Similar to FFPE tissues, processed cells also have been fixed during the CellSearch™ processing. Thus, the formation of protein cross-linking could hinder the access of antibodies to nuclear antigens. This might be the reason why all five methods mentioned above failed to demonstrate Ki-67 in processed cells. This can also explain why Ki-67 antigens were detected successfully in processed cells after pretreatment with AR following cold acetone fixation. (More details about AR will be discussed in part 4.5)

Previous results of du Manoir et al. (1991) and Suurmeijer et al. (1999) indicated that different Ki-67 staining patterns could reflect different cell cycle stages. In G0 and early G1 phase, no Ki-67 staining was detected. Ki-67 had presented since late G1 phase and the content increased with cell cycle progression. In later G1 phase, the staining was localized principally in nucleoli. In S phase, increasing nucleolar staining and increasing diffuse nuclear staining were observed. An additional strong diffuse and granular staining presented in G2 phase. In prophase, nucleoli are no longer

visible, instead a bright meshwork throughout the nucleoplasm was observed. In metaphase, peripheral staining of individual chromosomes could be demonstrated. In our study we found similar results. However, as a prognostic factor for breast cancer, more attention is paid to the Ki-67 index (percentage of cells staining positive) rather than different staining patterns of individual cells (Colozza et al. 2005). Although Ki-67 immunostaining cannot be used for detailed cell cycle analysis, it might give some hints for particular cell cycle phases in which the cells are. Nevertheless we should note that only optimally stained samples can demonstrate the entire spectrum of Ki-67 staining patterns, otherwise, only S-G2 or M-phase staining patterns could be seen (Suurmeijer et al. 1999).

4.3 Detection of apoptosis by M30 immunostaining

During apoptosis, the programmed cell death, a cell undergoes dramatic changes in morphology including cellular shrinkage, membrane blebbing, nuclear condensation and fragmentation (Mehes et al. 2001), which are due to a complete reorganization of its cytoplasmic and nuclear skeleton (van Engeland et al.1997). The activation of proteolytic caspase cascades is a critical feature during apoptosis which is responsible for the stepwise cleavage of many vital cellular proteins (Salvesen et al. 1997). Recently it was found that the cleavage of CK18 at the ^{393}DALD/S site by caspases 3, 7, and 9 is an early event, which generates a fragment of approximately 45kD. A second cleavage at the L1-2 linker region by caspase 6 is responsible for the final collapse of the keratin skeleton into large aggregates, which generates a fragment of 20 kD (Schutte et al. 2004). The monoclonal antibody M30 can identify these newly generated ~45kD and 20kD fragments by detecting a 10-residue epitope of CK18 on the liberated C terminus as a cleavage at ^{393}DALD/S site (Leers et al. 1999; Schutte et al. 2004). Consequently, detection of caspases-cleaved CK18 by M30 can show a wide spectrum of apoptosis from early stage to late stage as shown in our study.

Our study revealed a serial change in cytokeratin and M30 neoepitope expression as

well as morphological changes during apoptosis from early stage to late stage. The appearance of the M30 neoepitope in apoptotic cells is coincident with the gradual disappearance of cytokeratin and the appearance of cellular shrinkage and nuclear fragmentation. These findings are consistent with those resulting from a study of Grassi and colleagues (2004). In addition, the report of Grassi et al. (2004) suggested that M30 immunostaining is superior to the TUNEL assay in detection of apoptotic cells. TUNEL, an abbreviation for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling, is known as a standard technique to detect apoptosis (Walker et al. 2001), demonstrating the formation of DNA strand breaks as a result of DNA strand cleavage during apoptotic breakdown of nuclei (Morsi et al. 2000). In contrast to the TUNEL assay that may also show positivity in necrotic cells resulting from extensive DNA degradation (Furth et al.2006), M30 immunotaining embraces superior specificity for exclusive positivity in apoptotic cells, but not in intact or necrotic cells (Barret et al. 2001; Kadyrov et al. 2000). On the other hand, since the CK18 cleavage starts earlier than DNA cleavage, M30 immunostaining appears to detect the introduction of apoptosis earlier than TUNEL, and therefore a higher number of events can be detected by M30 immunostaining (Leers et al. 1999; Kadyrov et al. 2001; Grassi et al. 2004). Conclusively, the detection of the M30 antigen is superior and might be an alternative to TUNEL in detecting apoptotic cells since the former is easier to handle, more specific and sensitive (Carr et al. 2000; Kadyrov et al. 2000).

4.4 Effects of processing the blood within the CellSearch™ system on downstream experiments with CTCs

The problems appearing during the establishment of immunofluorescence and FISH for processed cells revealed that the fixation during the CellSearch™ system processing could have side effects on the penetration of antibodies or probes through cell membranes. The same fixation-permeabilization protocol can successfully demonstrate nuclear antigens in native cells while failing to work in processed cells.

Pretreatment with proteinase K led to hybridization signals in native cells, whereas it generated high background without recognizable FISH signals in nuclei of processed cells. The fixation performed within the CellSearch^{TM} system run could result in the formation of protein cross-linking and hamper the access of antibodies and probes to the target in nuclei. This explains the success of the application of antigen retrieval techniques in nuclear antigen immunostainings and FISH for processed cells. In addition, enhancing permeabilization using pepsin combined with RNase A instead of only proteinase K pretreatment also succeeded in hybridization signals in processed cells. Although the processing of the CellSearch^{TM} system had adverse effects on nuclear antigen immunofluorescence, it facilitated membranous antigen staining without any need for further pretreatment.

The cells processed by the CellSearch^{TM} system are labeled with anti-EpCAM ferrofluids, anti-CK-PE, anti-CD45-APC and all these antibodies derived from mouse. The binding of at least two of these antibodies to the cells of interest limit the selection of new antibodies for a further characterization of these cells. If we want to use further antibodies derived from the same species, we can only use directly labeled antibodies. Otherwise before the application of new antibodies a complete blocking of previously used antibodies should be performed to avoid cross-reactions due to secondary antibodies. Since the commercial availability of fluorescently-labeled primary antibodies is limited, some studies described labeling procedures for antibodies with fluorochromes (Brown et al. 2004; Ino 2004). However, labeling of antibodies is time-consuming, laborious and requires relatively large quantities of the purified antibody (Mao 1999). On the other hand, we should keep in mind that all these antibodies combined to processed cells are primary antibodies, serum blocking generally utilized in sequential double immunofluorescence with primary antibodies derived from same species is not applicable in this case because the target of serum blocking is the secondary antibody instead of the primary one. Thus to avoid secondary antibodies' cross-reaction, blocking with monovalent secondary antibodies, especially Fab fragments, has been suggested (Lewis Carl et al. 1993; Negoescu et al.

1994). This method may generate satisfactory results, but the procedure of establishment and optimization to obtain highly specific results might be time-consuming. Therefore, in our study, the use of directly labeled mouse monoclonal antibodies was preferred to Fab fragments blocking. Besides directly labeled antibodies, unlabeled antibodies derived from other than mouse species such as polyclonal antibodies produced in rabbit or goat or monoclonal rabbit antibodies are also good choices for avoiding cross-reactions. Thus, they will become a promising tool for further immunocytochemical characterization of CTCs.

Whether the CellSearch^{TM} processing interferes with the downstream molecular analysis of processed cells is a further important question we would like to answer by establishing a real time PCR protocol. In particular, the aim of our study was to quantify the HER2 gene copy number in processed cells. Therefore, both two to five hundred of processed and native SK-BR-3 cells were lysed to extract DNA for real time PCR. The ratios of HER2/Contr17 in processed and native SK-BR-3 cells were 5.0±0.8 and 3.9±0.1 respectively, both presenting HER2 amplified. The concordance of real time PCR results between processed cells and native cells indicates that the CellSearch^{TM} procedure does not strongly affect the real time PCR analysis. This is a prerequisite for the evaluation of the HER2 status in individual CTCs detected by the CellSearch^{TM} system using molecular analysis. In fact, nuclear dyes used in immunostainings can affect the downstream analysis of the sampled genetic material. Ehrig et al. (2001) examined the effect of four histological nuclear dyes (methyl green, hematoxylin, toluidine blue O, azure B) on real time PCR of genomic DNA from tissue sections after manual dissection or laser capture microdissection (LCM). Their results showed that when manually dissected tissue sections were analyzed, PCR of DNA after hematoxylin staining was inferior to that after staining with the other three dyes. In contrast, there was no influence of the staining method on DNA amplification when tissue was sampled by LCM. Most likely, this difference is related, at least partly, to the amount of tissue sampled (Ehrig et al. 2001). In our study we planed to detect HER2 amplification at single-cell or low cell level after processing blood

samples by the CellSearch^{TM} system. Thus, the effect of nuclear dye on PCR will be minimized.

4.5 Combined FISH and immunofluorescence by aid of antigen retrieval technique

In many published studies directed to evaluate the HER2 status of CTCs/DTCs, detection of tumor cells by CK immunofluorescence and HER2 FISH were conducted separately (Meng et al. 2004b; Meng et al. 2006; Rack et al.2006). Considering the cellular loss during subsequent analysis, not all cells of interest could be evaluated by both FISH and immunofluorescence. Therefore in our study we established a method for combined FISH and immunofluorescence using an antigen retrieval technique to detect and evaluate gene and protein status of CTCs simultaneously.

In our FISH protocol we replaced the enzymatic digestion step by a heat pretreatment to avoid antigen degradation. In order to improve the accessibility of target DNA to the probes, enzymatic digestion, using pepsin or proteinase K, is required in routine FISH protocol. However, it is likely that the enzymatic digestion will destroy the antigen or remove the antibody complex. Fehm and colleagues (2002) performed the FISH assay after immunofluorescence staining for cytokeratin on the same slides. Their results shown illustrated that the immunofluorescence was completely lost during the FISH procedure when pepsin was used in the pretreatment step. In our laboratory we got the same results when proteinase K was used in the FISH procedure after cytokeratin immunostaining. We also reversed the sequence of FISH and immunofluorescence, indicating that the immunostaining was impossible after pepsin pretreatment. Actually, the proteolytic digestion step can be omitted in combined FISH and immunostaining for preservation of antigenicity (Zaidi et al. 2000; Donadoni et al.2004). To achieve this, the antigen retrieval technique is introduced into combined FISH and immunofluorescence as permeabilization pretreatment instead. Many studies have been documented for simultaneous FISH and

immunofluorescence using antigen retrieval techniques on FFPE tissue sections (Martinez-Ramirez et al. 2004; Lottner et al. 2005; Ge et al. 2006), but no comparative analysis has previously been conducted on cytological specimens, especially for slides prepared after the CellSearch^{TM} system processing.

Antigen retrieval, also called heat-induced antigen retrieval, target retrieval or target unmasking, was firstly reported by Shi et al 1991. From then on several basic and important parameters of heat-induced antigen retrieval, such as composition and pH of retrieval buffer, heating temperature, duration of heating period, and the use of different heating sources, have been widely explored in various studies. The heating condition (temperature and period of heating) and the pH value of the retrieval buffer are the two major factors that influence the effectiveness of antigen retrieval (Werner et al. 1996; Shi et al. 2002). In the study of Emoto and colleagues (2005), results indicated that the pH value of retrieval buffer can result in dramatic differences in the intensity of immunostaining. Citrate buffer of pH 6.0, Tris-HCl of pH 2.0 or pH 9.0, and citraconic anhydride of pH 7.5 are often used as the retrieval solutions (Emoto et al. 2005; Namimatsu et al. 2005; Neves et al. 2005; Shi et al. 2007). Among them 10mM citrate buffer of pH 6.0 is the most popular solution for heat-induced antigen retrieval (Shi et al. 1999). It has been utilized in regular HER2 immunohistochemical staining and FISH assay (Shi et al. 2001; Press et al.2002). The most commonly used heating sources include microwave ovens, water bathes, steamers and pressure cookers. Neves et al. (2005) have compared the effects of different heating sources on immunostaining for estrogen receptor. The results shown illustrate that an automatic electric pressure cooker provided better results than microwave oven, steamer, and water bath. The main advantages of pressure cooker likely lie in the maintenance of a homogeneous heating environment since there are no “cold” or “hot” spots, there is no evaporation of the buffer solution, and there is no influence of electric beam variations. Thus, we took 10mM citrate buffer of pH 6.0 as a retrieval buffer and used an electric pressure cooker (Decloaking Chamber, Biocare Medical, Walnut Creek, CA) in our study.

Since we have set the retrieval buffer and the heating source, the optimal heating condition (temperature and period of heating), which is a crucial factor and is directly related to the effectiveness of target retrieval, should be established. In our study, the effects of different heating conditions on the results of combined FISH and immunofluorescence were examined in cytological specimens. Our results showed that heating slides at 120°C for 2.5min was the optimal condition to obtain a high intensity of FISH signals and immunofluorescence as well as the preservation of good nuclear morphology, both for native or processed cells. Increasing the temperature to 125°C or prolonging the heating period to 10min at 120°C did not only destroy the nuclear morphology in that the nuclei became pale and were invisible with DAPI staining, but also resulted in significantly decreasing FISH signals. However, on the other hand, at a lower temperature (100°C), even 10min were not sufficient to acquire a good equilibrium between FISH and immunofluorescence. Our finding is concordant to the result described by Suurmeijer and Boon (1999) in that the higher the temperature, the sooner an optimal staining could be obtained. The heating temperature and the heating time appear to be reversely correlated.

This optimized method was utilized for various kinds of specimens from native breast cancer cell lines, cell lines processed by the CellSearch™ system, FFPE pleural effusion cells from breast cancer patients, as well as to clinical samples prepared after the CellSearch™ system processing. Several antibodies such as anti-CK, anti-EpCAM, and anti-HER2 were tested as well as combinations with commercial or in-house HER2 FISH probes. The results showed that this method is a robust tool for phenotyping and genotyping individual cells simultaneously in cytological specimens. For the detection and characterization of CTCs, as shown in our study, tumor cells can be detected distinctively from blood cells by cytokeratin or EpCAM immunofluorescence, meanwhile, HER2 gene amplification and polysomy of chromosome 17 can be evaluated in individual cells. Furthermore, the analysis of the HER2 gene status in CTCs using this method could provide valuable information on standardization of HER2 testing by the CellSearch™ system, resulting from reliable

and precise comparisons of HER2 status in individual cells between the CellSearch™ system and FISH. Conclusively, this new approach of a combined FISH and immunofluorescence based on antigen retrieval technique for cytological specimens might be an extraordinarily useful tool for the detection of micrometastasis due to its capacity to improve the efficacy of the result interpretation.

4.6 Evaluation of HER2 gene amplification by real time PCR

As well-known, FISH remains the current gold-standard procedure for the assessment of HER2 gene amplification. However, the technique is time-consuming, laborious, and expensive. In the future, polymerase chain reaction (PCR)-based assays are likely to become more widely used for the detection of gene amplification because they are more sensitive, faster, and easier to perform (Konigshoff et al. 2003). In particular, real time PCR is suggested as an alternative method for measuring HER2 gene copy number changes in breast cancer (Decock 2006). In our study, HER2 gene amplification was analyzed with two different real time PCR methods, namely SYBR Green and TaqMan probe assays, in manually microdissected FFPE breast cancer tissue sections. Afterwards, the results obtained by PCR were compared with those obtained by FISH. The high concordance between PCR and FISH results suggested that both real time PCR methods were reliable, sensitive and specific tools for the detection of HER2 amplification. The obtained results and those achieved with the standard methods FISH as well as immunocytochemistry are concordant.

Real time PCR can amplify and detect the target simultaneously in contrast to conventional PCR which needs a post-PCR analytical step. In real time PCR, the products can be detected by using DNA-binding dyes or probes. SYBR Green is the most widespread non-specific dye (Bubner et al. 2004), which emits fluorescence when binding to double-stranded DNA during the annealing and extension step. The non-specific binding to double-stranded DNA results in some disadvantages. First, one cannot use this method to perform a multiplex PCR. For each sample DNA, two

reactions must be run in separate wells -one for the target and one for the reference gene- thereby increasing variations. The second problem is that both specific and non-specific products generate signal. Thus, any mis-priming event or primer-dimers will generate false positive signals. However, the primer-dimers can be distinguished by melting curves. Another aspect is that multiple dyes bind to a single amplified molecule. If the amplification efficiencies are the same, a longer product will generate more signal than a shorter one.

The most common probe systems are TaqMan probes (Bubner et al. 2004). The fluorescence is released from the reporter dye at the 5' end of the hybridization probe when it is separated from the quencher dye at the 3' end of the probe due to the hydrolysis of the probe by the 5'-nuclease activity of the DNA polymerase during extension step. One advantage of the TaqMan assay is that specific hybridization between probe and target is required to generate fluorescent signal. So, non-specific amplification due to mis-priming or prime-dimer artifacts does not generate a signal. Another advantage is that the probe can be labeled with different, distinguishable reporter dyes, subsequently, multiplex PCR can be done.

In our study, both SYBR Green monoplex PCR and TaqMan multiplex PCR used the same primers. The HER2 gene locates on 17q21, and polysomy of chromosome 17 is frequently observed in breast cancer. Thus, centromere probe 17 (CEP17) is introduced into the dual color FISH assay for correcting the HER2 copy number for the chromosome 17 copy number. In a recent study, it was reported that polysomy 17 was linked with HER2 protein overexpression, and increased HER2 copy numbers (Salido et al. 2005). However, tumors with polysomy 17 and IHC scores of 2+ frequently present with a HER2/CEP17 ration of <2.0 and therefore no HER2 gene amplification is indicated. To get a reliable HER2 gene quantification by real time PCR, a good reference gene is needed which fulfills the following criteria. It has to be amplified with a high efficacy and should not be localized in an amplified region. Although some reference genes mapped to chromosome 2, 6 and 12, which are least

frequently numerically altered in breast cancer, such as the IGF-1 gene (12q22) (Konigshoff et al. 2003) have been reported, an internal control for chromosome 17 is still strongly suggested (Decock 2006). We designed a pair of primers against chromosome 17q11.2 (Contr17), and the amplicon is located near the cetromere region. In studies of Willmore-Payne at al. (2006) and Nistor et al. (2006), internal controls for chromosome 17 were used, and the results from both studies indicated that the evaluation of the HER2 status by real time PCR results in gene copy numbers comparable with FISH. Willmore-Payne at al. designed primers for the WHN gene (17q11.2), which is 590kb nearer to the centromere region than our Contr17 primers. Nistor et al. performed PCR analysis with the LightCyler HER2/neu DNA Quantification kit (Roche Molecular Biochemicals, Mannheim, Germany), which used the gastrin gene (17q21.2) as reference housekeeping gene. Although the location of the reference region on chromosome 17 is different from those described in these two publications, our HER2 quantification results determined by real time PCR using Contr17 as reference region are also highly concordant with those obtained by the FISH assay.

The concordance between results of monoplex PCR and FISH, as well as between those of multiplex PCR and FISH was 100% (10/10) and 90.9% (10/11), respectively. A similarly high concordance between FISH and real time PCR has been reported in recent studies (Gjerdrum et al. 2004; Nistor et al. 2006; Willmore-Payne at al. 2006). Furthermore, manual microdissection or laser capture microdissection was utilized for DNA isolation in these studies just as we did. The results shown suggested that real time PCR analysis of HER2 amplification following microdissection represents a complementary or even an alternative method for establishing HER2 status in breast cancer tissues (Gjerdrum et al. 2004; Nistor et al. 2006).

Nevertheless, although both monoplex and multiplex PCR were useful tools to determine the HER2 gene status, we would prefer monoplex PCR when sufficient DNA material is available (Layfield et al. 2005). The optimization of multiplex

TaqMan real time PCR is difficult because of the increased chance for the formation of primer dimers and the primer-probe interaction (Brownie et al. 1997; Li et al. 2006). Preferential amplification of one target sequence over another is a known phenomenon in multiplex PCR reactions (Elnifro et al. 2000). Thus, the primers for the preferential target should be limited. In our study, the ratio of HER2 primers to Contr17 was decreased from 1:1 to 1:4 eventually. The other components such as the concentration of reaction buffer and the concentration of Mg^{2+} as well as PCR cycling conditions also need to be optimized (Henegariu et al. 1997). In other words, the optimization of a multiplex PCR strategy could encounter many difficulties, and overcoming the difficulties might be a challenge.

Conclusively, the choice of Contr17 as an internal control is an advance of this study, which is helpful to maximally eliminate the influence of polysomy 17 on reliable and accurate quantification of HER2 gene copy number by real time PCR. Our results suggest that real time PCR might be complementary or alternative to current standard FISH assays for determination of HER2 gene amplification in breast cancer. Real time PCR is a relatively simple, low-cost technique that could be used as a rapid screening method for establishing the HER2 gene copy number present in breast cancers (Lewis et al. 2004). For those cases with PCR ratios of around 2.0, the additional FISH assay could further confirm the HER2 gene status, providing pivotal information for the administration of trastuzumab therapy.

4.7 Effects of whole genome amplification on downstream real time PCR assays

Analyzing single targeted cells can eliminate the contamination of non-neoplastic cells and improve the accuracy of HER2 evaluation in CTCs by real time PCR. However, the scarce DNA could limit the performance of a PCR at single-cell level. Recently, several whole genome amplification (WGA) techniques have been presented as capable for amplifying DNA from trace quantities and with less error than traditional PCR (Barker et al. 2004), which could provide solutions to the

limitation of scarce DNA. According to the strategy of amplification, WGA methods can be categorized into two subgroups: PCR-based and multiple displacement amplification (MDA)-based methods. The PCR-based WGA methods, such as primer extension preamplification (PEP) (Zhang et al. 1992), and degenerate oligonucleotide primed PCR (DOP-PCR) (Telenius et al. 1992), generate DNA less than 1kb long that cannot be used in many applications. In contrast the MDA generated DNA product is more than 10kb long, and hence its performance is well suited for a variety of applications such as quantitative PCR, comparative genome hybridization, and assessment of loss of heterozygosity (Dean et al. 2002). In comparison to other WGA methods, MDA has provided the highest amplification yield, best genomic coverage, and lowest amplification bias (Barker et al. 2004; Dean et al. 2002; Hosono et al. 2003; Lovmar et al. 2006). Therefore, MDA has been increasingly often used for WGA since it was first described by Dean et al. in 2002. In our study, the GenomiPhi protocol (GE Healthcare), an MDA method, was used for WGA. This protocol utilizes bacteriophage Phi29 DNA polymerase and random hexamer primers to exponentially amplify linear DNA templates during an isothermal (30°C), strand displacement reaction (Dean et al. 2002).

In this study, we surprisingly found that using WGA amplified DNA as template, amplification products produced during the PCR cannot be detected by SYBR Green PCR while using the Taqman assay detection is possible. The difference is most likely due to the different principles underlying signal generation by the two methods. SYBR Green emits fluorescence when it unspecifically binds to double-stranded DNA during the annealing and extension step. The template DNA amplified by MDA consists of high molecular weight double-stranded DNA as our results showed, which can also bind to SYBR Green and generate signals resulting in a high background. Thus the amplification of target fragments cannot be distinguished from the high background. When templates were digested with restriction enzyme before initial PCR, DNA molecular weight became lower leading to higher reaction efficiency and signal-to-noise ratio, thereby generating interpretable amplification curves. In the

TaqMan assay, fluorescence can only be released when the probe specifically hybridizes to the target region and is hydrolyzed by the DNA polymerase. Thus, no background is generated from the non-complementary strand and the amplicons from amplified DNA can be distinctively detected by this assay. In many other studies, the TaqMan assay was also preferred to SYBR Green real time PCR for MDA amplified DNA (Dean et al. 2002; Hosono et al. 2003).

Complete genomic coverage and minimal amplification bias of WGA could be prerequisites for the downstream genetic analysis. A certain amount of sequence bias may be anticipated with any amplification method and may result from factors such as priming efficiency, template accessibility, GC content, and proximity to telomeres and centromeres (Dean et al. 2002). Hosono and colleagues (2003) analyzed bias in 47 loci using a TaqMan assay and a maximum of a six-fold amplification bias between loci was observed in MDA products. In contrast, an amplification bias of 10^3- to 10^6-fold was observed in DOP-PCR products, and 10^2- to 10^4-fold bias in PEP products (Dean et al. 2002). Comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) analyses revealed that the MDA method provided complete coverage (99.9%) of the genome with minimal amplification (Barker et al. 2004; Dean et al. 2002; Pinard et al. 2006). In our study, we analyzed amplified human genomic DNA with real time PCR. The ratios of HER2/Contr17 in amplified DNA varied from 1.2 to 6.0 relative to original control DNA, while an ideal ratio of HER2/Contr17 in human genomic DNA should be 1.0. Thus, for the accurate assessment of the HER2 gene status at single-cell level, the techniques must be further optimized.

4.8 Conclusions

In the present study, CTCs from peripheral blood of breast cancer patients were characterized for HER2 expression by different methods. The results obtained by the CellSearch™ system demonstrated the heterogeneity of HER2 expression in CTCs of

individual patients. Furthermore, in some cases discrepancies between the HER2 status in primary tumors and CTCs in peripheral blood were observed.

A set of methods including immunofluorescence, FISH, combined FISH and immunofluorescence, as well as real time PCR was established for the further characterization of CTCs detected by the CellSearch^{TM} system. The fixation during the CellSearch^{TM} run could have side effects on the penetration of antibodies or probes through cell membranes. The application of new antigen retrieval techniques can successfully overcome this challenge. However, the CellSearch^{TM} system did not significantly interfere with the downstream molecular analysis. Additionally, combined FISH and immunofluorescence protocols have been established by aid of antigen retrieval techniques. A real time PCR using an internal control located on chromosome 17 appeared to be comparable to the current standard FISH assay for evaluation of HER2 gene status in tissue sections of breast cancer patients. Nevertheless, the PCR conditions need to be further optimized for the oncoming aim of analysis HER2 amplification of CTCs at the single-cell level.

Using these established methods we can further characterize CTCs for other biomarkers which can provide new target molecules, such as EGFR and VEGF, besides HER2 and hormone receptor for biologic targeted therapy for breast cancer patients. The therapies involving lapatinib for both HER2 and EGFR, cetuximab for EGFR, or bevacizumab for EGFR are promising perspectives for patients with hormone receptor and HER2 negative breast cancer.

Summary

The presence of CTCs in peripheral blood has been proven to be an independent prognostic and predictive factor for patients with metastatic breast cancer. The major aims of the study were to detect and characterize CTCs in peripheral blood from breast cancer patients with the help of the CellSearch^{TM} system, as well as to establish and optimize a set of methods to further phenotypically and molecularly characterize CTCs.

CTCs were detected in 65 breast cancer patients with the CellSearch^{TM} system and additionally characterized for HER2 expression in 51 cases through processing blood samples. HER2-positive CTCs were detected in 15 of 51 patients (29.4%), and heterogeneity of HER2 expression in CTCs among individual patients was observed. Furthermore, previously described discrepancies between the HER2 status in primary tumors and CTCs in blood were confirmed by the present study.

In this study, for the further characterization of CTCs a set of methods including double immunofluorescence, FISH, combined FISH and IF, as well as real time PCR was established. The application of a new antigen retrieval technique prior to FISH analysis was the most important prerequisite for the establishment of a combined method for FISH and immunocytochemistry on CTCs. Thus far, there are no hints that processing the blood with the CellSearch^{TM} system significantly interferes with the downstream molecular analysis of CTCs.

Using these established methods, we confirmed the expression of CK, HER2 and hormone receptor as well as the HER2 gene status in seven breast cancer cell lines, presented various Ki-67 staining patterns, and revealed a serial change in CK and M30 expression as well as morphological changes during apoptosis from early stage to late stage. The newly developed method of combined FISH and IF was utilized for various kinds of specimens, and appeared to be a robust tool for phenotyping and genotyping individual cells simultaneously in pleural effusion, CTCs and other cytological specimens. Our real time PCR protocol using an internal control located on chromosome 17 appeared to be comparable to the current gold-standard FISH for the evaluation of HER2 gene status in tissue sections of breast cancer patients. For

real time PCR with DNA obtained by whole genome amplification, the TaqMan assay was superior to the SYBR Green assay because of the higher specificity of the former one. Although the number of clinical samples analyzed in the study is small, the feasibility of the established methods has been evident. All these methods might be employed in the oncoming large scale analyses of clinical samples. However, further investigations of primary tumors are needed to identify tumor-specific markers that can be used both for the detection of CTCs as well as for their application in therapeutic monitoring.

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Declaration

I hereby declare that I have done the work by myself and have not used other than stated sources and aids. Any used citation from literature is noted as well.

I further confirm that this thesis is not submitted to any other university to open the dissertation procedure.

Liling Zhang

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