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## **Clinical Relevance of Allelic Imbalances on Chromosome 10q in Brain Metastases Formation in Breast Cancer Patients**

### **Dissertation**

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# Table of contents

<b>Abbreviations and definitions .....</b>	<b>3</b>
<b>1. Introduction .....</b>	<b>5</b>
<b>1.1. Breast cancer .....</b>	<b>5</b>
<b>1.2. Prognosis and therapy .....</b>	<b>7</b>
1.2.1. Prognosis .....	7
1.2.2. Therapy .....	9
<b>1.3. Metastatic disease .....</b>	<b>10</b>
1.3.1. Brain metastases of breast cancer .....	11
<b>1.4. Genetic determinants of breast cancer .....</b>	<b>12</b>
1.4.1. Genetic alterations observed in breast cancer .....	13
1.4.2. Chromosomal instability .....	14
1.4.3. Genes involved in breast cancer tumorigenesis .....	16
<b>1.5. Genetic signatures of breast cancer metastases .....</b>	<b>18</b>
<b>1.6. Aims of the study .....</b>	<b>20</b>
<b>2. Materials .....</b>	<b>21</b>
<b>2.1. Blood and tissue samples .....</b>	<b>21</b>
2.1.1. Primary tumors .....	21
2.1.2. Brain metastases .....	23
2.1.3. Primary tumor tissues on tissue micro array (TMA) used for fluorescence <i>in situ</i> hybridization (FISH) .....	23
<b>2.2. Reagents .....</b>	<b>26</b>
<b>2.3. Buffers and solutions .....</b>	<b>26</b>
<b>2.4. Kits .....</b>	<b>28</b>
<b>2.5. Microsatellite PCR primers .....</b>	<b>29</b>
<b>2.6. DNA .....</b>	<b>29</b>
<b>2.7. Other materials .....</b>	<b>30</b>
<b>2.8. Appliances .....</b>	<b>30</b>
<b>2.9. Software .....</b>	<b>30</b>
<b>3. Methods .....</b>	<b>32</b>
<b>3.1. DNA isolation from blood leukocytes .....</b>	<b>32</b>
<b>3.2. Spectrophotometric assessment of quality and quantity of isolated DNA .....</b>	<b>33</b>
<b>3.3. PCR-Amplification with specific microsatellite primers .....</b>	<b>33</b>
3.3.1. PCR as a tool for detection of genomic aberrations .....	33
3.3.2. Primer selection .....	35
3.3.3. Gradient PCR for detection of optimal primer annealing temperature .....	35
3.3.4. PCR using combinations of primer pairs .....	36
<b>3.4. Separation of DNA fragments by agarose gel electrophoresis .....</b>	<b>36</b>
3.4.1. Gel scanning .....	37
<b>3.5. DNA analysis in Genetic Analyser .....</b>	<b>37</b>
3.5.1. DNA fragment analysis using fluorescent primers .....	37
3.5.2. Sample preparation .....	38
3.5.3. Capillary electrophoresis .....	38
3.5.4. Analysis .....	38
<b>3.6. Fluorescence <i>in situ</i> hybridization (FISH) .....</b>	<b>39</b>
3.6.1. FISH probes .....	40
3.6.2. FISH on paraffin-embedded TMA .....	41
3.6.3. Evaluation of FISH results .....	42
<b>3.7. Statistical analysis .....</b>	<b>43</b>
<b>4. Results .....</b>	<b>44</b>

<b>4.1. DNA isolation and quantification.....</b>	<b>44</b>
<b>4.2. Optimization of microsatellite analysis.....</b>	<b>45</b>
4.2.1. Detection of optimal primer annealing temperature .....	46
4.2.2. Detection of optimal combination of primers .....	50
<b>4.3. Analysis of allelic imbalances on patients DNA.....</b>	<b>51</b>
4.3.1. Detection and evaluation of allelic imbalances.....	51
4.3.2. Specification of a cut-off ratio in analysis of allelic imbalances .....	55
4.3.3. Frequencies of allelic imbalances and not informative (NI) samples in primary tumors and brain metastases.....	55
4.3.4. Defining the core regions of allelic imbalances on chromosome 10q .....	58
4.3.5. Allelic imbalances in primary tumors in comparison to brain metastases.....	59
4.3.6. Correlation of occurrence of allelic imbalances on chromosome 10q in primary tumors with clinical and pathological characteristics.....	61
<b>4.4. FISH analysis for detection of copy number changes in primary tumor and brain metastases DNA on chromosome 10q .....</b>	<b>62</b>
<b>4.4.1. Preparation of DNA probes for FISH analysis.....</b>	<b>62</b>
4.4.2. Detection of chromosomal alterations on chromosome 10q with FISH analysis .....	64
4.4.3. Correlation of FISH results with clinical and pathological parameters .....	66
<b>5. Discussion .....</b>	<b>68</b>
<b>5.1. Methodological aspects.....</b>	<b>68</b>
<b>5.2. Allelic imbalances in primary tumors and brain metastases.....</b>	<b>69</b>
<b>5.3. Candidate genes on chromosome 10q .....</b>	<b>72</b>
<b>5.4. Genomic aberrations detected by FISH analysis.....</b>	<b>74</b>
<b>6. Outlook .....</b>	<b>76</b>
<b>7. Summary .....</b>	<b>77</b>
<b>8. Bibliography .....</b>	<b>79</b>
<b>9. Attachment.....</b>	<b>95</b>
<b>Acknowledgements .....</b>	<b>107</b>
<b>Affidavit .....</b>	<b>108</b>



## Abbreviations and definitions

annealing	binding of primers to the template
AI	allelic imbalance
aneuploid	having an abnormal number of chromosomes
antisense	a DNA sequence complementary to a genomic region
antisense primer	PCR primer that binds the 3'-end of the DNA template
assay	test
bp	base pairs
BBR	blood-brain barrier
<i>BRCA1</i>	breast cancer 1 gene
<i>BRCA2</i>	breast cancer 2 gene
CGH	chromosomal genetic hybridization
CNS	central nervous system
CR	core region
DAPI	4,6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
denaturation	separation of DNA double strands into single strands
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
downstream	the DNA region towards the 3' end of the strand
dTTP	deoxythymidine triphosphate
elongation	growth of the DNA sequence from primer on with help of DNA polymerase
EtBr	ethidium bromide
ER	estrogen receptor
exon	a coding gene sequence
HEX	hexachloro-6-carboxy-fluorescein
HiDi	highly deionized
FAM	6-carboxy-fluorescein
FISH	fluorescence <i>in situ</i> hybridization
gene	a DNA region coding for a protein

haploid	having a single set of chromosomes
<i>HER2</i>	Human Epidermal Growth Factor Receptor 2 gene
intron	a non-coding gene sequence
ILC	invasive lobular carcinoma
LOH	loss of heterozygosity
MBC	metastatic breast cancer
min	minute
MSI	microsatellite instability
PCR	polymerase chain reaction
polyploid	having one or more extra sets of chromosomes
primer	oligonukleotide that binds to DNA and serves as a starting point for DNA synthesis
PR	progesterone receptor
<i>PTEN</i>	phosphatase and tensin homolog deleted on chromosome 10 gene
replication	the process of copying a double-stranded DNA molecule
RER	replication error
rpm	rotations per minute
RT	room temperature
sense	original DNA sequence
sense primer	PCR primer that binds the 5'-end of the DNA
template	DNA sequence that serves as a pattern for amplification
tetraploid	having four times the haploid number of chromosomes
TMA	tissue micro array
triploid	having three times the haploid number of chromosomes
TSG	tumor suppressor gene
upstream	the DNA region towards the 3' end of the strand

# 1. Introduction

## 1.1. Breast cancer

Breast cancer is the most common malignant tumor among women in the Western world. In year 2008 there were around 72.600 new breast cancer cases and 17.000 death cases from this malignancy in Germany [1]. The median age at a time of the diagnosis was 65 years. It is estimated that the number of breast cancer cases will grow to 74.500 in year 2012 [1].

A large number of risk factors, including age, gender, reproductive behavior, hereditary factors, have been identified that modulate the likelihood of a woman to develop the disease (table 1.1). Breast cancer incidence and death rates generally increase with age, with 75 % of women developing the disease after the age of 50. Breast cancer is uncommon in women younger than age 30 and only 5 % of all breast cancer patients are younger than the age of 40. During 2002-2006, the median age at the time of breast cancer diagnosis was 61 years; in 2008 it was 65 years [1, 2, 3].

Breast cancer in men is an uncommon disease. It represents 0.6 % of all breast carcinomas and <1 % of all malignancies in men. In women breast cancer is the most common neoplasm [2, 5]. It is believed to be primarily due to lifelong higher estrogen exposure in women as well as post-menopausal hormone replacement therapy [89].

There are differences among countries and geographical regions in the incidence of breast cancer. The risk to develop a breast neoplasia is higher in North America and Europe than in Asian countries. For example, the incidence of breast cancer in the United States is approximately 5-times higher than in Japan. These differences seem to be related to environmental factors rather than genetic factors since the migrants from low-incidence to high-incidence regions tend to acquire the risk rate of their adoptive countries and *visa versa* [4, 6].

The parity is a modifiable risk factor for breast cancer. The parity and the number of births have been shown to inversely correlate to the incidence of breast cancer [7]. Additionally, breastfeeding reduces the risk of breast cancer for each year that a woman breastfeeds [8].

About 5 to 10% of breast cancers arise as a result of specific inherited mutations [2]. Women are more likely to bear a mutation if they develop a breast cancer before

menopause, have bilateral cancer or an associated cancer like ovarian cancer [4]. A more than two-fold increase in risk for women with one first-degree relative with early onset breast cancer has been reported in many studies [9]. Approximately half of the women with a hereditary breast cancer bear a mutation in Breast Cancer 1 gene (*BRCA1*) and an additional one third in Breast Cancer 2 gene (*BRCA2*) gene [4, 9]. These mutations are present in far less than 1% of the general population. Women with *BRCA1* mutations are estimated to have a 57% risk for developing breast cancer by age 70; the corresponding risk for *BRCA2* mutations is 49%, both depending on amino acid position of the mutations in the respective genes [2].

Reproductive hormones are thought to influence breast cancer risk through effects on cell proliferation and DNA damage, as well as promotion of cancer growth. Early menarche (<12 years) and older age at menopause (>55 years) may increase a woman's risk of breast cancer by affecting the levels of reproductive hormones produced by her body [3,10]. Recent use of oral contraceptives may slightly increase the risk of breast cancer; however, women who have stopped using oral contraceptives for 10 years or more have the same risk as women who never used the pill [2]. Estrogen replacement therapy in postmenopausal women was shown to be associated with a moderate increase in incidence of breast cancer [10]. However, the risk to develop breast cancer appears to decline soon after the discontinuation of the hormonal treatment [11].

Many other risk factors, such as obesity, alcohol consumption, cigarette smoking have also been implicated in the development of breast cancer. Also ionizing radiation to chest has been associated with increases the risk of breast cancer. The magnitude of the risk depends on the radiation dose, the time since exposure and age [4]. The California Environmental Protection Agency has concluded that regular exposure to secondhand smoke is causally related to breast cancer diagnosed in younger, primarily premenopausal women [12]. Alcohol consumption was reported to increase the risk of breast cancer regardless of the type of alcoholic beverage consumed [13].

**Table 1.1. Breast cancer risk factors.**

Factor	Relative Risk
Well-Established Influences	
Geographic factors	Varies in different areas
Age	Increases after age 30 yr
Family History	
First-degree relative with breast cancer	1.2-3.0
Premenopausal	3.1
Premenopausal and bilateral	8.5-9.0
Postmenopausal	1.5
Postmenopausal and bilateral	4.0-5.4
Menstrual History	
Age at menarche <12 yr	1.3
Age at menopause >55 yr	1.5-2.0
Pregnancy	
First live birth from ages 25-29 yr	1.5
First live birth after age 30 yr	1.9
First live birth after age 35 yr	2.0-3.0
Nulliparous	3
Benign Breast Disease	
Proliferative disease	1.9
Proliferative disease with atypical hyperplasia	4.4
Lobular carcinoma in situ	6.9-12.0
Less Well-Established Influences	
Exogenous estrogens	
Oral contraceptives	
Obesity	
High-fat diet	
Alcohol consumption	
Cigarette smoking	

Vinay, Kumar et al. Robbins basic pathology. 7th Edition. Philadelphia. The Curtis Center, 2003. Print.

## **1.2. Prognosis and therapy**

Breast cancer usually presents as discrete solitary painless mass with or without palpable axillary nodes. Less common signs and symptoms include breast pain or heaviness; persistent changes to the breast, such as swelling, thickening, or redness of the breast's skin; and nipple abnormalities such as spontaneous discharge, erosion, inversion, or tenderness. Therefore, a regularly mammographic screening after the age of 50 is recommended in Germany by the Federal Ministry of health ([www.bmg.bund.de/ministerium](http://www.bmg.bund.de/ministerium)). Using mammographic screening tumors as small as 5 mm can be detected [132].

### **1.2.1. Prognosis**

The relative 5-year survival of patients with breast cancer in Germany was estimated in 2008 as 83-87 % (Deutsches Krebsregister, [www.gekid.de](http://www.gekid.de)). Despite advanced operation techniques, adjuvant or neoadjuvant treatment many women still suffer from

a local relapse or distance metastases, often also more than 5 years after the operation. The prognosis for women with primary breast cancer is determined by several clinical, histopathological and biochemical factors:

1. *The size of the primary carcinoma.* Invasive carcinomas smaller than 1 cm have an excellent survival (92% after 8 years) in the absence of lymph node metastases and may not require systemic therapy [90].
2. *Lymph node involvement and the number of lymph nodes involved by metastases.* The 5-year survival rate is close to 90% with no axillary node involvement and 72% with involvement of more than 4 lymph nodes [91]. The survival rate decreases with each involved lymph node and is less than 50% with 16 or more involved nodes [4]. Sentinel lymph node detection technique allows an estimation of local disease spread with a high negative predictive value (94.5%) and therefore the individual load of subsequent treatment [92].
3. *The grade of the carcinoma.* Most common grading systems for breast cancer evaluate tubule formation, nuclear grade, and mitotic rate and classify carcinomas into 3 categories. It has been shown that well-differentiated carcinomas (Grade I) have a significantly better prognosis compared with poorly differentiated carcinomas (Grade II and III) [93].
4. *The histologic type of carcinoma.* Tubular, medullary, lobular, papillary, and mucinous are generally less aggressive than ductal carcinomas. If the cancer presents as an inflammatory phenotype it often has a high S-phase fraction, is high-grade and aneuploid and lacks hormone receptor expression what promotes its invasive behavior [4, 94].
5. *Lymphovascular invasion.* The invasion of vascular spaces around the primary tumor is a poor prognostic factor, especially in the absence of lymph node metastases. Especially dermal lymphovascular invasion often presents as inflammatory carcinoma, which is related to a very poor prognosis [94].
6. *The presence or absence of estrogen or progesterone receptors.* The presence of hormone receptors confers a slightly better prognosis. However, the reason for determining their presence is to predict the response to therapy. The highest rate of response (~80%) to anti-estrogen therapy (oophorectomy or tamoxifen and aromatase inhibitors) is seen in patients whose tumors have both estrogen and progesterone

receptors. Lower rates of response (25% to 45%) are seen if only one of the receptors is present. If both are absent, very few patients (less than 10%) are expected to respond to the treatment [4, 131].

*7. Overexpression of Human Epidermal Growth Factor Receptor 2 (HER2/ERBB2).* *HER2* is a membrane receptor protein and its overexpression is mostly caused by amplification of the gene. Therefore, immunohistochemistry (identification of the *HER2* protein in tissue sections) or fluorescence *in situ* hybridization (FISH) (identification of the number of gene copies) are helpful in detection and assessment of the *HER2* status. Overexpression is associated with a poorer prognosis. However, the importance of evaluating *ERBB2* status is to predict response to a targeted treatment of *HER2*, with either monoclonal antibody to the gene (Herceptin®) or TKI inhibitors [95]. These new targeted treatments have significantly improved the prognosis of HER2 positive patients [133, 138].

The tumors are classified according to the TNM system developed by UICC (Union International Contre le Cancer), where T designates the size of the tumor, N lymph node involvement and M the presence of distant metastases. The staging based on the TNM classification helps to divide women in different prognostic groups for treatment decisions.

### **1.2.2. Therapy**

The current therapeutic regimen of breast cancer consists of surgical removal and neoadjuvant/adjuvant treatment, such as, chemo- and radiation therapy, hormonal treatment and application of antibodies against specific targets on tumor cells. But often the stage of the disease requires a multimodality treatment approach.

Operation is still a golden standard in treatment of breast carcinoma. Different operational techniques are employed to resect the focus of malignancy: breast-conserving surgery plus radiation therapy, mastectomy plus reconstruction, and mastectomy alone. Surgical staging of the axilla should also be performed.

Adjuvant therapy is given in addition to surgery where all detectable disease has been removed, but where there remains a statistical risk of relapse due to occult disease. It consists of radiation, chemotherapy, hormonal treatment and HER2 antagonizing treatment. The recommendations on the adjuvant treatment algorithm have been worked out by the experts at St. Gallen International Expert Consensus on the Primary

Therapy of Early Breast Cancer based on the assessment of the new studies and clinical trials on effectiveness of available treatment modalities [14].

Chemo- and Radiation therapy can be applied before surgical intervention to downgrade the volume of the tumor to a resectable size. In breast-conserving surgical treatment radiation is mostly applied since it was shown that this regiment produces survival and freedom-from-recurrence rates similar to the mastectomy [15, 16].

### **1.3. Metastatic disease**

Metastatic breast cancer (MBC) is a heterogeneous disease that has a variety of different clinical scenarios, ranging from solitary metastatic lesions to diffuse and multiple organ involvement. The 5-year survival rate of patients with localized disease confined to the primary tumor is 98%, with regional lymph node involvement 83%, but with metastases to distant sites only 23% [99]. Not the regional disease but the development of distant metastases in vital organs is the major cause of these deaths [17]. In addition, metastatic breast cancer at diagnosis constitutes 3%–7% of all new breast cancers [18].

Autopsy studies of women dying of breast cancer suggest that widespread metastatic disease with bones (70%), lungs (66%) and liver (61%) are the most common sites of spread [19]. The incidence of brain metastases in women with metastatic disease is estimated as 10-20 % [96].

Overall, survival of patients with MBC is slowly but steadily improving, the risk of death is decreasing by 1%–2% each year [20]. The greatest improvement is most probably related to the development and widespread availability of modern systemic therapies [21]. In addition, modern diagnostic tools allow the detection of early metastatic disease, which may be more responsive to treatment than late metastatic disease [18]. Notwithstanding the available therapeutic modalities and detection tools metastatic breast cancer remains, currently, almost incurable with a median survival in the range of 17 to 20 months [22].

Solid breast carcinoma can spread over lymphatic and hematogenous route. Lymph node involvement is considered a one of the predicting factors for the development of distant metastasis (see 1.2.1.) [19]. Although the presence of metastases in the axillary lymph nodes predicts the development of distant metastases, 20–30% of the patients with breast cancer that are free of axillary lymph-node metastases develop disease at



distant sites [23].

Thus, the lack of specific predictors or markers for the development of distant metastases in breast cancer leads to overtreatment of many patients who are not at risk of developing a metastatic disease as well as undertreatment of patients who will develop the systemic spread later in the course of disease but do not present clinically as candidates at risk at the moment of diagnosis and staging.

Therefore several clinical problems arise in the management of breast cancer and its metastatic complications:

1. Many women get treated with chemotherapy unnecessarily. The selection of patients who will receive the additional systemic treatment is at present based on their statistical risk of developing tumor recurrence, without knowing whether they actually harbor pro-metastatic or yet micrometastatic formations or not. For example, only 20-25 % of patients with breast cancer without overt lymph node metastases will suffer metastatic relapse within 10 years post-surgery, but more than 90% of these patients are currently receiving chemotherapy [23].
2. The prediction of metastatic spread to specific organs is not well defined and the treatment regimens remain systemic with severe side effects in most patients. The evolution of knowledge about biological and genetic properties of primary tumor and its metastases is crucial for early characterization of metastatic patterns in a particular patient and application of targeted treatment modalities.
3. There is a need for strong prognostic and predictive tools based on molecular cancer portrait and its metastatic profile that can be integrated into the clinical diagnostic procedure.

In summary, breast cancer patients should ideally get tailored treatment in line with the specific molecular profile of their cancerous disease but additional insights into the biology of solid tumors and metastases are urgently needed.

### **1.3.1. Brain metastases of breast cancer**

Breast cancer is the second most common cause of brain metastases, after lung cancer, and represents 14–20% of all cases of brain metastases [24, 25, 26, 27].

Overall incidence of symptomatic brain metastases in patients with breast cancer ranges from 10 to 20%, but the incidence of metastases reported in autopsy studies

reaches up to 30% [26, 28, 96].

On average, the median latency between the initial diagnosis of breast cancer and the onset of brain metastasis is 2–3 years suggesting that central nervous system (CNS) involvement typically occurs late in the course of disease [29]. In most cases, involvement of the lungs, liver, or bone precedes the diagnosis of CNS metastasis [29]. Risk factors for the development of CNS metastases from breast cancer include patient characteristics, such as young age and African-American ethnicity, and biological features of the tumor, including ER-negativity, basal like tumors, HER2-positivity, high tumor grade, and mutated *BRCA1* [30, 96, 97, 98].

The current treatment regimens are limited to brain irradiation and surgery.

Due to improvement of systemic therapy, the incidence of brain metastases in breast cancer patients is expected to rise. Brain metastases present a particular problem in the management of metastatic breast carcinoma for several reasons.

First, in contrast to patients with brain metastasis from other solid tumors who usually die from progression of systemic disease, about half of the patients with brain metastasis from breast cancer die from their neurological disease. Therefore, long-term survival in breast cancer cases with brain metastasis depends more on local control of brain metastasis than on control of other metastatic sites [26]. Second, there is a limitation in therapeutic access to brain metastases with available chemotherapeutics due to the poor penetration of drugs through blood-brain barrier (BBB). Additionally, hormone therapy and molecular-targeted drug therapy are not effective for the treatment of brain metastases. It has been suggested that Herceptin® therapy may be related to the increased risk for brain metastases [31]. Third, breast cancer metastases to the brain usually present as multiple satellite lesions what make the surgical removal particularly difficult.

Therefore, the development of the management strategies for brain metastases has become a growing clinical challenge. Understanding of biology and homing properties of brain metastases is important for prediction of patients at risk to develop brain metastases in order to provide them with adequate treatment as well as withhold the toxic therapeutic regimens in cases that are not at risk for brain metastases.

#### **1.4. Genetic determinants of breast cancer**

Breast cancer is a genetic disease. Like other human cancers it is thought to develop as

a result of accumulated genetic aberrations. These genetic alterations lead to deviation of gene expression profile of breast cancer cells from that of the normal progenitor cell what results in a cancer phenotype.

Genetic analyses have for long been performed in breast cancer research in order to unravel the molecular aberrations leading to tumor initiation and progression [32, 33]. In the last years, increasingly more coherent information about genetic aberrations in breast cancer has been generated and molecular techniques are slowly becoming part of the diagnostic and prognostic armamentarium available for pathologists and oncologists to tailor the therapy for breast cancer patients [32].

At present breast cancer management still relies on clinic pathological features (i.e. tumor size, histological grade and presence of axillary lymph node metastasis) and three immunohistochemical markers (estrogen receptor [ER], progesterone receptor [PR] and HER2) for treatment decision-making) [34]. Traditional clinicopathological factors are important in helping to determine risk of relapse, but do not account for the complexity of breast cancer. Hence, advances in gene expression analysis make an important biological and clinical contribution to development towards tailored therapies for breast cancer.

Molecular profiling technologies like allelotyping, CGH, CGH-array allow studying gene expression signature and have led to identification of different gene expression patterns in breast cancer. Subsequently, breast cancers were classified into five molecular subtypes based on their “intrinsic gene list”: luminal A, luminal B, HER2, basal-like and normal/claudin-low breast cancer entities. Representative markers for this classification are estrogen receptor (ER), HER2 and basal or myoepithelial markers, e.g. cytokeratin (CK) 5/6, CK17, CK14. Basal-like subtype and normal breast subtype are negative for ER or HER2, but the expression of basal/myoepithelial markers is positive in the former only. The HER2 and basal-like subtypes are correlated with aggressive clinical behavior, whereas luminal A subtype is correlated with better prognosis [35, 36, 37]. It's obvious that genetic studies have the potential to deliver a more accurate description of tumor biology.

#### **1.4.1. Genetic alterations observed in breast cancer**

Different molecular alterations can contribute to the process of malignant transformation of a cell: point mutations, amplifications, numerical aberrations in

chromosome arms, losses and translocations of genetic material. This usually results in: activation of oncogenes, inactivation of tumor suppressor genes, activation or inactivation of genes whose products interfere with genome stability, e.g. DNA repair enzymes, alterations in genes responsible for epigenetic DNA control mechanisms [38].

Usually, one aberrational event is not sufficient to convert a normal cell phenotype to a malignant one. That's why tumor formation is a multi-stage process, which is a result of interaction and cooperation between sequentially accumulated aberrant genetic events [39].

DNA amplifications suggest the existence within the amplified region of a dominantly acting gene. Conversely, recurrent DNA losses may point to tumor suppressor genes. Two methods have mostly been used to investigate these anomalies:

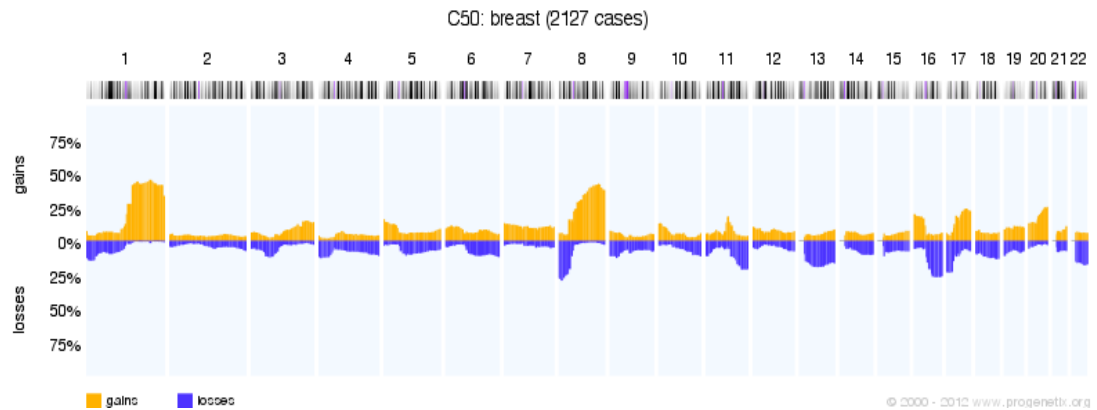
1. Allelotyping: the comparison of allelic status at chosen loci in tumor and normal DNA by the use of PCR with highly polymorphic markers. Allelotyping detects numerical alterations in chromosome arms as allelic imbalance (AI), also known as loss of heterozygosity (LOH). AI in cancer denotes the loss or gain of one or more alleles that are heterozygous at a locus [35, 39]. The genetic mechanisms involved in AI that may lead to different allelic ratios are monosomy, recombination events, or recombination followed by tetraploidization [40].

2. Chromosomal Genetic Hybridization (CGH): CGH involves *in situ* hybridization of labeled normal and tumor DNA to normal metaphase chromosomes or arrays (array-CGH). This method allows the detection of copy number changes across the tumor DNA.

LOH analysis may detect smaller aberrations in specific regions than CGH but LOH analysis cannot distinguish DNA losses from amplifications and cannot be applied on a genome wide scale.

#### **1.4.2. Chromosomal instability**

Although the genomes of clinically similar breast cancers are completely different, some regions of the genome are recurrently aberrant (figure 1.1).



**Figure 1.1. Genomic aberrations in breast carcinoma. CGH-array on 2127 cases. [www.progenetix.net](http://www.progenetix.net)**

Recurrent gains on chromosomes 1q, 8q are present in up to 40 % of cases. 11q, 16p, 17q and 20q are as well very common amplifications present in 15-20 % of breast cancers [39, 43, 44]. It has been shown that high-level amplifications are associated with poor outcome [43]. Further, several chromosomal regions were identified as differentially imbalanced in different breast carcinoma subtypes. For instance, gain of genetic material on chromosome 1q and losses on 16q are more characteristic for invasive lobular carcinoma (ILC) and can serve as important discriminators between invasive ductal and invasive lobular carcinoma subtypes [44]. Additionally, simultaneous presence of 1q gain and 16q loss has been shown to be associated with luminal A type of breast cancer and low proliferation rate and better prognosis in breast cancer [100, 102]. In contrast, gains on 8q were related to a poor prognosis of the disease [101].

Chromosomal deletions are very frequent events observed in breast cancer. The fact that the deletions are recurrently found from one tumor to another suggests the involvement of tumor suppressor genes (TSGs) located on the deleted loci. The loss of function of TSGs usually requires the inactivation of both parental copies of the gene, most often by a mutation of one allele and loss of the wild type allele [45]. Chromosomal arms: 1p, 3p, 7q, 6q, 8p, 9p, 11p, 16q, 17p are lost in 20-25 % of breast cancers [39, 44, 46]. DNA copy number losses in chromosomes 18q and 11p were shown to be associated with poor prognosis [47, 48].

Differences in chromosomal aberrations were also noted between primary tumors and metastases of breast cancer. Loss of genetic material on 11p has been reported in tumors exhibiting greater biological aggressiveness and invading the lymphatic system

[47]. It has been shown that loss of heterozygosity on 11p significantly correlates with the presence of metastases to lymph nodes [103]. Losses on 18q were as well characteristic for tumors with high rate of local recurrence and lymph node metastases [47, 104]. Another study linked allelic imbalances (AI) on 8p to the development of metastases to distant sites [105].

In some tumors, defects in mismatch repair enzymes lead to errors in the replication (RER) of simple nucleotide repeat segments. This condition is commonly known as microsatellite instability (MSI). It has been shown that MSI on chromosome 11p15.5 might play a role in development of breast cancer but the relation of this aberration to clinical parameters of breast cancer is not well defined and seems controversial [50, 51, 52].

### **1.4.3. Genes involved in breast cancer tumorigenesis**

The amplification and over-expression of several oncogenes has been describes in breast cancer. The human epidermal growth factor receptor 2 (*Her2*), epidermal growth factor receptor (*ERBB1/EGFR*), phosphoinositide-3-kinase (*PI3KCA*), myelocytomatosis viral oncogene homolog gene (*MYC*), and Cyclin D1 (*CCND1*) oncogenes are frequently deregulated in breast cancer [39].

For example, HER2, a receptor with tyrosine-kinase activity, is overexpressed in approximately 20 % of breast carcinomas and is related to poor prognosis [39, 95]. *MYC* gene encoding a transcription factor, which mediates cell growth, differentiation and apoptosis is altered in about 15-20 % of breast tumors [39].

*CCND1* located on chromosome 11q13 is a well-established oncogene. Deregulation of cyclin D1 gene expression or function contributes to the loss of normal cell cycle control during tumorigenesis. Yet, emerging evidence suggests that cyclin D1 might act in breast cancer, predominantly or at least in part, through pathways that do not involve its widely accepted function as a cell cycle regulator [108]. For example, cyclin D1 has been linked to mitogenic effects of estrogen. Since cyclin D1 is overexpressed preferentially in ER-positive breast cancer, it has been suggested that modulation of transcription via its action on ER probably underlies the oncogenic activity of cyclin D1 in breast cancer [109].

*PI3K/Akt1* pathway is integral to diverse cellular functions, including cellular metabolism and proliferation, differentiation, and survival [113]. Activating mutations

in *PIK3CA* gene are present in around 30% of breast tumors and are present very early in the development of breast cancer [117, 118]. While AKT1 overexpression plays a synergistic role in context of *ERBB1* driven cancer progression it has been as well shown to oppose tumor invasion and metastases in some studies [119, 120, 121].

Deletion or inactivation of tumor suppressor genes plays a crucial role in tumorigenesis. Several tumor suppressor genes have been related to breast cancer pathogenesis. Among them are well known genes like *TP53* (tumor protein 53), which is involved in many levels of DNA repair and genome stability (15-35 % of breast cancers); *RBI* (retinoblastoma protein 1) which controls the regulation of cell cycle (20% of breast cancers); *PTEN* (phosphatase and tensin homolog deleted on chromosome 10), coding for a cell cycle regulatory phosphatase, which promotes, once inactivated, breast cancer progression and aggressive phenotype in sporadic breast cancer [39]; *CDKN2A* (cyclin-dependent kinase inhibitor 2A), a cell cycle regulator working as a tumor suppressor gene and is lost in up to 40% of breast cancers [115, 116].

Deleted in breast cancer gene (*DBC2*) was detected as another tumor suppressor gene in breast cancer from chromosome 8p21.3. Its deletion was associated with breast cancer growth but the prognostic role is still not defined [106, 107].

It has been demonstrated that deficient tumor suppressor gene E-cadherin (*CDH1*) which is responsible for building bonds between cells, promotes peritumoral adhesion disruption events in ILC and may explain the scattered, multicentric pattern of this tumor subtype [110]. *CDH1* is mutated in 20-40% of lobular carcinomas through loss of wild type allele [39]. Loss of normal E-cadherin expression has been shown to indicate increased invasiveness and dedifferentiation in breast carcinoma [114].

Caveolin-1 (*Cav-1*), a protein found in invaginations of plasma membrane (caveolae) is thought to bind and hold in an inactive state several pro-proliferative proteins [such as the epidermal growth factor (EGF) receptor, ERBB2, and members of the growth-factor-activated Ras/44 mitogen-activated protein kinase (MAPK) pathway] as well as proteins of the prosurvival phosphatidylinositol 3-kinase/ Akt pathway [112]. Loss of *Cav-1* drives premalignant alterations in mammary epithelia, with abnormal lumen formation, EGF-independent growth, defects in cell substrate attachment, and increased cell invasiveness [111].

*BCL2* (B-cell lymphoma) gene, which plays a role in restraint of apoptosis, has been

shown to paradoxically exert a tumor suppressor effect, where its expression is associated with favorable prognostic and with reduction in the risk of death compared with BCL-2-negative cases [53, 54, 55].

### **1.5. Genetic signatures of breast cancer metastases**

A metastatic cell must complete a series of sequential steps in order to successfully colonize and grow at a distant site. It is still not well understood how the metastases progress and what characteristics determine the metastatic predilection to a specific organ. It has been proposed that metastatic phenotype is acquired at early stages of tumor formation and the same genetic alterations confer the tumor the replicative advantage as well as a proclivity to metastases [56]. Another model suggests that most primary tumor cells have a low metastatic potential and that during later stages of tumorigenesis rare cells acquire metastatic capacity through generation of additional somatic mutations [57]. Stromal factors and tumor environment seem to considerably contribute to the metastatic potential of the tumor [57]. All existing models explaining metastatic progression are conflicting and a better understanding of genetic and biochemical basis of breast cancer metastases is principal to more accurately identify patients who are at metastasis risk and to refine the prognostic classification of breast cancer.

Gene expression analyses have shown that pairs of human primary breast carcinomas and their distant metastases are highly similar in their expression signatures [58, 59]. However, detailed analyses have also revealed that a number of genes are consistently differentially expressed between primary tumors and metastases [60, 61, 62] and that metastases often show a greater variety of aberrations than the primary tumor [63, 64]. On chromosome levels some studies have shown larger differences between lymph node metastases and primary tumors [65, 66, 67, 68]. Whereas most of the analyses examined the genetic variation between local metastatic formation and the primary tumor [65, 66, 67, 68], data from the matched primary tumors and distant metastases is quite rare [69, 70, 71]. Still, the available data showed a high degree of discordance in genetic make-up between primary tumors and their matched metastases [72, 73]. This implies that dissemination of tumor cells does not obey the linear models of progression and specific constellations of genetic alterations might be causative of distant metastases and account for the proclivity of dissemination to a certain site. Metastatic cells of different cancer types exhibit patterns of organ tropism, which



cannot be explained by circulatory pathways alone. For example breast cancer disseminates preferentially to the bone and lung, less frequently to the liver and brain. A CGH analysis of primary breast tumors identified a set of 70 genes, which were highly predictive of occurrence of distant metastases in the course of disease [122]. Although the ‘poor prognosis’ genes may indicate whether a primary tumor is likely to develop distant metastases, expression of these genes does not explain the diversity of metastatic patterns exhibited by advanced breast cancer cells. A study by Massague et al. has shown that cells with the same ‘poor-prognosis’ signature exhibit different metastatic activity and different organ tropism [123]. Moreover Massague et al. identified a gene expression profile that enables the primary breast tumor to metastasize preferentially to the bone, lung, or the brain [123, 124]. This indicates that expression of ‘poor prognosis’ genes is not sufficient for metastasis and, consequently, that additional gene expression events must occur before cells gain a truly metastatic phenotype. Moreover, it seems that organotropism is controlled by an additional set of genes [136]. Identifying these genes might be useful for detection of high-risk patients and may have implications for the clinical management of their disease.

### ***Genetic alterations in brain metastases***

The mechanisms that determine the potential of certain tumors to develop brain metastases are not yet completely unraveled. Due to the poor availability of metastatic brain tissue most of the studies include unmatched samples of metastases or are based on a small number of samples [126, 127]. By means of classical CGH and AI analyses it has been shown that brain metastases of epithelial tumors (prostate, breast, lung, colon) accumulate a higher rate of microsatellite instability than their primary counterparts [73, 74, 125, 126]. The highest incidence of DNA gains in brain metastases of solid tumors was observed for the chromosomal regions 1q23, 8q24, 17q24-q25, 20q13 (>80% of cases) followed by the gain on 7p12 (77%). DNA losses were slightly less frequent with 4q22, 4q26, 5q21, 9p21 being affected in at least 70% of the cases followed by deletions at 17p12, 4q32q34, 10q21, 10q23-q24 and 18q21-q22 in 67.5% of cases. Two unusual narrow regional peaks were observed for the gain on 17q24-q25 and loss on 17p12 [127].

In breast cancer a significantly reduced post metastatic survival time has been observed in patients with brain metastases affected by multiple allelic losses [71]. The examination of a few cases has shown that samples of recurring brain metastases

exhibit accumulation of LOHs [73].

Candidate genes have been examined in a set of human brain metastases. Changes in E-cadherin gene were observed in the majority of brain metastases [75]. The accumulation of LOH in brain metastases has been detected on Adenomatous polyposis coli gene (*APC*) [76].

In breast cancer metastases an increase in p21 expression was reported as compared to primary tumors [128]. Reduced expression of tumor suppressor genes Kisspeptin (*Kiss1*), CD28 (*Kai1*), breast cancer metastases suppressor 1 (*Brms1*), and MAP kinase kinase 4 (*Mkk4*) was reported in breast cancer brain metastases as compared with unlinked primary tumors [27, 129]. Mouse models from Massague have in addition detected by means of gene expression analysis cyclooxygenase COX2 (also known as PTGS2), the epidermal growth factor receptor (EGFR) ligand HBEGF, and the alpha 2,6-sialyltransferase ST6GALNAC5 as mediators of cancer cell passage through the blood-brain barrier [133].

A recent study of brain metastases of breast cancer and their primary tumors conducted at the Institute of Tumor Biology (doctoral thesis of Niclas Detels, Institute of Tumor Biology, UKE, Hamburg), University Medical Center Hamburg-Eppendorf has shown by means of CGH a loss on chromosome 10q in 60-90% of the metastases whereas only 6% of the primary tumors harbored a loss in this region ( $p < 0.002$ ) (Wikman et al., 2012, see attachment). This finding is compatible with previous findings implicating involvement of 10q in formation of metastases [130]. Based on these findings it is possible that 10q plays an important role in determining the metastatic route to the brain.

## **1.6. Aims of the study**

In this study we perform LOH analysis of the chromosome region 10q of brain metastases and primary tumors of breast carcinoma to:

- validate the CGH analysis results on a bigger patients cohort;
- detect the minimal overlapping region of 10q deletion
- compare the genetic make-up on 10q of brain metastases and primary breast carcinomas;
- identify putative genes of brain metastases suppressor genes at chromosome 10q;
- establish a correspondence of the results with clinic-pathological parameters.

## **2. Materials**

### **2.1. Blood and tissue samples**

#### **2.1.1. Primary tumors**

Blood and primary tumor samples from 77 primary breast cancer patients were obtained from the Department of Gynecology, University Hospital Hamburg-Eppendorf (UKE, Prof. Dr. Volkmar Müller). For this thesis I analyzed the primary tumor tissues from 55 patients. Later the study was extended and 22 additional patients were analyzed. Table 2.1-A summarises the pathologic and clinical data of all the patients with primary tumors included in the study. The mean age of the patients was 77 years. From all women in the study 18 were praemenopausal, 45 postmenopausal and two perimenopausal. Invasive ductal carcinoma was the most common histological type of primary tumor and was detected in 51 cases, followed by invasive lobular carcinoma in 10 cases. Overexpression of either or both hormone receptors was found in 56 tumors, 25 tumors had overexpression of HER2 receptor and eight tumors had a tripple negative histological phenotype. The tumor size falled into pT2 category in 35 cases, whereas pT1 was represented in 24 cases and pT3 and pT4 combined were found in nine cases. Lymph node metastases were detected in 20 patients and involvement of distant organs was diagnosed in nine cases. Additionally, disseminated tumor cells were detected in the bone marrow of 18 patients. The differentiation grade GI was present in three tumors, GII in 26 tumors and GIII in 37 tumors. Ten of the primary tumors showed later a relapse to the brain and 17 to other sites.

The obtained primary tumor DNA was previously isolated from paraffin embedded samples using microdissection. DNA samples obtained by this method contained aminimum of 70% tumor cells in the sample (Wrage et al., Clin Cancer Res., 2009). As reference DNA we used the DNA previously isolated from non-malignant normal breast tissue and lymphocyte DNA.

**Table 2.1. Characteristics of patients with primary tumors (A) and brain metastases (B).**

<b>A</b>			<b>B</b>		
<b>PRIMARY TUMORS</b>	<b>n</b>	<b>%</b>	<b>BRAIN METASTASES</b>	<b>n</b>	<b>%</b>
<b>All</b>	77	100.0	<b>All</b>	21	100.0
<b>Histology</b>			<b>Histology</b>		
Inv. Ductal	51	66.2	Inv. Ductal	10	47.6
Inv. Lobular	10	13.0	Inv. Lobular	1	4.8
others	8	10.4	others	1	4.8
n.a.	8	10.4	n.a.	9	42.9
<b>Type</b>			<b>Age</b>		
ER+/PR+/HER-	29	37.7	<50	4	19.0
ER-/PR-/HER-	8	10.4	>50	17	81.0
HER2+	25	32.5	n.a.	0	0.0
n.a.	15	19.5	<b>Tumor stage</b>		
<b>Age</b>			pT1	6	28.6
mean	77		pT2	6	28.6
n.a.			pT3+pT4	2	9.5
<b>Tumor stage</b>			n.a.	7	33.3
pT1	24	31.2	<b>Lymph node status</b>		
pT2	35	45.5	pN neg.	6	28.6
pT3+pT4	9	11.7	pN pos.	8	38.1
n.a.	9	11.7	n.a.	7	33.3
<b>Lymph node status</b>			<b>Distant metastasis</b>		
pN neg.	46	59.7	M0	12	57.1
pN pos.	20	26.0	M1	1	4.8
n.a.	11	14.3	n.a.	8	38.1
<b>Distant metastasis</b>			<b>Grade</b>		
M0	55	71.4	GI	0	0.0
M1	9	11.7	GII	3	14.3
n.a.	13	16.9	GIII	8	38.1
<b>Grade</b>			n.a.	10	47.6
GI	3	3.9	<b>Bone marrow status</b>		
GII	26	33.8	neg	n.a.	
GIII	37	48.1	pos	n.a.	
n.a.	11	14.3	n.a.	21	100.0
<b>Bone marrow status</b>			<b>Tumor size</b>		
neg	32	41.6	<=2.0cm	n.a.	
pos	18	23.4	>2.0cm	n.a.	
n.a.	27	35.1	n.a.	21	100.0
<b>Tumor size</b>			<b>Hormone receptor</b>		
<=2.0cm	32	41.6	negative	8	38.1
>2.0cm	33	42.9	positive	11	52.4
n.a.	12	15.6	negative+positive*	2	9.5
<b>Menopausal status</b>			n.a.	0	0.0
perimenopausal	2	2.6	HER2 in prim. tum.		
praemenopausal	18	23.4	negative	8	38.1
postmenopausal	45	58.4	positive	4	19.0
n.a.	12	15.6	n.a.	9	42.9
<b>Hormone receptor</b>			<b>HER2 in brain</b>		
neg	11	14.3	negative	11	52.4
pos	56	72.7	positive	10	47.6
n.a.	10	13.0	n.a.	0	0.0
<b>HER2</b>			<b>Subtype**</b>		
neg	37	48.1	HR positive	5	23.8
pos	25	32.5	triple negative	5	23.8
n.a.	15	19.5	HER2 positive	11	52.4
<b>Ki-67</b>			n.a.		
<=20%	27	35.1	Ki-67		
>20%	37	48.1	<20%	n.a.	
n.a.	13	16.9	>20%	n.a.	
<b>Relapse</b>			n.a.	21	100.0
no	39	50.6	<b>Relapse</b>		
brain	10	13.0	no	0	0.0
other site	17	22.1	yes	21	100.0
n.a.	11	14.3	n.a.	0	0.0
<b>Course of disease</b>			<b>HR: hormone receptor</b>		
alive	48	62.3	* two tumors with different hormone receptor status		
died	13	16.9	** in brain metastasis patients classification based on the HER2 status determined in the brain metastases.		
n.a.	16	20.8	n.a.= clinical data not available		

### **2.1.2. Brain metastases**

Blood samples and tissue samples of brain metastases from 21 breast cancer patients were obtained from the Department of Neurosurgery, University Hospital Eppendorf (Prof. Dr. Katrin Lamszus and Prof. Dr. Manfred Westphal). In my experiments I worked with DNA from brain tumor tissues and from blood samples of nine patients. As the study was extended tissues and blood samples of 12 additional patients were added. The clinical and pathological characteristics of patients with brain metastases are presented in the Table 2.1-B. Ten patients had a primary ductal and one patient a lobular carcinoma phenotype. In six cases the tumor fell into pT1, in another six cases into pT2 and in two cases into pT3 or pT4 category. At the time of diagnosis six patients has no lymph node involvement (pN0), and eight patients had positive lymph node status (pN1). In one case distant metastases were detected at the time of diagnosis whereas no distant metastases were found in 12 cases. A differentiation grade GII was present in three cases and grade GIII in eight cases. Four women were younger than 50 years at initial presentation. In eight samples no overexpression of hormone receptors was detected but in 11 cases there was an chistochemical hormone receptor positivity. Two patients had both primary tumor subtypes, with and without overexpression of hormone receptors. The status of Her2 receptor was evaluated as positive in four primary tumors and 10 brain metastases and as negative in eight primary tumors and 11 brain metastases. All women suffered a relapse during the course of the disease.

Four pairs of matched primary and metastatic tumors were available.

### **2.1.3. Primary tumor tissues on tissue micro array (TMA) used for fluorescence *in situ* hybridization (FISH)**

To validate the results obtained in analysis of allelic imbalances on 10q we performed a screening for losses on 10q by means of fluorescence *in situ* hybridization. Tissues of primary tumors were available as a TMA. Fifty-five tumor tissues were used for analysis. The mean age of the patients was 58 years. From all women in this study 15 were praemenopausal, 37 postmenopausal and one perimenopausal. Invasive ductal carcinoma was the most common histological type of primary tumor and was detected in 31 cases, followed by invasive lobular carcinoma in 13 cases. In six tumors overexpression of either or both hormone receptors was found, 39 tumors had overexpression of HER2 receptor and four tumors had a tripple negative histological

phenotype. Based on the tumor size 26 tumors were classified into pT1, 24 into pT2 and five into pT3 or pT4 category. Out of all patients 32 patients had no detectable lymph node metastases and 53 had no distant metastases whereas 23 patients presented with lymph node involvement and two women had evidence of distant metastatic spread. Additionally, disseminated tumor cells were detected in the bone marrow of 14 patients. Three tumors were well differentiated (GI), 32 tumors were moderately differentiated (GII) and 10 tumors had a poor differentiation on histological exam. Six patients developed a relapse, in one case to the brain, and three patients died in the course of their disease (table 2.2).

**Table 2.2. Clinical and pathological characteristics of patients with primary tumors used for FISH analysis.**

<b>PRIMARY TUMORS</b>	<b>n</b>	<b>%</b>
<b>All</b>	55	100.0
<b>Histology</b>		
Inv. Ductal	31	56.4
Inv. Lobular	13	23.6
others	11	20.0
<b>Type</b>		
ER+/PR+/HER-	6	10.9
ER-/PR-/HER-	4	7.3
HER2+	39	70.9
n.a.	6	10.9
<b>Age</b>		
mean	58	
<b>Tumor stage</b>		
pT1	26	47.3
pT2	24	43.6
pT3+pT4	5	9.1
<b>Lymph node status</b>		
pN neg.	32	58.2
pN pos.	23	41.8
<b>Distant metastasis</b>		
M0	53	96.4
M1	2	3.6
<b>Grade</b>		
GI	3	5.5
GII	32	58.2
GIII	20	36.4
<b>Bone marrow status</b>		
neg	41	74.5
pos	14	25.5
<b>Tumor size</b>		
<=2.0cm	27	49.1
>2.0cm	28	50.9
<b>Menopausal status</b>		
perimenopausal	1	1.8
praemenopausal	15	27.3
postmenopausal	37	67.3
n.a.	3	5.5
<b>Hormone receptor</b>		
neg	12	21.8
pos	33	60.0
<b>HER2</b>		
neg	10	18.2
pos	39	70.9
n.a.	6	10.9
<b>Ki-67</b>		
<=20%	31	56.4
>20%	9	16.4
n.a.	5	9.1
<b>Relapse</b>		
no	46	83.6
brain	1	1.8
other site	5	9.1
n.a.	3	5.5
<b>Course of disease</b>		
alive	51	92.7
died	3	5.5
n.a.	1	1.8

## 2.2. Reagents

acetic acid	Merck, Darmstadt
ammonium acetate	Merck, Darmstadt
boric acid	Sigma, Deisenhofen
bromphenol blue	Merck, Darmstadt
DAPI (4',6-Diamidino-2-Phenylindole)	Sigma, Deisenhofen
desoxynucleoside triphosphate Set (PCR)	Roche, Mannheim
EDTA (ethylenediaminetetraacetic acid)	Sigma, Deisenhofen
ethanol	Merck, Darmstadt
ethidium bromide (10 mg/ml)	Sigma, Deisenhofen
HiDi formamide	Applied Biosystems, Freiburg
hydrochloric acid 5 M	Merck, Darmstadt
HPLC-H <sub>2</sub> O	Merck, Darmstadt
magnesium chloride	Sigma, Deisenhofen
nuclease free water	Promega, Mannheim
potassium dihydrogen phosphate	Merck, Darmstadt
sodium acetate	Merck, Darmstadt
sodium chloride	J.T. Baker, Deventer
sodium hydrogen carbonate	Merck, Darmstadt
SeaKem ME agarose	FMC Bioproducts
Trizma Base	Sigma, Deisenhofen
xylol	Fluka, Buchs

## 2.3. Buffers and solutions

AE Buffer	
Dulbecco's PBS w/o Calcium and Magnesium	Gibco BRL, Eggenstein



w/o Sodium Bicarbonat

ethanol 80 %, 96 %, 100 %

Hanks salt solution

H-Lyse buffer concentrate

Merck, Darmstadt

Biochrom AG

R&D-Systems,

Minneapolis

5x Loading Buffer

Karl Roth GmbH,

Karlsruhe

50% Glycerol.....5 ml

0,5 M EDTA.....1 ml

1 M Tris pH 7,5.....200 µl

H<sub>2</sub>O.....ad 10 ml

Bromphenolblau (1:3)

Xylene Cyanol (1:3)

10x PBS pH 7,4

90 g NaCl

14,33 g Na<sub>2</sub>HPO<sub>4</sub> x 2 H<sub>2</sub>O

2,67 g KH<sub>2</sub>PO<sub>4</sub>

H<sub>2</sub>O ad 1 l

5x TBE (Tris-Borat EDTA)

Trizma Base.....54 g

boric acid.....27,5 g

0,5 M EDTA pH 8.....20 ml

H<sub>2</sub>O.....ad 1 l

TE-Buffer

10 mM Tris-Cl, pH 7.5

1 mM EDTA

10xTAE-Buffer

Trizma Base.....48,4 g

glacial acetic acid.....11,4 ml (17,4 M)

EDTA.....3,7 g

ad 1L deionized water

TBS DAKO, Hamburg

50 mM Tris-HCl

150 mM NaCl

pH 7,6

1 M Tris pH 7,5, pH 9,5

Cell lysis buffer

155 mM NH<sub>4</sub>Cl.....8.29 g

10 mM KHCO<sub>3</sub>.....1 g

0.1 mM NA<sub>2</sub>EDTA.....0.034 g

H<sub>2</sub>O.....ad 1 l

Spot-Light Tissue Heat Pretreatment buffer Zymed

Hybridization buffer (pH=7) for FISH

Dextran.....4 g

Formamid.....10 ml

20xSSC.....4 ml

H<sub>2</sub>O.....ad 20 ml

20xSSC (saline-sodium citrate buffer), pH=7

3 M NaCl.....177 g

0.3 M sodium citrate....77,4 g

## 2.4. Kits

AmpliTaq Gold (200 µl, 1000 U, 5 U/µl) Applied Biosystems,  
 mit GeneAmp 10x PCR Gold Puffer Mannheim  
 (1,5 ml, 150 mM Tris-HCl, pH 8,0, 500 mM KCl)  
 und 25 mM MgCl<sub>2</sub>-Lösung (1,5 ml)

QIAamp DNA Mini Kit (250) Qiagen, Hilden

Proteinase K solution

Buffer AL – lyse puffer

Buffer AW1 – wash buffer 1

Buffer AW2 – wash buffer 2

Buffer AE – elution buffer

R&D Human Erythrocyte Lysing Kit

10x H-lyse buffer

10x wash buffer

10x fixative

Taq DNA Polymerase

Qiagen, Hilden

PCR-Buffer 10x

## 2.5. Microsatellite PCR primers

The characteristics of the primers used in this study are presented in the table 2.3.

**Table 2.3. Fluorescence PCR-Primers for the microsatellite analysis.**

Marker	Forward primer (5')	Reverse primer (3')
<b>Microsatellite primers</b>		
D10S219	[6-FAM]TCTTTCTACCACCCCCC	GCAGAGAACCTAAAGCATCC
D10S1765	[6-FAM]ACACTTACATAGTGCTTTCTGCG	CAGCCTCCCAAAGTTGC
D10S541	[6-FAM]CACCACAGACATCTCACAACC	CCAGTGAATAGTTCAGGGATGG
D10S1692	[6-FAM]GAGATTGTGCCATTGCAG	GGAAAGCCCAGATGTTGATG
D10S173	[6-FAM]CTGATTTTCTGCTGGTCT	TGTTTCTGAAGCATTTCCTTG
D10S1236	[HEX]ATACTCTTTCACTTTTCAACC	ACATATGACAAAGCCCAGA
D10S190	[HEX]GTGTTTGGGTCATGGAGATG	AGGCAAAGCAGGAGCAGG
D10S212	[HEX]GAAGTAAAGCAAGTTCTATCCAG	TCTGTGTACGTTGAAAATCCC

Marker	Chromosome band	Base pair position	Size of PCR product	Type of repeat	Heterozygosity	Reference
<b>Microsatellite primers</b>						
D10S219	10q22.3	80,522,854	89-103	CA	0.78	NCBI
D10S1765	10q23.2	89,491,516	166-184	CA	0.83	NCBI
D10S541	10q23.31	89,981,472	153-169	CA	0.78	Schmidt et al.2006
D10S1692	10q24.32	104,578,854	182-211	CA	0.88	own primer
D10S173	10q25.2	112,010,137	155	CA	0.82	NCBI <sup>b</sup>
D10S1236	10q26.11	119,799,027	121-137	TATC	nd	NCBI <sup>c</sup>
D10S190	10q26.11	119,835,725	203-219	CA	0.84	NCBI
D10S212	10q26.3	134,299,490	189-201	CA	0.72	NCBI

nd= not described

a= modified forward and reverse

b= modified forward primer

c= own reverse primer

## 2.6. DNA

Human Cot-1 DNA

Roche, Mannheim

Chromosome 10 centromeric DNA

Vysis, Downer Glove,  
USA

## 2.7. Other materials

Super Frost glass slides

Menzel-Gläser, Stuttgart

DNA Molecular Weight Marker VIII

Roche, Mannheim

## 2.8. Appliances

centrifuge, Biofuge fresco Heraeus

Kendro, Langselbold

BioPhotometer

Eppendorf, Hamburg

centrifuge, Heraeus 3SR

Kendro, Langselbold

fluorescence microscope

Leica

gel imager

INTAS, Goettingen

electrophoresis equipment

Bio-Rad Laboratories,  
Munich

gel chambers, ComPor L Mini

Bioplastics RV, Landgraaf,

Genescan ABI Prism 310, Genetic Analyzer

Applied Biosystems,  
Mannheim

microwave

Promicro, Munich

Mastercycler+Mastercycler gradient

Eppendorf, Hamburg

MJ Research PTC-200 Peltier Thermal Cycler

Biozym

Nanodrop ND-1000 spectrometer

PeqLab, Erlangen

pH meter

inoLab WTW, Heidelberg

pipettes

Hirschmann Laborgeräte,  
Eberstadt

thermocycler

Techne, Staffordshire, UK

bench top centrifuge, Heraeus B12

Kendro, Langselbold

scale

Sartorius AG, Goettingen

Vortex Genie 2

Scientific Industries

## 2.9. Software

ABI PRISM® 3100 Gene Scan®

analysis Software

Applied Biosystems,  
Mannheim

GeneMapper 3.7

Applied Biosystems,  
Mannheim

Nanodrop operating software 3.3.0

Thermo scientific, PeqLab

Gel analysis software Gel iX Imager

Excel 12.0

Excel 14.0

PowerPoint 14.0

Photoshop CS 5

EndNote X4

Papers 1.9.3

R statistical environment 2.11.0

Erlangen

INTAS, Goettingen

Microsoft Office 2007

Microsoft Office 2011

Microsoft Office 2011

Adobe

Thomson Reuters

Mekentosj BV

The R Foundation for  
Statistical Computing)

### **3. Methods**

#### **3.1. DNA isolation from blood leukocytes**

Leukocyte DNA was isolated from peripheral blood. The blood samples were drawn in EDTA coated tubes. To optimize the DNA isolation and receive a pure leukocyte pellet and higher DNA yields we explored different ways of DNA isolation. We pre-treated whole blood samples from two selected patients with either a commercial ready made H-lyse erythrocytes lysis buffer (R&D, option 1) or our self made erythrocyte lysis buffer (option 2) or did no pre-treatment and isolated leukocyte DNA out of whole blood samples with QIAamp DNA Blood kit (Qiagen, option 3).

Option 1: 8 ml of 1:2 diluted commercially available stock of H-lyse buffer was mixed and incubated with 2 ml of whole blood for 10 min and centrifuged after at 500 x g for 5 min minutes, washed twice with 2 ml of 1x R&D wash buffer and centrifuged again after each washing for 5 minutes at 500 x g. The leukocytes were resuspended in 1 ml of wash buffer at last.

Option 2: 6 ml of the own lysis buffer was incubated on ice with 2 ml of whole blood for 30 minutes and centrifuged after at 1200 rpm for 10 minutes at 4°C, washed twice and centrifuged again after each washing. The pellets were collected into new tubes.

Option 3 and end path of option 1 and 2: 200 µl of each pellet or whole blood were mixed with 20 µl of proteinase K and 200 µl of AL buffer, mixed for 15 s and incubated at 56 °C for 10 minutes. 200 µl ethanol were added to each sample, mixed and transferred into the QIAamp Spin Column (in a 2 ml collection tube), closed and centrifuged at 8000 rpm for 1 min. The contents of QIAamp Spin Columns were then sequentially treated with 500 µl buffers AW1 and AW2 and centrifuged at 8000 rpm for 1 min and at 14000 rpm for 3 min after adding the respective buffer and ultimately at 14000 rpm for 1 min. For each centrifugation the QIAamp Spin Columns were place into clean collection tubes. At last 100 µl of TE buffer was added into each spin column, incubated at room temperature (20 °C) for 3 min and centrifuged at 8000 rmp for 1 min and collected the first elute of DNA.

For the second elution step further 100 µl of TE buffer were added to each spin column and incubated at room temperature (20 °C) for 3 min and centrifuged at 8000 rmp for 1 min.

The rest of the samples were processed using H-lyse erythrocyte lysis buffer (R&D) and QIAmp DNA Blood kit due to better DNA yields (see Results).

### **3.2. Spectrophotometric assessment of quality and quantity of isolated DNA**

The purity and concentration of the isolated DNA samples was measured with help of spectrophotometry. This method employs the measurement of light transmission and reflexion by comparing various wavelengths of the light. In the NanoDrop ND-1000 spectrophotometer (PeqLab) a little sample droplet bridges between a light source and a detector due to high surface tension. The light transmission and reflexion are analysed after passing through the sample ([www.nanodrop.com](http://www.nanodrop.com)).

The spectrometer was blanked with 1 µl of water. Then 1 µl of the respective DNA sample was subsequently placed on top of the detection surface and scanned at range of 200-750 nm. The results of the measurements were graphically presented by an assisting software in form of a light absorbance curve by the sample at different wave lengths and a legend including calculated DNA concentration in ng/µL based on absorbance at 260 nm, absorbance values at 260, 230 and 280 nm and the ratio of absorbance at 260 and 280 nm (260:280) and the ratio of absorbance at 260 and 230 nm. The ratio of absorbance 260:280 makes it possible to assess the purity of DNA sample since DNA has its absorption maximum at 260 nm. The ratio of ~1,8 is generally accepted as indicative of a pure DNA sample. Values lower than 1,6 are suggestive of a protein contamination of the probe. The ratio 260:230 is a secondary measure of nucleic acid purity and lies in the range of 1,8-2,2 for a pure DNA sample. If the ratio is appreciably lower, it may indicate the presence of co-purified contaminants [139, 140].

### **3.3. PCR-Amplification with specific microsatellite primers**

#### **3.3.1. PCR as a tool for detection of genomic aberrations**

PCR is employed to selectively amplify a segment of DNA by *in vitro* enzymatic replication using a set of primers, consisting of a sense- (5') and an anti-sense-primer (3'). Primers are synthetic single-stranded DNA fragments, mostly 18 to 25 base pairs (bp) long (oligonucleotides) that flank the sequence of interest and serve as a starting

point for DNA replication by the DNA-polymerase. They are essential for the initiation of DNA synthesis since the polymerase is capable to enzymatically assemble a new DNA strand by only adding nucleotides to an already existing DNA sequence. The polymerase starts replication at the 3'-end of the primer, and copies the opposite strand. Over several cycles of primers extension in contrary directions, including denaturation, primer annealing and elongation, specific DNA regions are exponentially cumulated whereby the generated DNA serves as a template for further replication cycles. For the efficient activity of DNA-polymerase  $Mg^{2+}$ -ions are necessary, therefore magnesium chloride ( $MgCl_2$ ) is a further compound of a PCR-setup and is added along with PCR-Buffer. A solution of deoxynucleoside triphosphates, comprising dATP, dCTP, dGTP and dTTP (dNTPs), provides the building blocks of the newly synthesized DNA. For amplification of DNA we used a thermostable DNA-polymerase AmpliTaq Gold which remains in its inactive conformation at room temperature and is activated by heat [141].

Annealing temperature is one of the most important parameters that need adjustment in the PCR reaction. The length and composition of a base sequence in a primer determine its annealing temperature in the PCR. The standard PCR was set up as follows in the table 3.1.

**Table 3.1. Standard PCR set-up.**

<b>Reagents</b>	<b>Concentration</b>	<b>Volume, <math>\mu</math>l</b>
PCR Gold Buffer	10x	1
$MgCl_2$	25 mM	1
dNTP	8 mM	1
Forward Primer	10 pmol	0,5
Reverse Primer	10 pmol	0,5
AmpliTaq Gold	5 U/ $\mu$ l	0,1
$H_2O$		3,9
DNA	5ng/ $\mu$ l	2
<b>TOTAL</b>		<b>10</b>

We used a ThermoCycler to run the PCR (Mastercycler Gradient, Eppendorf). In a first cycle the inactive AmpliTaq Gold DNA-polymerase is activated for 10 min at 95 °C. The amplification of DNA follows whereby the sequence of denaturation, annealing and elongation of the template are repeated in each cycle. While heated to 95 °C for 30 seconds the double-stranded DNA is denatured into single-stranded



templates. After that the temperature drops for 30 seconds to the level where optimal annealing of the primer is expected and the primers bind to DNA single strands. Next, the elongation of the primer operated by the AmpliTaq Gold DNA-polymerase occurs within 30 sec at 72 °C. The end cycle of PCR runs 7 min at 72°C after all cycles of DNA template replication have been completed (table 3.2).

**Table 3.2. Standard PCR conditions.**

Step	Temperature	Time	Number of cycles	Description
1	95°C	10 min	1	activation of AmpliTaq Gold DNA-polymerase
2	95°C	30 sec		denaturation of DNA
3	X °C	30 sec	35	annealing of primers
4	72°C	30 sc.		elongation
5	72°C	7 min	1	end of elongation

*X: specific annealing temperature depends on target*

### 3.3.2. Primer selection

To study potential chromosomal aberrations we used markers that covered the chromosomal region 10q22.3-10q26.11. We selected primers with highest possible frequency of heterozygosity to achieve higher informativity. The primers were labeled with fluorescence on their 5'-end (FAM, HEX). In my experiments I worked with 8 sets of primers: D10S219, D10S1765, D10S541, D10S1692, D10S173, D10S1236, D10S190, and D10S212. Later the study was extended and primers D10S185, D10S562, and D10S587 were included into LOH analysis.

### 3.3.3. Gradient PCR for detection of optimal primer annealing temperature

To avoid non-specific secondary bands formation PCR conditions must be optimized. The selection of the annealing temperature is one of the most critical step for optimizing the specificity of a PCR reaction. Primers have individual melting temperatures ( $T_m$ ) depending on the CG content and length of the oligonucleotide. Gradient PCR is a technique that allows the empirical determination of an optimal annealing temperature of a primer to DNA template during replication [142]. For gradient PCR we chose a temperature range that comprises the calculated primer

annealing temperatures. Each position in the thermal cycler was set for a specific annealing temperature from the tested range in an increasing manner. The rest of the PCR conditions remained the same.

After the completion of PCR the products were separated by means of agarose gel electrophoresis to estimate the specificity of the PCR bands as well as the quality and quantity of PCR products at each particular temperature. This way we could detect the optimal annealing temperature for each pair of primers.

### **3.3.4. PCR using combinations of primer pairs**

Due to scarcity of available patient material we tested PCR set ups using several combination consisting of two or three primer pairs in order to gain several PCR products in one PCR run. We combined the primers based on the similarity of the optimal annealing temperatures and clean separability of their PCR products. We combined primers D10S1236 and D10S1692, D10S212 and D10S219, D10S541 and D10S1236.

## **3.4. Separation of DNA fragments by agarose gel electrophoresis**

Agarose gel electrophoresis is a method used to separate a mixed population of DNA fragments by length.

Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. The phosphate backbone of DNA molecules gives them a uniform charge per unit length. Therefore upon electrophoresis through agarose gel molecules will migrate at rates largely independent of their sequences. The retarding forces the gel exerts on the migrating molecules increase sharply with the length of the DNA, so that the larger the molecule, the slower it migrate through a gel [143].

Following electrophoresis, the location of specific DNA fragments can be detected by staining. The most common dye used to visualize DNA bands is ethidium bromide (EtBr). If added into the liquid agarose it intercalate with DNA strands and fluoresces upon exposition to UV light [144]. To identify the size of the separated DNA fragments and verify the separation efficiency on the gel DNA ladders are employed as reference. They are commercially available solutions of linear double-stranded DNA fragments of known size [145].

Samples containing PCR-products were admixed in 1:5 ratio with 5x loading buffer and transferred into the wells of the agarose gel. The loading buffer contained bromphenol blue and xylencyanol as a dye and Glycerol to render the samples denser than the running buffer, so that the samples sink in the well [146]. To prepare a gel we boiled up 2 g SeaKem Agarose (2 % w/v) in 100 ml 1x TAE running buffer and let it cool down to about 50 °C. Then we added 4 µl of EtBr into the liquid agarose, let it homogeneously admix under gentle shaking, poured the solution into the gel container and inserted the comb to create wells for the DNA samples. After the gel solidified we removed the comb and transferred the gel into the electrophoretic chamber filled with 1xTAE running buffer so that the buffer completely covered the gel. The DNA samples were admixed in a 1:5 proportion with the 5x loading buffer and transferred into the wells. Additionally, we used 5 µl of the “DNA Molecular Weight Marker VIII” (0.5 µg DNA) as a reference to estimate the size of the separated DNA fragments. We ran the electrophoresis at 100 V and 40 mA approximately 45 min till the bromphenol blue reached the bottom edge of the gel.

### **3.4.1. Gel scanning**

The gels with separated DNA fragments were scanned in the Intas gel imager using the Intas gel analysis software (Gel iX Imager) and processed in Adobe Photoshop CS 5.

## **3.5. DNA analysis in Genetic Analyser**

### **3.5.1. DNA fragment analysis using fluorescent primers**

The Genetic Analyzer is an automated capillary electrophoresis system that can separate, detect and analyze fluorescent-labeled DNA fragments with a high sensitivity and acuity of a few base pairs. Specifically, it can provide a profile of separated PCR products according to the length and relative concentration of each fragment in a sample. Due to different fluorescence labels and different size of the PCRs products (HEX, FAM, TAM), we could analyzed two different PCR products simultaneously. Thus, we performed a concomitant analysis of markers D10S1236 (HEX) and D10S1692 (FAM), D10S212 (HEX) and D10S219 (HEX), D10S541 (FAM) and D10S1236 (HEX).

The analyzed was performed on the GeneScan ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

### **3.5.2. Sample preparation**

For the PCR sample preparation 5'-primer of each pair was labeled at its 5'-end with a fluorescence-tag (HEX or FAM). 3'-primers were left untagged. PCR reactions were performed under standard conditions (Table 1). After the PCR run has been completed we diluted PCR products with HPLC-H<sub>2</sub>O in 1:20 ratio. To prepare the injection samples for capillary electrophoresis we mixed together 1 µl of each diluted PCR products labeled with different fluorescence markers, 20 µl HiDi formamide (ultra pure) and 0.1 µl of GenescanRox 500 (internal size standard).

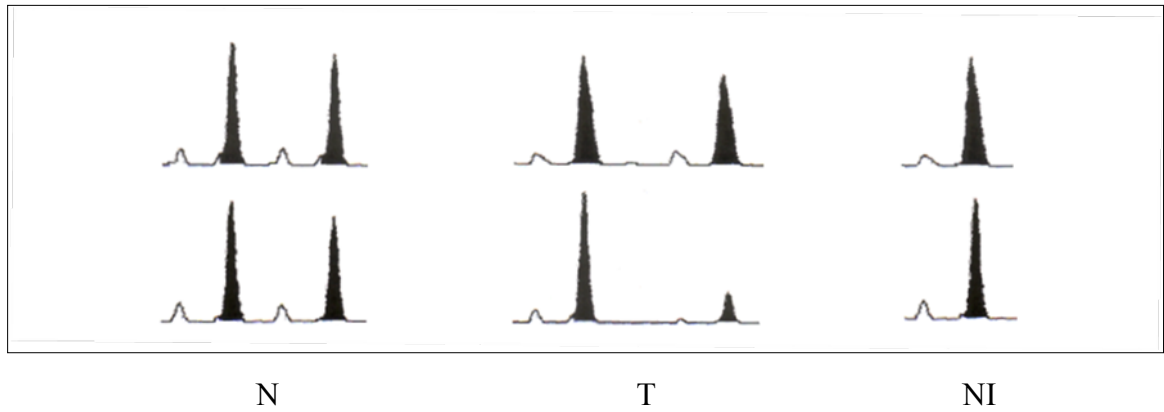
The samples were denatured at 94°C for 2 min and then cooled down on ice for 10 minutes. After that the samples were transferred into a 96-well plate and placed in a tray in the instrument's auto-sampler.

### **3.5.3. Capillary electrophoresis**

During the electrophoresis the auto-sampler successively brings each sample into contact with an electrode at the end of a glass capillary filled with polymer. A portion of the sample enters the capillary as current flows between the electrodes. The negatively charged DNA fragments travel towards the anode located at the end of the capillary. When the DNA fragments reach a detector window in the capillary coating, an argon laser excites the fluorescent dye labels. Emitted fluorescence from the dyes is collected by a CCD (charged coupled device) camera and can be digitally processed upon completion of the electrophoresis. The assisting software generates an electropherogram, which represents a calculation of the size or quantity of the fragments from the fluorescence intensity at each data point [147].

### **3.5.4. Analysis**

Analysis of the results of capillary electrophoresis was performed with GeneMapper software (Applied Biosystems), which processes the peak values of electropherogram and allowed the graphical and tabular presentation of the fragments profiles according to peak height and area under curve (figure 3.1).



**Figure 3.1. Electropherogram of a microsatellite marker in DNA from healthy (normal=N, left panel), tumor (tumor=T, middle panel) and not informative sample (not informative=NI, right panel).**

Based on the obtained data we calculated the ratio between the areas under highest peaks of tumor DNA to those of normal DNA and determined the ratios of allelic imbalance (AI).

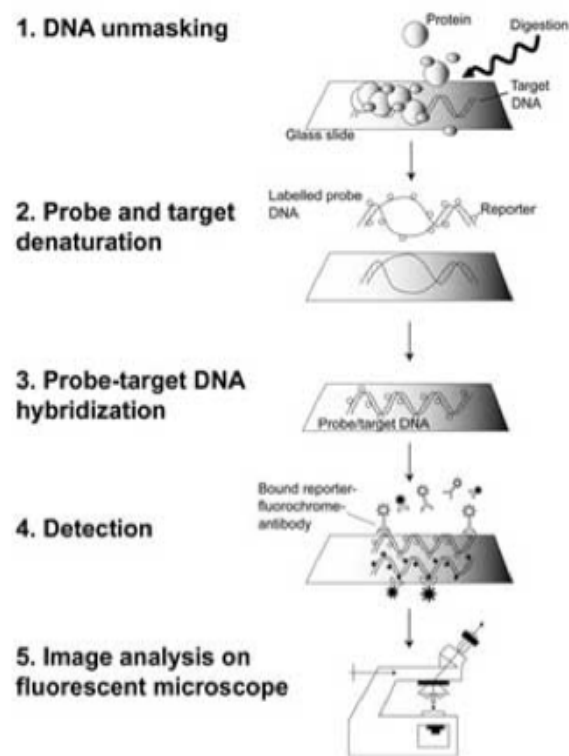
$$\text{Ratio} = \frac{\frac{\text{Peak Height of allele 1 in tumor sample}}{\text{Peak Height of allele 2 in tumor sample}}}{\frac{\text{Peak Height of allele 1 in healthy sample}}{\text{Peak Height of allele 2 in healthy sample}}}$$

The ratio values  $\geq 2$  or  $\leq 0.5$  were defined as AI. We determined a relatively high ratio in comparison to the literature due to a high content of tumor DNA in the studied samples (> 70%). Values < 2 were considered to be normal.

### **3.6. Fluorescence *in situ* hybridization (FISH)**

Fluorescence *in situ* hybridization is a cytogenetic technique used to detect the presence or absence of specific DNA sequences on chromosomes. It allows identifying or quantifying numeric or structural alterations of chromosomal regions. The sample DNA (metaphase chromosomes or interphase nuclei) and a fluorescently labeled probe of interest are first denatured and then hybridized at the target site upon re-annealing back into a double helix. The probe signal can then be visualized with help of fluorescence microscopy and the sample DNA can be scored for the presence or absence of the signal. The principle of FISH analysis is shown in the figure 3.1. The set up of FISH depends on the type of the fluorescent probe and its size; the probe

should penetrate the nucleus without causing any alterations in original tissue characteristics [148, 149, 150].



**Figure 3.1. Principle of fluorescence *in situ* hybridization (Oliveira and French 2005).**

To validate the results obtained by LOH analysis we performed FISH with the probe for the chromosomal region 10q23 (PR11-318C4) on a paraffin section of a tissue microarray (TMA) containing tissue punches of patients with primary tumors.

### 3.6.1. FISH probes

The BAC-probes cloned in recombinant *E. coli* were previously amplified and isolated in the lab from *E. coli* culture. The isolated probes were labeled with spectrum Orange marked dUTPs. The centromer probes labeled with spectrum Aqua were obtained from Vysis (Downer Grove, USA) (Table 3.3) (Wrage et al., Clin Cancer Res 2009).

**Table 3.3. BAC-Clone and FISH probes.**

Clone/Probe*	Binding site	Label
RP11-318C4	10q25.3-26.1	spectrum orange
Cen 10	centromer	spectrum aqua

\*Cen: centromeric probe

### 3.6.2. FISH on paraffin-embedded TMA

A 3 µm thick slice of paraffin-embedded TMA was placed on a glass slide (Superfrost plus) and incubated overnight at 37 °C in a drying cabinet. To dissolve the paraffin we incubated the slide with xylol overnight and dehydrated with 100% and then 80 % ethanol. The tissue was subsequently fixed for 10 min at -20 °C in 2% formaldehyde solution in methanol and then washed it in PBS 3 times for 3 min at room temperature. To denature the slide we incubated it in Spot-Light Tissue Heat Pretreatment buffer (Zymed) for 10 min at 90 °C and then cooled it down at room temperature for 15 min. Next we washed the slide in PBS 3 times for 2 min and subsequently coated it with pepsin to digest and remove the excess of cytoplasmic proteins and incubated in a humidity cabinet for 10 min at 37 °C. Finally, we washed it with PBS 3 times for 2 min at room temperature and the dehydrated with 70%, 80 % and 100 % ethanol sequentially.

During the incubation of the slides a hybridization mix containing both the target probe (SpectrumOrange-labeled) and a reference probe (centromere 10, SpectrumAqua-labeled) was prepared. We performed the hybridization of DNA with the labelled probe using the following probe mix:

	Volume, ul
human Cot-1 DNA	2
spectrum orange labeled DNA probe RP11-318C4	4
spectrum aqua labeled centromer probe	1
hybridization buffer	8

Hybridization buffer: 70% Formamid, 50% Dextransulfat., 4 x SSC, pH 7

Cot1-1 DNA was used as a short competitor DNA to suppress repetitive sequences in the target DNA that can often interfere with the visualization of the signal of interest. Due to Cot-1 DNA we aimed to decrease in the amount of background noise associated with non-specific binding of repetitive DNA and this way to enhance the signal of the labeled probe.

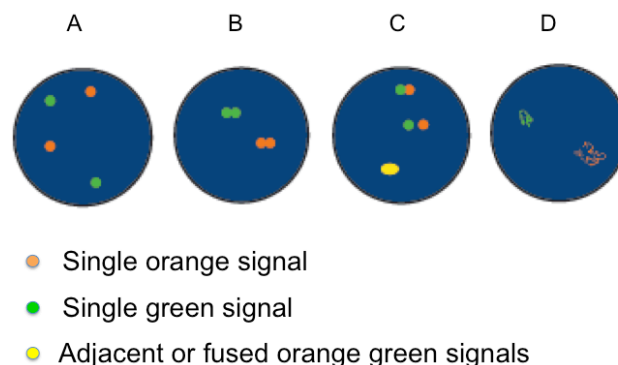
The hybridization mix was transferred onto the dehydrated TMA and the slide was covered with a thin glass and sealed with fixogum. The TMA slide with the

hybridization solution was denatured for 3 min at 95 °C and subsequently hybridized for 16 h at 37 °C. After that the cover was removed in 2x SSC/ 0.3% NP-40 buffer and subsequently washed in it at 70 °C for 2 min. Finally we washed the tissue microarray for dehydration in 70%, 80 % and 100 % ethanol in series, covered the slide with Vectashield mounting medium containing DAPI for the nuclear detection and placed the slide for storage into a dark room at -4 °C.

### 3.6.3. Evaluation of FISH results

The area of interest on the slide after staining was analysed using a fluorescence microscope (Zeiss, Jena). Using DAPI filter we first identified not overlapping, morphologically intact areas of tumor cells. Then with help of respective filters for spectrum orange and spectrum aqua we analysed the hybridization signals for each probe. The analysis was performed at 630- and 1000-fold magnification. We evaluated 31 cells on average per tumor sample, ranging from 6 to 66 analysed cells. Each tissue was scored by using Vysis signal enumeration guidelines [151]. Figure 3.2 represents the rules of signal enumeration we applied.

The number of centromeric and telomeric signals in each cell was counted, and a ratio of telomeric to centromeric signals was determined. The ratio between the number of signals from DNA probe and centromer probe was first calculated in 100 normal cells. Based on the variation found in the normal cells telomeric/centromeric signal ratio of less than or equal to 0.75 was interpreted as representing loss of chromosome 10q23, ie, allelic loss on chromosome 10q. A telomeric/centromeric signal ratio of greater than or equal to 1.5 was interpreted as DNA gain on 10q23. Ratio values between 0.75 and 1.5 were considered as representing no evidence of allelic loss on chromosome 10q, ie, 10q intact.



**Figure 3.2. Enumeration of signals in FISH analysis.**



### **3.7. Statistical analysis**

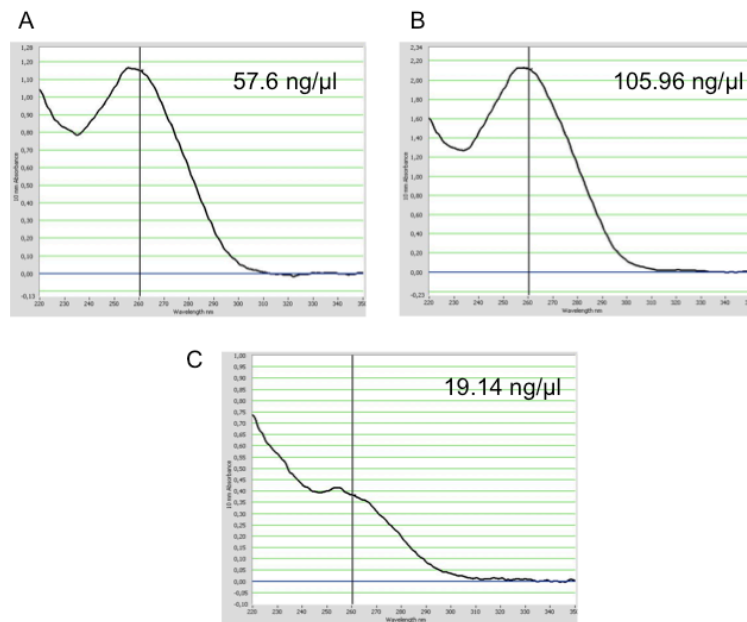
Statistical analyses were performed using the R statistical environment (Version 2.11.0, The R Foundation for Statistical Computing). The relationship between the microsatellite markers and clinical factors was examined by means of the means of the  $\chi^2$  -test and test of independence. Differences between primary tumors, brain metastases in relation to allelic imbalance were calculated with the Fischer's exact test.

## **4. Results**

### **4.1. DNA isolation and quantification**

Breast cancer tissue is very heterogeneous and consists apart from tumor cells out of stromal fibroblasts and leukocytes. The primary tumor DNA of 77 patients was previously isolated so that the obtained samples contained at least 70 % of tumor DNA. DNA from 21 samples brain metastases as well previously isolated from surgical specimen was available for the experiments. The reference DNA from blood leukocytes of 9 patients was isolated in this study. The concentrations of obtained DNA were determined with help of a photospectrometer measuring the absorbance (optical density) of DNA samples. Knowing the elution volume total amount of DNA was calculated for each sample (Table 4.1).

Different methods of DNA isolation can be employed to higher the yield and quality of obtained DNA. The pre-treatment of whole blood samples with erythrocyte lysis buffer permits the preferential lysis of red blood cells what leads to concentration of white blood cells. Additionally there are commercially available kits that allow DNA isolation in a fixed step-by-step process. Due to a scarcity of available patient blood samples we first explored three different protocols of DNA isolation out of whole blood on abundantly available control blood material to determine the most efficient way (see 3.1). The quantity of the isolated DNA was not uniform. Isolation of DNA out of whole blood using an erythrocyte lysis buffer (R&D, Minneapolis, USA) and QiAMP Blood kit yielded a higher DNA quantity with respective DNA concentrations of 57.6 and 105.96 ng/ $\mu$ l in 100  $\mu$ l elution volume. In contrast pre-treatment with own erythrocyte lysis buffer was less efficient with obtained DNA concentration of 19.14 ng/ $\mu$ l in 100  $\mu$ l elution volume (figure 4.1).



**Figure 4.1. DNA yield using different protocols of DNA isolation out of blood leukocytes.**  
 DNA isolation with erythrocyte lysis buffer (R&D) A), with QiAMP Blood kit B), with self made erythrocyte lysis buffer C).

The isolation of the patients blood leukocyte DNA was therefore performed using R&D erythrocyte lysis buffer followed by isolation with QiAMP Blood kit. The DNA yield is described in table 4.1.

**Table 4.1. DNA Concentrations and total contents.**

Sample ID	DNA concentration ng/ul	total DNA ng/ 100 µl elution volume	A260	A280	260/280
BrM-21	<b>33.4</b>	3340	0.668	0.359	1.86
BrM-1	<b>15.7</b>	1570	0.313	0.152	2.07
BrM-6	<b>17.5</b>	1750	0.35	0.175	2
BrM-17	<b>4.9</b>	490	0.097	0.051	1.89
BrM-4	<b>10.3</b>	1030	0.206	0.103	2.01
BrM-5	<b>17.7</b>	1770	0.354	0.176	2.01
BrM-3	<b>18.5</b>	1850	0.37	0.195	1.9
BrM-7	<b>106.0</b>	10600	2.1	1.1	1.9
BrM-2	<b>33.9</b>	3390	0.7	0.3	2

## 4.2. Optimization of microsatellite analysis

An important component of microsatellite analysis is the need for optimization of PCR conditions for every new primer pair. The reason is that the amplification of DNA may be influenced by many factors such as different types/brands of thermocyclers,

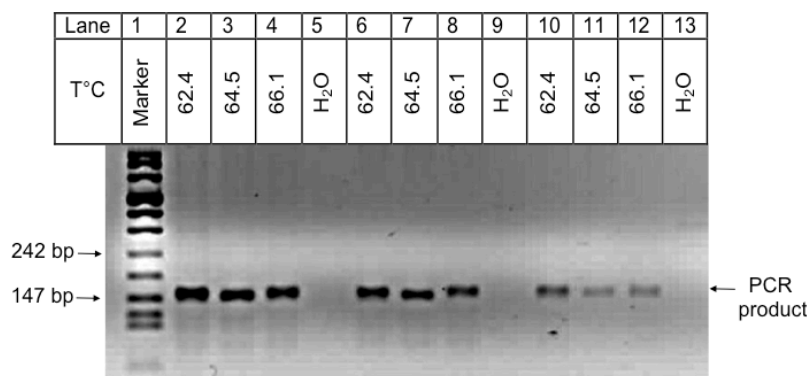
reaction components, or even minor differences in thickness of walls of PCR tubes. In addition, the quality and quantity of template DNA obtained with different DNA extraction protocols may also affect the PCR results.

In this study due to the limited quantity of available patients primary tumor DNA the number of PCR-runs and the amount of used DNA had to be sparing but still generating clean and solid results. That's why it was important to first work out the ideal PCR conditions, including the detection of optimal annealing temperature for each primer pair (see 4.2.1), and subsequently optimize the PCR for a combination of primers (see 4.2.2) to save on valuable patient material and avoid PCR-by-products.

#### 4.2.1. Detection of optimal primer annealing temperature

We used 8 different sets of primers. We determined the optimal annealing temperature of 6 primer pairs (D10S212, D10S219, D10S190, D10S1765, D10S1692, D10S173) using a gradient PCR (see 3.4.3). The annealing temperatures for primers D10S1236 and D10S541 were previously optimized in the laboratory and therefore were not optimized *de novo*.

After the PCR at different annealing temperatures we separated the PCR products on a 1.5 % agarose gel (see 3.4) and determined the optimal annealing temperature.



**Figure 4.2. Gradient PCR with the primer D10S173.**

Lane 5, 9, 13: water control

Lane 1: size marker VIII

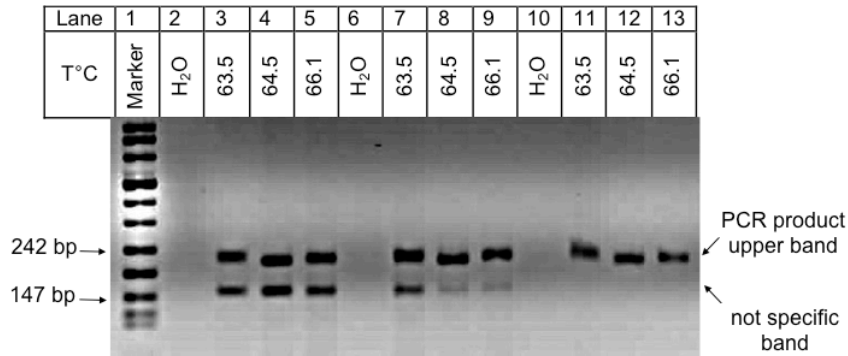
Lane 2-4: amplification of DNA sample 1

Lane 6-8: amplification of DNA sample 2

Lane 10-12: amplification of DNA sample 3

The temperature around 62 °C was considered optimal for the primer D10S173 since there is the strongest amplification of DNA at the expected size of 155 bps. At higher

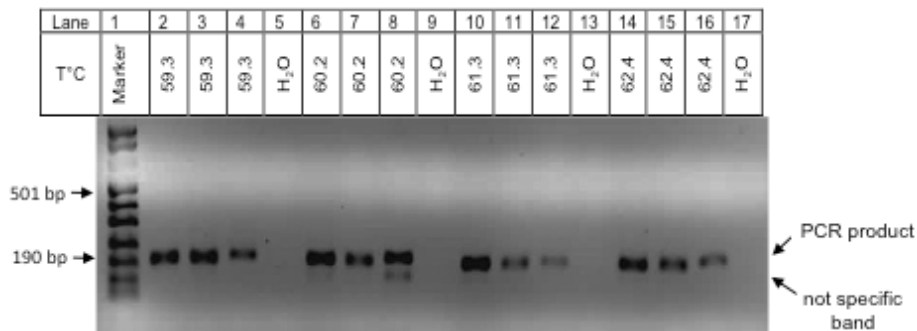
temperatures the PCR product gets less intense. There is a negligible formation of primer dimers and the water control demonstrates absence of contamination.



**Figure 4.3. Gradient PCR with the primer D10S190.**

Lane 5, 9, 13: water control  
 Lane 1: size marker VIII  
 Lane 2-4: amplification of DNA sample 1  
 Lane 6-8: amplification of DNA sample 2  
 Lane 10-12: amplification of DNA sample 3

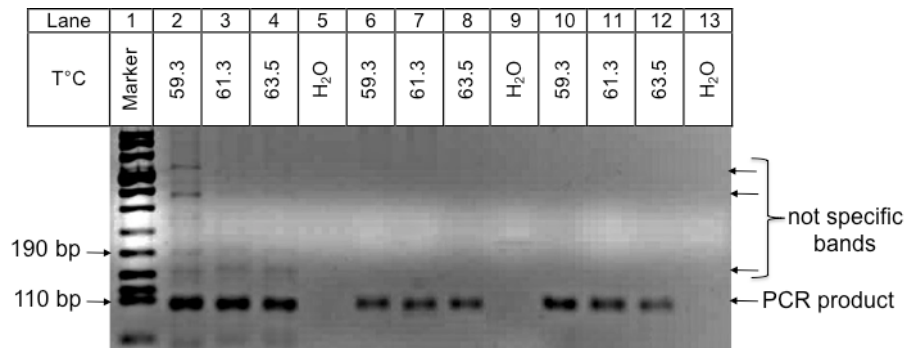
The temperature around 66 °C was selected as optimal for the primer D10S190 since it provided a good DNA amplification along with minimal formation of not specific PCR products. Amplification of DNA samples 1 and 2 at lower annealing temperatures produced pronounced additional bands, which were less intense at 66 °C. The obtained PCR product corresponded to the expected size of 203-219 bps. The control run with a water sample proved no external contamination.



**Figure 4.4. Gradient PCR with the primer D10S212.**

Lane 5, 9, 13, 17: water control  
 Lane 1: size marker VIII  
 Lane 2, 6, 10, 14: amplification of DNA sample 1  
 Lane 3, 7, 11, 15: amplification of DNA sample 2  
 Lane 4, 8, 12, 16: amplification of DNA sample 3

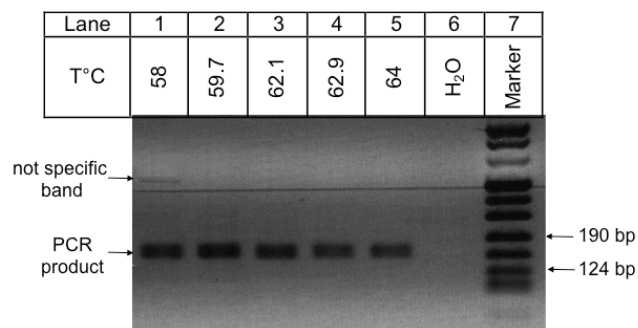
The temperature 60 °C was considered optimal for the primer D10S212 because the PCR product showed a strong amplification and minimal formation of by-products. The PCR product corresponded the expected size of 203-219 bps. The water control showed that the samples were not contaminated with external DNA.



**Figure 4.5. Gradient PCR with the primer D10S219.**

Lane 5, 9, 13: water control  
 Lane 1: size marker VIII  
 Lane 2-4: amplification of DNA sample 1  
 Lane 6-8: amplification of DNA sample 2  
 Lane 10-12: amplification of DNA sample 3

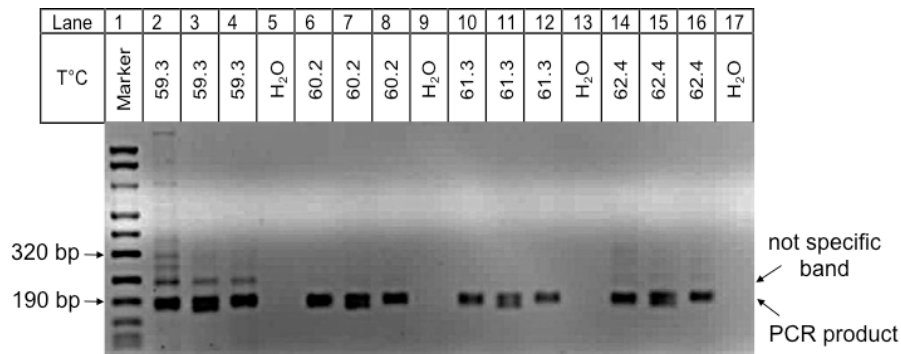
The temperature 61°C was determined as optimal for the marker D10S219 because it shows the strongest amplification of the template DNA but at the same time a lesser formation of PCR-by-products. The PCR size of the PCR product corresponded to the expected size of 83-103 bps. The control PCR with clean water proved no contamination of the samples.



**Figure 4.6. Gradient PCR with the primer D10S1692.**

Lane 6: water control  
 Lane 7: size marker VIII  
 Lane 1-5: amplification of DNA sample 1

The temperature range 62-64 °C was considered optimal for the primer D10S173 since there is a strong amplification of DNA at the expected size of 182-211 bps and a lesser building of PCR by-products. At 58°C there is a detectable non-specific product. The water control demonstrates absence of contamination.



**Figure 4.7. Gradient PCR with the primer D10S1765.**

Lane 5, 9, 13, 17: water control  
 Lane 1: size marker VIII  
 Lane 2, 6, 10, 14: amplification of DNA sample 1  
 Lane 3, 7, 11, 15: amplification of DNA sample 2  
 Lane 4, 8, 12, 16: amplification of DNA sample 3

The temperature of 60 °C was selected as the optimal annealing temperature for the primer D10S1765 because of the strongest DNA amplification at expected size of 164-184bps and minimal PCR by-products formation. A parallel run with water demonstrated absence of PCR samples contamination.

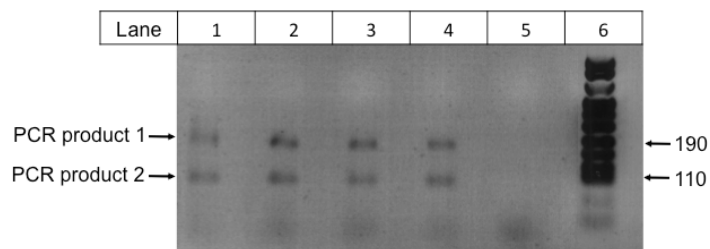
The summary of optimized primer conditions is presented in the table 4.2.

**Table 4.2. Optimal annealing temperatures of primer pairs.**

Primer	Locus	Size of PCR product	Optimal annealing temperature, °C
<b>Microsatellite primers</b>			
<b>D10S219</b>	10q22.3	89-103	61
<b>D10S1765</b>	10q23.2	166-184	60
<b>D10S541</b>	10q23.31	153-169	56
<b>D10S1692</b>	10q24.32	182-211	62-64
<b>D10S173</b>	10q25.2	155	62
<b>D10S1236</b>	10q26.11	121-137	56
<b>D10S190</b>	10q26.11	203-219	66
<b>D10S212</b>	10q26.3	189-201	60

#### 4.2.2. Detection of optimal combination of primers

Due to the scarcity of some DNA samples we used combinations of primers in one PCR run. Based on similarity of optimal annealing temperatures and/or distinct separability of their PCR products we combined markers D10S219 and D10S212, D10S1236 and D10S541, D10S1692 and D10S1236. Other combinations of markers either did not result in well distinguishable PCR products or had many not specific bands, that is why primers D10S1765, D10S190, D10S173 were used separately in analysis.



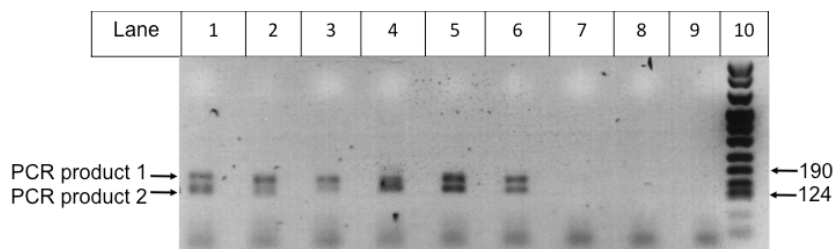
**Figure 4.8. PCR with combination of primers D10S212 and D10S219.**

Lane 1-4: amplification of DNA samples with primer D10S212 (PCR product 1) and D10S219 (PCR product 2)

Lane 5: water control

Lane 6: molecular size marker VIII

PCR at 61 °C showed 2 PCR bands at expected sizes. The amplification with sterile water instead of gDNA was used as control to rule out a contamination of PCR products.



**Figure 4.9. PCR with combination of primers D10S541 and D10S1236.**

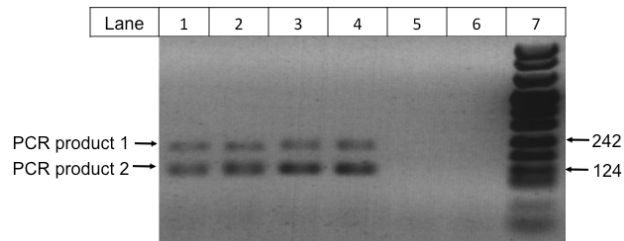
Lane 1-6: amplification of DNA samples with primer D10S541 (PCR product 1) and D10S1236 (PCR product 2)

Lane 7-9: water control

Lane 7: molecular size marker VIII

PCR at 56 °C showed 2 PCR bands at expected sizes of around 160 bps (D10S541) and 130bps (D10S1236). The amplification with sterile water instead of gDNA was used as control to rule out a contamination of PCR products.





**Figure 4.10. PCR with combination of primers D10S1236 and D10S1692.**

Lane 1-4: amplification of DNA samples with primer D10S1692 (PCR product 1) and D10S1236 (PCR product 2)

Lane 5, 6: water control

Lane 7: molecular size marker VIII

The amplification with two primer pairs D10S1236 and D10S1692 shown in this figure produced in electrophoresis two distinct bands of expected size and a clean control samples with sterile water.

### 4.3. Analysis of allelic imbalances on patients DNA

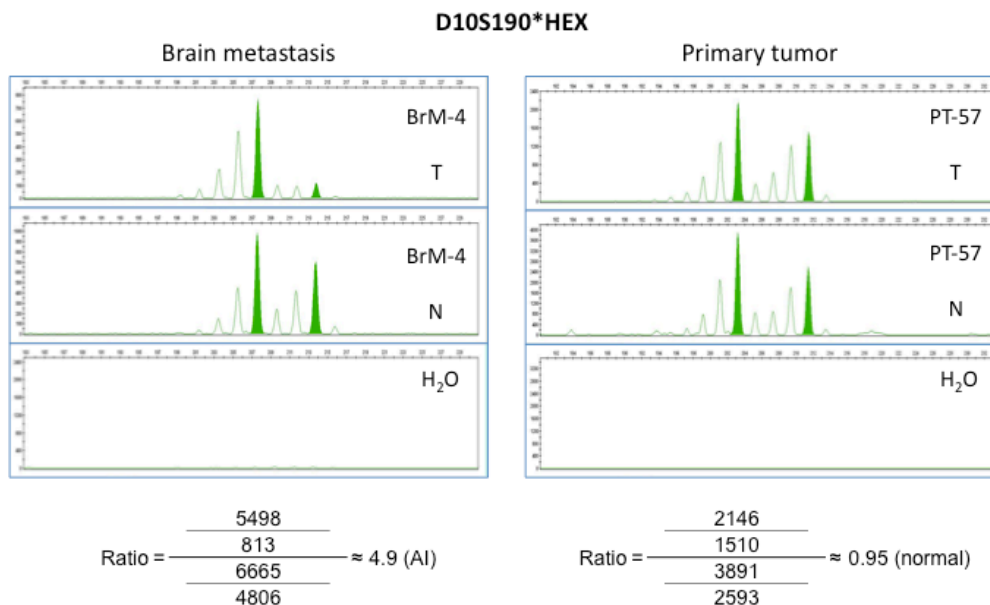
#### 4.3.1. Detection and evaluation of allelic imbalances

After optimizing the PCR conditions and primer combinations we performed analysis of patients DNA for tumor associated alterations, specifically allelic imbalances (AI). For PCR we used genomic DNA from 77 primary tumors, 21 brain metastases and blood leukocytes as templates (see 2.1). As a reference to primary tumor DNA we used both normal breast tissue and leukocyte DNA, whereas leukocyte DNA served as a reference to DNA from brain metastases.

For the PCR the sense-primer (5') was marked with a fluorescence tag at 5' end. The separation of the fluorescence marked PCR products was performed by means of capillary electrophoresis in Genetic Analyser 3010 (ABI Prism). The results were evaluated with help of GeneScan software.

Figure 4.11 demonstrates an example of data assessment for allelic imbalances after the separation of PCR products amplified with primer D10S190. The two highest peaks correspond the two alleles. They are located in the area between 180 and 215 bp what corresponds the expected size of the amplification product with the marker D10S190. The left diagram shows 3 panels, the upper one demonstrating amplification of brain metastasis DNA (T=tumor) of the patient BrM-6, the middle one the leukocyte control DNA (N=normal) of the same patient and the lower panel shows the control PCR run with sterile water. The left diagram presents the amplification of the

primary tumor DNA of the patient PT-57 in the upper panel (T=tumor), normal breast tissue DNA of the same patient as reference in the middle panel (N=normal) and the control PCR run with sterile water in the lower panel. The right peak in the brain metastasis DNA is much lower than the left peak in comparison to the respective peaks of the control DNA whereas in primary tumor DNA the difference between peak sizes is approximately the same like in the respective control DNA. This finding speaks for allelic imbalance in the brain metastasis DNA. To objectify this finding the ratios between the peaks were calculated as described in chapter 3.6.4. The ratios with calculations are presented beneath respective diagrams. As aforementioned the cut-off ratio for AI was determined as  $\geq 2$ . As a result the ratio of 4.9 in brain metastasis DNA related to the control results in AI. In contrary the ratio of 0.95 in primary tumor DNA is determined as normal (without allelic imbalance). The control run with sterile water shows no amplification product because of absent contamination with external DNA template.



**Figure 4.11. PCR with fluorescence tagged primer D10S190\*HEX (green).**

A) The brain metastasis DNA (T) and leukocyte DNA (N) of the patient BrM-4, and B) the primary tumor DNA (T) and normal breast tissue DNA (N) of the patient PT-57 were analyzed in Genetic Analyzer and evaluated with GeneScan software.

Analysis for allelic imbalance was performed for all DNA sample of brain metastases, primary tumors and their respective controls with other seven primer pairs used in this study: D10S219, D10S1765, D10S541, D10S1692, D10S173, D10S1236, D10S190,

and D10S212. The graphical examples of this analysis with calculated peak ratios for each primer pair are presented in the Figure 4.12. The analysis with additional three primer pairs: D10S185, D10S562, and D10S587 was performed upon the later extension of the study and is not presented here. In the left column microsatellite analysis of PCR products of brain metastases (BrM, T=tumor) and reference leukocyte DNA (BrM, N=normal) are presented. The right column contains the microsatellite analysis of primary tumor DNA (PT, T=tumor) and normal breast tissue as control (PT, N=normal)

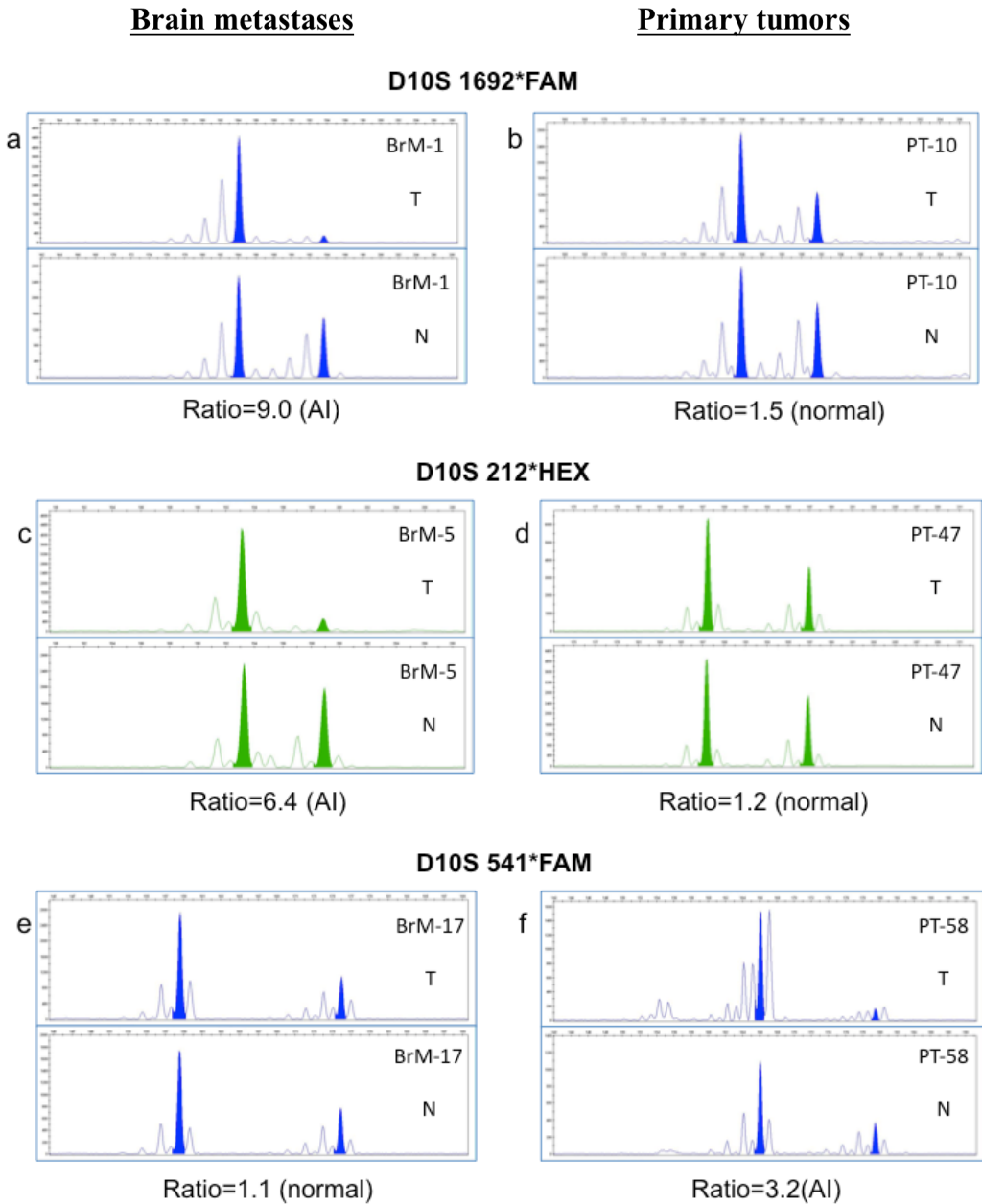
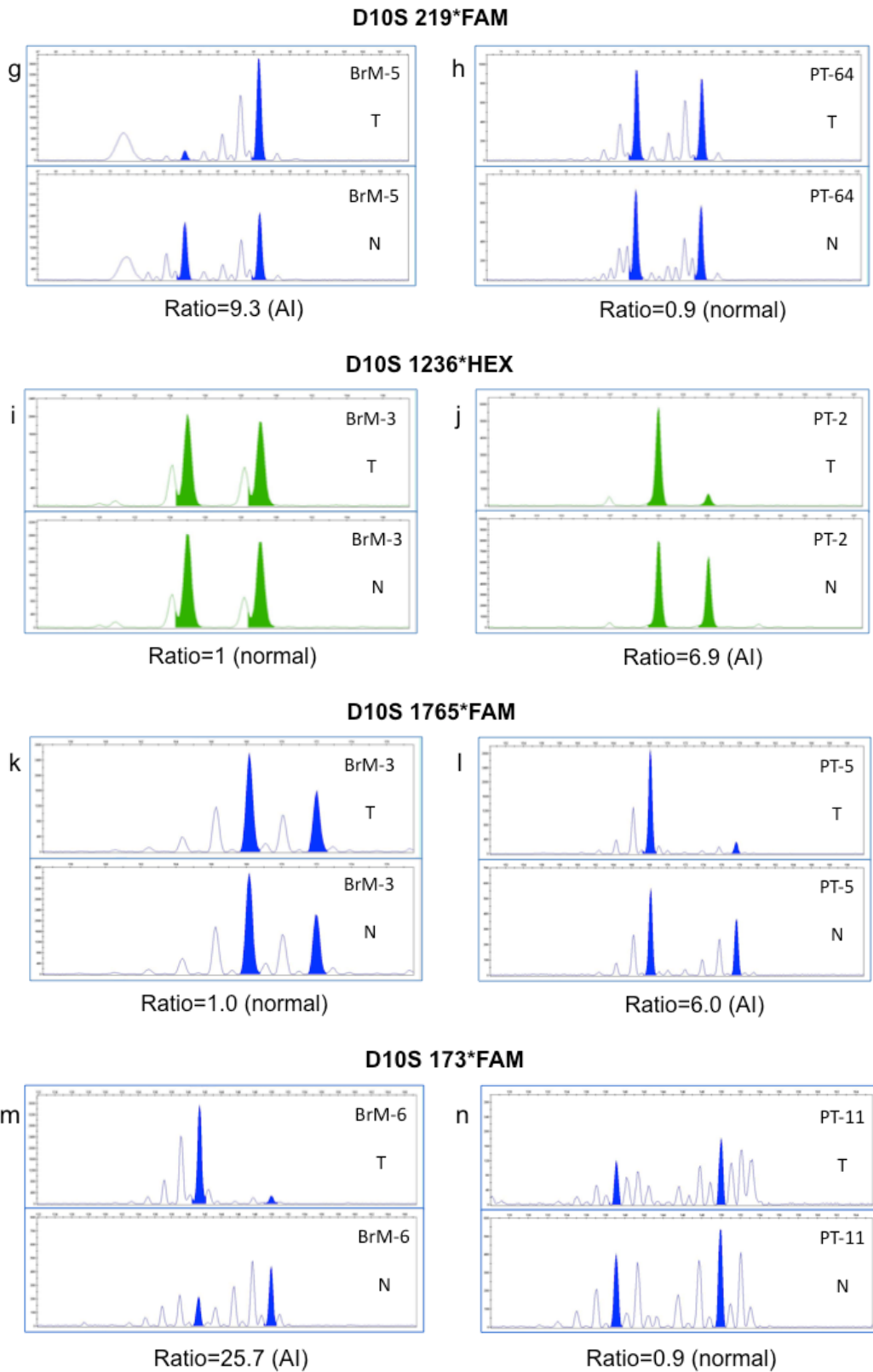


Figure 4.12. Microsatellite analysis of primary tumor and brain metastases DNA with fluorescence tagged primers (continue on page 54).



**Figure 4.12. Microsatellite analysis of primary tumor and brain metastases DNA with fluorescence tagged primers (beginning on page 53).**

#### **4.3.2. Specification of a cut-off ratio in analysis of allelic imbalances**

We defined that AI is present when the fluorescence intensity of one allele of tumor DNA is in comparison to control DNA at least 50 % lesser than the intensity of a reference allele (see 3.6.4). Abnormal results were demonstrated twice with equivalent results. This degree of AI indicates that a substantial proportion of the cells within a sample contains the same DNA abnormality and likely represents the presence of a clonal population. Tumors are heterogenic consisting of normal breast cells and stromal fibroblasts as well as leukocytes next to malignant cells. The detection of the clonal population containing allelic imbalance can fail if the original tumor sample contains a high proportion of non-neoplastic cells who's DNA quantitatively mask the minority of abnormal DNA. Depending on the quality of the tumor DNA sample it is important to define an AI ratio cut-off resulting in best possible identification of allelic imbalances minimizing false negative values. In the literature different cut-off values defining LOH in breast cancer have been used for analysis ranging from 1.25 to 5 whereas most commonly LOH cut-off ratios between 1.5 and 2 were used [152, 153, 154, 155, 156, 157, 158]. The cut-offs were based on the tumor cells isolation and DNA extraction methods, leading to different tumor DNA fraction in the sample, with lower cut-off for e.g. macrodissected tissue samples and the highest cut-offs for laser microdissection techniques [157, 158]. In this study tumor DNA was previously extracted from cells isolated by means of microdissection of tumors cells under light microscope from paraffin embedded tumor slices with result of at least 70% content of tumor cells in each sample. Due to high tumor DNA fraction in the samples we specified the ratio for allelic imbalance as  $\geq 2$ .

#### **4.3.3. Frequencies of allelic imbalances and not informative (NI) samples in primary tumors and brain metastases**

Microsatellite analysis for allelic imbalances was performed on 55 samples of primary tumor DNA and 9 samples of brain metastases DNA. As the study was extended later additional 22 primary tumor and 12 brain metastases DNA samples were analyzed so that we obtained 77 analyzed primary tumor samples and 21 brain metastases samples. The microsatellite analysis was performed to validate the CGH results previously gained in our laboratory and assess the extent of loss on 10q (doctoral thesis of Niclas Detels, Institute of Tumor Biology, UKE, Eppendorf). A 54 MBp region on 10q was screened with eight markers for microsatellite instability. Table 4.3 summarizes the

frequencies of allelic imbalance for each investigated region on chromosome 10q in primary tumors and brain metastases. Screening with the marker D10S219 resulted in allelic imbalances in 50% of brain metastases and 19% of primary tumors samples. Markers D10S541 and D10S1765 covering approximately the same region revealed 47.4 % of allelic imbalances in brain metastases and 22.4 % of allelic imbalances in primary tumors. Screening with microsatellite marker D10S1692 demonstrated an allelic imbalance in 44.4 % of brain metastases and in 20.4 % of primary tumors. With the marker D10S173 allelic imbalance in 36.8 % of brain metastases and in 42.9 % of primary tumor samples could be detected. In the chromosomal region on 10q26 examined with markers D10S190 and D10S1236 allelic imbalances were found in 47.1 % of brain metastases and in 29.1 % of primary tumors. Examination with the microsatellite marker D10S212 showed allelic imbalances in 38.5 % of brain metastases and in 17.8 % of primary tumors.

Not informative cases with two alleles of the same size that could not be differentiated in capillary electrophoresis were present in 46.5 %, 22.4 %, 20.4 %, 42.9 %, 29.1 % and 17.8 % of all DNA samples for the respective markers D10S219, D10S541 and D10S1765, D10S1692, D10S173, D10S190 and D10S1236, and D10S212.

**Table 4.3. Frequences of allelic imbalance in brain metastases (A) and primary tumors (B) (Wikman et al., Breast Cancer Res, 2012).**

*Legend:*

1,2,3,4= matching primary tumors and brain metastases; a: loss from bp:100000000; b: loss from bp: 95000000; c: loss from bp:114000000; d: gain until bp:100000000; e: loss from 80-90000000; n.d.= not determined; NI= non informative; CR 1= core region 1; allelic imbalance around makers D10S173 and D10S190; CR 2= core region 2; allelic imbalance around markers D10S541 and D10S1765.

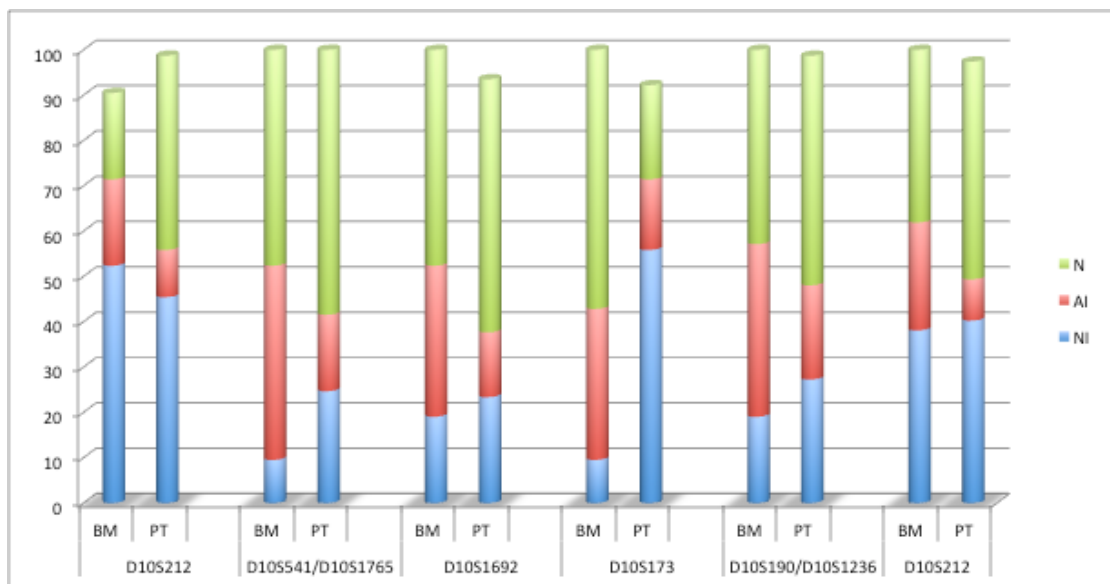
**A**

Patient identifier	Pairs	HER2	Microsatellite analysis at 10q						Relapse	Result AI	Result CGH
			D10S219	D10S541 D10S1765	D10S1692	D10S173	D10S190 D10S1236	D10S212			
<b>BRAIN METASTASES</b>											
BrM-1	1	pos	NI	NI	AI	AI	AI	AI		CR 1 + 2	loss
BrM-2	2	pos	NI				NI	NI		normal	normal
BrM-5	3	neg	AI	AI	AI	AI	AI	NI		CR 1 + 2	loss <sup>a</sup>
BrM-20	4	neg	NI	AI	AI	AI	NI	NI		CR 1 + 2	n. d.
BrM-21		neg	AI	NI	NI	NI	AI	AI		CR 1 + 2	loss
BrM-6		neg	AI	AI	AI	AI	NI	AI		CR 1 + 2	loss
BrM-7		neg	NI	AI	AI	AI	AI	NI		CR 1 + 2	loss
BrM-14		neg		AI	AI	AI	AI	NI		CR 1 + 2	n. d.
BrM-9		neg	NI	AI	AI		AI	AI		CR 1 + 2	n. d.
BrM-4		pos		AI		AI	AI	NI		CR 1 + 2	loss
BrM-18		pos	NI	AI	AI					CR 1	n. d.
BrM-11		n. d.	AI	AI	NI					CR 1	n. d.
BrM-3		neg	n. d.				AI	AI		CR 2	loss
BrM-10		pos	NI					AI		1x AI	n. d.
BrM-16		neg	NI							normal	n. d.
BrM-13		neg								normal	n. d.
BrM-19		neg								normal	n. d.
BrM-8		pos	n. d.					NI		normal	n. d.
BrM-12		pos	NI							normal	n. d.
BrM-15		pos	NI			NI	NI			normal	n. d.
BrM-17		pos	NI		NI			NI		normal	n. d.
% AI			50.0	47.4	44.4	36.8	47.1	38.5			

**B**

Patient identifier	Pairs	HER2	Microsatellite analysis at 10q						Relapse	Result AI	Result CGH
			D10S219	D10S541 D10S1765	D10S1692	D10S173	D10S190 D10S1236	D10S212			
<b>PRIMARY TUMORS</b>											
PT-29	1	neg	NI	NI	AI	AI	AI	AI	brain-rel.	CR 1 + 2	n. d.
PT-27	2	pos	NI		n. d.		NI	NI	brain-rel.	normal	n. d.
PT-30	3	n. d.		AI	AI	AI	AI	NI	brain-rel.	CR 1 + 2	n. d.
PT-39	4	neg	NI	AI	AI	AI	NI	NI	brain-rel.	CR 1 + 2	n. d.
PT-13		pos		NI	AI	AI	NI	AI	chest wall-rel.	CR 1 + 2	loss <sup>b</sup>
PT-67		neg	NI	AI	AI	AI	NI	NI	n.d.	CR 1 + 2	n. d.
PT-47		neg	AI	AI			AI		none	CR 1 + 2	n. d.
PT-8		pos	NI	AI	NI	NI	AI	NI	none	CR 1 + 2	normal
PT-23		pos		NI	NI	NI	AI	NI	none	CR 1 + 2	normal
PT-26		pos	AI	AI	AI	AI	AI	n. d.	none	CR 1 + 2	n. d.
PT-58		pos		AI	NI	NI	AI	NI	none	CR 1 + 2	n. d.
PT-5		n. d.		AI	NI	NI	NI	NI	none	CR 1 + 2	normal
PT-24		neg	NI	NI	AI	AI	AI	AI	none	CR 1 + 2	n. d.
PT-38		neg	AI	NI	NI	AI	n. d.	AI	brain-rel.	CR 1 + 2	n. d.
PT-66		neg	NI	AI			AI	NI	n.d.	CR 1 + 2	n. d.
PT-40		n. d.	AI	AI	AI	AI	AI	NI	brain rel.	CR 1 + 2	n. d.
PT-18		neg	AI	AI	NI	NI			chest wall-rel.	CR 1	loss <sup>c</sup>
PT-36		neg	AI	AI	AI	AI			n.d.	CR 1	gain <sup>d</sup>
PT-55		neg		AI			NI	NI	n.d.	CR 1	n. d.
PT-42		neg				AI	NI	NI	mult.-rel.	CR 2	n. d.
PT-2		neg	NI	NI	AI	NI	AI	AI	n.d.	CR 2	n. d.
PT-15		neg				NI	AI		none	CR 2	normal
PT-1		neg	NI		NI	NI	AI		none	CR 2	n. d.
PT-22		neg	NI			NI	AI	AI	none	CR 2	loss <sup>e</sup>
PT-34		pos			AI	n. d.	AI	AI	none	CR 2	n. d.
PT-41		neg	NI		n. d.	AI	AI	NI	bone-rel.	CR 2	n. d.
PT-54		neg	NI			AI			none	1x AI	n. d.
PT-49		neg	AI			NI	NI	NI	mult.-rel.	1x AI	n. d.
PT-62		pos	NI			AI			n.d.	1x AI	n. d.
PT-21		neg	AI			NI			none	1x AI	normal
PT-59		neg				NI			bone-rel.	normal	n. d.
PT-73		neg	NI		n. d.	n. d.			brain-rel.	normal	n. d.
PT-56		pos	NI			NI	NI		n.d.	normal	n. d.
PT-45		neg		NI		NI		NI	none	normal	n. d.
PT-48		neg	NI	NI		NI	NI	NI	none	normal	n. d.
PT-50		neg	NI		NI	NI		NI	none	normal	n. d.
PT-51		neg	NI		NI	NI			none	normal	n. d.
PT-53		neg	NI	NI		NI			visceral rel.	normal	n. d.
PT-76		neg	NI			NI		NI	bone-rel.	normal	n. d.
PT-74		neg			n. d.	NI	NI	n. d.	bone-rel.	normal	n. d.
PT-65		neg	NI	NI		NI		NI	bone-rel.	normal	n. d.
PT-17		pos		NI		NI		NI	breast rel.	normal	normal
PT-60		neg				NI			mult.-rel.	normal	n. d.
PT-61		neg	NI			NI			mult.-rel.	normal	n. d.
PT-14		neg				NI			none	normal	normal
PT-3		neg							none	normal	n. d.
PT-4		neg		NI	NI	NI			none	normal	normal
PT-11		neg		NI					none	normal	n. d.
PT-16		neg	NI	NI			NI		none	normal	normal
PT-19		neg	NI		NI	NI			none	normal	normal
PT-20		neg		NI			NI	NI	none	normal	normal
PT-43		neg		NI			NI		none	normal	n. d.
PT-37		neg		NI				NI	none	normal	n. d.
PT-63		neg	NI		NI	n. d.			none	normal	n. d.
PT-6		neg	NI			NI			none	normal	normal
PT-35		neg		NI		NI	NI		none	normal	n. d.
PT-7		pos				NI	NI		none	normal	normal
PT-9		pos			NI	NI	NI		none	normal	n. d.
PT-12		pos			NI	NI			none	normal	n. d.
PT-32		pos	NI						none	normal	n. d.
PT-33		pos	NI					NI	none	normal	n. d.
PT-57		pos	NI					NI	none	normal	n. d.
PT-68		pos	NI	NI			NI		none	normal	n. d.
PT-69		pos	NI		NI	NI			none	normal	n. d.
PT-46		neg			NI	NI			none	normal	n. d.
PT-25		neg				NI		NI	none	normal	n. d.
PT-10		pos				NI	NI		visceral rel.	normal	gain
PT-31		pos					NI		visceral rel.	normal	n. d.
PT-71		neg	NI		NI			NI	bone-rel.	normal	n. d.
PT-77		neg			NI	n. d.	NI	NI	bone-rel.	normal	n. d.
PT-70		neg			n. d.	n. d.		NI	brain-rel.	normal	n. d.
PT-72		neg					NI	NI	mult.-rel.	normal	n. d.
PT-75		neg	NI			n. d.	NI		none	normal	n. d.
PT-28		n. d.						NI	brain-rel.	normal	n. d.
PT-44		neg					NI	NI	n.d.	normal	n. d.
PT-64		neg						NI	n.d.	normal	n. d.
PT-52		neg	NI						n.d.	normal	n. d.
<b>% AI PT</b>			<b>19.0</b>	<b>22.4</b>	<b>20.4</b>	<b>42.9</b>	<b>29.1</b>	<b>17.8</b>			
<b>% NI all</b>			<b>46.5</b>	<b>20.4</b>	<b>21.4</b>	<b>50.0</b>	<b>26.2</b>	<b>39.8</b>			

The prevalence of marker informativity is presented in Table 4.3 and graphically depicted in the figure 4.13 as procentually related to number of cases with AIs and normal findings. The fraction of non-informative cases in brain metastases and primary tumors was approximately the same for all markers apart from D10S173. In brain metastases the frequency of non-informative cases at D10S173 was lower than in primary tumors. The discrepancy in informativity here can be attributed to the relatively small number of brain metastases samples in comparison to the number of primary tumors. The detected frequencies of non-informative cases were similar to those found in literature (Table 2.3, <http://www.ncbi.nlm.nih.gov/mapview/>).



**Figure 4.13. Informativity of genetic markers.** N=normal, AI=allelic imbalance, NI=not informative, BM=brain metastases, PT=primary tumors.

#### 4.3.4. Defining the core regions of allelic imbalances on chromosome 10q

The frequency of allelic imbalances for individual markers varied between 18-43% in the primary tumors and 37-50 % in brain metastases. The AI did not cover the entire examined region but accumulated around two core regions (figure 4.14). The first core region (CR1) was found around *PTEN* locus (markers D10S541 and D10S 1765) and the second region (CR2) was detected around the markers D10S173 and D10S190. The AI in CR1 were observed in 52.4% of brain metastases and in 23.4% of primary tumors. In the CR2 47.6% of brain metastases and only 28.6% of primary tumors contained AI. In both regions concomitantly AI were found in 42.8% of brain metastases and in 19.5% of primary tumors.



	D10S219	D10S1765 + D10S541	D10S185	D10S1692	D10S173	D10S562	D10S1236+ D10S190	D10S587	D10S212	RESULT
<b>PTEN (CR 1)</b>										
PT-13										CR 1
PT-58										CR 1
PT-30										CR 1
BrM-14										CR 1
PT-23										CR 1
BrM-11										CR 1
PT-47										CR 1
PT-66										CR 1
PT-8										CR 1
BrM-4										CR 1
OM-4										CR 1
PT-5										CR 1
PT-55										CR 1
<b>REGION 2 (CR 2)</b>										
BrM-3										CR 2
PT-15										CR 2
BrM-9										CR 2
PT-67										CR 2
PT-1										CR 2
PT-23										CR 2
PT-47										CR 2

non-informative or AI  
 normal

**Figure 4.14. Accumulation of allelic imbalances in two core regions**  
(Wikman et al., Breast Ca Res 2012).

#### 4.3.5. Allelic imbalances in primary tumors in comparison to brain metastases

Based on this distribution we compared the frequencies of allelic imbalances between primary tumors and brain metastases in the two core regions as well as at any locus. The majority of the brain metastases (62%) demonstrated AI at any locus on 10q. In contrast to this only 38% of primary tumors carried an AI on examined region (table 4.4). We detected significant differences in frequency of AI between the brain metastases and primary tumors without relapse ( $p=0.05$ ). Among brain tumor samples 62 % carried an AI whereas 33% of primary tumors without relapse exhibited an AI at any locus. Especially, AIs around the *PTEN* locus were significantly more often observed in brain metastases (52%) than in primary tumors from patients without later relapse (18%) ( $p=0.003$ ) or relapse to other organs (12%) ( $p=0.006$ ).

Notably, primary tumors without a subsequent history of relapse (33%) and primary tumors with metastases to the sites other than brain (29%) showed a lower frequency of allelic imbalances than primary tumors from patients who developed brain metastases in the later course of their disease (50%). We observe this finding as well for each core region. Especially around *PTEN* locus primary tumors without relapse

(18%) and primary tumors with later relapse to organs other than brain (12%) showed much less frequent AIs than primary tumors with later brain metastases (50%).

The frequency of AI at any locus was not significantly different between brain metastases (62%) and primary tumors with a later brain relapse (50%). Similarly, the difference in frequency of AI between brain metastases and primary tumors with a later brain relapse was not observed for each core region.

**Table 4.4. Frequencies and *P* values for AI at chromosome 10q in primary breast tumors and metastases.**

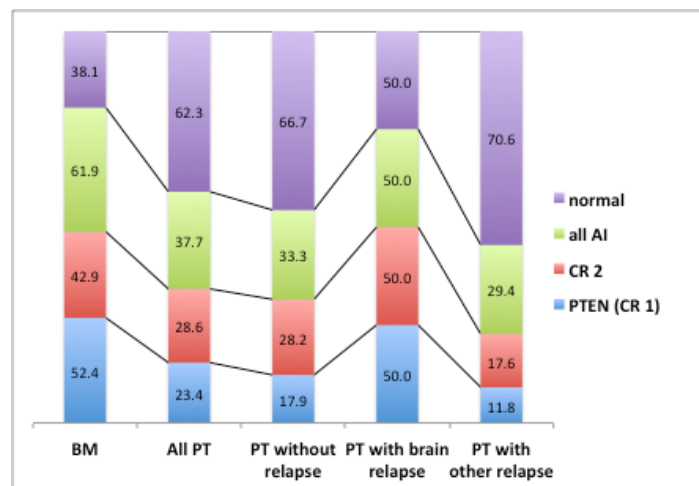
	Brain metastases (n=21)		All primary tumors (n=77)			Primary tumors without relapse (n=39)			Primary tumors with brain relapse (n=10)			Primary tumors with other relapse (n=17)		
	n	%	n	%	p-value <sup>a</sup>	n	%	p-value <sup>a</sup>	n	%	p-value <sup>a</sup>	n	%	p-value <sup>a</sup>
PTEN (CR 1)	12	52.4	18	23.4	<b>0.006</b>	7	17.9	<b>0.003</b>	5	50.0	<b>1.000</b>	2	11.8	<b>0.006</b>
CR 2	9	42.9	22	28.6	0.289	11	28.2	0.268	5	50.0	1.000	3	17.6	0.161
all AI	13	61.9	29	37.7	0.080	13	33.3	<b>0.055</b>	5	50.0	0.701	5	29.4	0.058
normal	8	38.1	48	62.3	-	26	66.7	-	5	50.0	-	12	70.6	-

a= p-values calculated with the Fischer's exact test ( brain metastases versus other groups).

PTEN (CR1)= allelic imbalance around markers D10S1765 and D10S541

CR 2= allelic imbalance around markers D10S190 and D10S1236

Figure 4.15 presents the results of analysis on frequencies of AI in brain metastases and primary tumors. From the figure it can be seen that brain metastases and primary tumors with brain relapse have a similar AI profile whereas primary tumors without relapse or with relapse to other organs exhibit a much lower frequency of AI than brain metastases.



**Figure 4.15. Frequencies of AI at chromosome 10q in primary breast tumors and metastases at detected core regions.**

Additionally, we compared the frequencies of allelic imbalances between primary tumors and brain metastases for each marker separately (Table 4.5). In line with the results obtained for the core regions it could be shown that AI on the locus covered by markers D10S541 and D10S1765 were significantly more frequent in brain metastases (43%) than in primary tumors without relapse (13%) ( $p=0.013$ ) or relapse to distant sites other than brain (6%) ( $p=0.012$ ). For markers D10S1692 and D10S173 we detected as well a significantly more frequent occurrence of AI in brain metastases (33% for each marker) than in primary tumors without relapse (8% for each marker) ( $p=0.025$  for each marker). On the locus covered by markers D10S190 and D10S1236 a significantly higher frequency of AI was detected in metastases to the brain (38%) compared to primary tumors with metastases to other distant sites ( $p=0.028$ ). For all markers no difference in frequency of AI between brain metastases and primary tumors with later brain relapse could be detected.

**Table 4.5. Frequencies and *P* values for AI at chromosome 10q in primary breast tumors and metastases.**

	Brain metastase (n=21)		All primary tumors (n=77)			Primary tumors without relapse (n=39)			Primary tumors with brain relapse (n=10)			Primary tumors with other relapse (n=17)		
	n	%	n	%	p-value <sup>a</sup>	n	%	p-value <sup>a</sup>	n	%	p-value <sup>a</sup>	n	%	p-value <sup>a</sup>
D10S219	4	19.0	8	10.4	0.280	3	7.7	0.226	2	20.0	1.000	2	11.8	0.672
D10S541/D10S1765	9	42.9	13	16.9	<b>0.018</b>	5	12.8	<b>0.013</b>	3	30.0	0.697	1	5.9	<b>0.012</b>
D10S1692	7	33.3	11	14.3	0.059	3	7.7	<b>0.025</b>	4	40.0	1.000	1	5.9	0.053
D10S173	7	33.3	12	15.6	0.115	3	7.7	<b>0.025</b>	5	50.0	0.447	3	17.6	0.460
D10S190/D10S1236	8	38.1	16	20.8	0.15	10	25.6	0.381	3	30.0	0.712	1	5.9	<b>0.026</b>
D10S212	5	23.8	7	9.1	0.125	3	7.7	0.114	2	20.0	1.000	1	5.9	0.197
normal	8	38.1	48	62.3	-	26	66.7	-	5	50.0	-	12	70.6	-

a= p-values calculated with the Fischer's exact test (brain metastases versus other groups).

#### 4.3.5.1. Matched samples of primary tumors and brain metastases

In four cases matched primary tumor and brain metastases samples were available for microsatellite analysis. Three cases showed identical aberration patterns at 10q, one normal and two AI, whereas in the third case the AI was larger, i.e. in the metastases the marker D10S219 was also affected by AI.

#### 4.3.6. Correlation of occurrence of allelic imbalances on chromosome 10q in primary tumors with clinical and pathological characteristics

We analyzed whether a specific clinical or pathological feature of a primary tumor would correlate with a higher frequency of AI. The clinical and pathological characteristics analyzed are presented in the table 4.6. None of the explored

characteristics was significantly associated with AI. As shown in the table 4.4 only presence of brain metastases correlated significantly with AI on 10q.

**Table 4.6. 10q allelic imbalances and association to clinical factors in primary tumors.**

	Normal		PTEN (CR 1)			Region 2 (CR 2)			all AI		
	n	%	n	%	p-value	n	%	p-value	n	%	p-value
<b>All</b>	<b>40</b>	<b>62.5</b>	<b>16</b>	<b>23.4</b>		<b>22</b>	<b>28.6</b>		<b>29</b>	<b>37.7</b>	
<b>Histology</b>					0.966			0.523			0.549
Ductal	32	74.4	11	73.3		27	87.1		19	73.1	
Lobular	5	11.6	2	13.3		2	6.5		5	19.2	
others	6	14.0	2	13.3		2	6.5		2	7.7	
<b>Age</b>					1.000			0.805			1.000
<mean 57.1	24	52.2	9	50.0		12	54.5		15	51.7	
>mean 57.1	22	47.8	9	50.0		10	45.5		14	48.3	
<b>Tumor stage</b>					0.739			0.473			0.974
pT1	15	34.9	5	35.7		7	36.8		9	36.0	
pT2	22	51.2	8	57.1		11	57.9		13	52.0	
pT3+4	6	14.0	1	7.1		1	5.3		3	12.0	
<b>Lymph node status</b>					0.088			0.229			0.583
pN0	28	66.7	12	92.3		15	83.3		18	75.0	
pN1	14	33.3	1	7.7		3	16.7		6	25.0	
<b>Distant Metastasis</b>					1.000			0.098			0.470
M0	28	66.7	11	91.7		17	100.0		21	91.3	
M1	14	33.3	1	8.3		0	0.0		2	8.7	
<b>Grade</b>					0.582			0.308			0.182
GI	3	7.3	0	0.0		0	0.0		0	0.0	
GII	18	43.9	5	35.7		6	31.6		8	32.0	
GIII	20	48.8	9	64.3		13	68.4		17	68.0	
<b>Bone Marrow status</b>					0.463			0.523			0.540
neg	19	59.4	5	50.0		11	73.3		13	72.2	
pos	13	40.6	5	50.0		4	26.7		5	27.8	
<b>Tumor size</b>					0.764			0.587			0.612
<median 2.5	19	46.3	7	53.8		10	55.6		13	54.2	
>median 2.5	22	53.7	6	46.2		8	44.4		11	45.8	
<b>Menopausal status</b>					0.535			0.600			0.659
perimenopausal	1	2.6	1	7.7		1	5.6		1	4.2	
praemenopausal	8	20.5	3	23.1		6	33.3		8	33.3	
postmenopausal	30	76.9	9	69.2		11	61.1		15	62.5	
<b>Hormone receptor</b>					0.105			0.264			0.304
neg	9	20.9	0	0.0		1	5.6		2	8.3	
pos	34	79.1	13	100.0		17	94.4		22	91.7	
<b>Her-2</b>					0.326			0.365			0.579
neg	25	61.0	5	45.5		7	46.7		12	57.1	
pos	16	39.0	6	54.5		8	53.3		9	42.9	
<b>Ki-67</b>					0.747			1.000			0.576
<20%	18	43.9	6	50.0		8	44.4		9	39.1	
>20%	23	56.1	6	50.0		10	55.6		14	60.9	
<b>Relapse</b>					0.550			1.000			1.000
no	26	59.1	7	50.0		11	57.9		13	56.5	
yes	18	40.9	7	50.0		8	42.1		10	43.5	
<b>Course of disease</b>					0.707			0.486			0.517
alive	32	82.1	9	75.0		12	70.6		16	72.7	
died	7	17.9	3	25.0		5	29.4		6	27.3	

#### 4.4. FISH analysis for detection of copy number changes in primary tumor and brain metastases DNA on chromosome 10q

##### 4.4.1. Preparation of DNA probes for FISH analysis

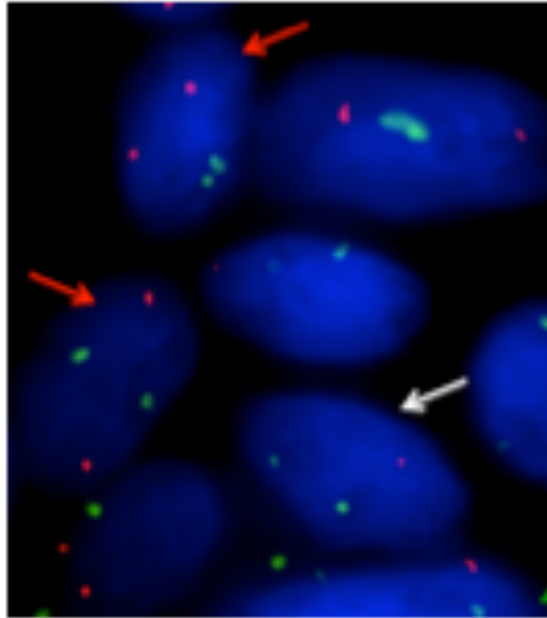
Previously performed CGH screening and analysis of 10q for AI lead to identification of AIs in two core regions on chromosome 10q (doctoral thesis of Niclas Detels,

Institute of Tumor Biology, UKE, Hamburg; figure 4.14). To verify the frequency of occurrence of genetic alterations on 10q we performed a FISH analysis on a tissue microarray containing primary tumor tissue of a distinct cohort of patients (2.1.3). The specific DNA-probe used for FISH-analysis was previously amplified and isolated from a BAC-clone and marked with a fluorescence-tagged dUTPs by means of random priming. The specificity of the probes was examined on leukocyte metaphase chromosomes of a healthy test person. As prerequisite for a specific hybridization the probe had to penetrate the cell without changing its morphology. Clearly discernible signals and low background noise of the image served as additional criteria to define high hybridization specificity. The binding site of the BAC-probe RP11-318C4 was located at 25.3-26.1 and corresponded the core region CR2 detected in the study on AI (4.3.5).

As a reference probe a commercial centromeric probe for the chromosome 10q was used. A centromeric probe binds to repetitive sequences in or around the centromere of a specific chromosome. Depending on naturally occurring polymorphisms in these regions the specificity of the probe binding can be compromised. The centromeric probe specifically at 10q was selected for several reasons. First, if BAC-probe and centromeric probe both bind at chromosome 10 then in case of chromosomal numeric aberrations on chromosome 10 deletions or gains of chromosomal material would falsely not be detected. Second, since copy number variations around cen10 are rare, centromeric probe both binding at chromosome 10 would give more reliable information on polyploidy status of the tumor.

For the evaluation of FISH a ratio between the signals of DNA-probe and centromeric probe was determined. Based on comparison to the ratio in normal cells the ratio  $\geq 1.5$  was determined as a DNA gain and the ratio of  $\leq 0.75$  as a DNA loss.

Figure 4.16 shows an example of FISH analysis with loss of DNA copy number. The cell pointed with the white arrow contains two green and one orange signal, so the ratio of green to orange signals  $2:1=0.5$  defines here the loss of copy number. The cells marked with red arrows possess equal number of green and orange signals so that there is neither loss nor gain of genetic material on investigated region on 10q here. (Due to a breakage of the original TMA the picture presented in the Figure 4.16 was kindly provided by Dr. Wrage, Institute of Tumor Biology, University Hospital Eppendorf, Hamburg).



**Figure 4.16. Detection DNA copy number loss by means of FISH analysis.**

#### **4.4.2. Detection of chromosomal alterations on 10q with FISH analysis**

From all the tissue samples on the TMA it was possible to evaluate 55 cases. All the samples with not clearly discernible results were excluded from the evaluation. Specifically, we excluded the samples having no intact cells or having only overlapping cells as well as samples containing cells with fused signals or no centromeric signals. The majority of cases (92.7%) demonstrated no change in copy number on 10q. In 4 cases (7.3 %) we detected DNA copy number changes (losses). There were no gains found in the evaluation of the 55 samples. Table 4.7 summarizes the results of FISH evaluation analysis and includes the number of signals detected as well as the ratio calculation.

In the analysis of AI we detected a much higher occurrence of DNA copy number changes than in FISH analysis. The range of AI was often very small (sometimes covering only one microsatellite marker), what explains better detection rates in LOH analysis. Nonetheless the results of FISH analysis are compatible with the LOH analysis since they show a low rate of genetic imbalances in primary tumors.

Further, 29.1% of all samples were found to be polyploid (tri- or tetraploid) what reflects a high level of ploidity, a well known characteristic of cancer cells. In constast there were only a few aneuploid samples (5.5%). Among the four samples with deletions on 10q three were tri- or tetraploid (polyploid) and only one sample was diploid, what speaks for a higher genomic instability of these tumors.

**Table 4.7. Evaluation of FISH analysis.**

Nr.	Number of signals in RP11-318C4	Number of signals Cen 10	Number of cells per sample	FISH Ratio	RESULT	Ploidy ratio	Ploidy description
1	26	29	12	0.89	NORM	2.4	diploid
2	70	59	32	1.18	NORM	1.8	diploid
3	43	43	24	1.00	NORM	1.8	diploid
4	59	59	32	1.00	NORM	1.8	diploid
5	25	25	19	1.00	NORM	1.3	monoploid
6	21	38	14	0.55	DEL	2.7	triploid
7	85	88	50	0.97	NORM	1.8	diploid
8	57	69	28	0.81	NORM	2.5	diploid
9	17	13	6	1.29	NORM	2.2	diploid
10	24	24	17	1.00	NORM	1.4	monoploid
11	44	39	15	1.13	NORM	2.6	triploid
12	47	59	26	0.79	NORM	2.3	diploid
13	133	121	50	1.09	NORM	2.4	diploid
14	102	139	46	0.46	DEL	3.0	triploid
15	98	113	50	0.87	NORM	2.3	diploid
16	45	57	24	0.79	NORM	2.4	diploid
17	147	165	38	0.89	NORM	4.3	tetraploid
18	29	29	11	1.00	NORM	2.6	triploid
19	70	76	28	0.90	NORM	2.7	triploid
20	61	120	27	0.5	DEL	4.4	tetraploid
21	65	77	29	0.84	NORM	2.7	triploid
22	59	57	26	1.03	NORM	2.2	diploid
23	59	62	25	0.95	NORM	2.5	diploid
24	51	53	24	0.95	NORM	2.2	diploid
25	86	95	41	0.90	NORM	2.3	diploid
26	140	176	50	0.79	NORM	3.5	triploid
27	64	60	28	1.06	NORM	2.1	diploid
28	193	165	48	1.15	NORM	3.4	triploid
29	90	90	50	1.00	NORM	1.8	diploid
30	104	99	50	1.10	NORM	2.0	diploid
31	86	84	50	1.02	NORM	1.7	diploid
32	99	99	38	1.00	NORM	2.6	triploid
33	97	88	27	1.14	NORM	3.3	triploid
34	70	75	35	0.90	NORM	2.1	diploid
35	55	52	27	1.05	NORM	1.9	diploid
36	101	98	38	1.04	NORM	2.6	triploid
37	51	47	25	1.09	NORM	1.9	diploid
38	54	51	26	1.06	NORM	2.0	diploid
39	56	52	25	1.08	NORM	2.1	diploid
40	52	58	35	0.90	NORM	1.7	diploid
41	61	60	24	1.02	NORM	2.5	diploid
42	80	87	38	0.92	NORM	2.3	diploid
43	41	39	23	1.05	NORM	1.7	diploid
44	37	33	19	1.12	NORM	1.7	diploid
45	112	107	30	1.04	NORM	3.6	triploid
46	77	82	25	0.94	NORM	3.3	triploid
47	51	46	24	1.11	NORM	1.9	diploid
48	49	46	45	1.07	NORM	1.0	monoploid
49	109	105	35	1.04	NORM	3.0	triploid
50	75	78	38	0.96	NORM	2.1	diploid
51	62	65	31	0.95	NORM	2.1	diploid
52	179	135	66	1.32	NORM	2.0	diploid
53	36	45	19	0.80	NORM	2.4	diploid
54	31	46	27	0.68	DEL	1.7	diploid
55	24	29	17	0.82	NORM	1.7	diploid

#### **4.4.3. Correlation of FISH results with clinical and pathological parameters**

In the FISH analysis 4 cases with DNA copy number losses were detected. The clinical and pathological characteristics of the samples were analyzed and summarized in the Table 4.8. It was found that all of the four tumors were smaller than 2 cm and belonged to the pT1 tumor staging category. Three of four patients had no lymph node involvement, no bone marrow dissemination and had a low proliferation rate (Ki-67) as well as were moderately well differentiated. None of the patient presented with distant metastases in the time of diagnosis and only one patient developed a relapse. In all tumors hormone receptor overexpression was detected. In summary all 4 had features related to a good prognosis, like hormone receptor positivity, small size, low proliferation rate and absence of distant metastases. If compared with the whole population of examined 55 tumor samples it can be concluded that the majority of the samples had similar features of good prognosis like the four tumors with deletions on 10q in FISH. Namely, T1 or T2 stage was attributed to more than 90% of tumor samples, almost 60% were lymph node negative, 96% had no distant metastases at time of diagnosis, in 60% of case positive hormone receptors were detected and 72% had a low or moderate proliferation rate (Ki-67), the relapse in the course of the disease occurred in 16% of patients. It can be concluded that the four tumors with deletion on 10q did not differ in their clinical and pathological features from other samples examined in FISH analysis.



**Table 4.7. Correlation of losses on 10q in FISH analysis with clinical and pathological characteristics of the tumors.**

<b>PRIMARY TUMORS</b>	<b>n</b>	<b>%</b>
<b>All</b>	4	100.0
<b>Histology</b>		
Inv. Ductal	1	25.0
Inv. Lobular	1	25.0
others	2	50.0
<b>Type</b>		
ER+/PR+/HER-	0	
ER-/PR-/HER-	2	50.0
HER2+	2	50.0
n.a.	1	25.0
<b>Age</b>		
mean	62	
<b>Tumor stage</b>		
pT1	4	100.0
pT2	0	0.0
pT3+pT4	0	0.0
<b>Lymph node status</b>		
pN neg.	3	75.0
pN pos.	1	25.0
<b>Distant metastasis</b>		
M0	4	100.0
M1	0	0.0
<b>Grade</b>		
GI	0	0.0
GII	3	75.0
GIII	1	25.0
<b>Bone marrow status</b>		
neg	3	75.0
pos	1	25.0
<b>Tumor size</b>		
<=2.0cm	4	100.0
>2.0cm	0	0.0
<b>Menopausal status</b>		
perimenopausal	0	0.0
praemenopausal	0	0.0
postmenopausal	4	100.0
<b>Hormone receptor</b>		
neg	0	0.0
pos	4	100.0
<b>HER2</b>		
neg	1	25.0
pos	2	50.0
n.a.	1	25.0
<b>Ki-67</b>		
<=20%	3	75.0
>20%	1	25.0
<b>Relapse</b>		
no	3	75.0
brain	0	0.0
other site	1	25.0
<b>Course of disease</b>		
alive	4	100.0
died	0	0.0

## **5. Discussion**

Breast carcinoma is the most common malignancy among the women in the Western countries. The detection techniques and management of this disease have improved over the past years and the relative 5-year survival is around 85% [1]. Metastatic spread is the main reason of death in patients with breast carcinoma and dramatically reduces their survival rates [17, 99]. CNS metastases are a frequent complication in breast carcinoma and a particularly ominous one, due to a bad surgical accessibility, multifocal growth and blood brain barrier, which hinders penetration of anti-cancer drugs to the site of the tumor. Brain metastases occur in a range of solid malignancies with a notably higher incidence in certain cancer types like small cell lung cancer (40%), breast cancer (15-20%) and much lower frequency in other types like prostate cancer (1-5%), and gastrointestinal cancers (<4%) [159, 160]. The factors determining brain tropism of metastases of certain tumors are not well understood. Allegedly, brain metastases possess features allowing them to penetrate restrictive blood brain barrier, escape the selection pressure and meet the survival requirements in the CNS microenvironment. Currently there are no specific detection markers to identify patients with high risk for brain metastases in breast carcinoma and the satellites in the brain are detected only after they settled down and started to grow in brain tissue. In this study our goal was to identify molecular markers related to metastatic spread of breast carcinoma to CNS. By means of analysis of tumor DNA for microsatellite associated alterations we compared the frequency of allelic imbalances in brain metastases and primary tumors on chromosome 10q and identified regions associated with CNS-metastatic phenotype. Apart from this, the results of this study promote a better understanding of breast cancer and biology of metastatic dissemination.

### **5.1. Methodological aspects**

The possibility to use high polymorphism microsatellite markers for detection of allelic imbalances with PCR and subsequent allele discrimination using fluorescent markers has been widely used as a molecular tool in cancer studies [161]. Allelic imbalances in tumor tissue are indicative for a possible deletion of that chromosomal region. Deletions of specific chromosomal regions have been described in almost all types of cancer [162, 163, 164, 165, 166, 167, 168]. These deletions often involve allelic imbalances of either paternal or maternal allele [169]. The high frequency of AI

in certain regions has been detected in many cancer types including breast cancer and potential clinical implication have been delineated [167,169].

A thorough choice of patients' material is very important to achieve a representative selection and good comparability of results. Breast carcinoma is a heterogeneous tumor comprising stromal fibroblasts and leukocytes next to tumor cells. To ensure a similar content of tumors DNA in each probe, the DNA samples used in this study were prepared by means of microdissective isolation of tumors cells from fresh frozen and paraffin embedded tissues sections [170, 170].

Eight microsatellite markers spanning a region on chromosome 10q23 on chromosome 10q were chosen based on their high incidence of LOH. We tried to select markers with low non-informativity levels shown in previous studies of primary tumors and metastases (Table 2.3, <http://www.ncbi.nlm.nih.gov/mapview/>).

The analysis of DNA for allelic imbalances, specifically for LOH, using PCR analysis is a good method to detect losses of genetic material and implicate regions harboring tumor suppressor genes. Nonetheless this method has some limitations and LOH results had to be interpreted cautiously [172, 173]. For example detected LOH could be caused by events other than the loss of one allele, such as the differential amplification of the other allele [173]. If an observed LOH were accompanied by no change of copy number, then this would suggest that the LOH event was caused by copy-neutral events such as mitotic non-disjunction followed by duplication of one parental chromosomal. However, if an LOH event were accompanied by significant increase in copy numbers, then this would suggest preferential amplification of one parental allele that may be masking the presence of the other allele. Distinguishing between these possibilities is important conceptually, but would not change the data analysis. In context of the previously obtained CGH results that indicated deletions on 10q we assumed that the large majority of AI detected in this study are losses of genetic material.

## **5.2. Allelic imbalances in primary tumors and brain metastases**

In this study we found a significantly higher number of AI in brain metastases than in primary tumors at virtually all examined loci at 10q. The CGH screening data available in the literature as well as previously published data from our lab seldom reported deletions on 10q in primary and locally advanced breast cancer but were

shown in other types of cancer [77, 78, 79]. For example genetic alteration on 10q have been shown to confer prostate cancer and squamous cell lung cancer a metastatic phenotype [80, 81, 82]. Notably, LOH on 10q is the most frequent genetic alteration in a primary very aggressive CNS tumor - glioblastoma (up to 80%) and is as well common in other primary brain tumors indicating that genes located on 10q might be involved into regulation of tumor survival in CNS environment [83, 84, 85, 86, 87]. High mutation rate of 10q region in primary brain tumors and high frequency of 10q deletions in brain metastases of breast cancer suggests a role of this chromosomal area in determining brain tropism and/or selective survival advantage in brain microenvironment of the cells bearing aberrations on 10q.

The genesis of the specific genetic aberrations found almost exclusively in brain metastases shown in this study can be explained by different hypotheses [23, 174].

It is plausible that there is a small subset of tumor cells containing the aberrations within the bulk of primary tumor but the much higher quantity of the remaining tumor masks the detection of small population.

It is as well credible that single tumor cells gaining a specific mutation acquire the ability to metastasize and detach from the primary tumor to travel to a remote target organ.

Another possibility is that metastatic cells acquire additional genetic aberration at the site of a distant organ, the so-called *de novo* mutation, which renders them capable of assimilating into the remote organ microenvironment.

Alternatively, it is probable that only a specific subset of primary tumors carry the mutation, which renders this cancer entity prone to metastasize specifically to the brain. The majority of samples used in this study were unmatched primary and metastatic tumors. Only four matched sample pairs were available for examination. The analysis for AI in these cases demonstrated a similar pattern of AI in three out of four matched sample pairs. This finding favors the suggestion that the metastatic phenotype originates in the primary tumor tissue and determines the proclivity of the tumor to metastasize to the brain. For validation of this hypothesis a larger study with matched primary and metastatic samples is needed.

Ultimately, there is a possibility that many cells with different genetic entities metastasize to the brain but only those carrying alterations on 10q can withstand the

selective pressure in the brain microenvironment. A recent study of Ding *et al.* demonstrated in a massive parallel DNA sequencing of matched primary breast tumors, brain metastasis, peripheral blood samples and primary tumor xenografts that the metastasis were very similar to the primary tumors but contained two *de novo* mutations and a large deletion not present in the primary tumor [175]. Another study evaluated corresponding primary breast tumors and their distant metastases and demonstrated that in comparison with the corresponding primary tumor, additional LOH events are frequently found in metastases and that the incidence of combined LOH in the primary tumor, plus the occurrence of additional LOH events in the distant metastases, correlated significantly with decreased postmetastatic survival [175].

In this study only four pairs of matched primary and metastatic tumors were available for analysis. We found corresponding AI patterns in three of four pairs, although in one of the three brain metastasis samples AI was larger involving AI of an additional marker. Similarly, we found no significant difference in AI between brain metastases and primary tumors with a later relapse. In contrast, brain metastases carried a significantly higher number of AI if compared to primary tumors without later relapse. These findings indicate that a certain subset of primary tumors initially harbors AI on 10q, which are then detectable as well in their metastatic satellites. Additionally, we could show that the accumulation of AI among the primary tumor patients did not correlate with any other clinical or pathological parameters other than brain metastases, so that it can be concluded that AI on 10q play a role in development of brain metastases of breast carcinoma.

Further, the results of this study revealed a “hot spot” locus covered with markers D10S541 and D10S1765 where AI were accumulated in brain metastases as opposed to all primary tumors with or without later metastatic dissemination except for those with later brain relapse. It seems that AI in this region play a role in brain tropism of breast cancer.

Based on this study it still remains open whether alterations on 10q are associated with specifically brain tropism of metastases and whether spread to other distant organs is determined by a different genetic profile or 10q is a general culprit in metastatic disease of breast cancer. It is as well possible that a compound signature of several genetic alterations is pivotal for formation of metastatic properties and itineraries. Although several studies described a role of other chromosomal regions like 7q, 8p,

14q involved into metastatic process in breast carcinoma, the alterations on chromosome 10q were not described for metastases to other sites than brain [177, 178, 179, 180].

### **5.3. Candidate genes on chromosome 10q**

The AIs identified in the study were concentrated around two core regions, one 6.3 MBp-large AI core region around the markers D10S1765 and D10S541 comprising *PTEN* locus (*PTEN/CR1*), and the other 6.4 MBp-large AI site around markers D10S1236 and D10S190 (*CR2*). There are several genes located at these regions. After the completion of the full study on AI on 10q described in this thesis the *in silico* expression analysis of 10q23.3-qter was performed by Wikman et al. to identify transcripts downregulated in brain metastases compared to unmatched primary tumors (Wikman et al. 2012, see attachment). All together 49 gene transcripts were identified which were downregulated in brain metastases in comparison to brain tumors. The results showed that the CR1 contained nine gene transcripts, which were significantly downregulated compared to the primary tumors. The tumor-suppressor gene *PTEN* was among these mostly uncharacterized genes (Wikman et al. 2012, see attachment). Genes *IFIT5* and *HECTD2* were described to play a role in brain related diseases, their role in cancer has been not well established [181, 182].

To see whether a difference exists in *PTEN* expression patterns among different primary tumor patients with different relapse patterns, the GSE14020 data set was later analyzed by Wikman et al. Significance analysis of microarrays (SAM) revealed a significant downregulation of *PTEN* expression in patients with brain relapse compared with patients with bone relapse. Interestingly, the *PTEN* expression was not significantly different between patients with lung or brain relapse (Wikman et al. 2012, see attachment). These findings indicate that *PTEN* might be important in the development of distant metastases in particular brain metastases.

The *PTEN* gene, (phosphatase and tensin homolog deleted on chromosome 10), located at 10q23.31, is a well known tumor suppressor gene and appear to be mutated at considerable frequency in human cancers such as CNS, breast, kidney and prostate cancer [88]. An LOH in this region is especially often found in advanced cancer [183, 184]. *PTEN* plays an important role in the signal transduction and catalyses the inactivation of phosphatidylinositol-(3,4,5)-triphosphate, which is built by the

phosphatidylinositol-3-kinase (PI3K). This reaction leads to inactivation of further target proteins in the signal cascade, e.g. protein kinase B (PKB/Akt), decreases cell proliferation rates and survival and promotes apoptosis [185, 186]. *PTEN* is directly and through PI3K/AKT pathway involved into the regulation of cell cycle and prevents the cell progression from G<sub>1</sub>-into the S-Phase. In addition, recent studies showed the intriguing roles of *PTEN* in regulating genomic instability, DNA repair, stem cell self-renewal, cellular senescence, and cell migration and/or metastasis [187]. Interestingly, specifically *PTEN* mutations are often found in primary brain tumors like glioblastoma multiforme [137, 188]. *PTEN* was shown to be related to a worse outcome and metastasis in breast cancer as well as in prostate and bladder cancer [189, 190]. Recently, a moderate decrease in *PTEN* expression to 80% of the normal level has been shown to increase susceptibility to develop cancer in mice, particularly in mammary tissue [191]. A recent study of Adamo et al. on brain metastases of breast carcinoma evaluated the prognostic implications of *PTEN* expression in brain metastases of breast carcinoma. The study demonstrated that *PTEN* was expressed at lower levels in brain metastases of breast cancer compared with other distant metastatic sites, indicating that *PTEN* might play an important role determining brain tropism of CNS metastases of breast cancer. This finding is consistent with systematic AI in *PTEN* locus detected in our study. Additionally lower levels of *PTEN* expression were found to be associated with a shorter time to distant and brain recurrence, showing that *PTEN* loss favors the formation and progression of brain metastases [180]. The study of Adamo et al. examined 12 matched primary breast tumors and brain metastases for deletions of *PTEN* and could show an 83% concordance of *PTEN* deletions in the matched pairs [180]. This finding is in line with our results on matched tumor samples and suggests that *PTEN* deletion is present in a fraction of primary tumors with high risk of developing brain metastases. Given this finding *PTEN* status in primary breast tumors can be potentially used as prognostic tool potentially predictive of distant and CNS recurrence. Confirming these findings in a larger cohort of patients with primary breast tumors and matched brain metastases would certainly be valuable for definitive statement.

In the second AI core region (CR2) eight different gene transcripts were detected that were significantly downregulated in brain metastases (Wikman et al. 2012, see attachment) including the recently described tumor-suppressor gene *HTRA1* (high-temperature requirement A serine peptidase 1) and three other genes (*GFRA1* (GDNF

family receptor alpha 1), *HSPA12A* (heat shock 70kDa protein 12A), *RGS10* (RGS10 regulator of G-protein signaling 10)) known to be involved in brain-related diseases [192, 193, 194, 195].

Apart from genetic aspects the local brain microenvironment is one of the determining factors of disease progression in the CNS [196]. It appears that a joint action of AI on 10q and several other factors and genes concomitantly involved into metastatic process is responsible for CNS spread in breast cancer. Further studies are needed to understand the details of molecular signature of the brain metastases of breast cancer.

#### **5.4. Genomic aberrations detected by FISH analysis**

For the FISH analysis the probe binding at 10q25.3-26.1 was used to investigate genetic alteration on 55 primary breast tumors. In the microsatellite analysis this region corresponded the core region 1 (CR1) and was covered with markers D10S173, D10S190 and D10S1236.

In the FISH analysis performed on 55 primary breast cancer cases, we demonstrated that the majority of primary tumors (92.7%) carried no change in copy number on 10q. In four cases (7.3 %) we detected DNA copy number changes. All these changes were a heterozygous loss of one allele. These results are generally compatible with results on AI, suggesting that primary tumors carry a low frequency of chromosomal alterations on 10q. Detected losses in four samples did not correlate with a distant or brain metastases and were characterized as having good prognostic clinical and pathological features. At first sight this finding seems to be in conflict with the statement that losses on 10q might favor brain metastases. But it can be explained with several arguments. First, the clinical and pathological characteristics of most of the patients involved in the FISH analysis show features of good prognosis: 90.9% having a tumor stage pT1 or pT2 and only 9% having a more advanced tumor stage of pT3 or pT4, 96.4% having no distant metastases, only 1.8 % having metastases to the brain and a great majority having positive hormone receptor status (60%) and no bone marrow involvement (75%). It is not surprising that four samples exhibiting deletions fall into the category of tumors with good prognosis. Possibly, they reflex incidence of 10q deletions within normal variation range. Second, it is possible that allelic imbalances were present in primary tumors examined but were small enough to escape detection by FISH but would possibly be detected by PCR at 10q25.3-26.1. Third, the



number of four tumors exhibiting deletions is too small to be representative and allow judgment on correlation with clinical and pathological parameters. Finally, the factor of experience and subjectivity in evaluation of FISH signals might have influenced the results outcome.

Our study shed a further light on understanding the molecular mechanisms of breast cancer progression and specifically biology of brain metastases of breast carcinoma. Additional knowledge has yet to be collected to unravel the complexity of cancer genesis.

A FISH study on brain metastases and on matched pairs of primary tumors and brain metastases would be helpful to investigate the concordance of allelic imbalances what could promote the understanding on development and chronology of genetic aberration in breast cancer and its metastases.

## 6. Outlook

Currently the detection of brain metastases is performed by means of imaging methods like MRI after primary tumor has already spread. Presently, no specific markers identifying patients at risk for brain metastases have been implemented into clinical practice.

By means of microsatellite analysis we could detect specific genomic alterations on 10q that correlate with occurrence of brain metastases in patients with breast cancer. Especially genetic region containing *PTEN* gene was predominantly affected leading to a significant correlation with formation of brain metastases. The specific role of *PTEN* can be further investigated with help of knock-out models and studies on matched primary and metastatic tumors.

However, the results of this study do not fully unravel the complexity of metastatic cascade. Other studies available in the literature demonstrate that other allelic alterations might be as well involved into the formation of brain metastases of breast cancer. For example, it has been demonstrated that in carcinomas of the breast, there was a significant difference in LOH frequencies on chromosome 15 between non-metastatic tumors and brain metastases of breast cancer [197]. Additionally, several genes and different mechanisms of their functional modification were suggested as contributors to the metastatic progression of breast cancer to the brain [198, 199, 200, 201]. It seems that a constellations of several genes rather than one gene determines the genetic signature of brain metastases of breast cancer. Further knowledge is needed on additional genes that might play a role in the metastatic cascade.

The detection of target genes responsible for brain tropism of breast cancer would allow screening of primary tumors by established laboratory methods like PCR defining a subgroup of patients at high risk of developing brain metastases. Moreover, more knowledge on genetics of brain metastases could lead to new therapeutic strategies involving molecular targets with the aim of preventing brain metastases formation.

## 7. Summary

Breast cancer is the most common malignancy in women with particularly high mortality rate in patients who develop brain metastases in course of the disease [17, 99]. The biology of breast cancer and its metastases has been extensively studied but is still not fully understood. The multistep process of metastases formation involves detachment from the primary tumor, migration, penetration of blood vessels, extravasation at the distant site and a successful growth at the foreign environment of the distant organ. Different hypotheses on chronology and origin of metastases have been proposed [23]. Especially brain metastasis encounter a challenge of trespassing the blood-brain barrier [134]. Genetic alterations leading to a loss of function in tumor suppressor genes play an important role in tumorigenesis of breast cancer [39].

In this study we examined and compared 77 primary breast tumors and 21 brain metastases for allelic imbalances on chromosome 10q by means of PCR with fluorescence-tagged microsatellite markers and subsequent capillary electrophoresis. The goal was to detect genetic alterations in the primary tumors which are associate with metastatic spread to the brain and which could serve as a specific marker to identify breast cancer patients at high risk for brain metastases.

We found a significantly higher number of AI in brain metastases than in primary tumors at virtually all examined loci at 10q. The AI accumulated around two core regions. The first core region (CR1) was found around *PTEN* locus (markers D10S541 and D10S 1765) and the second region (CR2) was detected around the markers D10S173 and D10S190. The AI in these loci were significantly more frequent in brain metastases than in primary tumors without relapse. Interestingly, primary tumors with later relapse to brain exhibited similar frequencies of AI at 10q like brain metastases of breast cancer. The four matched primary tumors and brain metastases showed as well similar patterns of AI. These results favor the hypothesis that a certain subset of primary tumors initially harbors AI on 10q, which are then detectable as well in their metastatic satellites.

*PTEN* was identified as a tumor suppressor gene that is likely to be involved in formation of brain metastases of breast cancer. The locus containing *PTEN* carried a much higher number of AI in brain metastases than in primary tumors without relapse or relapse to other organs other than brain. According to our study this locus can be defined as a “hot spot” of AI on 10q in brain metastases. Interestingly, loss of *PTEN* is

generally not present in epithelial tumors but is very common in primary brain tumors like glioblastoma [83]. AI on this locus may confer the epithelial cells features supporting their survival in brain microenvironment.

In conclusion, the present results show that brain metastases of breast cancer carry very complex patterns of genetic alteration. Still certain regions and genes, such as *PTEN*, are predominantly affected and might play an important role in dissemination of breast cancer to the brain. Further studies like functional analysis of these genes are needed to determine their specific role in the metastatic cascade of breast cancer.

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## **9. Attachment**

The findings of the thesis served as integral part for the publication of a scientific article “**Relevance of PTEN loss in brain metastasis formation in breast cancer patients**”. Herewith is the attachment of this document as evidence of valuable scientific contribution in cancer research.

RESEARCH ARTICLE

Open Access

## Relevance of PTEN loss in brain metastasis formation in breast cancer patients

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### Abstract

**Introduction:** With the improvement of therapeutic options for the treatment of breast cancer, the development of brain metastases has become a major limitation to life expectancy in many patients. Therefore, our aim was to identify molecular markers associated with the development of brain metastases in breast cancer.

**Methods:** Patterns of chromosomal aberrations in primary breast tumors and brain metastases were compared with array-comparative genetic hybridization (CGH). The most significant region was further characterized in more detail by microsatellite and gene-expression analysis, and finally, the possible target gene was screened for mutations.

**Results:** The array CGH results showed that brain metastases, in general, display similar chromosomal aberrations as do primary tumors, but with a notably higher frequency. Statistically significant differences were found at nine different chromosomal loci, with a gain and amplification of *EGFR* (7p11.2) and a loss of 10q22.3-qter being among the most significant aberrations in brain metastases ( $P < 0.01$ ; false discovery rate (fdr)  $< 0.04$ ). Allelic imbalance (AI) patterns at 10q were further verified in 77 unmatched primary tumors and 21 brain metastases. AI at *PTEN* loci was found significantly more often in brain metastases (52%) and primary tumors with a brain relapse (59%) compared with primary tumors from patients without relapse (18%;  $P = 0.003$ ) or relapse other than brain tumors (12%;  $P = 0.006$ ). Loss of *PTEN* was especially frequent in HER2-negative brain metastases (64%). Furthermore, *PTEN* mRNA expression was significantly downregulated in brain metastases compared with primary tumors, and *PTEN* mutations were frequently found in brain metastases.

**Conclusions:** These results demonstrate that brain metastases often show very complex genomic-aberration patterns, suggesting a potential role of *PTEN* and *EGFR* in brain metastasis formation.

### Introduction

Breast cancer is the most common malignancy in women, with the mortality rate being especially high in patients in whom brain metastases develop. Approximately 15% to 20% of breast cancers metastasize to the brain, although incidence rates are increasing [1]. The incidence of metastases is thought to be increasing because of the improved treatment of metastases at other distant sites and advances in imaging techniques,

leading to improved detection of central nervous system (CNS) metastases [2].

Metastasis formation is a highly selective, multistep process, involving complex interactions between tumor and host cells. To metastasize, tumor cells must disengage from the primary tumor, invade the stroma, and penetrate into vessels, where they disseminate, extravasate, and start to grow at distant organ sites. As a distant metastatic site, the brain forms a special challenge for tumor cells because of the blood-brain barrier [3]. In addition, all other steps have to be successfully completed for the tumor cell to survive and expand. The molecular basis for all of these steps is still unclear, and several models have been suggested [4,5].

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Comparative gene-expression analyses on primary breast tumors and lymph node metastases have indicated that, in general, metastases have very similar expression signatures compared with their parent tumors [6,7]. However, detailed analyses have also revealed that a number of genes are consistently differentially expressed between primary tumors and metastases [8-10] and that metastases often show a greater variety of aberrations than the primary tumor [11,12]. At the chromosomal level, even greater differences have been described between primary breast tumors and their derived metastases. Most of the relevant studies compared the chromosomal aberrations in matched primary breast tumors and lymph node metastases [13-16], and only a very few studies on distant metastases exist [17-19]. In general, all of these studies showed that metastases harbor more and also new aberrations that could not be found in the corresponding primary tumors (reviewed in [20]). These results imply that the clonal evolution of a tumor is more complex than would be predicted by linear models, highlighting the importance of investigating distant metastases as the end point of the metastatic cascade.

In this study, the patterns of chromosomal aberrations of primary tumors and brain metastases from breast cancer patients were compared with array-comparative genomic hybridization (CGH) and microsatellite analysis. The goal was to identify genetic alterations in the primary breast tumors associated with metastatic spread to the brain to be able to define subgroups of high-risk breast cancer patients. Our results indicate that loss of 10q and especially phosphatase and tensin homologue (*PTEN*) could be predictive factors for the development of brain metastases. Interestingly, whereas loss of *PTEN* is generally very rarely seen in most epithelial tumors, it is one of the most frequent aberrations found in primary glioblastomas [21] and other CNS malignancies [22,23], indicating that loss of *PTEN* might be an important factor for breast tumor cell survival in the CNS environment.

## Materials and methods

### Patient collection

All samples were collected from female patients who underwent surgical resection at the University Medical Center, Hamburg-Eppendorf, Germany. For array CGH profiling, unmatched fresh-frozen tumor samples were collected from 30 primary breast cancer patients, with 10 breast cancer samples that had metastasized to the brain. All primary tumors were of an early stage, and none relapsed to the brain at a later stage (mean follow-up of 58.8 months). The mean age of the patients at brain metastases surgery was 57 years, with an average of 11 years (range, 4 to 30 years) between primary tumor diagnosis and brain relapse (Additional file 1).

For allelic-imbalance (AI) analysis, 77 primary breast tumor (55 fresh-frozen and 22 paraffin-embedded samples) and 21 brain metastases (all fresh-frozen) samples were analyzed. To avoid misleading results by analyzing unmatched samples, the primary tumor samples were matched for the main clinicopathologic characteristics in the AI analysis. Ten of the primary tumors later showed a relapse to the brain, and 17, to other sites. Eighteen of the primary tumor cases and eight metastases overlapped with the array samples, and five pairs of primary and corresponding metastases were available (Additional file 1). Four pairs of matched primary tumor and brain metastases were also available.

This study was approved by the Ethics Committee of the Chamber of Physicians, Hamburg, Germany, and sample donors gave written informed consent.

### HER2 status

The HER2 status was first assessed in both the primary tumors and the brain metastases with immunohistochemical tests (Dako HercepTest; Dako, Glostrup, Denmark) according to the manufacturer's instructions. All cases with an immunoscore of 3+ were considered to be HER2 positive, whereas all cases with a score of 2+ were reevaluated with fluorescence *in situ* hybridization (FISH) (PathVysion Kit Vysis; Abbott, Abbott Park, IL, USA). Tumor tissue in which the HER2-FISH signal to centromere 17 ratio was > 2 was also considered HER2 positive.

### Array CGH

Genomic DNA was isolated (QIAmpDNA MicroKit; Qiagen, Hilden, Germany) from fresh cryosections for the array CGH analysis and part of the microsatellite analysis. If necessary, manual microdissection was performed to obtain a tumor-cell content of at least 70% [24]. Total volumes of 300 ng of tumor DNA and 300 ng of reference DNA (pooled leukocyte DNA of 10 healthy men) were labeled with Cy3- and Cy5-labeled dCTP, respectively, and co-hybridized on 30 K oligonucleotide CGH microarrays. The array contains 60 mer oligonucleotides representing 28,830 unique genes designed by Compugen (Human Release 2.0 oligonucleotide library; San Jose, CA, USA) [25]. The raw signal intensities were obtained by using Agilent's Feature Extraction software program after bad-quality spots were removed by using BlueFuse Version 3.1 (BlueGenome, Cambridge, UK). We used a sex-mismatch in the hybridization (that is, DNA of the opposite gender was used as a reference). Subsequently, the X and Y chromosomes were excluded for downstream data analysis. The log<sub>2</sub> ratios were centered to a median of zero, and the resulting log<sub>2</sub> ratio values for each probe were segmented by using GLAD in R [26]. All probes within the genomic bounds of a given GLAD-derived segment were given the

mean copy-number value of the probes within that segment. Copy-number values  $> 0.05$  log<sub>2</sub> ratio units represented a gain and values  $< -0.05$  log<sub>2</sub> ratio units represented a loss. The cut-off points were based on the variance found in the X and Y reference chromosomes. After segmentation, the log<sub>2</sub> ratios were centered to a median of zero by using only normal segments, and the segmentation was repeated. All CGH data are available at ArrayExpress [27].

#### Microsatellite analysis

Microsatellite analysis was carried out to verify the loss of 10q and to reveal the extent of the aberration in 77 primary and 21 brain metastases. Tumor DNA was isolated in 22 cases from paraffin-embedded samples, also using macrodissection to achieve a minimum of 70% tumor cells in the samples. The DNA was extracted by using the InnuPREP DNA Microkit (AnalytikJena, Jena, Germany). For reference DNA, DNA samples isolated from peripheral blood mononuclear cells or nonmalignant normal breast tissue were used. Allelic imbalance in the chromosomal region 10q22.3-qter (chr10:80,522,854-134,299,490; 53.8 MBp) was first assessed by using eight microsatellite markers with an approximate spacing of 10 MBp. Three additional microsatellite markers were analyzed in samples that contained enough DNA and showed a partial AI (Additional file 2). The FAM or HEX end-labeled polymerase chain reaction (PCR) products were analyzed with a Genetic Analyzer 3130 (Applied Biosystems, Darmstadt, Germany). GeneScan software (Applied Biosystems) was used to study the lengths of the allele fragments and fluorescence intensity. The allelic imbalance (AI) was determined for heterozygous markers by calculating the ratio of the peak heights of the tumor and normal alleles. Ratios of 2.0 or higher were scored as AI.

#### PTEN mutation analysis

For the 10 brain metastases samples, the entire *PTEN* coding region was screened for mutations by sequencing *PTEN* cDNA. For an additional 10 samples, exons 3 and 5 were sequenced for mutations (Additional file 2). For the sequencing of cDNA total RNA was extracted from fresh-frozen tissue by using the Qiagen Minikit (Qiagen, Hilden, Germany), and then the total RNA was reverse transcribed by using a First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). First, the complete coding region was amplified, followed by sequencing PCR for both DNA strands. When multiple PCR products were detected, the respective bands were gel-purified by using the GelExtract Mini Kit (5Prime, Hamburg, Germany), and 40 ng of the purified product was used for the sequencing PCR by using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Freiburg, Germany). For genomic DNA, PCRs were performed by using exon-spanning

primer pairs for exons 3 and 5, as described in Danielsen *et al.* [28]. The PCR products were purified by using the PCRExtract Mini Kit (5Prime), and subsequent sequencing PCR was performed by using 30 ng of DNA. The sequences were determined in a Genetic Analyzer 3130 (Applied Biosystems).

#### Gene-expression analysis of the 10q gene in primary tumors and brain metastases

Array data from the 32 untreated primary breast tumors without relapse included in GEO DataSet [GSE21974] (including 10 basal-like and 22 non-basal-like tumors) and nine brain metastases samples (the same samples as used for CGH analysis, except for one sample from which no RNA could be extracted) were compared for differentially expressed genes. The datasets, which both were analyzed on the Agilent Whole Human Genome Microarray  $4 \times 44K$ , were combined, quantile normalized, and checked for systematic differences between the two array groups. Subsequently, differentially expressed genes were selected by using the significance analysis of microarrays (SAM) algorithm with a false-discovery rate (fdr) of 5%. To narrow the results, in a second step, only transcripts with an expression level at the 25th percentile or greater of the overall expression level, located on chromosome 10 with a fold change (FC)  $> 2$ , were taken into account.

In addition, a data set from Zhang *et al.* [11] GEO DataSet [GSE14020] was analyzed to see whether a difference in the *PTEN* expression exists among different primary tumor patients with different relapse patterns. The data set consist of primary breast tumors with 22 cases of brain relapse, 20 cases with lung relapse, and 18 cases with bone relapse. The CEL files were processed by using GCRMA. Differentially expressed genes (brain versus bone relapse and brain versus lung relapse) were identified by repeated permutation testing with the SAM algorithm by using a 5% fdr.

#### Statistical analysis

Statistical analyses were performed within the R statistical environment. For array CGH data, differences between primary tumors and brain metastases with respect to copy-number changes were determined per probe region by using CGHMultiarray, which is based on the Wilcoxon rank-sum test and is implemented in the CGHtest R-package [29,30]. Raw *P* values were corrected for multiple testing by using Benjamini and Hochberg's False Discovery Rate method [31].

The relation between microsatellite markers and clinical factors was examined by means of the  $\chi^2$  test and of independence. Differences between primary tumors and brain metastases in relation to allelic imbalance were calculated with the Fisher Exact test.



## Results

### Comparative analysis of primary and metastatic breast tumors with array CGH

To identify chromosomal aberrations that function as molecular markers for brain metastasis, array CGH screening was performed on both primary breast tumors and brain metastases. Copy-number changes were found in all samples ( $n = 40$ ) and on each chromosome arm. The most frequently observed gains (> 30% of cases) in the primary tumors were found at 1q, 8q, 16p, 17, and 20, and the most common losses were at 1p, 8p, 11q, 13, 16q, 17p, and 22 (Figure 1a). In the brain metastases, a high degree of aberration was found on almost every chromosome, with the most common gains (> 30% of cases) at 1q, 5p, 7, 8q, 10p, 11p, 12p, 15q, 18, 20, and 22, and losses at 3p, 8p, 9p, 10q, 11, 12p, 13, 15, 17, and 18q (Figure 1b).

The results showed a very similar pattern of genetic aberrations in both primary and metastatic breast tumors (that is, the metastases carried the majority of genetic alterations present in the corresponding primary tumors but with a significantly higher frequency). Whereas 20 loci were gained or amplified and 17 lost in brain metastases (with > 30% difference), only gains of 1p and 16p and a loss of 16q were most frequently found in the primary tumors.

After correction for multiple testing ( $\text{fdr} < 0.04$ ), nine regions were found to differ significantly ( $P < 0.05$ ) between the primary tumors and metastases (Table 1). The most striking difference between the primary tumors and metastases was found at chromosome 10q, showing a loss of 10qter in 60% to 90% of the metastases, whereas only none to 13% of the primary tumors harbored a loss ( $P < 0.002$ ). The terminal arm of chromosome 10p was often gained in the metastases (40%) compared with primary tumors, where no such gain was found (none) ( $P = 0.005$ ). Gains of 7p22.2-p15.3 and 7p11.2 (EGFR) were found in 70% to 80% of the metastases but in only 10% to 13% of the primary tumors ( $P = 0.001$  to 0.003, respectively). Interestingly, deletion at chromosome 17 in the primary tumors involved only the p-arm, whereas in brain metastases, most of the q-arm also was involved, except for the HER2 locus.

Twenty loci in the primary tumors showed a high-level amplification, whereas in the brain metastases, 10 loci harbored a high-level amplification. In general, no significant difference could be found in the number and distribution of high-level amplifications between the primary and metastatic tumors. The most common high-level amplification was the HER2 amplification at 17q21, which was found in 6% of primary and 20% of metastatic tumors (Additional file 3).

### Microsatellite analysis at 10q

Microsatellite analyses for allelic imbalances (AIs) were carried out to verify the CGH results and to assess the

extent of loss on 10q. Between eight and 11 microsatellite markers spanning a 54-Mbp region on 10q were used to screen a total of 21 brain metastasis and 77 primary tumor samples, including four matched pairs of primary tumors and metastases (Figure 2). The primary tumor cohort was matched for the main clinicopathologic characteristics.

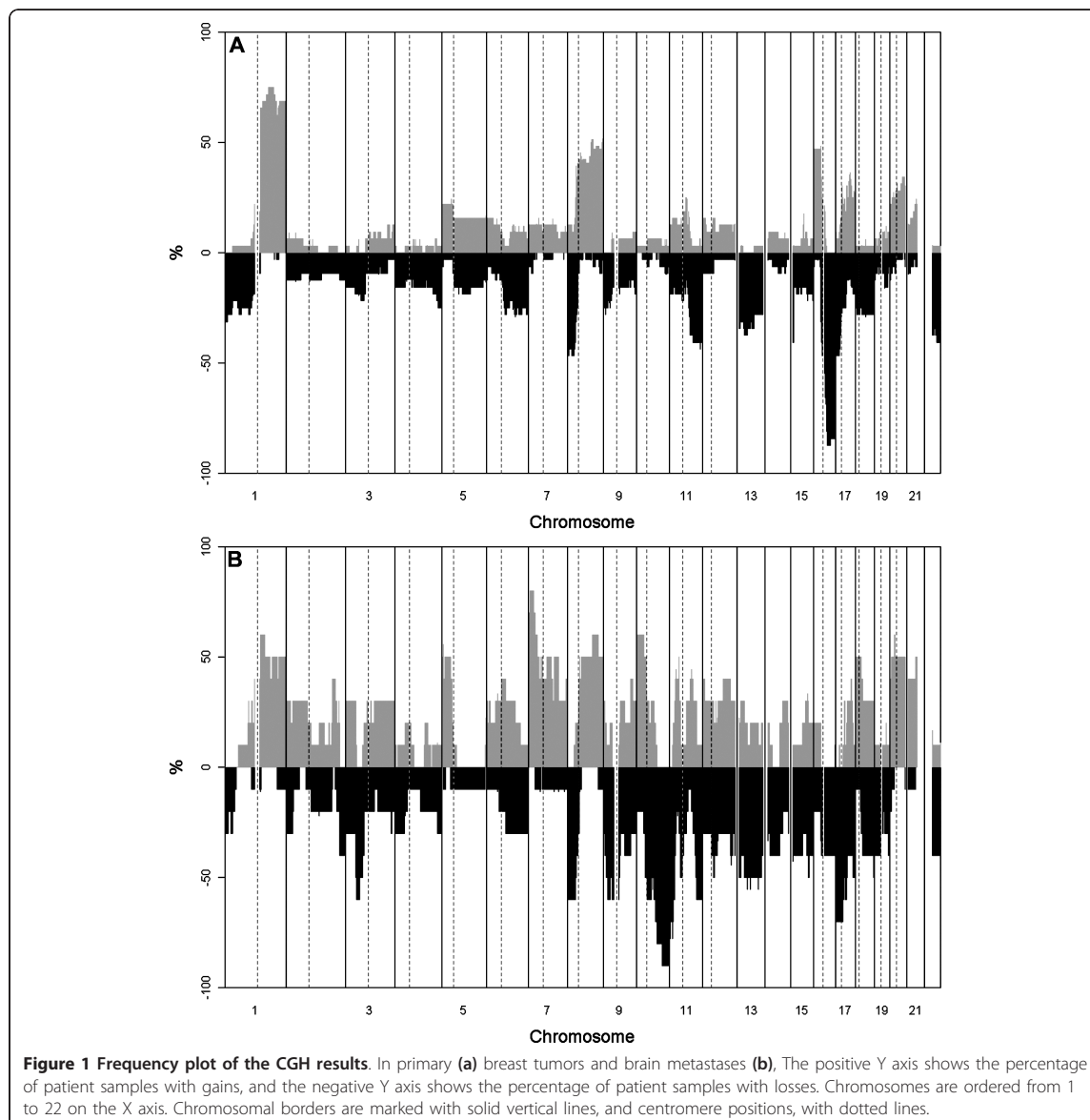
Significant differences in AI frequencies were found between the brain metastases and primary tumors without relapse ( $P = 0.05$ ) (Table 2): 62% of brain metastases and 38% of the primary breast tumors were found to be carriers of AI in the 10q region. Interestingly, samples from primary tumors without a history of subsequent brain relapse or from patients with a relapse to organs other than the brain (28% and 18%, respectively) showed fewer AI than did primary tumors from patients with relapse to the brain (50%).

The frequency of AI for individual markers varied between 18% and 43% in the primary tumors and 37% and 50% in the brain metastases. Interestingly, the AI did not cover the entire region but was concentrated around two core regions (Additional file 4). The first core region (CR1) was found around the *PTEN* locus (markers D10S541 and D10S1765), and the second region (CR2) was detected around markers D10S173 and D10S190. In particular, the AI in the CR1 (around the *PTEN* locus) was observed significantly more often in brain metastases than in primary tumors from patients without later relapse ( $P = 0.003$ ) or relapse to organs ( $P = 0.006$ ) other than the brain (Table 2). Furthermore, AI around the *PTEN* locus (CR1) was more common in HER2-negative brain metastases (seven of 11; 64%) compared with HER2-positive brain metastases (three of nine; 33%). The HER2 status was not inversely associated with AI at the *PTEN* locus in the primary tumors (19% and 25% in HER2-negative and HER2-positive primary tumors, respectively) (Figure 2).

The size of AI was often quite small (sometimes covering only one microsatellite marker) in many of the primary tumors, which could explain its rare detection by CGH. In general, however, allelic-imbalance detection was in good accordance with the results indicated by the CGH array (Figure 2).

Matched primary-tumor and brain-metastasis samples were available for AI analysis in four cases. Three cases showed identical aberration patterns at 10q, one normal and two AI, whereas in the fourth case, the AI was larger (that is, in the metastases, the distal marker was also affected by AI (Figure 2)).

In the primary tumors, AI at any locus was not significantly associated with any clinical or histopathologic factors (including hormone-receptor and HER2 status) other than brain relapse (Additional file 5).



#### Differentially expressed genes at 10q loci

In *in silico* gene-expression analysis, we identified 49 transcripts residing in 10q22.3-qter that were significantly downregulated in the brain metastases samples compared with unmatched primary breast tumors without relapse. The first 6.3 MBp-large AI core region around markers D10S541 and D10S1765 (*PTEN/CR1*) contained nine different transcripts that were significantly downregulated. The tumor-suppressor gene *PTEN* was among these mostly uncharacterized genes. The second 6.4-MBp-large AI hot spot around the markers

D10S173 and D10S190 (*CR2*) contained eight different transcripts, including the recently described tumor-suppressor gene *HTRA1* [32] and three other genes (*GFRAL*, *HSPA12A*, *RGS10*) known to be involved in brain-related diseases (Additional file 6) [33-35].

To see whether a difference exists in *PTEN* expression patterns among different primary tumor patients with different relapse patterns, the GSE14020 data set was analyzed. SAM analysis revealed a significant downregulation of *PTEN* expression in patients with brain relapse compared with patients with bone relapse (Additional file 7).

**Table 1 Statistically significantly different regions between primary breast tumors and brain metastases**

Chrom. region <sup>a</sup>	bp start	bp end	Size (Mbp)	% del PT	% gain PT	% del MET	% gain MET	P value	Fdr
1 p22.1-21.2	94472000	101828000	7.4	25.8	3.2	0.0	20.0	0.005	0.04
7 p22.1-15.2	5383000	27787000	22.4	3.2-12.9	12.9	0.0	70.0-80.0	0.001-0.002	0.01-0.02
p11.2	54565000	55475001	0.5	0.0	9.7-12.9	0.0	70.0	0.001-0.003	0.01-0.02
10 pter-p12.1	173000	28895000	28.7	0.0	0.0	20.0	40.0	0.005	0.04
q11.22-q21.1	49685000	59935000	10.3	3.2	6.5	60.0	20.0	0.005	0.04
q22.1-qter	71226000	134848000	63.6	0-12.9	3.2-6.5	60.0-90.0	0.0-10.0	0.001-0.002	0.02
11 pter-p15.4	188000	9693000	9.5	12.9-19.4	9.7-12.9	70.0	0.0	0.001-0.005	0.01-0.04
16 q24.2	87416000	87465000	0.0	87.1	0.0	30.0	0.0	0.002	0.02
17 q11.2	26591000	29500000	2.9	16.1-22.6	25.8-29.0	70.0	0.0	0.001-0.005	0.01-0.04

fdr, false discovery rate; MET, brain metastasis; PT, primary tumor. <sup>a</sup>The complete region that was statistically significantly different between the two groups (primary versus metastasis). Therefore, some break points in some patients might occur within the region, causing a variation in the frequencies of deletions/amplifications within a region and P values and fdr.

Interestingly, the PTEN expression was not significantly different between patients with lung or brain relapse (data not shown).

#### PTEN mutation screening in brain metastases

The entire coding region in 10 brain-metastasis samples was sequenced for *PTEN* mutations. In addition, 10 samples were used to sequence exons 3 and 5 for mutations. Table 3 shows the three tumor samples (15%) with mutations. Two patients were carriers of a splice-site mutation. In one tumor, the cDNA lacked a complete exon 4. In the second tumor, both alleles were mutated, resulting in one product with a deletion of exons 4 to 6 and the second product with a complex translocation and duplication of exon 3. The deletion of exon 4 in the cDNA was shown to be caused by a 41-bp deletion in the intron 3-exon 4 junction of *PTEN*. Because of the complex nature of the translocations and deletions of both alleles in the second case, no clear sequencing product on genomic DNA could be obtained. The third mutation was detected in exon 5 (c.389G > T). This base-pair substitution causes an amino acid change of arginine to lysine (p. R130L) in the active-site pocket of the phosphatase domain, which is essential for catalysis [36]. This mutation was previously described as causing a loss of PTEN protein expression [37].

#### Discussion

Central nervous system metastases are a frequent complication of many solid tumors. Approximately 15% of all epithelial tumors metastasize to the brain, with incidence rates highly dependent on the primary tumor type. Whereas prostate cancer very rarely metastasizes to the brain (1% to 5%), small-cell lung cancer (40%) and breast cancer (15% to 20%) commonly metastasize to the brain [38]. Apparently, the brain microenvironment is especially permissive for the growth of disseminated tumor cells from some carcinomas but not from others. The mechanisms by which metastatic tumor cells adapt to the

selection pressure exerted by the brain microenvironment are still unknown.

In this study, our aim was to identify putative molecular markers associated with the development of brain metastases in breast cancer. First, we screened for chromosomal aberrations by array CGH. The most prominent finding of the loss of 10q in brain metastases was validated in a larger study population, and the tumor-suppressor gene *PTEN* was found to be the potential target gene in this region.

Overall, the array CGH results of the primary breast tumors were in agreement with those described previously [39-41]. In general, the brain metastases showed aberration patterns similar to those of the primary tumors. However, in the brain metastases, a remarkably higher frequency of gains and losses was found at almost every chromosomal locus. Only a gain at 1p and a loss at 16q, described as being typical of luminal breast tumors and as markers of a favorable prognosis, were more common in the primary tumors [39-41]. This finding is not surprising, as most of the primary tumors were hormone receptor (HR) positive, whereas 38% of the brain metastases were HR negative. Statistically significant differences were found between the primary breast tumors and brain metastases at nine different loci on six different chromosomes.

The most significant differences were found at chromosomes 7 and 10. Chromosome 7 contains two regions, 7p22-p15 and 7p11.2, that were gained or amplified in more than 70% of the metastases and gained in only 3% to 13% of the primary tumors. Whereas the 22-Mbp region 7p22.1-p15.3 contains many genes, the second gained region on chromosome 7 contains only one gene, the epidermal growth factor receptor (*EGFR*); *EGFR* is a well-known and important gene in breast cancer initiation and progression [42]. Recently, Gaedcke *et al.* [43] reported a *de novo* protein expression of *EGFR* in the brain metastases of matched primary and metastatic breast cancer cases, and mouse models have shown the

Patient Identifier	Pairs	HER2	Microsatellite analysis at 10q					Relapse	Result AI	Result CGH	
			D10S541 D10S219	D10S1765 D10S1692	D10S173	D10S190 D10S1236	D10S212				
<b>BRAIN METASTASES</b>											
B/M-1	1	pos	AI	AI	AI	AI		AI	CR 1 + 2	loss	
B/M-2	2	pos	AI	AI	AI	AI		AI	CR 1 + 2	loss	
B/M-3	3	neg	AI	AI	AI	AI		AI	CR 1 + 2	loss	
B/M-4	4	neg	AI	AI	AI	AI		AI	CR 1 + 2	loss	
B/M-5	5	neg	AI	AI	AI	AI		AI	CR 1 + 2	loss	
B/M-6	6	neg	AI	AI	AI	AI		AI	CR 1 + 2	loss	
B/M-7	7	neg	AI	AI	AI	AI		AI	CR 1 + 2	loss	
B/M-8	8	neg	AI	AI	AI	AI		AI	CR 1 + 2	loss	
B/M-9	9	neg	AI	AI	AI	AI		AI	CR 1 + 2	loss	
B/M-10	10	neg	AI	AI	AI	AI		AI	CR 1 + 2	loss	
B/M-11	11	pos	AI	AI	AI	AI		AI	CR 1	n.d.	
B/M-12	12	pos	AI	AI	AI	AI		AI	CR 1	n.d.	
B/M-13	13	pos	AI	AI	AI	AI		AI	CR 2	loss	
B/M-14	14	pos	AI	AI	AI	AI		AI	CR 2	loss	
B/M-15	15	pos	AI	AI	AI	AI		AI	CR 2	loss	
B/M-16	16	pos	AI	AI	AI	AI		AI	CR 2	loss	
B/M-17	17	pos	AI	AI	AI	AI		AI	CR 2	loss	
%			50.0	47.4	44.4	38.6	47.1	38.5			
<b>PRIMARY TUMORS</b>											
PT-26	1	neg	NI	AI	AI	AI	AI	AI	brain-rel.	CR 1 + 2	n.d.
PT-27	2	pos	NI	AI	AI	AI	AI	AI	brain-rel.	normal	n.d.
PT-30	3	n.d.	AI	AI	AI	AI	AI	AI	brain-rel.	CR 1 + 2	n.d.
PT-39	4	neg	AI	AI	AI	AI	AI	AI	brain-rel.	CR 1 + 2	n.d.
PT-13	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-67	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-47	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-8	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-23	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-29	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-58	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-5	n.d.	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-24	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-38	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-66	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-40	n.d.	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-18	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-36	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-55	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-42	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-12	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-15	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-11	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-22	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-34	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-41	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-54	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-49	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-62	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-21	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-99	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-73	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-56	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-48	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-50	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-51	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-53	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-76	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-74	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-65	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-17	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-60	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-61	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-14	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-3	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-4	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-11	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-16	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-19	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-20	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-43	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-37	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-63	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-6	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-35	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-7	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-9	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-12	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-32	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-33	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-97	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-68	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-69	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-46	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-25	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-10	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-31	pos	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-71	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-77	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-70	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-72	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-75	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-29	n.d.	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-44	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-64	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
PT-52	neg	AI	AI	AI	AI	AI	AI	AI	chest wall-rel.	CR 1 + 2	loss
% AI PT			19.0	22.4	20.4	42.9	29.1	17.9			
% NI all			46.5	20.4	21.4	50.0	36.2	39.9			

**Figure 2** Microsatellite analyses for AI on 10q in primary breast cancers and metastases. Base-pair position and the markers used are indicated on the top line. The result for each marker is shown as follows: AI, black; noninformative, light gray; unavailable measurement, dark gray; and informative without changes, white box. 1, 2, 3, 4, matching primary tumors and brain metastases; a: loss from bp:100000000; b: loss from bp: 95000000; c: loss from bp:114000000; d: gain until bp:100000000; e: loss from 80-90000000; n.d., not determined; NI, noninformative marker; CR 1, core region 1; allelic imbalance around markers D10S173 and D10S190; CR 2, core region 2; allelic imbalance around markers D10S541 and D10S1765.

EGFR ligand HBEGF to be one of the key mediators of cancer-cell passage through the blood-brain barrier [5]. Most of the long arm of chromosome 10 was lost in 60% to 90% of the brain metastases, but only in none to 13% of the primary tumors. Numerous previously published large-array CGH datasets, also including locally advanced breast cancer, seldom showed a loss of 10q

[39-41]. The loss of 10q is, in general, rarely seen in most epithelial tumors, whereas it is the most common aberration found in glioblastomas, and it also is common in other CNS malignancies [21,22]. Similarly, the gain of 7p is among the most frequently found gains in astrocytomas [44]. Both aberrations also are present in melanomas, which often metastasize to the brain

**Table 2 Frequencies and P values for AI at chromosome 10q in primary breast tumors and metastases**

	Brain metastases (n = 21)		All primary tumors (n = 77) <sup>a</sup>		P value <sup>b</sup>	Primary tumors without relapse (n = 39)		Primary tumors with brain relapse (n = 10)			Primary tumors with other relapse (n = 17)			
	n	%	n	%		n	%	n	%	P value <sup>b</sup>	n	%	P value <sup>b</sup>	
<b>PTEN (CR1)<sup>c</sup></b>	11	52.4	18	23.4	<b>0.006</b>	7	17.9	<b>0.003</b>	5	50.0	n.s.	2	11.8	<b>0.006</b>
<b>CR2<sup>c</sup></b>	10	47.6	22	28.6	n.s.	11	28.2	n.s.	5	50.0	n.s.	3	17.6	n.s.
<b>all AI</b>	13	61.9	29	37.7	n.s.	13	33.3	0.055	5	50.0	n.s.	5	29.4	n.s.
<b>normal</b>	8	38.1	48	62.3	-	26	66.7	-	5	50.0	-	12	70.6	-

<sup>a</sup>Relapse pattern not recorded for 11 patients. <sup>b</sup>P values calculated with the Fisher Exact test (brain metastases versus other groups). <sup>c</sup>CR1 and CR2 are included in all AI. AI, allelic imbalance; CR2, core region 2; allelic imbalance around markers D10S190 and D10S1236; n.s., not significant; PTEN (CR1), core region 1 allelic imbalance around markers D10S1765 and D10S541.

(Additional file 8). The origin of these brain-specific aberrations in metastases could be explained by different hypotheses [45]. The first one is that only few cells carried these genetic alterations in the primary tumors, and thus they could not be detected when the tumors were analyzed in bulk. Subsequently, cells with these alterations selectively metastasized to the brain and formed the bulk of the metastases. Alternatively, these additional alterations might have occurred at the distant site of metastasis, and therefore represent *de novo* mutations that were not present in the primary tumors. The third scenario is that only a small fraction of the primary tumors contained these aberrations and that these tumors are specifically prone to relapse in the brain. Finally, another possibility is that several cells metastasize to the brain (for example, as a tumor-thrombus), but only those with alterations at 10q and/or 7p are able to survive in the brain environment, giving rise to metastases. Recently, by massively parallel DNA sequencing, Ding *et al.* [46] showed that metastases were indeed significantly enriched for shared mutations, which supports the last model.

Matched primary and metastatic tumor samples could be investigated in four cases by microsatellite analysis. Three cases showed identical aberration patterns, whereas in the fourth case, the AI imbalance was larger (that is, in the metastases, the distal marker was also affected by AI). Furthermore, primary tumor samples from patients in whom brain metastasis later developed showed a frequency of AI at 10q that was almost as high as in the

brain metastases, but AI was rarely seen in primary tumors without brain relapse or other distant metastases. These results indicate that the loss of 10q does exist in a fraction of primary tumors with a high risk of developing brain metastases. The size of the aberration can expand in metastases and thus become more detectable by, for example, CGH. Because aberrations in 10q were not associated with any clinical factor other than brain relapse, this implies that loss of 10q is a specific marker of brain metastasis and is thus needed for the outgrowth of the breast tumor in the brain. However, this hypothesis must be validated in future studies, both functionally and on independent larger cohorts of patients.

The AIs were concentrated around two core regions, the first one around markers D10S1765 and D10S541 containing the *PTEN* locus, and the second around markers D10S1236 and D10S190 at 10q26. The *PTEN* gene located at 10q23.31 is a well-described tumor-suppressor gene, also in breast cancer; *PTEN* functions as an important tumor suppressor by negatively regulating the PI3K-mediated cell-signaling pathway [47]. The present microarray analysis showed that *PTEN* is significantly downregulated in brain metastases compared with nonmetastatic primary tumors. Furthermore, mutation screening of the *PTEN* gene in brain tumors showed that the frequency of the mutation was much higher (15%) in primary breast tumors (none to 5%) than previously described [48,49].

Several studies have shown that ERBB2/HER2 and the basal subtype of breast cancer are the predominant types of breast cancer that metastasize to the brain

**Table 3 PTEN mutations in brain metastases**

Sample	Mutations in gDNA				Mutations in cDNA	AI result	CGH (PTEN) result
	Exon3	Exon4	Exon5	Exon6			
BrM-6	Wt	g.del [72586_72627]	wt	nd	c.[del1241_1284]	AI	Het. loss
BrM-7	Wt	No product	wt	wt	Allele 1: c.[del1241_1665] Allele 2: [del1524_1665; dup1196_1240, 950_1240con1196_1523]	AI	Het. loss
BrM-8	wt	nd	c.389C > A	nd	nd	Normal	nd

het. Loss, heterozygous loss; nd, not determined; wt, wild type.



[43,50,51]. To avoid misleading results by analyzing unmatched samples, the primary tumor sample cohort was matched for the main clinicopathologic characteristics in our AI analysis. When we classified the primary tumors as being ER/PR-positive, triple-negative, and HER2-positive tumors, no association was found between PTEN status (or loss of 10q) and breast cancer subtype, indicating that loss of PTEN is an independent predictor of brain metastases. In a recent publication, the protein expression of PTEN was analyzed in 54 brain metastasis samples from breast cancer patients [52]; no correlation was found between PTEN loss and subtype in this study. Furthermore, a high concordance rate (83%) of PTEN expression was found among the 12 matched pairs. The authors also investigated the role of PTEN expression in primary breast cancer progression by using *in silico* expression datasets with 855 patients [52]. They showed that, in all patients, lower levels of PTEN expression were associated with a poor prognosis and shorter time to brain recurrence, irrespective of hormone-receptor and HER2 status after 5 years. Also, this analysis was independent of subtype. In addition, we found, by using the same data set, a significant downregulation of PTEN expression among primary tumor patients with brain relapse compared with patients with bone relapse, but not to the lung. This finding is in line with the findings from Bos *et al.* [4,5], who found a significant overlap of brain with lung-relapse signature, but not with the bone signature, which argues for the important role of environment interaction in metastasis formation in different organs.

Interestingly, the two predominant genes derived from this breast cancer study on brain metastases also play a prominent role in the development and progression of primary brain tumors. The *PTEN* gene is one of the key tumor-suppressor genes found in primary glioblastomas, and it is often (15% to 40%) silenced through mutations (reviewed in [21]). Interestingly, also in glioblastomas, *PTEN* inactivation does not seem to be required for tumor initiation, but its loss is a hallmark for progression to highly malignant cancer [53]. Together with *EGFR* amplification, the loss of *PTEN* is the most frequent alteration observed in primary glioblastomas. Thus, these two genes, which are both involved in the PI3K kinase pathway, may play a key role in the growth of malignant cells in the brain environment and therefore might be suitable targets for therapeutic intervention.

## Conclusions

In conclusion, the present results show that brain metastases in breast cancer often carry very complex genomic aberration patterns. Nevertheless, certain target genes, such as *PTEN* and *EGFR*, are predominantly affected and might therefore play an important role in brain metastasis

formation. The genetic analyses of these genes might contribute to defining a subgroup of breast cancer patients who are at high risk of developing brain metastases. Moreover, increasing knowledge about the genetics of brain metastasis with regard to therapeutic targets and pathways may eventually lead to new antimetastatic strategies.

## Additional material

**Additional file 1: Table S1: Clinicopathologic characteristics of the patients.**

**Additional file 2: Table S2: Primers used for the AI analysis and *PTEN* mutation analysis.**

**Additional file 3: Table S3: High-level amplifications detected in primary and metastatic breast tumors.**

**Additional file 4: Figure S1: Chromosome 10q AI hot-spot region detection.**

**Additional file 5: Table S4: 10q allelic imbalances and association to clinical factors in primary tumors.**

**Additional file 6: Table S5: Differentially expressed genes at 10q between primary tumors and brain metastases.** Genes found significantly differentially expressed between primary tumors and brain metastases by using SAM analysis. Genes marked in bold reside in regions defined as hot spots by using the microsatellite analysis.

**Additional file 7: Table S6: Genes significantly differently expressed between primary breast cancer patients with bone and brain relapse.**

**Additional file 8: Figure S2.** CGH copy-number patterns in different tumor entities showing a high additional gain of 7p and loss of 10q. Frequencies and plots of gains and losses were retrieved from a CGH data base [progenetix](http://www.progenetix.net) <http://www.progenetix.net>.

## Abbreviations

AI: allelic imbalance; CGH: comparative genetic hybridization; CNS: central nervous system; CR: core region; fdr: false discovery rate; FISH: fluorescence *in situ* hybridization; HR: hormone receptor.

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## Authors' contributions

HW, ND, LU, MW, IH, and KY carried out the molecular genetic studies, CB, DK, and MZ carried out the statistical analyses. DK and BY performed the microarray analyses. HW, KL, NW, GS, MG, VM, and KP participated in the design of the study and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

#### Competing interests

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For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely my own.

## **Affidavit**

I, Liubou Uslar, hereby declare that I wrote the thesis “ Clinical relevance of allelic imbalances on chromosome 10q in brain metastases formation in breast cancer patients” on my own and without the use of any other than the cited sources and tools and all explanations that I copied directly or in their sense are marked as such, as well as that the thesis has not yet been handed in neither in this nor in equal form at any other official commission.

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## **Eidesstattliche Erklärung**

Ich, Liubou Uslar, versichere hiemit, dass ich die vorliegende Arbeit mit dem Titel „Klinische Relevanz von allelischen Imbalancen auf dem Chromosome 10q in Entstehung von Gehirnetastasen in Patienten mit Mammakarzinom“ selbstständig ohne fremde Hilfe und nur unter Benutzung der angegebenen Quellen und Hilfsmittel angefertigt habe.

Mit der Veröffentlichung dieser Arbeit erkläre ich mich einverstanden.

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