A Genetic Basis for Luminal and Basal-Type Breast Cancer

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A Genetic Basis for Luminal and Basal-Type Breast Cancer

Een genetische basis voor luminale en basale typen borstkanker

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General Introduction

1.1 BREAST CANCER EPIDEMIOLOGY AND ETIOLOGY

Cancer is the second leading cause of death, after heart disease, and a major health issue in the western world. In the Netherlands, 35% of women will develop cancer during their lifetime of which one third is diagnosed with breast cancer. This means that 1 out of 8 women in the Netherlands will develop breast cancer during her lifetime, making breast cancer the most frequently diagnosed cancer in women [1]. Breast cancer is not only the second leading cause of cancer death in western countries, after lung cancer, it is also the leading cause of overall mortality in women aged 35-55 years. In 2003, 11687 women were diagnosed with breast cancer in the Netherlands and 3361 women died resulting from the disease (http://www.ikcnet.nl/index.php). Currently, the 5-year survival for breast cancer in the Netherlands is 85% [1]. Fortunately, breast cancer mortality rates are declining in the Netherlands as a result of earlier diagnosis, through increased awareness and the breast cancer screening program on the one hand, and improved treatment on the other [2, 3].

Breast cancer incidence rates strongly vary around the world. The highest incidences are found in western countries of Europe and North America, whereas in the developing countries of South America and Africa incidence rates are relatively low [1, 4, 5]. Breast cancer incidence is dependent on the presence or absence of certain risk factors. The major risk factors for breast cancer include female gender, increasing age, western culture, positive family history of breast or ovarian cancer in first-degree relatives, germline mutation in a high-risk breast cancer susceptibility gene, prior diagnosis of breast cancer, benign breast disease with atypical hyperplasia, and exposure to ionizing radiation in young women. Other risk factors include early age at menarche, late age at menopause, nulliparity, late age at first child birth, small number of children, use of oral contraceptives and hormone replacement therapy, high socioeconomic status, dense tissue on mammography and postmenopausal obesity [6-8].

In the Netherlands, breast cancer incidence rates are still rising (http://www.ikcnet.nl/index.php, [9]). It is believed that this is due to the increasing average age of the population, resulting in a higher percentage of women in the age group where breast cancer is most commonly diagnosed [9]. Another reason for the increasing breast cancer incidence rates is the introduction of the national breast cancer screening program in 1989 for women aged 50 to 69 years. As a result, there was a substantial increase of breast cancer incidence in women aged 50-69 years after 1989, but not of women younger than 50 years or 70 years and older [2, 9]. Additionally, amongst women aged 50-69 years there was an increase in the rate of stage I cancers, and a decrease in stage III+ cancers [2]. As of 1998, women aged 70-74 years were therefore also invited for the national screening program [2, 9].

1.2 CLASSIFICATION OF BREAST CANCER

Fifty percent of women diagnosed with breast cancer will survive the disease without recurrence, whereas 15% of the patients will survive the disease despite a recurrence within 15 years. However, one third of breast cancer patients will die of metastases of the primary cancer within 15 years from diagnosis. It is therefore important to distinguish patients with a good prognosis that do not need additional therapy from patients with a poorer prognosis that will benefit from additional therapy. Reliable prognostic and predictive factors that classify breast tumors accurately are thus imperative for the clinician and have been a major focus in breast cancer research.

1.2.1 TRADITIONAL PROGNOSTIC AND PREDICTIVE FACTORS

TNM stage

As yet, the most powerful predictor of breast cancer recurrence is tumor stage. The TNM method for tumor staging is based on three tumor characteristics at the time of diagnosis: tumor size (T), axillary lymph node involvement (N) and the presence of metastases (M). Together these three factors define tumor stages I through IV (Table 1.1). Almost 90% of all patients with stage I cancers survive at least 5 years after diagnosis, whereas 5-year survival rates for stage II and III cancers are 60-80% and 40-50%, respectively [10]. Patients who have a stage IV cancer have a very poor 10-year survival of less than 5% [11]. The TNM stage of the tumor is thus a very strong indicator of the 5-year survival of the patient.

Axillary lymph node status

The extent of axillary lymph node involvement at the time of diagnosis is one of the most reliable independent prognostic factors for breast cancer. Patients with tumor free lymph nodes have a far better prognosis than patients with positive lymph nodes, with about 15-45% of node-negative patients having a disease recurrence compared to 50-70% of node-positive patients. Additionally, the risk of disease recurrence as well as mortality increases with an increasing number of lymph nodes involved [13-15]. The lymph node status is determined by the sentinel node procedure for staging purposes, followed by an axillary node dissection when metastases are present in sentinel nodes [16, 17].

Tumor size

The size of the tumor is a very strong prognostic factor, even after 20 years of follow-up [18, 19]. Although some pathologists measure the macroscopic size or the microscopic size of the tumor including both the invasive part and the *in situ* components, only the microscopic size of the invasive part of the tumor is clinically significant. Tumor size is directly correlated to

Table 1.1 TNM stage classification (adapted from [12])

T	Primary tumor size
T0	No evidence for primary tumor
Tis	Carcinoma in situ
T1	Tumor of 2 cm or less in greatest dimension
T2	Tumor larger than 2 cm, but not more than 5 cm in greatest dimension
T3	Tumor larger than 5 cm in greatest dimension
T4	Tumor of any size with direct extension to chest wall of skin

N	Regional lymph node involvement
N0	No regional lymph node metastases
N1	Metastases in movable ipsilateral axillary lymph node(s)
N2	Metastases in fixed ipsilateral axillary lymph node(s) or in clinically apparent ipsilateral internal mammary lymph node(s) in the absence of clinically evident axillary lymph node involvement
N3	Metastases in ipsilateral infraclavicular lymph node(s) with or without axillary lymph node involvement; or in clinically apparent ipsilateral internal mammary lymph node(s) in the presence of clinically evident axillary lymph node metastases

M	Presence of distant metastases
M0	No distant metastases
M1	Distant metastases

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

The size of the primary tumor (T), involvement of regional lymph nodes (N) and the presence of distant metastases (M) together define the stage of the breast tumor at the time of diagnosis.

axillary lymph node involvement, as larger tumors frequently have more positive lymph nodes. However, larger tumor size is correlated with a worse prognosis independent of lymph node status [20-22]. This is mainly because lymph node-negative patients with a tumor smaller than 1 cm have a far better prognosis than patients with a tumor larger than 2 cm (80% versus 65%, respectively) [23].

Histological tumor grade

Although not as strong as TNM stage, lymph node status or tumor size, the histological grade of a tumor is a good prognostic marker for breast cancer patients. Tumor grade is determined by the Scarf-Bloom-Richardson Grading system, modified by Elston and Ellis [24, 25]. According this grading system 1, 2 or 3 points are given for each of the following tumor characteristics: tubule formation, nuclear pleomorphism, and mitotic count. The sum of these points forms a score of 1 to 9 that determines the grade or differentiation status of the tumor (Table 1.2). Tumors with a low grade are well differentiated and predict a more favorable prognosis for the patient than poorly differentiated tumors with a high grade. The ten-year survival of patients with the lowest grade is 90-95% as opposed to 30-80% for patients with the highest grade [26, 27]. Additionally, higher grade is associated with negative hormone receptor status and low grade with positive hormone receptor status. Therefore, histological grade is correlated with response to either endocrine therapy (low grade) or chemotherapy (high grade) [28, 29]. Importantly, the distinct patterns of chromosomal loss between grade I versus grade III tumors has led to the assumption that the majority of grade I tumors do not progress to grade III tumors, but are likely to follow distinct genetic pathways [30].

Table 1.2 Histological grade (adapted from [12])

Score	Tubule formation
1	More than 75% of the tumor has tubule formation
2	10% to 75% of the tumor has tubule formation
3	Less than 10% of the tumor has tubule fromation

Score	Nuclear pleomorphism
1	Nuclei are small and uniform in size and shape
2	Nuclei are moderate in nuclear size and variation
3	Nuclei have marked variation, are relatively large, and have prominent or multiple nucleoli

Score	Mitotic count (per 10 high power fields with field area of 0.274 mm2)
1	0-9 mitoses
2	10-19 mitoses
3	More than 20 mitoses

Grade	Differentiation status	Total score
I	Well differentiated	3-5 points
II	Moderately differentiated	6-7 points
III	Poorly differentiated	8-9 points

A score of one, two or three points is given for each of the three tumor characteristics: tubule formation, nuclear pleomorphism and mitotic count. The sum of these three scores determines the grade or differentiation status of the tumor.

ER and PR status

Estrogens and progestins are important regulators of proliferation and differentiation of the mammary gland. These nuclear transcription factors exert their function by binding to their respective receptors: the estrogen receptor (ER) and the progesterone receptor (PR). Two thirds of breast cancers express both ER and PR, 10% are ER-positive and PR-negative, 5% are ER-negative and PR-positive and 20% of all breast cancers are negative for both ER and PR [31]. Patients with ER-negative tumors initially have a shorter survival than patients with ER-positive tumors. However, from 3 years of follow-up these differences in survival diminish, thereby losing the prognostic significance of ER [32]. The preferential site of distant metastases is different for ER-positive and ER-negative tumors, as ER-positive tumors tend to metastasize to bone and soft tissue, whereas ER-negative tumors metastasize more frequently to the liver, lung and central nervous system [33]. Importantly, ER-positive tumors respond better to endocrine therapy, whereas ER-negative tumors respond better to chemotherapy [34, 35]. About 50% of ER-positive tumors respond to endocrine therapy compared to less than 10% of ER-negative tumors. This can be even further refined by combining ER and PR, with up to 80% of patients with ER and PR-positive tumors responding to endocrine therapy compared to less than 10% of patients with ER and PR-negative tumors [36]. Therefore, the real power of ER and PR lies in their ability to predict the most appropriate class of systemic therapy.

ERBB2 overexpression

The ERBB2 receptor tyrosine kinase is a member of the epidermal growth factor receptor (EGFR) family and is also referred to as HER2/neu. About 25% of invasive breast cancers have amplification of the *ERBB2* gene and/or overexpression of the ERBB2 protein [37-39]. As a prognostic factor, ERBB2 overexpression is most valuable to lymph node-positive patients and associated with a less favorable clinical outcome in the pre-trastuzumab era. Lymph node-positive patients without ERBB2 overexpression have a ten-year survival of 65%, whereas those with ERBB2 overexpression had a ten-year survival of 50% [40, 41]. For node-negative patients ERBB2 overexpression has no significant prognostic value [26, 40, 42]. However, ERBB2 overexpression is able to predict response to trastuzumab (Herceptin) antibody therapy in both patient groups, but only 50% of the advanced patients appear to respond [43-45]. Activation of the PI3K pathway in patients with ERBB2 overexpressing breast cancer appears to be a major determinant of the resistance to trastuzumab [46]. Similar to the hormone receptors, the value of ERBB2 thus lies in its prediction of targeted therapy response.

1.2.2 HISTOPATHOLOGY

1.2.2.1 HISTOLOGY OF THE NORMAL BREAST

The functional unit of the mammary gland is a hormone responsive tubulo-alveolar gland of which the functional component is the terminal ductal lobular unit (TDLU) connected to an excretory system consisting of a large duct system (Figure 1.1). These components are embedded in the stromal tissue. The TDLU is the secretory part of the gland and composed of lobules and terminal ductules that discharge into the large duct system and excrete via the nipple. The epithelium that lines the entire lobular-ductal system is composed of two layers, an inner secretory luminal epithelial layer and an outer contractile basal myoepithelial layer. The inner epithelial cells are columnar or cuboidal shaped and form a polarized continuous layer that lines the lumen. The outer myoepithelial cells are typically elongated when cut longitudinally and triangular in cross section, and reside between the luminal epithelial layer and the basement membrane. In the ducts these myoepithelial cells form a nearly continuous layer, whereas in the lobules they form a discontinuous basket-like structure around the acini [47].

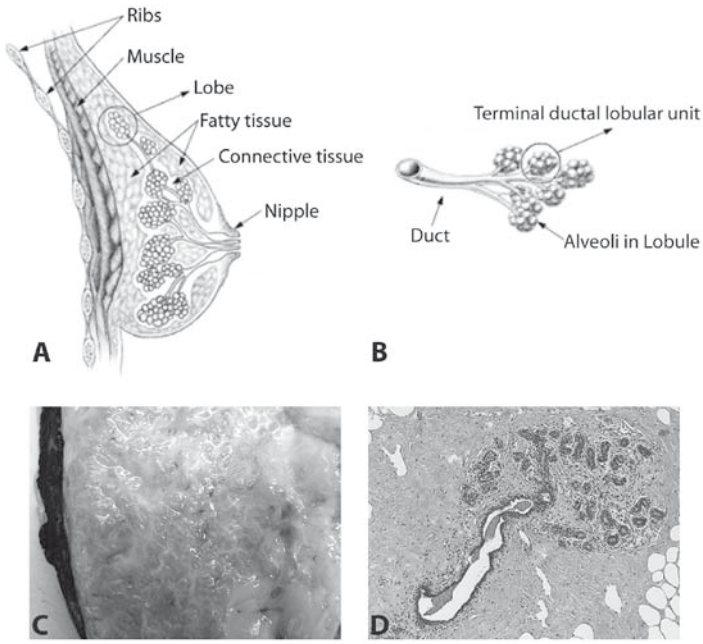

Figure 1.1 (A), schematic representation of the anatomy of the breast; (B), schematic representation of a breast lobe; (C), macroscopic view of the normal breast parenchyma; (D), microscopic view of a normal terminal ductular lobular unit of the breast. (A) and (B) were adapted from http://www.blogsforcompanies.com/TTimages/dcis_in_situ.jpg. This figure is also available in color in the appendix.

The luminal epithelial cells and the basal myoepithelial cells have distinct features and can be distinguished on the basis of expression of various cytokeratin (CK) proteins and smooth muscle actin (SMA). Luminal cytokeratins CK7, CK8, CK18, and CK19 are typically expressed in the luminal epithelial cells, but not in the basal myoepithelial cells. SMA and basal cytokeratins CK5, CK14, CK17, on the other hand, are expressed in the myoepithelial cells, but not in the luminal epithelial cells [48-50]. The two distinct epithelial cell lineages of a TDLU are clonally related and arise from a single mammary stem cell [51-57]. In addition, two types of luminal-restricted and one myoepithelial-restricted progenitor have been identified [58-60]. Still, the exact cellular hierarchy present in the breasts' epithelium is only partially understood.

1.2.2.2 HISTOPATHOLOGY OF BREAST CANCER

The vast majority of breast cancers arise in the epithelial cells of the TDLU, and are therefore classified as carcinoma. Breast carcinomas are classified pathologically on the basis of their morphology and growth pattern. The majority of the breast carcinomas (about 60%), however, can not be classified satisfactorily according to specialized pathological subtypes and are designated as invasive ductal carcinoma (IDC) not otherwise specified (NOS). Another common name for these tumors is IDC of no special type (NST). The "special type" pathological subtypes of breast carcinoma include invasive lobular carcinoma (ILC), medullary breast carcinoma (MC), mucinous breast carcinoma, tubular breast carcinoma, and metaplastic breast carcinoma (MBC). ILC is the most common of the special types, accounting for 10-15% of the breast cancers, and is thus the second most common pathological subtype after IDC NOS. The remainder of the special types of breast carcinoma each do not account for more than 5% of breast tumors, with metaplastic breast cancer being the rarest (less than 1% of breast carcinomas).

Invasive ductal carcinoma

Invasive ductal carcinoma not otherwise specified (IDC NOS) is a very heterogeneous group of tumors which includes all breast carcinomas that cannot be classified as a special pathological subtype (Figure 1.2 A and B). The tumors are classified as IDC mixed-type tumors when a special type component of more than 50% is present in addition to the IDC NOS component. In 80% of the IDC cases a precursor lesion of ductal carcinoma *in situ* (DCIS) is present, often of high grade comedo type. Although IDC NOS is generally considered to be a diverse group of breast carcinomas that can not be assigned to one of the currently-known specialized pathological subtypes, many breast pathologists would agree that there may be one or more specialized subtypes still to be defined in this subgroup of carcinomas.

Invasive lobular carcinoma

The classical pattern of invasive lobular carcinoma (ILC) is characterized by small rounded cells with scant cytoplasm, that diffusely grow through the stroma, often in strings of cells called "Indian files" (Figure 1.2 C; [12]). In most ILC cases, a lobular carcinoma *in situ* (LCIS) component is present, although DCIS has also been observed [61, 62]. In addition to classical ILC, other variants of ILC have been described including pleomorphic, alveolar, and solid lobular carcinoma [63-66]. Classical ILC tumors are frequently low grade tumors because of the morphologically uniform cells and a low mitotic index. Therefore, they have a more favorable prognosis than the ILC variants that have more marked nuclear pleomorphism and thus are of higher grade [61, 67, 68]. Complete loss of expression of the cell adhesion molecule E-cadherin has been observed for the majority (about 80%) of the ILCs [69-73], and has been associated with truncating mutations of the *E-cadherin* gene in 50% of ILC breast cancers [74-76].

Medullary carcinoma

Medullary carcinomas (MC) are poorly differentiated carcinomas with a syncitial growth pattern, absence of glandular structures, moderate to marked nuclear pleomorphism, complete histological circumscription of the tumor, and diffuse lymphocytic infiltrate (Figure 1.2 D; [12]). MCs are typically high grade tumors and are mostly ER-negative. However, the prognosis of MC is remarkably favorable and better than common IDC NOS, with 10-year survival rates of 50-90% depending on the criteria used. This probably is because less than 10% of the patients present with lymph node metastases [77-85]. Notably, 11% of MCs carry *BRCA1* germline mutations, which is about seven times more frequent than among breast cancers as a whole [86]. Reciprocally, 13-20-% of *BRCA1* mutant tumors are medullary carcinomas or carcinomas with medullary features [86-88].

Mucinous carcinoma

Mucinous or colloid carcinoma of the breast is characterized by clusters of small and uniform cells floating in a sea of extracellular mucin (Figure 1.2 E; [12]). DCIS is found to be present in 60-75% of mucinous carcinomas and may have any of the conventional patterns of DCIS (cribriform, comedo, papillary or micropapillary). Mucinous carcinomas are typically ER-positive and mostly also PR-positive [89, 90]. Mucinous carcinoma has a very good prognosis with ten-year survival rates of 80-100%, although patients with mixed variants of mucinous carcinoma tend to do worse [91-94]. The favorable prognosis probably is because pure mucinous carcinomas infrequently metastasize [90].

Tubular carcinoma

Tubular carcinomas are very well differentiated tumors that are characterized by single-layered open tubules, absence of necrosis and/or very few mitoses and minor nuclear pleomorphism (Figure 1.2 F; [12, 95]). Almost two third of these tumors have a low-grade DCIS component, usually

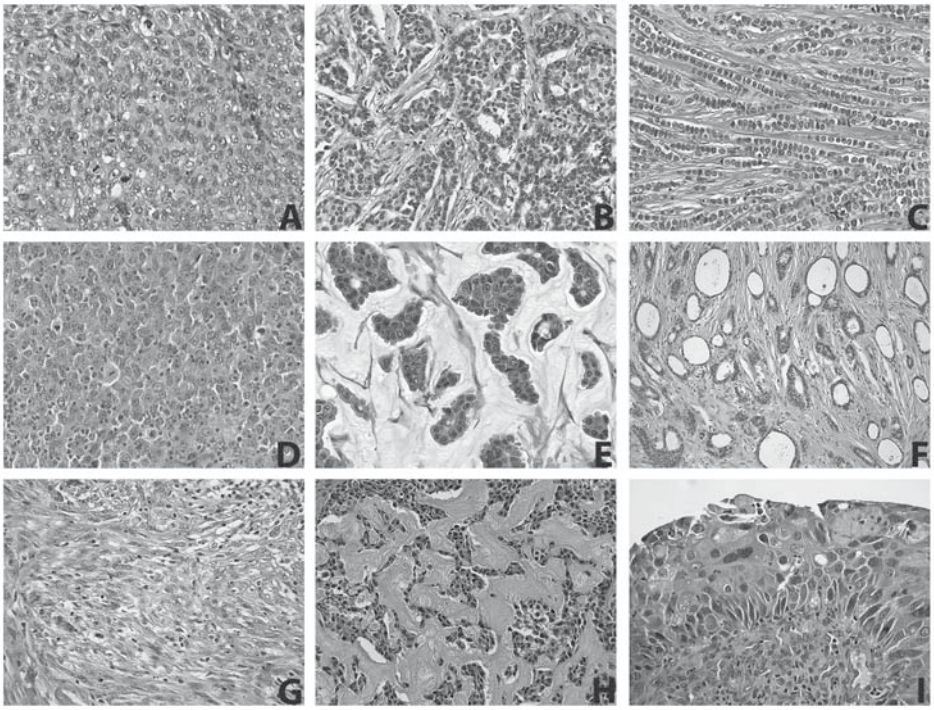

Figure 1.2 Microscopic views of histopathological subtypes of breast cancer. (A) and (B), ductal carcinoma of high and low grade, respectively; (C), lobular carcinoma with strings of cells called "Indian files"; (D), medullary carcinoma; (E), mucinous carcinoma; (F), tubular carcinoma; (G) through (I), metaplastic carcinoma of the breast with spindle, matrix-producing and squamous differentiation, respectively. This figure is also available in color in the appendix.

of cribriform or micropapillary type [96]. In comparison with IDC NOS, tubular carcinomas are more frequently ER and PR positive and EGFR and ERBB2 negative [90, 97, 98]. Similar to mucinous carcinomas, tubular carcinomas also have a particularly favorable prognosis. Although mixed variants do worse than the pure form, their prognosis still is better than IDC NOS [78, 95, 99-105]. Probably, this favorable prognosis is because tubular carcinomas tend to be smaller in size and present with less lymph node involvement at the time of diagnosis.

Metaplastic breast carcinoma

Metaplastic breast carcinoma (MBC) is a very heterogeneous group of carcinomas that are characterized by one or more prominent metaplastic components. These components can either be epithelial (squamous) or mesenchymal (matrix, spindle cell, osseous, chondroid and sarcomatous) (Figure 1.2 G through I; [12]). The mesenchymal components of MBCs frequently express vimentin and other mesenchymal markers, but in some cases epithelial characteristics are also retained, suggesting transdifferentiation rather than collision tumors [106, 107]. Importantly, genetic studies have shown that the different components in MBCs are indeed clonally

related [108-110]. MBCs are mostly ER, PR and ERBB2 negative and, as a whole, tend to have a less favorable prognosis than common IDC NOS [12]. Moreover, expression of the epidermal growth factor receptor (EGFR) is frequently observed in metaplastic breast cancers, suggesting that patients with this type of breast tumor may benefit from EGFR targeted therapy. [111].

1.2.3 GENE EXPRESSION PROFILING

Intrinsic subtypes of breast cancer

Global gene expression profiling of breast tumors has allowed additional classification of breast cancers. Large gene expression differences exist between ER-positive and ER-negative breast cancers and further molecular subclasses have been identified within these two groups [112-119]. Based on an 'intrinsic' gene set, consisting of genes that were least variably expressed between paired tumor samples from the same patient and most variably between tumors from different patients, five distinct intrinsic subtypes of breast cancer were defined. Importantly, these intrinsic subtypes were identified without supervision on the biology or clinical parameters of the tumors. The five intrinsic subtypes included the ER-negative basal-like, normal-like and ERBB2+ subtypes and the ER-positive luminal A and luminal B subtypes [115-117]. The basal-like and normal-like subtypes consist of tumors that have a high expression of the basal gene cluster (including *KRT5*, *KRT17*, *ANXA8*, *CX3CL1* and *TRIM29*) and a low expression of luminal gene cluster (including *ER*α, *GATA3*, *XBP1*, *TFF3*, *HNF3*α and *LIV1*). In addition, basal-like tumors have high expression of a novel set of genes whose coordinated function is not known (including *GGH*, *LAPTMB4*, *NSEP1* and *CCDE1*), whereas tumors of the normal-like subtype have high expression of the adipose and non-epithelial gene cluster (including *FACL2*, *AKR1C1*, *PIK3R1*). The ERBB2+ subtype is defined by tumors with high expression of genes from the *ERBB2* amplicon at chromosome 17q (including *ERBB2*, *GRB7* and *TRAP100*) and have low expression of the luminal gene cluster. Of the two luminal subtypes, tumors in the luminal A group have the highest expression of the luminal gene cluster, compared with moderate to low expression of these genes in luminal B tumors. Additionally, luminal B tumors have a relatively high expression of the novel set of genes whose coordinated function is not known, similar to basal-like tumors [116]. Importantly, these five intrinsic subtypes are conserved among different microarray platforms, different patient series and different races [120, 121].

Evidence is accumulating that the intrinsic subtypes have clinical significance (Figure 1.3). Patients with basal-like tumors had the worst overall survival, reflected by the abundance of triple negative (ER-negative, PR-negative and ERBB2-negative) tumors in this subtype, as did patients with tumors of the ERBB2+ subtype [116]. Tumors from these two intrinsic subtypes are also more sensitive to neoadjuvant chemotherapy than normal-like and luminal subtypes [114]. In addition, among the luminal subtype of tumors, the luminal B tumors had a less favorable outcome than luminal A tumors [112, 116]. The intrinsic subtypes of breast cancer also associated

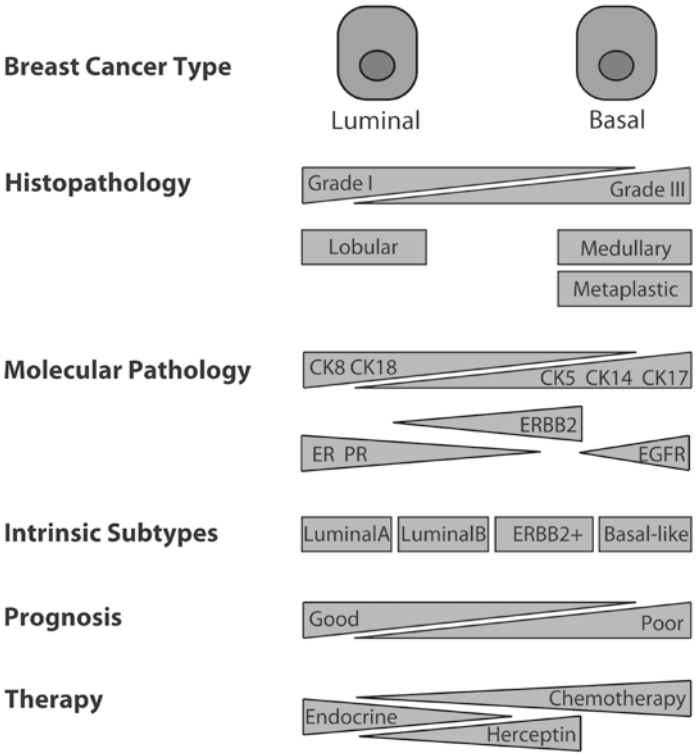

Figure 1.3 Presumed relations between breast cancer subtypes and their histopathological subtypes, expression of histological markers, intrinsic subtypes, prognosis and suggested therapy. Freely adapted from [29].

with different sites of distant metastases [122]. Breast tumors of the luminal intrinsic subtypes more frequently metastasized to bone and pleura, whereas tumors of the intrinsic basal-like and ERBB2+ subtypes more frequently metastasized to the brain. Lung metastases were most frequently observed among basal-like and luminal B breast tumors. Still, the relevance of the intrinsic gene subset lies not in its prognostic or predictive significance, but rather in its ability to capture breast cancer heterogeneity.

Prognostic gene expression profiles

Many gene expression profiles have been defined by taking clinical outcome of breast cancer patients into account. To reduce overtreatment of lymph node-negative patients with a low risk of developing metastases, a 70-gene prognostic signature was defined on tumors of lymph node-negative breast cancer patients younger than 55 years, most of whom had not received systemic treatment [123]. Partially independent validation of this gene signature showed that it was the strongest predictor of metastasis-free survival for lymph node-negative as well as lymph node-positive patients. In multivariate analysis, the 70-gene signature was independent

of the contribution of traditional prognostic factors [124]. For the same purpose, a 76-gene prognostic signature was established on tumors from lymph node- negative breast cancer patients of all age groups who had not received any adjuvant systemic treatment [125]. In contrast to the previous study, ER-positive and ER-negative tumors were analyzed separately. The 76-gene signature was a strong independent prognostic factor for metastasis-free and overall survival. Independent multicenter validation of this signature showed comparable hazard ratios to the original study [126, 127]. The 70-gene and 76-gene signatures both outperformed the NIH [128] and St. Gallen criteria [129], which are both classical clinical pathological prognostic indices, by reducing overtreatment in patients with a good prognosis [126, 130]. In addition, both signatures were as effective in selecting those high-risk patients who would be candidates for adjuvant systemic therapy as the NIH and St. Gallen criteria.

To predict metastasis in ER-positive breast cancer patients that had received adjuvant hormonal treatment, but had no local or distant metastases at the time of diagnosis, a 21-gene recurrence score (RS) was defined [131, 132]. According the RS, patients are classified in low, moderate or high-risk groups. Moreover, this signature predicted which patients in the NSABP B20 clinical trial would benefit from additional chemotherapy, showing that the prognostic signature also had relevant predictive value [133]. Currently, large prospective validation studies are underway the test the prognostic power of the 70-gene signature (MINDACT) and the 21-gene RS (TAILORx), which will provide definitive information on whether these signatures provide sufficient improvement in prognostic classification to be used for everyday clinical practice.

Prognostic gene expression signatures established by biological rather than prognostic criteria, include the genomic grade index (GGI) and the wound healing signature [134-136]. The 97-gene GGI signature was established on low versus high grade breast cancers and showed that grade II breast cancers are a mixture of grade I and III tumors rather than an intermediate grade. Importantly, the GGI signature had prognostic value for grade II breast cancers [134]. The 512-gene wound healing signature was established on the serum response of human fibroblasts but showed prognostic value in many human tumors, including breast cancer [135, 136]. The signature identified a subset of low-risk breast cancer patients among the presumed high-risk patient group and also outperformed the NIH and St. Gallen criteria in patients who had not received chemotherapy. Importantly, patients with both the wound healing signature and the poor prognosis 70-gene signature had a higher risk of metastases compared with patients without the wound healing signature and a poor prognosis 70-gene signature (47% versus 78% 10-year distant metastasis-free survival probability; [136]). This observation strongly indicates that combining various signatures may aid in risk stratification.

A valid concern regarding the various prognostic gene expression signatures was that they showed very little overlap. However, analysis of five distinct gene signatures on the same group of breast cancer patients showed a high concordance in risk stratification of patients among poor and good prognosis groups [137]. In addition, it has been shown that gene signatures that classify patients according the same clinical endpoint may include different genes but often

represent similar biological pathways, indicating that the differences among gene signatures most likely reflect differences in microarray platform and/or methodology [138].

Predictive gene expression profiles

In addition to the 21-gene recurrence score, numerous predictive gene signatures have been defined for response to hormonal treatment as well as to chemotherapy [139-147]. Patients with primary or metastatic ER-positive breast cancer are most frequently treated with the anti-estrogen tamoxifen. In the adjuvant setting, tamoxifen therapy results in a 5% and 13% improvement in survival in ER-positive lymph node-negative and lymph node-positive patients, respectively [148]. In the metastatic setting, approximately half of the patients with ER-positive tumors show intrinsic therapy resistance, while the other half shows an objective response to tamoxifen therapy. However, also almost all of the responding patients develop acquired therapy resistance at some time and eventually die of the disease. Therefore, reliable predictive factors are needed to predict the type of patients' response to tamoxifen. A 2-gene ratio of HOXB13 and IL17BR was claimed to predict the response to adjuvant tamoxifen treatment better than current clinical predictors [139]. However, this study did not have a control group of untreated patients, and the observations could have been the result of an association of the 2-gene ratio with prognosis, prediction, or both [149, 150]. Validation of this 2-gene ratio in a retrospective study then showed that it associated with both tumor aggressiveness and failure of tamoxifen treatment [149]. Similarly, a 44-gene predictor significantly correlated with the type of response to tamoxifen treatment for metastatic disease and predicted progression-free survival in multivariate analysis [140]. This tamoxifen profile was then validated in an independent series of ER-positive primary breast cancers and associated significantly with time to progression after adjusting for ER and PR [151]. For the prediction of response to chemotherapeutics, many gene expression profiles have been established. Two gene signatures have been established on tumors from patients who had received neoadjuvant docetaxel chemotherapy for primary breast cancer or locally advanced disease [141, 143]. Likewise, predictive gene signatures have been established for the response to neoadjuvant treatment with paclitaxel followed by 5-fluoracil, doxorubicin and cyclophosphamide [142, 144], neoadjuvant treatment with paclitaxel and doxorubicin [146], treatment with doxorubicin and cyclophosphamide [145] and neoadjuvant treatment with epirubicin and cyclophosphamide [147]. These predictive gene signatures are relevant for the understanding of therapy resistance and in defining the optimal treatment for breast cancer patients, thereby reducing unnecessary treatment and toxicity.

1.3 MOLECULAR GENETICS OF BREAST CANCER

Cancer is a genetic disease that involves accumulation of genetic alterations in multiple genes. These mutations enable the cell to replicate limitlessly, evade apoptosis, become insensitive

to anti-growth stimuli and self sufficient in growth signals and promote angiogenesis, invasion and metastasis [152]. Recent re-sequencing efforts of most human protein encoding genes in twenty-two breast and colorectal cancers suggested there may be as many as 15 somatic oncogenic driver mutations present in a single breast tumor [153, 154]. Importantly, the number of genes with oncogenic mutations was similar in breast cancers and colorectal cancers. Also, for both tumor types there were far more genes involved that had a low mutation frequency (gene hills) than genes with a high mutation frequency (gene mountains). However, colon cancers had more gene mountains than breast cancers [154]. This appears consistent with the fact that there is as yet no high prevalent breast cancer specific gene identified.

At least ten percent of all breast cancer cases have a family history of breast cancer. Depending on the number of affected first-degree relatives, a family history of breast cancer implies increased risk ratios of 1.5 to more than five-fold [155]. Only about 25% of the familial predisposition to breast cancer is explained by a germline mutation in one of the high-risk breast cancer susceptibility genes *BRCA1*, *BRCA2*, *p53* or *PTEN* or in the moderate-risk genes *CHEK2*, *ATM*, *BRIP1* and *PALB2* [156-163]. Recently, a genome-wide association study using single nucleotide polymorphisms identified five new breast cancer susceptibility loci, showing that some of the variation in breast cancer risk is due to common alleles [164]. As genetic linkage studies had failed to identify additional high-risk breast cancer genes, the existence of a polygenic model of breast cancer inheritance in which multiple low-risk genes act additive or multiplicative has gained much interest. Indeed, the currently known moderate-risk and low-risk susceptibility alleles all appear to operate in such a polygenic setting.

The E-cadherin/catenin complex

The *E-cadherin* tumor suppressor gene encodes a transmembrane glycoprotein that localizes to the adherence junctions of epithelial cells. Here, it mediates homophilic cell-cell adhesion between adjacent epithelial cells and thus integrity of epithelial tissues. This is established by interaction of the extracellular part of the E-cadherin molecule on one cell with the extracellular part of another E-cadherin molecule on an adjacent cell, resulting in a zipper-like structure. The C-terminal intracellular domain of E-cadherin binds to either β-catenin or γ-catenin (also known as plakoglobin), which are both proteins of the armadillo protein family and are mutually exclusive in the E-cadherin-catenin protein complex. The vinculin related α-catenin protein, in its turn, interacts with either the actin cytoskeleton or with β-catenin or γ-catenin in a dynamic fashion. The armadillo protein p120ctn also binds directly to the intracellular domain of E-cadherin, but more proximal to the cell membrane, and it stabilizes the complex [165-174].

E-cadherin reportedly is a suppressor of tumor invasion *in vitro* as well as *in vivo* and aberrant E-cadherin expression has been seen in many epithelial tumor types, including breast cancer [175-177]. The vast majority of breast cancers of the lobular subtype have lost E-cadherin protein expression, whereas in ductal breast cancers E-cadherin protein expression is mostly retained or only heterogeneously reduced [69, 70, 73, 178, 179]. Importantly, inactivating mutations of the

E-cadherin gene, located at 16q22, have only been identified in the lobular subtype of breast cancer, the diffuse subtype of gastric cancer and a small number of gynecological cancers [75, 76, 180-183]. About half of all lobular breast cancers have somatic mutations in the *E-cadherin* gene [75, 76]. In fact, mutations of *E-cadherin* in lobular breast cancer are already present in the premalignant carcinoma *in situ* stage, which makes it an early event in breast tumorigenesis [179]. The presence of *E-cadherin* somatic mutations in both lobular-type breast cancer and diffuse-type gastric cancer, which have a morphologically similar diffuse growth pattern of small rounded cells with scant cytoplasm, suggests that E-cadherin has a profound effect on cell morphology and may be causally involved in the observed characteristic histopathology. Indeed, conditional *E-cadherin* mutations in p53 knock-out mice resulted in breast carcinomas reminiscent of human invasive lobular breast cancer [184]. The causality of *E-cadherin* mutations for the lobular breast cancer phenotype makes one wonder about the absence of *E-cadherin* mutations in the other half of lobular breast cancers.

Germline mutations of *E-cadherin* have been identified in about 30% of families with predisposition to hereditary diffuse-type gastric cancer (HDGC) [185, 186]. The penetrance of autosomal dominant inherited *E-cadherin* mutations is very high and results in a cumulative life time risk of diffuse-type gastric cancer of 67% in men and 83% in women [187]. The presence of lobular breast cancers in families with HDGC suggested a role for *E-cadherin* germline mutations in families with a history of breast cancer [187-192]. However, to date only a single non-HDGC associated breast cancer family with an *E-cadherin* germline mutation has been identified [193]. Also, no *E-cadherin* germline mutations have been found in patients with LCIS, which is associated with an elevated familial breast cancer risk [194].

Also unexpected was the absence of *E-cadherin* gene mutations among carcinomas from other anatomical sites, or in the remaining breast cancers and gastric cancers. Loss of E-cadherin expression in these carcinomas was suggested to involve transcriptional silencing in association with methylation of CpG islands in the *E-cadherin* promoter region or with transcriptional repression [195-198]. The latter has been associated with expression of several transcriptional repressors of E-cadherin: Snail, Slug, SIP1, δEF1, E47 and Twist [198-205]. Specific chromatin remodeling complexes are recruited by some of these transcriptional repressors during tumorigenesis, suggesting that hypermethylation and transcriptional repression of E-cadherin is coupled [206]. The expression of E-cadherin's transcriptional repressors has been observed for various carcinoma types and has been associated with a more aggressive clinical course and with epithelial mesenchymal transition (EMT) [198]. EMT involves the conversion of polarized epithelial cells in motile cells with a mesenchymal phenotype which normally occurs during the gastrula stage of the development of an organism. In addition, this process has also been proposed to play a role in cancer metastasis [207, 208]. Loss of E-cadherin expression is considered to be one of the hallmarks of EMT, also involving (the crosstalk of) multiple pathways including the TGFβ, BMP, Wnt, RAS and PI3K pathways [207]. Interestingly, induction of EMT by ectopic expression of Snail or Twist in immortalized human mammary epithelial cells was shown to generate cells

with properties of stem cells, including acquisition of a mesenchymal phenotype, expression of stem cell markers and an increased ability to form mammospheres [209].

Besides its role in E-cadherin-mediated cell adhesion, β-catenin also plays a central role in the canonical Wnt (Wingless/INT-1) pathway. In the absence of Wnt signaling, the cytoplasmic pool of β-catenin is targeted for proteosomal degradation by a destruction complex that consists of the tumor suppressors APC en Axin and the kinases GSK3β and CK1. Activation of Wnt signaling inhibits the destruction complex. As a result, β-catenin is free to translocate to the nucleus where it interacts with TCF/LEF factors and facilitates transcription of TCF target genes [210]. In colorectal cancer, mutations of either APC, β-catenin or Axin-2 induce constitutive Wnt pathway activation, which is associated with a crypt stem cell or progenitor cell phenotype [211, 212]. The dual role of β-catenin in E-cadherin-mediated cell adhesion as well as Wnt signaling had led to the assumption that the Wnt pathway might be constitutively activated by the free β-catenin pool in E-cadherin deficient tumors [213]. However, breast cancer cell lines with either *E-cadherin* mutation or hypermethylation were shown not to have constitutive activation of the canonical Wnt signaling pathway, suggesting that aberrant activation of the canonical Wnt pathway is not of major importance in breast tumorigenesis [214].

The p53 signaling pathway

The transcription factor p53 is activated in response to DNA damage or hypoxia through phosphorylation, by among others, CHEK2 kinase. CHEK2 regulates the response to DNA damage by phosphorylating multiple substrates, including p53, CDC25C, CDC25A and BRCA1. Upon activation, p53 tetramerizes and is able to activate the transcription of downstream targets. This leads to either delayed cell cycle arrest at the G1-S cell cycle checkpoint until damage is repaired or sustained cell cycle arrest and apoptosis [215-218]. p21 is an important downstream transcription target of p53 and accumulates to levels capable of inhibiting Cyclin E and CDK2, which in turn promote progression through the G1/S checkpoint [219]. HDM2 is another protein induced by p53, which antagonizes the p53 response by binding p53 and targeting it for ubiquitination and degradation [220]. However, p14ARF is able to inhibit the interaction of HDM2 with p53 by binding HDM2, thereby stabilizing the p53 protein [221]. Cells with mutant *p53* are not capable of G1-S cell cycle arrest and its associated apoptosis, resulting in replication of damaged DNA and thus the accumulation of genetic alterations. Alternative ways of inactivating p53 are by overexpression of HDM2 or by inactivation of CHEK2 or p14ARF [221].

Mutations of the *p53* tumor suppressor gene, located on 17p13, are found in virtually every cancer type, stressing the pivotal role of p53 in different cell types and carcinogenesis. About 30-40% of clinical breast cancers have a mutation in *p53*, making *p53* the most frequently mutated gene in human breast cancer [222, 223]. Aberrant expression of p53 associates with breast cancers of the ductal subtype, a higher grade and poorer prognosis [224, 225]. In familial breast cancer patients, *p53* germline mutations are present in less than 1% of the cases [223]. Notably, germline mutations of *p53* have only been identified in families with the rare Li-Fraumeni

syndrome, that is characterized by an increased risk of breast cancer, sarcomas, brain tumors, leukemia's and adrenal tumors [226, 227].

CHEK2, located on chromosome 22q12, was first identified in association with wild-type *p53* Li-Fraumeni kindreds, suggesting that germline *CHEK2* mutations are an alternative genetic defect of Li-Fraumeni syndrome [228, 229]. As *CHEK2* founder mutations also appeared to be present at low frequency in healthy individuals it became clear that *CHEK2* could not be a susceptibility gene for Li-Fraumeni syndrome. In fact, the *CHEK2* 1100delC variant was shown to be significantly more frequent in breast cancer families than in healthy controls and it confers a modest two-fold increased breast cancer risk. *CHEK2* 1100delC thus was the first moderate-risk breast cancer susceptibility allele identified. [159, 160]. In addition, *CHEK2* 1100delC was associated with a hereditary breast and colon cancer (HBCC) phenotype, an elevated risk of bilateral breast cancer and an elevated risk for male breast cancer [159, 160, 230, 231]. *CHEK2* 1100delC breast cancers are mostly ER-positive tumors of the luminal intrinsic subtype and are of a higher grade than tumors from non-*CHEK2* 1100delC carriers (Nagel et al, submitted for publication; [232, 233]).

Amplification or overexpression of *HDM2*, located at chromosome 12q13, and mutations and deletions in *p14ARF*, located on chromosome 9q21, reportedly are uncommon in breast cancer, although hypermethylation of *p14ARF* is observed in one quarter of human breast cancers [234-236]. However, in contrast to some human cancers, inactivation of *p14ARF* frequently coincided with mutation of *p53* in breast cancer [236]. This suggests that in breast cancer *p14ARF* inactivation is not biologically similar to *p53* mutation.

The PI3K signaling pathway

The phosphatidylinositol 3-OH kinase (PI3K) pathway is pivotal for the regulation of cellular processes, including growth, proliferation and survival of cells. The main players in this pathway are PIK3CA, PTEN and the three AKT proteins. PIK3CA or p110α is released from its inhibitor p85α upon PI3K pathway activation, usually by signaling through receptor tyrosine kinases (RTKs) on the plasma membrane. Once activated, PIK3CA is able to convert phosphatidylinositol-4,5-diphosphate (PIP2) to its active form, phosphatidylinositol-3,4,5-triphosphate (PIP3). Upon production of PIP3, AKT translocates to the plasma membrane and becomes activated by phosphorylation at Thr308 and Ser473 by PDK1 and PDK2. The conversion from PIP2 to PIP3 is counteracted by the lipid phosphatase PTEN, thereby blocking the activation of AKT [237]. As constitutive signaling through the PI3K pathway is oncogenic, *PIK3CA* and *AKT* are oncogenes and *PTEN* is a tumor suppressor gene by virtue of their roles in the pathway.

PIK3CA, located at chromosome 3q26, was identified in a mutation screen of PI3K and PI3K-like genes [238] and has since been found mutated predominantly in liver, colon and breast tumors [239]. In breast cancer, *PIK3CA* is the second most frequently mutated gene after *p53*, and has been found mutated in 20-40% of breast tumors [239]. *PIK3CA* mutations have been associated with ER and PR positive tumors and tumors with ERBB2 overexpression. Importantly, mutations

of *PIK3CA* were found to be mutually exclusive with loss of PTEN expression, which is consistent with their opposing function in PI3K signaling [240].

The *PTEN* tumor suppressor gene, located on chromosome 10q23, was identified in a screen of breast cancers and glioblastomas but was found to be mutated less frequently in breast cancer (10-20%) than *PIK3CA* [241-243]. Germline mutations of *PTEN* are found in patients with Cowden disease and Bannayan-Zonana syndrome, predisposing to hamartomatous lesions and conferring an increased risk of cancer [244, 245]. Recently, loss of PTEN expression had been associated with sporadic basal-like breast cancer. In addition, large structural mutations of the *PTEN* locus, in contrast to small intragenic sequence alterations of *PTEN*, associated with *BRCA1* mutations, suggesting a role for PTEN in both sporadic as well as hereditary basal-like breast cancers [246]. In addition, a PTEN gene signature obtained from PTEN negative tumors has been associated with metastasis and poor survival of breast cancer patients [247].

AKT has three homologues in mammals of which AKT1 and AKT2 have been implicated in breast cancer. Increased AKT1 kinase activity has been observed in about 40% of human breast tumors and recently an oncogenic mutation in *AKT1* (E17K), located on chromosome 14q32, was identified in 8% of breast tumors [248, 249]. *AKT2*, located on chromosome 19q13, has been found amplified in only a minority of human breast cancers (<5%), however increased AKT2 kinase activity is present in 40% of human breast cancers [250, 251]. These data combined suggest mutational activation of the PI3K pathway in 40-70% of human breast cancers.

The RAS signaling pathway

The RAS pathway is activated by stimulation of receptor tyrosine kinases and regulates proliferation and differentiation of cells, as well as cytoskeletal rearrangements. RAS proteins are activated by guanine nucleotide exchange factors (GNEFs) that convert GDP-bound RAS to the GTP-bound state. Once activated, RAS can signal through multiple pathways, including the MAPK pathway via the kinases RAF, MEK and ERK, and the PI3K pathway via the kinase p110. Inactivation of RAS is regulated by GTPase activating proteins (GAPs) of which the most relevant for tumorigenesis is the neurofibromin1 (NF1) protein. Mutational inactivation of the *NF1* tumor suppressor gene leads to accumulation of activated GTP-bound RAS and ultimately to tumorigenesis in tissues derived from the neural crest and myeloid malignancies [252]. Germline mutations of *NF1* cause neurofibromatosis type 1, characterized by neurofibromas, iris hamartomas, café-au-lait spots and an increased risk of developing cancers, including brain tumors and leukemia's [253].

Three isoforms of human *RAS* genes exist that are implicated in human cancer: *KRAS*, *HRAS* and *NRAS*, located on chromosomes 12p12, 11p15 and 1p13 respectively. Point mutations of codons 12, 13 and 61 of the *RAS* genes have been identified in a wide variety of human tumor types, resulting in constitutively activated RAS and its downstream pathway [252, 254]. For breast cancer however, mutations in the *RAS* genes were infrequently identified (less than 10%). This

is in contrast to colon and pancreatic cancers that have mutated *KRAS* genes in 40-50% and 90-95% of the tumors, respectively [255-257].

Oncogenic hotspot mutations in *BRAF*, located on chromosome 7q34, lead to increased kinase activity, providing an alternative route to RAS pathway activation [258]. This is nicely illustrated by the mutual exclusiveness of *KRAS* and *BRAF* mutations in colorectal cancers [259]. Mutations in *BRAF* are most frequent in melanomas and colorectal tumors, but infrequent in human breast tumors (~5%, [258]). There was no evidence for mutational activation of *ARAF* and *CRAF*, the other two *RAF* isoforms in cancer [260, 261]. Interestingly, germline mutations of the RAS/MAPK pathway cause two clinically overlapping syndromes characterized by heart defects, mental retardation and distinctive facial appearances. Costello syndrome is caused by germline mutations in the *HRAS* gene, whereas germline mutations in *KRAS*, *BRAF*, *MEK1* and *MEK2* cause cardiofaciocutaneous syndrome [262-264]. Importantly, activation of the RAS pathway appears to be an infrequent (< 15%) event in breast cancer.

The RB signaling pathway

The retinoblastoma (RB) pathway is important in regulating the G1 to S-phase transition of the cell cycle. Mitogenic stimulation during the G1 phase accumulates complexes of Cyclin D1 with either CDK4 or CDK6 cyclin-dependent kinases in the cell nucleus. These complexes, together with Cyclin E/CDK2 complexes phosphorylate the RB1 gene product, inducing the subsequent release of RB1 from the E2F transcription factor. Release of RB1 from E2F initiates E2F-dependent transcription of genes, which is necessary for DNA replication and S-phase entry. Actions of the Cyclin D1/CDK complexes on RB1 are inhibited by the p16 protein. Therefore, loss of either p16 or the RB1 tumor suppressors, as well as amplification and overexpression of Cyclin D1 or CDK4 have been implicated in human tumorigenesis [265].

Germline mutations of *p16*, located on chromosome 9q21, predispose an individual to familial multiple melanoma [266, 267]. Loss of p16 is observed in about 30% of human breast cancers and occurs mainly through promoter hypermethylation, not mutation or deletion of the gene [268-271]. This is in contrast to many other tumor types that have inactivated *p16* through either deletions or point mutations, including 80% of pancreatic cancers [272-275].

Mutations of the *RB1* gene, located on chromosome 13q14, were first identified in the germline of patients with retinoblastoma, a rare childhood eye tumor. In time, somatic mutations of the gene were found in various tumor types, including osteosarcomas, small cell lung cancers and 10% of breast cancers [276-281]. In breast cancer, loss of RB1 protein expression is associated with ER-negativity and grade III ductal tumors [282].

Amplification and translocation of *Cyclin D1*, located on chromosome 11q13, has been found in various human tumors [279]. In breast cancer, *Cyclin D1* is amplified in 25% of the tumors and overexpressed in half of the tumors. Also, overexpression of Cyclin D1 is significantly associated with ER-positive breast cancers [283, 284].

Amplification of the *CDK4* gene, located on chromosome 12q13-14, is found frequently in sarcomas and gliomas and in about 15% of human breast tumors [279, 285]. An alternative mechanism of CDK4 activation is mutation of the p16 binding domain in the *CDK4* gene which is found incidentally in sporadic melanoma (less than 5%) and in melanoma-prone families (six families reported to date) [286-289].

Cyclin E and the CDK inhibitor p27kip are not known to be mutationally involved in breast cancer, but their expression (Cyclin E high/p27kip low) alone or together is associated with worse outcome of breast cancer patients [290, 291]. Overall, mutational inactivation of the RB pathway has been implicated in more than 80% of human breast cancers [292].

The BRCA1 and BRCA2 genes

BRCA1 and BRCA2 are caretakers of preserving genomic stability through their role in DNA damage signaling and repair, particularly of double strand breaks (DSBs). Both BRCA1 and BRCA2 deficient cells are unable to repair DSBs by error-free homologous replication (HR). This results in rerouting of repair by the still intact but error-prone non-homologous end-joining (NHEJ) pathway. BRCA1, however, appears to have a much broader role in DNA damage repair than BRCA2. In addition to its involvement in the BRCA1, BRCA2 and Rad51 complex, BRCA1 is also part of the BRCA1-associated genome-surveillance complex (BASC), which includes ATM, RAD50, MRE11 and NBS1 and the mismatch repair proteins MLH1, PMS2, MSH2 and MSH6. ATM, like ATR, CHEK1 and CHEK2 are able to phosphorylate BRCA1 in response to different types of DNA damage. BRCA1 is also implicated in transcription-coupled nucleotide excision repair, chromatin remodeling at DNA damage sites and ubiquitination via the BRCA1/BARD1 heterodimeric complex [293-297].

Germline mutations of the *BRCA1* gene, located on chromosome 17q21, and the *BRCA2* gene, located on chromosome 13q12, predispose a woman to an average cumulative breast cancer risk by age 70 years of 65% and 45%, respectively, when she was unselected for family history [298]. These breast cancer risk estimates are even higher in women from families with multiple breast cancer cases, as their risk may be modified by other genes. Indeed, it was shown that common breast cancer susceptibility alleles may act multiplicatively on the breast cancer risk in *BRCA1* and *BRCA2* mutation carriers [299]. Besides breast cancer, women with a *BRCA1* or *BRCA2* germline mutation have an increased risk of ovarian cancer and men have an increased risk of prostate cancer. In addition, *BRCA2* mutation carriers also have an increased risk of other cancer types such as breast cancer in men, pancreas cancer, gall bladder cancer, bile duct cancer, stomach cancer and melanoma [300]. Despite similarities in function and disease spectrum, *BRCA1* and *BRCA2* mutant tumors are very distinctive. *BRCA1* mutant tumors are frequently ER-negative, of the basal-like intrinsic subtype and more frequently of medullary histology than sporadic breast tumors [88, 117]. *BRCA2* mutant tumors, in contrast, are more frequently ER-positive. However, both *BRCA1* and *BRCA2* mutant tumors have a higher frequency of *p53* mutations than sporadic breast cancers and a higher degree of aneuploidy [87, 301]. Somatic *BRCA1* or *BRCA2*

mutations are rare, although reduced BRCA1 expression has been observed in sporadic breast cancers [302]. In these tumors, hypermethylation of CpG islands in the *BRCA1* promoter region has been observed [303, 304].

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Chapter 2

Aims and Outline of the Thesis

AIMS AND OUTLINE OF THE THESIS

Breast cancer not only is the most frequently diagnosed cancer in Western women, but also is the second leading cause of cancer death in the Western world [1]. Clinically, breast cancer has for long been recognized to be a heterogeneous disease. Currently, about two-thirds of breast cancer patients survive their disease, whereas, one-third of breast cancer patients will die of metastases of the primary cancer within 15 years from diagnosis. Therefore, it is important for clinicians to accurately predict the prognosis and most appropriate therapy for each breast cancer patient. Recent advances in large scale gene expression analysis have significantly improved prognostic and predictive stratification of the patients. Importantly, these analyses also identified five molecular subtypes of breast cancer, in concordance with the notion that breast cancer is a heterogeneous disease [2-26]. In this respect, the recent development of targeted trastuzumab therapy has indeed improved the survival of a subset of breast cancer patients that have tumors overexpressing the ERBB2 receptor tyrosine kinase [27-29]. However, appropriate molecular targets have as yet not been identified for most breast cancer subtypes, implying suboptimal treatment for a significant fraction of the breast cancer patients. Thus, a better understanding of the disease is needed to improve upon current methods to treat breast cancer patients.

Breast cancer is a genetic disease in which gene mutations may be inherited or acquired somatically. Recently, large scale re-sequencing efforts have suggested that there are as many as 15 oncogenic driver mutations present in a single breast tumor [30, 31]. One of the genes that is frequently inactivated in breast cancer is the tumor suppressor gene *E-cadherin* [32, 33]. The E-cadherin protein is essential in maintaining epithelial tissue integrity through intercellular cell adhesion [34]. Loss of E-cadherin in human breast cancer can be achieved by either mutation of the *E-cadherin* gene, hypermethylation of its gene promoter or transcriptional silencing by its repressors [35, 36]. Mutations of *E-cadherin* were shown to be causal for the lobular phenotype of breast cancer [37]. However, silencing of *E-cadherin* by promoter hypermethylation or by transcriptional repression has not properly been assessed. Notably, a discrepancy exists between the loss of E-cadherin expression and the presence of *E-cadherin* gene mutations in breast cancer. First, only half of lobular breast cancers have mutated the *E-cadherin* gene although most lobular breast cancers have lost E-cadherin protein expression. Second, E-cadherin protein expression is absent or aberrant in a significant fraction of breast cancers of non-lobular pathology, but no mutations of *E-cadherin* have been found in non-lobular breast cancers. It is not clear how the various inactivation mechanisms of *E-cadherin* are involved human breast carcinogenesis, and certainly not in relation with particular subtypes of breast carcinoma.

The aim of this thesis was to gain insight in the various mechanisms of E-cadherin inactivation in human breast cancer. For this purpose, we have evaluated the inactivation mechanisms of E-cadherin using human breast cancer cell lines as a model in **chapter 3**. Gene expression profiling and gene reconstitution experiments revealed that E-cadherin inactivation by gene

mutation represents a distinct biological mechanism from E-cadherin inactivation by promoter hypermethylation and transcriptional repression. The identification of two distinct modes of E-cadherin inactivation that associate with distinct (histopathological) subtypes of breast cancer *in vitro* as well as *in vivo* is important as it challenges the paradigm that genetic and epigenetic inactivation of a tumor suppressor gene are biologically similar. The results of this study may also explain recurrent controversies in E-cadherin research and calls for re-evaluation of functional E-cadherin studies as well as studies on the clinical outcome of patients with E-cadherin-negative breast cancers.

In **chapters 4-7**, we sought to identify the genetic mechanisms that underlie the breast cancer subtypes that associated with the two different modes of E-cadherin inactivation. Although mutations of *E-cadherin* were shown to be causal for the lobular subtype of breast cancer, only half of lobular breast cancers had mutated *E-cadherin* genes [32, 33, 37]. Therefore, we have pursued the identification of other E-cadherin pathway members as a tumor suppressor gene causal in lobular breast cancers without *E-cadherin* mutations. In **chapter 4**, we provide evidence that α-*catenin* is a putative new tumor suppressor gene. The results of this study underline the importance of the E-cadherin/catenin protein complex in cancer, as α-catenin is already the third member of this complex of which mutations contribute to the development of cancer.

Breast cancer cell lines of the basal-type were associated with epigenetic inactivation of E-cadherin and EMT, through expression of E-cadherin's transcriptional repressors. Notably, EMT involves (the crosstalk of) multiple signaling pathways, including the PI3K and RAS pathways [36]. Also, mutations of the *BRCA1* gene have been associated with basal breast tumors [26]. Therefore, we investigated whether mutations in genes of the PI3K and RAS pathways and the *BRCA1* gene might be associated with breast cancer cell lines that had inactivated E-cadherin through epigenetic mechanisms. In **chapters 5 and 6**, we performed mutation analysis of the genes of the PI3K and RAS pathways and the *BRCA1* gene in all breast cancer cell lines. Interestingly, we found an association of mutations of genes of the RAS pathway and the *BRCA1* gene, but not genes of the PI3K pathway with epigenetic E-cadherin inactivation and basal-type breast cancer. The results of these studies may provide some clues to the underlying genetic events of the basal-type breast cancers and EMT.

Finally, we set out to provide a genetic basis for the two major breast cancer subtypes that associated with the two different modes of E-cadherin inactivation. In **chapter 7**, we have performed protein and gene expression analyses and large scale mutation screens of 20 other known cancer genes in the breast cancer cell lines. This study resulted in the identification of two distinct mutation profiles that associated with luminal and basal-type breast cancer cell lines. The results of this study may provide a further refinement of current molecular breast cancer classification and aid the development of new treatment modalities that target the here identified potential drug targets.

The results of this thesis are summarized in **chapter 8** and further discussed in **chapter 9**.

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Epigenetic Silencing and Mutational Inactivation of E-cadherin Associate with Distinct Breast Cancer Subtypes

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Chapter 4

α-Catenin is a Putative New Tumor Suppressor Gene

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Chapter 5

Phosphatidylinositol-3-OH Kinase or RAS Pathway Mutations in Human Breast Cancer Cell Lines

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ABSTRACT

Constitutive activation of the PI3K and RAS signaling pathways are important events in tumor formation. This is illustrated by the frequent genetic alteration of several key players from these pathways in a wide variety of human cancers. Here, we report a detailed sequence analysis of the *PTEN*, *PIK3CA*, *KRAS*, *HRAS*, *NRAS* and *BRAF* genes in a collection of 40 human breast cancer cell lines. We identified a surprisingly large proportion of cell lines with mutations in the PI3K or RAS pathways (54% and 25%, respectively), with mutants for each of the six genes. The *PIK3CA*, *KRAS* and *BRAF* mutation spectra of the breast cancer cell lines were similar to those of colorectal cancers. Unlike in colorectal cancers, however, mutational activation of the PI3K pathway was mutually exclusive with mutational activation of the RAS pathway in all but one of 30 mutant breast cancer cell lines (p=0.001). These results suggest that there is a fine distinction between the signaling activators and downstream effectors of the oncogenic PI3K and RAS pathways in breast epithelium and those in other tissues.

INTRODUCTION

The phosphatidylinositol-3-OH kinase (PI3K) and RAS signaling pathways are pivotal to the transduction of extracellular signals to intracellular targets. Both signaling pathways may be activated by growth factors or nutrients in the cell's environment. The subsequent signaling events regulate cell metabolism, cell survival, cell cycle progression and cell growth. Upon activation, usually via receptor tyrosine kinases, PI3K converts phosphatidylinositol-4,5-diphosphate (PIP_2) to its active form, phosphatidylinositol-3,4,5-triphosphate (PIP_3). This lipid second messenger then transduces the activation signal to downstream targets, most notably members of the AKT family of serine/threonine kinases. The PIP_2 to PIP_3 conversion is counteracted by PTEN phosphatase, thus serving a negative feedback for PI3K signaling (reviewed in [1-4]). The RAS proteins are also major effectors of growth factor signaling through RTKs. Ligand-induced activation of receptor tyrosine kinases generates a cascade of signaling events, during which the RAS GTPase proteins are converted from the inactive GDP-bound state to the active GTP-bound state. Activated RAS proteins confer signals to downstream effectors, including members of the RAF family protein kinases, through interaction with their RAS binding domain. RAF kinases, in turn, further transduce the signals upon the mitogen-activated protein kinase pathway or a number of other possible effectors (reviewed in [5-7]).

Cross-talk between the PI3K and RAS signaling pathways may occur at several stages. GTP-bound RAS proteins may directly activate PI3K [8]. Further downstream, activation of the AKT pathway, through PI3K signaling, may converge with signals from the mitogen-activated protein kinase pathway, through RAS signaling, on mammalian target of rapamycin kinase [5, 9]. There are ample downstream effectors of the PI3K and/or RAS pathways, with a variety of signaling routes. Specificity of the signal transduction is determined by the activating extracellular signaling molecules, with an apparent additional specificity related to cell type and cell activation status. Particularly the unraveling of the regulation of this specificity within the PI3K and RAS signaling pathways is currently a major research challenge.

The importance of the PI3K and RAS signaling pathways for cellular processes is illustrated by their frequent mutational activation in human cancers. Cancer is a genetic disease driven by the accumulation of genetic abrogations in pathways that regulate the growth of cells, their survival and their integrity. After the *p53* tumor suppressor, members of the PI3K pathway are most frequently mutated in human cancers. Most prevalent are activating mutations in the *PIK3CA* gene, which encodes the p110α catalytic subunit of PI3K, and inactivating mutations in the *PTEN* tumor suppressor gene. *PIK3CA* amplification is found in ovarian, cervical and thyroid carcinoma [10-12], while mutations are found predominantly in liver, colon and breast tumors [13-15]. Most *PIK3CA* mutations are located in three mutational hot-spot regions in the gene sequence, which result in increased kinase activity of PI3K [13, 16]. The *PTEN* tumor suppressor gene was originally identified by genetic screens of breast cancers and glioblastomas [17, 18], but it soon became apparent that its mutational involvement also includes many other tumor types [2].

Importantly, germline *PTEN* mutations were identified in patients with Cowden Disease [19], and in patients with Bannayan-Zonana syndrome [20] (OMIM #158350 and #153480), two cancer predisposition syndromes that share clinical symptoms such as benign hamartomatous lesions. Similar symptoms are characteristic for the Tuberous Sclerosis and Peutz-Jeghers syndromes, which have been associated with germline mutations in the *TSC1*, *TSC2* and *LKB1* genes [21-24]. Each of these genes encodes downstream effectors from the PI3K signaling pathway, illustrating both the ubiquitous involvement of this pathway and its tissue specificity.

Mutational activation of the RAS signaling pathway in human cancers is mainly achieved by mutations in the *RAS* and *BRAF* genes. Although many RAS GTPases have been identified, activating oncogenic mutations have been reported for only three *RAS* isoforms: *KRAS*, *HRAS* and *NRAS*. Oncogenic *RAS* mutations appear restricted to codons 12, 13 and 61 of the proteins, resulting in constitutive active RAS GTPase. *RAS* mutations have been identified in a wide variety of human tumor types, and display tissue specificity [25]. *KRAS* is frequently mutated in pancreatic cancers and colorectal cancers, whereas mutations in *NRAS* appear to be more pronounced in melanoma and hematological cancers. Activating *BRAF* mutations are also found in many different tumor types, but their mutational involvement is particularly pronounced in melanoma [6, 26]. Oncogenic *BRAF* mutations are restricted mainly to exons 11 and 15 of the gene, and hotspot mutations have been shown to result in increased kinase activity of BRAF [26].

Oncogenic mutations in the PI3K and RAS signaling pathways have been instrumental in deciphering the biology of these pathways. Conversely, knowledge of the functional implications of oncogenic mutations has increased our understanding of human carcinogenesis, through the commonalities as well as the differences between tumor types. Few studies, however, have addressed the mutational activation of both the PI3K pathway and the RAS pathway in a single cohort of human tumor samples. Here, we report a detailed sequence analysis of six genes (*PTEN*, *PIK3CA*, *KRAS*, *HRAS*, *NRAS* and *BRAF*) that are of major importance for the PI3K and RAS signaling pathways in a collection of 40 human breast cancer cell lines.

MATERIALS AND METHODS

Breast cancer cell lines

The 40 human breast cancer cell lines used in this study are listed in Table 5.2. Cell lines EVSA-T, MPE600, and SK-BR-5/7 were kind gifts of Dr. N. de Vleesschouwer (Institut Jules Bordet, Brussels, Belgium), Dr. H.S. Smith (California Pacific Medical Center, San Francisco, CA), and Dr. E. Stockert (Sloan-Kettering Institute for Cancer Research, New York, NY), respectively. The SUM cell lines were generated in the Ethier laboratory (available at http://www.asterand.com). Cell line OCUB-F was obtained from Riken Gene Bank (Tsukuba, Japan), and all other cell lines were obtained from ATCC (Manassas, VA). All cell lines are unique and monoclonal as shown by extensive analysis of nearly 150 polymorphic microsatellite markers [27]. We were unsuccessful in

obtaining constitutional normal tissues or tumor blocks from the cell lines, precluding assessment of the somatic or germline nature of mutations.

Mutational analysis

The complete coding sequences and intron-exon boundaries of *PTEN* (ENSG00000171862) and *PIK3CA* (ENSG00000121879), as well as exons 2 and 3 of the *RAS* genes (ENSG00000133703, ENSG00000174775, ENS00000168638) and exons 7, 11 and 15 of *BRAF* (ENSG00000157764) were analyzed for genetic alterations. For each of the six genes, intronic primers were used to PCR-amplify gene-specific fragments from genomic DNA. *PTEN* transcripts were amplified from total RNA, using the Qiagen (Hilden, Germany) one-step reverse transcription-PCR kit and gene-specific exonic primers (with or without inclusion of gene-specific *HPRT* primers). For sequence analysis, amplification products were incubated with Shrimp Alkaline Phosphatase and Exonuclease-I enzymes, and subsequently sequenced with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) using an ABI3100 Genetic Analyzer. All sequence variants identified were validated by sequencing an independently amplified PCR product, and for *PTEN* mutants also by transcript sequencing. Allelic loss at the *PTEN* chromosomal locus was determined by microsatellite analysis, using markers *D10S1765, D10S1687* and *D10S1744*. Forward microsatellite primers contained a M13 sequence at their 5' end. Amplification products were obtained by using both the microsatellite primers and a FAM-labeled complementary M13 sequence in a single reaction. Product lengths were determined on an ABI3100 Genetic Analyzer. Primer sequences are available as Supplementary Data. Amplification of the *PIK3CA* locus at chromosome 3q and the *AKT2* locus at chromosome 19q was established from SNP array data that were available for 19 cell lines at www.sanger.ac.uk/cgi, with an intensity ratio cut-off of 1.5 for low-level amplification (equivalent to 3 allele copies).

Gene cloning

PTEN transcripts of cell line CAMA-1 were amplified with the Qiagen one-step reverse transcription-PCR kit, using gene-specific primers designed to include either a *Bam*HI or *Eco*RI restriction site and to span both mutations in CAMA-1 (Supplementary Data). The RT-PCR products were digested with these restriction enzymes and subsequently cloned in the multiple cloning site of the pcDNA3.0 vector (Invitrogen, Paisley, Scotland). Inserts from 14 single colonies were PCR amplified and sequenced using vector-specific primers.

Methylation analysis

Exponentially growing cells were seeded at a density of approximately 1 million cells per T75 flask, in RPMI 1640 with 10% FCS. On each of the following three days, 10 μM filter-sterilized 5-aza-2'-deoxycytidine (Sigma, Steinhein, Germany) was added to the cell cultures. On the fourth day, cells were washed with PBS at 37°C, harvested by lysis in the flask, and total RNA was isolated. As a control, cultures untreated with 5-aza-2'-deoxycytidine were included.

RESULTS

We analyzed forty human breast cancer cell lines for mutations in the *PTEN*, *PIK3CA*, *RAS* and *BRAF* genes, by direct sequencing of PCR-amplified genomic DNA fragments. Mutational analysis of all nine exons of the *PTEN* tumor suppressor gene revealed eight mutant cell lines (Table 5.1). One cell line had a homozygous deletion of exons 1 through 9 of *PTEN*, three cell lines had truncating mutations (IVS4+1G>T, 821delG, 951delACTT), and four cell lines had missense mutations (D92H, L108R, C136Y, E307K). The IVS4+1G>T splice site mutation resulted in the exact deletion of exon 4 from the encoded transcript, predicting a change in the protein sequence after codon 71 with four additional amino acids followed by a stop codon. This splice site mutation has also been identified in the germline of a patient with Cowden Disease, in two endometrial carcinomas, and in a glioblastoma [28], rendering it highly likely that this mutation is relevant for tumorigenesis. The 821delG mutation is also presumed to be oncogenic, as it resulted in a premature stop at codon 275 that has been identified in eight endometrial carcinomas [28]. The 951delACTT mutation resulted in a premature stop at codon 319 that was also found in the germline of a patient with Cowden Disease and in seven endometrial carcinomas, three glioblastomas and a prostate carcinoma [28]. The D92H and C136Y missense mutations are both presumed oncogenic, as a mutation at codon 92 was found in an endometrial carcinoma and C136Y was found in the germline of a patient with Cowden Disease [28]. The L108R mutation has never been reported in clinical cancer samples but is likely oncogenic, as it is located in the phosphatase domain of PTEN which is frequently mutated in Cowden Disease patients, Bannayan-Zonana patients, and in endometrial carcinoma [28]. The E307K mutation also has not been reported, but it is located in the C2 domain of PTEN, and neighboring codons have been found mutated in a Cowden Disease patient and in two endometrial carcinomas [28]. However, the functional significance of the E307K mutation in cell line MDA-MB-453 is unclear, as this mutation is heterozygous and we did not identify additional *PTEN* sequence alterations in this cell line. All other *PTEN* mutant breast cancer cell lines had lost the other *PTEN* allele, except for cell line CAMA-1. CAMA-1 carried the D92H mutation at one allele and had a second mutation at the other allele, where an insertion of four base pairs at position 802 was followed by a deletion of four base pairs at position 834, predicting the exchange of twelve amino acids within the PTEN protein sequence (D268_F279delins12; Figure 5.1). The biallelic nature of the mutations in CAMA-1 was confirmed by transcript analysis and by cloning and sequencing of transcript fragments, both only identifying the D92H mutation. We also identified a possible primer site polymorphism in cell line UACC893, as we were unable to PCR amplify exon 2 from genomic DNA even though sequence analysis revealed expression of the wild-type *PTEN* transcript. Analysis of *PTEN* transcript expression by RT-PCR revealed that cell lines HCC1937, MDA-MB-436 and SUM149PT did not express *PTEN* transcripts (Figure 5.2). Whereas cell line HCC1937 had a homozygous deletion of the *PTEN* gene, both MDA-MB-436 and SUM149PT had a wild-type *PTEN* gene sequence (Table 5.2). We excluded transcriptional silencing through

Table 5.1 Mutations identified in the *PTEN, PIK3CA, RAS and BRAF* genes in human breast cancer cell lines

Breast Cancer Cell Line	Affected Gene	Gene sequence	Transcript sequence	Predicted protein effect*	Oncogenic
HCC1937†	*PTEN*	HD Ex. 1-9‡	not detectable	no expression	yes
MDA-MB-468†	*PTEN*	IVS4+1G>T‡	c.del210_253 (Ex. 4)	A72fsX5	yes
BT549†	*PTEN*	821delG‡	821delG	V275X	yes
EVSA-T	*PTEN*	951delACTT‡	951delACTT	T319X	yes
CAMA-1	*PTEN*	274G>C §	274G>C	D92H	yes
		802insTAGG/ 834delCTTC §	not detectable	no expression	yes
ZR-75-1†	*PTEN*	323T>G‡	323T>G	L108R	likely
MDA-MB-415†	*PTEN*	407G>A‡	407G>A	C136Y	yes
MDA-MB-453	*PTEN*	919G>A	919G>A	E307K	likely
BT474†	*PIK3CA*	333G>C	na	K111N	yes
BT20†	*PIK3CA*	1616C>G	na	P539R	yes
BT483†	*PIK3CA*	1624G>A	na	E542K	yes
MCF-7†	*PIK3CA*	1633G>A	na	E545K	yes
MDA-MB-361†	*PIK3CA*	1633G>A	na	E545K	yes
MDA-MB-361	*PIK3CA*	1700A>G	na	K567R	likely
BT20†	*PIK3CA*	3140A>G	na	H1047R	yes
MDA-MB-453†	*PIK3CA*	3140A>G	na	H1047R	yes
OCUB-F	*PIK3CA*	3140A>G‡	na	H1047R	yes
SK-BR-5	*PIK3CA*	3140A>G	na	H1047R	yes
SUM102PT†	*PIK3CA*	3140A>G	na	H1047R	yes
SUM185PE†	*PIK3CA*	3140A>G‡	na	H1047R	yes
SUM190PT†	*PIK3CA*	3140A>G	na	H1047R	yes
T47D†	*PIK3CA*	3140A>G	na	H1047R	yes
UACC893†	*PIK3CA*	3140A>G	na	H1047R	yes
SUM159PT†	*PIK3CA*	3140A>T	na	H1047L	yes
MDA-MB-134VI†	*KRAS*	34G>C	na	G12R	yes
SK-BR-7	*KRAS*	34G>T	na	G12C	yes
SUM229PE	*KRAS*	35G>A	na	G12D	yes
MPE600	*KRAS*	35G>T	na	G12V	yes
MDA-MB-231†	*KRAS*	38G>A	na	G13D	yes
Hs578T†	*HRAS*	35G>A	na	G12D	yes
SUM159PT	*HRAS*	35G>A	na	G12D	yes
SK-BR-7	*NRAS*	182A>G	na	Q61R	yes
ZR-75-30†	*BRAF*	977T>C	na	I326T	unknown
MDA-MB-231	*BRAF*	1391G>T	na	G464V	yes
DU4475	*BRAF*	1799T>A	na	V600E	yes
MDA-MB-435s	*BRAF*	1799T>A	na	V600E	yes

Abbreviations: HD, homozygous deletion; Ex, exon; IVS, intervening sequence; del, deletion; ins, insertion; NA, not analyzed.

* Frameshift mutations are indicated by the first changed codon and the number of newly encoded codons, including premature termination codon X.

† cell lines were reported to be mutated in [17, 26, 38, 45-49].

‡ Mutations were homozygous based on sequence analysis and confirmed with polymorphic markers.

§ Mutations are heterozygous, but located on different alleles.

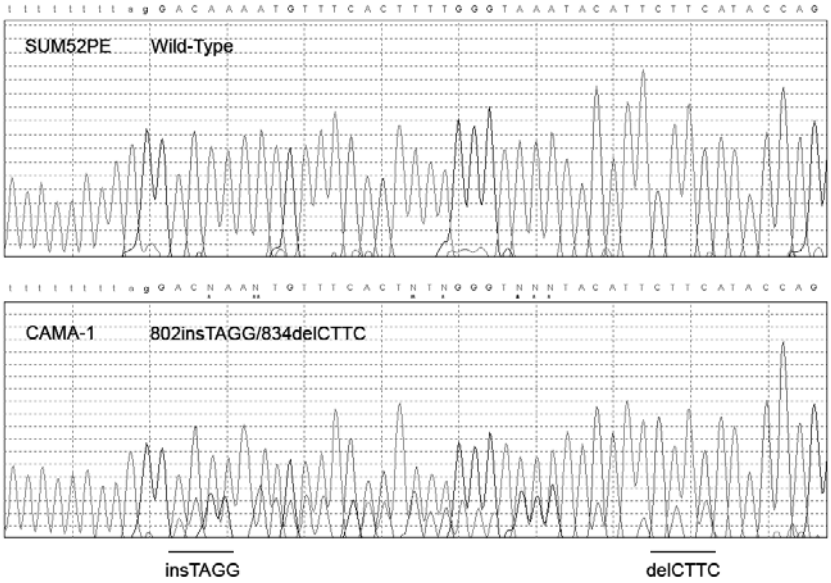

Figure 5.1 Identification of the *PTEN* 802insTAGG/834delCTTC mutation in cell line CAMA-1 by PCR amplification and sequencing of genomic DNA (bottom electropherogram). The wild-type *PTEN* gene sequence is shown for comparison (top electropherogram). This figure is also available in color in the appendix.

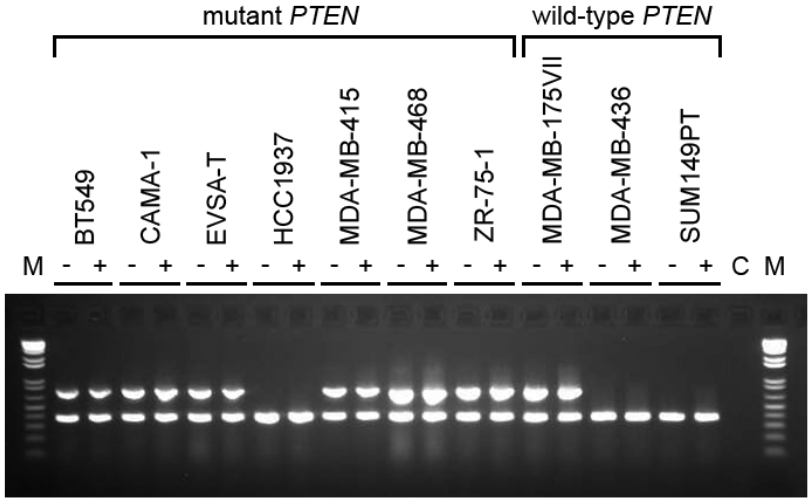

Figure 5.2 Analysis of *PTEN* transcriptional silencing through promoter methylation by cell culture in the presence (+) or absence (-) of 5-azacytidine. Reverse transcription-PCR amplification products are shown from seven mutant and three wild-type *PTEN* breast cancer cell lines, using primers specific for *PTEN* (top fragments) and the *HPRT* housekeeper (bottom fragments). These cell lines included the three cell lines without detectable *PTEN* expression, but there was no indication of *PTEN* promoter methylation. C, template negative control; M, size marker (1 kb+ DNA ladder, Invitrogen, Paisley, Scotland).

hypermethylation of the *PTEN* promoter region as a probable cause, by culturing the cell lines in the presence of the demethylating agent 5-azacytidine. As a result, neither MDA-MB-436 nor SUM149PT re-expressed *PTEN* transcripts even though 5-azacytidine did induce expression of *E-cadherin* transcripts in both cell lines (Figure 5.2 and chapter 3). Together, seven of 38 (18%) breast cancer cell lines had biallelic inactivating *PTEN* mutations, one cell line had a monoallelic missense mutation and two cell lines did not express *PTEN* transcripts for reasons unknown.

Mutational analysis of the complete coding sequence of the *PIK3CA* oncogene revealed 16 missense mutations in 14 breast cancer cell lines (Table 5.1). The mutations K111N, P539R, E542K, K567R and H1047L were each identified in one cell line, the E545K mutation was found in two cell lines, and the H1047R mutation was found in nine cell lines. All mutations except for K111N and K567R were previously identified in colon carcinomas [13], and functional analysis of the E542K, E545K and H1047R mutations had shown that these mutations were oncogenic [16, 29]. Although the K111N mutation was not previously reported in a primary cancer, this codon was found deleted in a colon carcinoma [13], suggesting that the K111N mutation is oncogenic. The K567R mutation has also not been reported, but its location in the helicase domain of PIK3CA suggests that it may have functional implications. Notably, we identified the K567R mutation in cell line MDA-MB-361, that also carried the oncogenic E545K mutation. Similarly, cell line BT20 carried both the P539R and H1047R mutations. All *PIK3CA* mutations were heterozygous, except for the H1047R mutations in cell lines OCUB-F and SUM185PE. In addition, we identified the as yet unreported synonymous 363C>T alteration in cell line MDA-MB-231, and the 1173A>G single nucleotide polymorphism in five cell lines (SNP rs3729680; heterozygous in SUM52PE, T47D and ZR-75-30, and homozygous in MDA-MB-231 and SUM149PT). Available SNP array data for nineteen cell lines revealed a single low-level amplification of 4 copies at the *PIK3CA* locus for the mutant cell line T47D and no amplifications at the *AKT2* locus, suggesting that *PIK3CA* and *AKT2* amplification is uncommon in breast cancer (average intensity ratio for *PIK3CA* was 1.1, range 0.7 to 1.9; average intensity ratio for *AKT2* was 1.0, range 0.7 to 1.4; www.sanger.ac.uk/cgi). Together, we identified activating *PIK3CA* mutations in 14 of 39 (36%) breast cancer cell lines.

Mutational analysis of exons 2 and 3 of the three human *RAS* oncogenes revealed eight heterozygous *RAS* mutations in seven of 40 breast cancer cell lines (18%; Table 5.1). We identified five cell lines with each a different *KRAS* mutation (G12C, G12D, G12R, G12V and G13D). The *HRAS* G12D mutation was found in two cell lines and the *NRAS* Q61R mutation was found once. The latter mutation was identified in cell line SK-BR-7, that also carried the *KRAS* G12C mutation. In addition to these well described oncogenic *RAS* mutations, we identified the synonymous *HRAS* 81T>C SNP in 15 cell lines (SNP rs12628; heterozygous in BT483, MDA-MB-175VII, MDA-MB-415 and SK-BR-3, and homozygous in BT20, BT474, CAMA-1, HCC1937, MDA-MB-453, MPE600, SK-BR-5, SK-BR-7, SUM149PT, SUM159PT and T47D).

Mutational analysis of exons 7, 11 and 15 of the *BRAF* oncogene revealed four of 40 breast cancer cell lines with a heterozygous *BRAF* mutation (10%; Table 5.1). We identified the I326T

Table 5.2 Mutational activation of the PI3K and RAS pathways is mutually exclusive in human breast cancer cell lines

Breast Cancer Cell Lines	PTEN	PIK3CA	KRAS	BRAF	HRAS	NRAS
BT549	V275X					
CAMA-1	D92H					
EVSA-T	T319X					
HCC1937	no protein					
MDA-MB-415	C136Y					
MDA-MB-468	A72fsX5					
ZR-75-1	L108R					
MDA-MB-453	E307K	H1047R				
BT20		P539R / H1047R				
MDA-MB-361		E545K / K567R				
BT474		K111N				
BT483		E542K				
MCF-7		E545K				
OCUB-F		H1047R				
SK-BR-5		H1047R				
SUM102PT		H1047R				
SUM185PE		H1047R				
SUM190PT	na	H1047R				
T47D		H1047R				
UACC893		H1047R				
SUM159PT		H1047L			G12D	
Hs578T					G12D	
SK-BR-7			G12C			Q61R
MDA-MB-134VI			G12R			
MPE600			G12V			
SUM229PE			G12D			
MDA-MB-231			G13D	G464V		
MDA-MB-435s				V600E		
DU4475				V600E		
ZR-75-30				I326T		
MDA-MB-157						
MDA-MB-175VII						
MDA-MB-330						
MDA-MB-436						
SK-BR-3						
SUM149PT						
SUM225CWN	na	na				
SUM1315MO2						
SUM52PE						
UACC812						
Mutation Rate	8 of 38 (21%)	14 of 39 (36%)	5 of 40 (13%)	4 of 40 (10%)	2 of 40 (5%)	1 of 40 (3%)

Note: overview of mutations that were identified in 40 human breast cancer cell lines.
The mutations are detailed in Table 5.1.
Abbreviation: NA, not analyzed.

and G464V mutations each in a single cell line and the V600E mutation was found in two cell lines. The V600E mutation is the most frequently identified oncogenic mutation in the *BRAF* gene. The G464V mutation is less frequently identified, but also considered to be oncogenic as it is located within the highly conserved G loop region [26]. Importantly, the G464V and V600E mutations both resulted in an increased activity of BRAF kinase [26]. So far, the I326T variant has only been identified in the ZR-75-30 breast cancer cell line and its functional effect is yet unknown [26]. It is important to note that the *BRAF* mutant MDA-MB-435s cell line was recently shown to be genetically identical to the M14 melanoma cell line, although it had not conclusively been investigated which of the two cell lines was correct ([30] and references therein). Since *BRAF* mutations typically associate with melanoma, one could perhaps also wonder on the origin of the other three *BRAF* mutant breast cancer cell lines. Based on gene expression and methylation profiles, there is no reason to doubt the breast origin of MDA-MB-231 [31-33]. No profiles have been reported for ZR-75-30 and DU4475, but our recent identification of a truncating *E-cadherin* mutation in cell line ZR-75-30 renders it likely that this cell line indeed is of breast origin (chapter 3). We can not be certain on DU4475, as we have as yet not identified breast-specific mutations in this cell line. But then, one never can be sure about the origin of a cancer cell line. Even so, we identified four *BRAF* mutant breast cancer cell lines or, when MDA-MB-435s and DU4475 would turn out not to be of breast origin, two *BRAF* mutants were identified.

DISCUSSION

We performed a mutational analysis of six major cancer genes from the PI3K and RAS signaling pathways in a collection of 40 human breast cancer cell lines. We identified 26 unique mutations: nine mutations in *PTEN*, seven mutations in *PIK3CA*, five in *KRAS*, one each in *HRAS* and *NRAS*, and three in *BRAF*. Four of these mutations have not yet been described in the literature (Table 5.1). In total, 30 of the 40 breast cancer cell lines had mutations in any of these six genes, 40% of which had not yet been reported (Table 5.1). This detailed mutational analysis of the PI3K and RAS pathway genes is complemented by our previously reported mutational analyses of the *E-cadherin*, *MKK4*, *p53* and *BRCA1* genes, rendering this collection of breast cancer cell lines a valuable model for functional and pharmacological studies [34-37].

Mutational activation of the PI3K signaling pathway was detected in 21 breast cancer cell lines (Table 5.2). Two cell lines were *PIK3CA* double mutants. Cell line BT20 carried the P539R and H1047R mutations, for which kinase assays had shown that the H1047R mutation resulted in a substantially higher PI3K activity [13, 16]. Cell line MDA-MB-361 carried the E545K and K567R mutations, of which only the E545K mutation had been previously identified and had been shown to increase PI3K activity [16]. It is conceivable that these *PIK3CA* double mutants reflect a progression of tumorigenesis through further mutational activation of PI3K. In this scenario,

the more oncogenic H1047R and E545K mutations would have been the second hit of the PI3K pathway in the original breast cancers. Indeed, *PIK3CA* double mutant tumors have previously been reported for three primary breast cancers and a gastric cancer [38, 39], suggesting that a two-hit mutational activation of the PI3K pathway may not be uncommon. Similarly, we identified the highly oncogenic *PIK3CA* H1047R mutation together with the *PTEN* E307K mutation in cell line MDA-MB-453. Importantly, MDA-MB-453 had retained a wild-type *PTEN* allele. As the *PTEN* E307K mutation is located in a mutational hot-spot domain [28], it appears that *PTEN* is haploinsufficient in cell line MDA-MB-453. Mutation of *PIK3CA* at its critical H1047 residue would then have been the second hit to full activation of the PI3K pathway in cell line MDA-MB-453. Of course, a two-hit activation of the PI3K signaling pathway awaits further confirmation in primary cancer specimens, allowing dissection of tumor progression by mutational analysis of the earlier premalignant tumor lesions. Either way, our observation of mutational activation of the PI3K pathway in half of human breast cancer cell lines suggests that this signaling pathway may be more important for breast carcinogenesis than currently perceived.

Mutational activation of the RAS signaling pathway was detected in 10 breast cancer cell lines (Table 5.2). We were somewhat surprised by the 13% *KRAS* mutation frequency among the breast cancer cell lines, given the general conviction that *KRAS* mutations are relatively rare in human breast cancers [25]. Two *RAS* double mutant cell lines were identified. Cell line SK-BR-7 carried the *KRAS* G12C mutation and the *NRAS* Q61R mutation, whereas cell line MDA-MB-231 carried the *KRAS* G13D mutation and the *BRAF* G464V mutation. The *BRAF* G464V mutation was shown to be a less potent activator of BRAF kinase than the more prevalent *BRAF* V600E mutation (2 and 10 times wild-type kinase activity, respectively) [26]. One again can conceive a two-hit activation of the RAS pathway, through the *BRAF* G464V mutation and subsequent mutation of *KRAS* G13D. In agreement, only *KRAS* and *BRAF* V600E mutations were reportedly mutually exclusive in colorectal cancers, and one of the four reported double mutants harbored the same combination of *KRAS* G13D with *BRAF* G464V [26, 40].

We identified an unexpected large proportion of breast cancer cell lines with mutational activation of the PI3K and RAS signaling pathways (54% and 25%, respectively). Perhaps even more surprising was that only one of the 30 mutant cell lines had mutations in both pathways (*PIK3CA* H1047L and *HRAS* G12D; Table 5.2), suggesting that mutational activation of the PI3K pathway is essentially mutually exclusive with mutational activation of the RAS pathway in breast cancer (χ^2 p=0.0012, with exclusion of SUM225CWN from the analysis, and p=0.0043 when MDA-MB-435s and DU4475 were also excluded; Table 5.2). This could imply that signals critical for breast carcinogenesis converge through the PI3K and RAS pathways, targeting a single downstream effector. Concurrent mutational activation of both the PI3K and RAS pathways would then not be observed, as double mutants would not have a selective growth advantage over single mutants. In this respect, it is of interest that the mutation spectra of genes from the PI3K and RAS pathway may differ among tumor types. For example, the *BRAF* mutation spectra of breast cancers, colorectal cancers and melanomas are each dominated by the V600E mutation.

However, these three tumor types differ in that activating *RAS* mutations occur predominantly in the *NRAS* gene in melanomas and in the *KRAS* gene in breast cancers and colorectal cancers [25]. Similarly, breast cancers and colorectal cancers share a *PIK3CA* mutation spectrum that is dominated by the H1047R, E545K and E542K mutations, whereas *PIK3CA* mutations are rare in melanomas [41]. Breast cancers and colorectal cancers thus have similar *PIK3CA*, *BRAF* and *KRAS* mutation spectra. Yet, *PIK3CA* mutations are coincident with RAS pathway mutations in colorectal cancers [42], whereas we found that in breast cancers mutational activation of the PI3K pathway was mutually exclusive with mutational activation of the RAS pathway. In melanoma on the other hand, *PTEN* mutations are coincident with *BRAF* mutations, but not with mutations of *NRAS* [43, 44]. Such differences in PI3K and RAS pathway mutations among human tumor types suggest that there is a tissue-specific distinction in the activation and transduction of signals through these oncogenic pathways, at the very least for the skin and epithelia of the colon and breast.

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Chapter 6

BRCA1 Mutation Analysis of 41 Human Breast Cancer Cell Lines Reveals Three New Deleterious Mutants

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ABSTRACT

Germline mutations of the *BRCA1* gene confer a high risk of breast cancer and ovarian cancer to female mutation carriers. The BRCA1 protein is involved in the regulation of DNA repair. How specific tumor-associated mutations affect the molecular function of BRCA1, however, awaits further elucidation. Cell lines that harbor *BRCA1* gene mutations are invaluable tools for such functional studies. Up to now, the HCC1937 cell line was the only human breast cancer cell line with an identified *BRCA1* mutation. In this study, we identified three other *BRCA1* mutants from among 41 human breast cancer cell lines by sequencing of the complete coding sequence of *BRCA1*. Cell line MDA-MB-436 had the 5396+1G>A mutation in the splice donor site of exon 20. Cell line SUM149PT carried the 2288delT mutation and SUM1315MO2 carried the 185delAG mutation. All three mutations were accompanied by loss of the other *BRCA1* allele. The 185delAG and 5396+1G>A mutations are both classified as pathogenic mutations. In contrast with wild-type cell lines, none of the *BRCA1* mutants expressed nuclear BRCA1 proteins as detected with Ab-1 and Ab-2 anti-BRCA1 monoclonal antibodies. These three new human *BRCA1* mutant cell lines thus seem to be representative breast cancer models that could aid in further unraveling of the function of BRCA1.

INTRODUCTION

Germline mutations of the *BRCA1* breast cancer susceptibility gene predispose female carriers to develop breast cancer and ovarian cancer (OMIM 113705; http://www.ncbi.nlm.nih.gov/omim/). The BRCA1 protein normally resides in a nuclear multiprotein complex, including BRCA2, BARD1, and RAD51, and the DNA damage repair proteins MSH2, MLH1, MSH6, ATM, NBS1, MRE11, RAD50, BLM, and RFC. This BRCA1-associated genome surveillance complex functions as a sensor of abnormal DNA structures, such as double-strand breaks and base pair mismatches. BRCA1 has been suggested to have a pivotal function within BRCA1-associated genome surveillance complex by coordinating the actions of damage-sensing proteins and executive repair proteins. BRCA1 may also act as a transcriptional regulator of genes involved in checkpoint reinforcement and, in complexes with BARD1, as a ubiquitin ligase (reviewed in [1-4]). Thus, mutations of *BRCA1* likely impair the repair of damaged DNA, thereby rendering the mutant cells prone to malignant transformation. To fully unravel the function of BRCA1 in DNA damage responses, cell lines with naturally occurring mutations of the gene provide invaluable research tools as they allow extensive analyses and in vitro manipulation. Only a single human *BRCA1* mutant breast cancer cell line had thus far been described (HCC1937; [5]). To identify additional mutants, we screened 41 human breast cancer cell lines for alterations in the *BRCA1* gene sequence.

MATERIALS AND METHODS

Breast cancer cell lines

The 41 human breast cancer cell lines used in this study are listed in Table 6.1. The SUM-series were generated in the Ethier laboratory (available at www.asterand.com). Cell lines EVSA-T, MPE600 and SK-BR-5/7 were kind gifts of Dr. N. de Vleesschouwer (Institut Jules Brodet, Brussels, Belgium), Dr. H.S. Smith (California Pacific Medical Center, San Francisco, CA) and Dr. E. Stockert (Sloan-Kettering Institute for Cancer Research, New York, NY), respectively. Cell line OCUB-F was obtained from Riken Gene Bank. All other cell lines were obtained from American Type Culture Collection. Extensive analysis of near 150 polymorphic microsatellite markers had shown that all cell lines are unique and monoclonal [6].

Mutation analysis

The complete coding sequence and exon-intron boundaries of *BRCA1* (Genbank U14680) were analyzed for genetic alterations in all cell lines, except for SUM44PE and ZR-75-30 (only exons 11-15 and exons 3-7 and 11-15 were analyzed, respectively). Exons 1a through 11 and 16 through 24 were PCR-amplified from genomic DNA templates and exons 12 through 15 were amplified from RNA templates, as described [7]. Amplification products were then analyzed for sequence

alterations with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), using an ABI 3100 Genetic Analyzer. All unique sequence alterations were confirmed by sequencing of an independently amplified template. This approach may allow mutations to go undetected in cell lines without allelic loss, specifically deletions when analyzing DNA and truncating mutations that result in down-regulated transcripts when analyzing RNA. Allelic loss of the *BRCA1* gene was determined by PCR-based microsatellite analysis, as described [7]. *BRCA1* and *HPRT* transcripts were concurrently amplified from RNA templates, using the Qiagen Onestep reverse transcription-PCR (RT-PCR) kit and gene-specific primers. Primer sequences are available upon request.

Immunocytochemistry

Cell lines were cultured to optimal cell density in eight T162 flasks and medium was refreshed 24 hours before harvesting. Cells were harvested by scraping, washed twice with PBS, and fixed in PBS with 2% formalin for 12 to 72 hours. Cells were then washed once with PBS, resuspended in liquidized PBS with 2% agarose, and embedded in paraffin by routine diagnostic procedures. Paraffin sections (4 μm) on Starfrost microscope slides (Knittel Gläser, Braunschweig, Germany) were routinely deparaffinized and dehydrated, and epitopes were retrieved in Tris-EDTA (pH 9.0) for 30 minutes at 100°C in a microwave oven. Slides were blocked with 2% BSA in PBS for 30 minutes at room temperature and then incubated overnight at 4°C with antibodies diluted in Normal Antibody Diluent (Scytek Laboratories, Logan, UT). Anti-BRCA1 mouse monoclonal antibodies Ab-1 (Clone MS110; 1:100 or 1 μg/ml) and Ab-2 (Clone MS13; 1:320 or 0.6 μg/ml) were both purchased from Calbiochem (Darmstadt, Germany) and isotype-matched control monoclonal antibody X0931 (1:100 or 1 μg/ml) from Dako (Glostrup, Denmark). Slides were developed using the DakoCytomation Envision System horseradish peroxidase (3,3'-diaminobenzidine) kit, with omission of the antiperoxidase treatment. Slides were counterstained for 5 seconds with hematoxylin. Both anti-BRCA1 antibodies were titrated in two-step serial dilutions on *BRCA1* wild-type cell lines. At the presumed optimal antibody dilution, both antibodies showed distinct nuclear staining and Ab-1 also gave slight cytoplasmic staining. More diluted antibodies showed only nuclear staining for both antibodies and less diluted antibodies were aspecific (examples of wild-type and mutant cell lines at several antibody dilutions are provided as Supplementary Data).

RESULTS AND DISCUSSION

Sequencing of *BRCA1* revealed eighteen different alterations in the gene sequence among 41 human breast cancer cell lines (Tables 6.1 and 6.2). Alterations were presumed to be non-pathogenic polymorphisms when they were described as such in the Breast Cancer Information Core (BIC) mutation database (http://research.nhgri.nih.gov/bic/). Together, 11 *BRCA1*

Table 6.1 *BRCA1* mutation analysis of 41 human breast cancer cell lines.

Breast cancer cell line	*BRCA1* allelic loss	*BRCA1* gene variants*	*BRCA1* mutation status	*BRCA1* transcript expression†	
BT20	Loss	-	Wild-type	+	Unmethylated
BT474	Loss	6,7,9,10,11,13,14,15	Wild-type	++	Unmethylated
BT483	No loss	-	Wild-type	++	
BT549	Loss	-	Wild-type	++	Unmethylated
CAMA-1	No loss	3,5,6,7,9,10,11,13,14,15	Wild-type	++	Unmethylated
DU4475	No loss	6,7,9,10,11,13	Wild-type	+	Unmethylated
EVSA-T	Loss	-	Wild-type	+	Unmethylated
HCC1937	Loss	17	5382insC	++	
HS578T	Loss	3	Wild-type	++	Unmethylated
MCF-7	Loss	-	Wild-type	+/-	Unmethylated
MDA-MB-134VI	No loss	3	Wild-type	++	
MDA-MB-157	Loss	10	Wild-type	++	Unmethylated
MDA-MB-175VII	No loss	7,9,10,11,13,15	Wild-type	++	Unmethylated
MDA-MB-231	Loss	3,5	Wild-type	++	Unmethylated
MDA-MB-330	No loss	3,4,5	Wild-type	++	
MDA-MB-361	Loss	7,9,10,11,13,14,15	Wild-type	++	Unmethylated
MDA-MB-415	Loss	3	Wild-type	++	
MDA-MB-435S	Loss	-	Wild-type	++	Unmethylated
MDA-MB-436	Loss	7,9,10,11,13,14,15,18	5396+1G>A	++‡	
MDA-MB-453	Loss	-	Wild-type	++	Unmethylated
MDA-MB-468	Loss	10	Wild-type	++	Unmethylated
MPE600	No loss	3,5	Wild-type	++	Unmethylated
OCUB-F	Loss	2	Wild-type	++	
SK-BR-3	Loss	7,9,10,11,13,14,15	Wild-type	++	Unmethylated
SK-BR-5	Loss	3	Wild-type	++	
SK-BR-7	No loss	3	Wild-type	++	
SUM44PE	Loss	14	Wild-type	++	
SUM52PE	Loss	7,9,10,11,13,14,15	Wild-type	++	
SUM102PT	No loss	7,9,10,11,13,15	Wild-type	+/-	
SUM149PT	Loss	8,10	2288delT	+	
SUM159PT	No loss	3,5	Wild-type	++	
SUM185PE	Loss	3	Wild-type	+	
SUM190PT	Loss	-	Wild-type	++	
SUM225CWN	Loss	7,9,10,11,13,14,15	Wild-type	++	
SUM229PE	No loss	7,9,10,11,13,14,15	Wild-type	++	
SUM1315MO2	Loss	1,7,9,10,11,13,14,15	185delAG	+	
T47D	No loss	-	Wild-type	++	Unmethylated
UACC812	Loss	10,12,16	Wild-type	++	Unmethylated
UACC893	Loss	-	Wild-type	++	Unmethylated
ZR75-1	No loss	-	Wild-type	++	Unmethylated
ZR-75-30	Loss	7,9,10,11,13,14	Wild-type	+	

* Identified *BRCA1* sequence variants are detailed in Table 6.2.

† Transcript expression based on five experiments (see text): ++, Normal transcript levels; +, Low transcript levels; +/-, Barely detectable transcripts.

‡ Two transcript lengths that both differ from the wild-type sequence (see text).

Unmethylated, no hypermethylation at the *BRCA1* promoter region, as reported elsewhere [8]

Table 6.2 *BRCA1* sequence variants among 41 human breast cancer cell lines.

Variant	Nucleotide change*	Exon	Predicted protein effect†	Type of variant‡	No. in cell lines§	No. in BIC db§
1	185delAG	2	E23fsX17	Path	1	1642
2	233G>A	3	K38K	Poly	1	5
3	561–34C>T			Poly	11	18
4	788+3G>A			UV	1	1
5	1186A>G	11	Q356R	Poly	5	57
6	2196G>A	11	D693N	Poly	3	16
7	2201C>T	11	S694S	Poly	13	25
8	2288delT	11	N723fsX13	Mut	1	0
9	2430T>C	11	L771L	Poly	13	39
10	2731C>T	11	P871L	Poly	17	38
11	3232A>G	11	E1038G	Poly	13	48
12	3537A>G	11	S1140G	UV	1	27
13	3667A>G	11	K1183R	Poly	13	41
14	4427T>C	13	S1436S	Poly	11	49
15	4956A>G	16	S1613G	Poly	11	51
16	5106–20A>G			UV	1	17
17	5382insC	20	Q1756fsX74	Path	1	1676
18	5396+1G>A		E1731del28 I1760insX8	Path	1	46

* Numbering of nucleotide changes according *BRCA1* Genbank sequence U14680 and nomenclature according the BIC mutation database (http://research.nhgri.nih.gov/bic/).

† Frame shift and insertion mutations are indicated by the first changed codon and the number of newly encoded codons, including premature termination codon X. Predicted effect of variant 18 is based on sequence analysis from both transcript lengths (see text).

‡ Path, pathogenic variant according the BIC mutation database; Poly, polymorphism or non-pathogenic variant according the BIC; UV, unclassified variant according the BIC; Mut, variant not previously reported but presumed mutant as it generates a frame shift with a premature termination codon in the transcripts and because the cell line does not express nuclear BRCA1 proteins.

§ Number of cell lines with a particular *BRCA1* variant and number of citations of the variant in the BIC mutation database by July 2005.

polymorphisms were identified among 29 of the breast cancer cell lines. Three other *BRCA1* variants had been described as unclassified variant in the BIC mutation database and were each detected once in the cell lines (788+3G>A in MDA-MB-330, and S1140G and 5106-20A>G both in UACC812). Deleterious *BRCA1* mutations were identified in four breast cancer cell lines (Tables 6.1 and 6.2). The insertion of a cytosine residue at position 5382 of *BRCA1* in cell line HCC1937 had been reported [5]. In cell line MDA-MB-436, we identified the 5396+1G>A mutation in the splice donor site of exon 20 (Figure 6.1). Analysis of *BRCA1* transcripts from MDA-MB-436 identified two transcript lengths. Sequencing revealed that one transcript had skipped exon 20, predicting an in-frame deletion of 28 amino acids in the encoded BRCA1 proteins, whereas the other transcript had spliced at a cryptic splice site in intron 20 (5396+88/89), predicting an insertion of seven amino acids encoded by intron sequences followed by a termination codon. The patient from whom MDA-MB-436 was generated had been diagnosed with adenocarcinoma

of the breast at age 39 [9], an early onset that is suggestive for hereditary breast cancer. The original tumor was not available for analysis, but the 5396+1G>A mutation has been reported 46 times in the BIC mutation database and is classified as pathogenic. In cell line SUM149PT, we identified the deletion of a thymine residue at position 2288 of *BRCA1* (Figure 6.1). The 2288delT mutation predicts a shift in the *BRCA1* reading frame with an insertion of 12 new amino acids after codon 723 followed by a termination codon. The patient was diagnosed at age 35 years with inflammatory breast carcinoma and she had a single known second-degree relative with postmenopausal breast cancer. The 2288delT mutation was not present in the germline of the patient as we did not detect the mutation in a DNA sample from her blood. Of note, the identity of the donor was confirmed by analysis of 10 microsatellite markers from three chromosomes, with heterozygosity ratios of $\geq$ 0.80 for all markers ($P < 10^{-7}$). The original tumor was not available for analysis, but the 2288delT mutation was detected in all available passages of the SUM149PT cell line. It is thus unclear whether the mutation was somatically acquired during tumorigenesis in the patient or *in vitro* during establishment or propagation of the SUM149PT cell line. Importantly, we detected the 2288delT mutation in the earliest available passage P16 and cells were only distributed to other laboratories after this passage. We identified an AG dinucleotide deletion at position 185 of *BRCA1* in cell line SUM1315MO2, predicting a shift in the *BRCA1* reading frame with an insertion of 16 new amino acids after codon 22 followed by a termination codon (Figure 6.1). The patient was diagnosed with invasive ductal carcinoma of the breast but the age at diagnosis nor the cancer history of her family is known. The original tumor was not available for analysis, but the 185delAG mutation is a well-described pathogenic *BRCA1* mutation that is prevalent in the Ashkenazi Jewish population (http://research.nhgri.nih.gov/bic/).

Allelic loss of the *BRCA1* gene was determined by PCR-amplification of microsatellite markers *D17S1321*, *D17S932*, *D17S855*, *D17S1327* and *D17S1325*. These markers are located within a 0.7-Mb chromosomal region encompassing the *BRCA1* gene at 17q21. Analysis of the markers

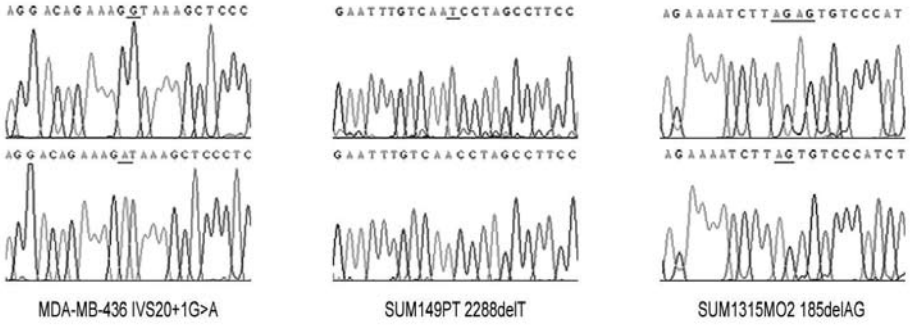

Figure 6.1 Identification of three new *BRCA1* mutant breast cancer cell lines by PCR amplification and direct sequencing. Top, electropherograms displaying the wild-type sequence. Bottom, electropherograms displaying the mutations. This figure is also available in color in the appendix.

on germline DNA's from 25 randomly selected Dutch individuals revealed heterozygosity ratios of 0.61, 0.76, 0.60, 0.55 and 0.88, respectively. Allelic loss of the *BRCA1* locus was presumed when each of the five markers had a single allele size, resulting in a reliability of $P = 0.002$ [6]. None of the 25 control DNA's had a homozygous allele pattern at the *BRCA1* locus, thus validating this statistical approach. Of the 41 breast cancer cell lines, 28 (68%) had allelic loss of the *BRCA1* locus, including the four *BRCA1* mutants (Table 6.1). Similar allelic loss frequencies have been reported for primary breast cancer specimens [10-12]. It is important to note that several regions at 17q are frequently amplified in human breast cancers. Allelic losses at 17q are therefore often underestimated, as karyotype-based methods do not detect loss when the retained allele is amplified or reduplicated [6, 13]. Indeed, we did not identify loss of the *BRCA1* locus in three *BRCA1* mutant cell lines that we analyzed by array CGH (data not shown), whereas our microsatellite analysis revealed allelic loss in all of them. Conclusively, all *BRCA1* mutants were homozygous in the sequence analysis (Figure 6.1).

BRCA1 transcript expression was analyzed by semi-quantitative RT-PCR using five overlapping PCR fragments (Table 6.1). Cell lines HCC1937 and MDA-MB-436 had *BRCA1* transcript expression levels that were comparable to those of most other cell lines, SUM149PT had variable but always lower expression levels and SUM1315MO2 had consistently low expression of *BRCA1* transcripts. In contrast with wild-type cell lines, nuclear BRCA1 protein expression was not detectable in any of the four mutant cell lines, as determined by immunocytochemistry on paraffin-embedded cells using anti-BRCA1 monoclonal antibodies Ab-1/MS110 and Ab-2/MS13 (Figure 6.2 and Supplementary Data).

We thus describe four cell lines with a *BRCA1* mutation from among 41 human breast cancer cell lines, three of which had not previously been reported. All *BRCA1* mutants had lost the other *BRCA1* allele, in accordance with the tumor suppressor function of BRCA1. Three mutations generated a premature termination codon in the *BRCA1* transcript, whereas the fourth mutation resulted in two transcripts of which one had an in-frame deletion and the other generated a premature termination codon. Three of the *BRCA1* mutations have been classified as pathogenic mutations and none of the *BRCA1* mutant cell lines expressed nuclear BRCA1 proteins. In an ongoing effort to characterize our panel of breast cancer cell lines, we identified biallelic mutations of the *p53* tumor suppressor gene in each of the four *BRCA1* mutants [14], consistent with the notion that *BRCA1* mutant tumors frequently harbor *p53* mutations (reviewed in [15]). Pending further mutational data, these *BRCA1* mutant breast cancer cell lines already are a valuable asset in pinpointing the BRCA1 functions that are critical in the suppression of breast tumorigenesis.

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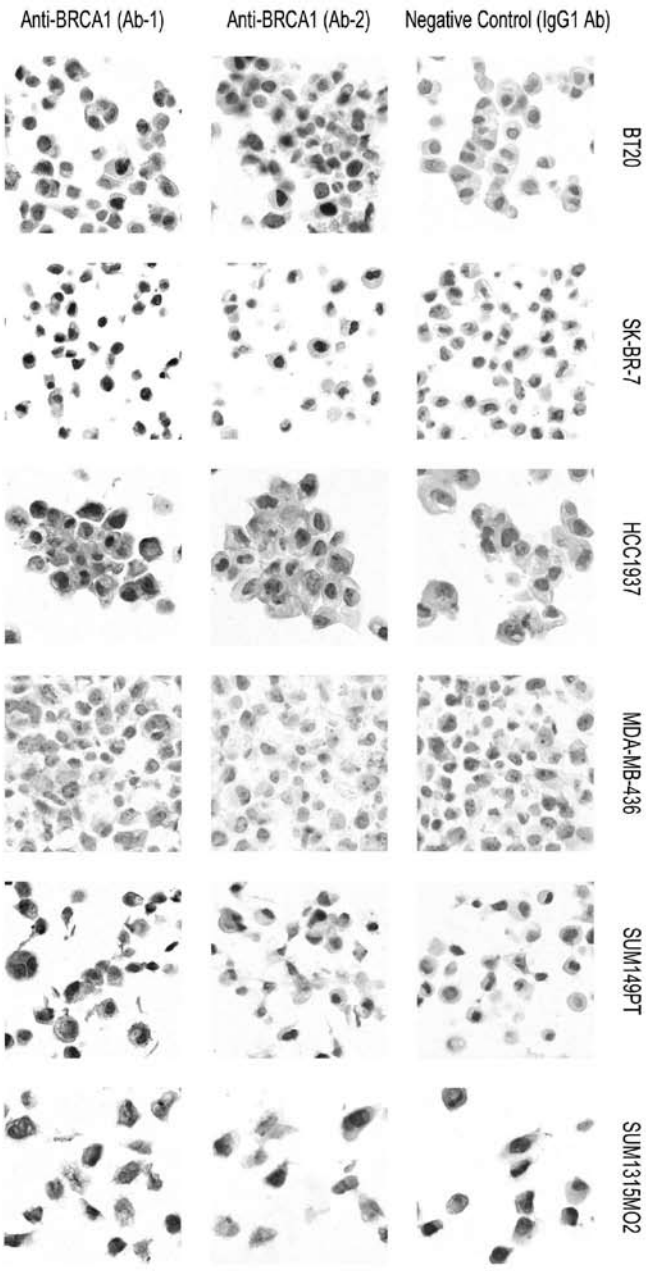

Figure 6.2 BRCA1 immunocytochemistry in *BRCA1* mutant and wild-type breast cancer cell lines. In contrast with the two wild-type cell lines (BT20 and SK-BR-7), none of the four *BRCA1* mutants had nuclear BRCA1 staining with either of the two anti-BRCA1 monoclonal antibodies Ab-1 and Ab-2. There is some cytoplasmic staining of unclear significance in all samples with Ab-1, which is not observed with more diluted Ab-1 antibodies nor with Ab-2 (see also Supplementary Data). The negative control antibody is an IgG1 isotype-matched antibody. Magnification 40X. This figure is also available in color in the appendix.

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Chapter 7

Distinct Gene Mutation Profiles Among Luminal and Basal-Type Breast Cancer Cell Lines

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Summary and Samenvatting

SUMMARY

E-cadherin is a well-known tumor suppressor gene that encodes a cell adhesion molecule involved in maintaining integrity of epithelial tissues. In breast cancer, E-cadherin was found to be inactivated by mutation of the gene in half of breast cancers of the lobular subtype. Alternatively, E-cadherin may be inactivated by hypermethylation of CpG islands in the *E-cadherin* promoter region and transcriptional repression through its repressor proteins, but this had not been associated with a particular breast cancer subtype. The studies described in this thesis were aimed at improving our understanding of E-cadherin inactivation mechanisms in breast carcinogenesis, by using a model of 41 human breast cancer cell lines. Furthermore, we sought to identify the genetic basis of the breast cancer subtypes that associated with the two different modes of E-cadherin inactivation.

Chapter 1 comprises a general introduction on the epidemiology, classification and molecular genetics of breast cancer and **chapter 2** describes the aim and outline of this thesis.

In **chapter 3** we have studied inactivation mechanisms of *E-cadherin* in the breast cancer cell lines. Mutations of *E-cadherin* were found solely in cell lines with a rounded cell morphology. In contrast, hypermethylation of *E-cadherin* and expression of its transcriptional repressors associated with cell lines with a spindle cell morphology. Gene reconstitution experiments revealed that inactivation of *E-cadherin* was causal for the rounded cell morphology but not for the spindle cell morphology. Concordantly, transcriptional profiling of the cell lines showed that cell lines with genetic and epigenetic inactivation of *E-cadherin* had vastly different gene expression programs, implying that the two inactivation mechanisms involve distinct biological pathways. Indeed, we observed mutations of *E-cadherin* only in lobular breast cancers, whereas epigenetic inactivation of *E-cadherin* associated with clinical breast cancers of the metaplastic pathological subtype. These results challenge the paradigm that inactivation of a tumor suppressor gene by promoter hypermethylation is biologically similar to mutational inactivation of the gene.

Next, we have evaluated the genetic basis of the two inactivation modes of E-cadherin in the breast cancer cell lines. In **chapter 4** we have searched for another cancer gene in the E-cadherin mutational tumor suppressor pathway. Like in lobular breast cancers, not all breast cancer cell lines with the rounded cell morphology had a mutant *E-cadherin* gene. Because we had shown that E-cadherin was causal for the rounded cell phenotype, we hypothesized that another gene in the E-cadherin pathway was mutated in the rounded cell lines with wild-type *E-cadherin*. Expression and mutation analyses of the E-cadherin-associated proteins α-, β-, γ-catenin and p120ctn in the cell lines revealed that α-*catenin* was genetically inactivated in the rounded cell lines with wild-type *E-cadherin*, suggesting that α-*catenin* is a new tumor suppressor gene involved in lobular breast cancer. Indeed, loss of α-catenin protein expression specifically associated with clinical breast cancers of the lobular subtype. These results thus suggest α-*catenin* as a putative new tumor suppressor gene.

In **chapter 5** we have analyzed the breast cancer cell lines for mutations of PI3K and RAS pathway genes. Inactivation of the PI3K pathway, through mutations of *PIK3CA* and *PTEN*, was identified in 21 cell lines, but the mutations were equally prevalent among *E-cadherin* mutant and methylated cell lines. RAS pathway mutations, in *KRAS*, *HRAS*, *NRAS* and *BRAF*, were identified in nine cell lines. Interestingly, eight of the eleven RAS pathway mutations were found in *E-cadherin* methylated cell lines, suggesting an association with this inactivation mechanism of E-cadherin. Moreover, we found that unlike in colorectal cancers, mutational activation of the PI3K pathway was mutually exclusive with mutational activation of the RAS pathway. This suggests that there is a fine distinction between the signaling activators and downstream effectors of the oncogenic PI3K and RAS pathways in breast epithelium and colonic epithelium.

In **chapter 6** all breast cancers cell lines were analyzed for mutations in the *BRCA1* gene. We identified three new human *BRCA1* mutant cell lines that seem to be representative breast cancer models that could aid in further unraveling of the function of BRCA1. Importantly, three of the four cell lines with deleterious *BRCA1* mutations had epigenetic inactivation of E-cadherin, suggesting an association of *BRCA1* mutation with *E-cadherin* methylation.

In **chapter 7** we have performed a large scale molecular characterization of the breast cancer cell lines. Protein and transcript expression analysis revealed that the cell lines resemble two major subtypes of luminal and basal-type breast cancers. Mutation analysis of 27 well-known cancer genes identified 146 oncogenic mutations, including 92 inactivating mutations among 12 tumor suppressor genes and 52 activating mutations among 12 oncogenes. The mutational data combined revealed two gene mutation patterns among the cell lines. First, we identified frequent mutations among genes of the p53, PI3K and RB pathways. Second, we identified distinct mutational profiles specific for the luminal and basal-types of breast cancer. The luminal mutation profile was observed among 21 of 25 luminal-type cell lines and involved the mutation of *E-cadherin* and *MAP2K4*, as well as amplification of *ERBB2*, *CyclinD1* and *HDM2*. The basal mutation profile was observed among 14 of the 15 basal-type cell lines and involved *RB1*, *BRCA1*, *RAS* and *BRAF* gene mutations and *p16/p14ARF* deletions. The identification of two subtype-specific mutation profiles among the breast cancer cell lines provides a genetic basis for luminal and basal-type breast cancer.

SAMENVATTING

E-cadherine is een bekend tumorsuppressorgen dat codeert voor een celadhesiemolecuul dat de integriteit van epitheliale weefsels bewaakt. In borstkanker wordt E-cadherine geïnactiveerd door mutatie in de helft van de borstkankers van het lobulaire subtype. Als alternatief kan E-cadherine geïnactiveerd worden door hypermethylatie van CpG-eilanden in de promotorregio van het *E-cadherine* gen of door transcriptionele onderdrukking door repressoreiwitten, maar dit was nog niet geassocieerd met een bepaald subtype van borstkanker. De studies die zijn beschreven in dit proefschrift beogen het verbeteren van ons begrip van deze E-cadherine inactivatiemechanismen in borstkanker door onderzoek te doen aan 41 humane borstkankercellijnen, die model staan voor borstkanker bij de mens. Daarnaast hebben we getracht andere genetische veranderingen te identificeren in de borstkankersubtypen die associeerden met de twee verschillende manieren van E-cadherine inactivatie.

Hoofdstuk 1 omvat een algemene inleiding in de epidemiologie, classificatie en moleculaire genetica van borstkanker en in **hoofdstuk 2** worden de doelstellingen en de opzet van dit proefschrift beschreven.

In **hoofdstuk 3** wordt verslag gedaan van onderzoek naar de inactivatiemechanismen van *E-cadherine* in de borstkankercellijnen. Mutaties in het *E-cadherine* gen werden enkel gevonden in borstkankercellijnen met een rondcellige morfologie. Dit in tegenstelling tot hypermethylatie van *E-cadherine* en expressie van de transcriptionele repressoreiwitten, die associeerden met borstkankercellijnen met een spoelcellige morfologie. Genreconstitutie experimenten lieten zien dat inactivatie van *E-cadherine* oorzakelijk was voor de rondcellige morfologie, maar niet voor de spoelcellige morfologie. In overeenstemming met deze bevinding werd gevonden dat de genexpressieprofielen van de cellijnen met genetische en epigenetische inactivatie van *E-cadherine* immens verschillen. Dit impliceert dat de twee inactivatiemechanismen verschillende biologische routes betreffen. We vonden mutaties van *E-cadherine* inderdaad alleen in lobulaire borstkankers, terwijl epigenetische inactivatie van *E-cadherine* associeerde met borstkanker van het metaplastische subtype. Deze resultaten ondermijnen het paradigma dat inactivatie van een tumorsuppressorgen door promotorhypermethylatie biologisch gelijk is aan inactivatie door mutatie van het gen.

Vervolgens hebben wij de genetische basis van de twee manieren van E-cadherine inactivatie in de borstkankercellijnen geëvalueerd. In **hoofdstuk 4** wordt de zoektocht naar andere kankergenen in de E-cadherine tumorsuppressorroute beschreven. Net als in lobulaire borsttumoren hadden niet alle borstkankercellijnen met een rondcellige morfologie een mutatie in het *E-cadherine* gen. Omdat wij hebben laten zien dat E-cadherine oorzakelijk is voor de rondcellige morfologie was onze hypothese dat een ander gen in de E-cadherine signaaltransductieroute gemuteerd moest zijn in cellijnen met rondcellige morfologie zonder een mutatie in het *E-cadherine* gen. Expressie en mutatie analyse van de met E-cadherine geassocieerde eiwitten α-, β-, γ-catenine en p120ctn in de cellijnen liet zien dat α-*catenine* juist genetisch

geïnactiveerd is in de cellijnen met rondcellige morfologie zonder een gemuteerd *E-cadherine* gen. Dit suggereert dat α-*catenine* een tumorsuppressorgen is dat genetisch is geïnactiveerd in lobulair borstkanker. Inderdaad, verlies van α-catenine eiwitexpressie werd specifiek gevonden in borstkanker van het lobulaire subtype. Deze resultaten suggereren dat α-*catenine* vermoedelijk een nieuw tumorsuppressorgen is.

In **hoofdstuk 5** rapporteren wij de analyse van mutaties in genen van de PI3K en RAS signaaltransductieroutes in borstkankercellijnen. Inactivatie van de PI3K signaaltransductieroute, door mutaties in de genen *PIK3CA* en *PTEN*, werd geïdentificeerd in 21 cellijnen, maar de mutaties kwamen even vaak voor in *E-cadherine*-mutante en gemethyleerde cellijnen. Mutaties in genen van de RAS signaaltransductieroute werden gevonden in negen cellijnen. Interessant was dat acht van de elf mutaties in genen van de RAS signaaltransductie route gevonden werden in *E-cadherine* gemethyleerde cellijnen, wat een associatie met dit E-cadherine inactivatiemechanisme suggereert. Daarnaast hebben we gevonden dat, in tegenstelling tot darmtumoren, activatie door mutatie van de PI3K en de RAS signaaltransductieroutes elkaar uitsluit. Dit suggereert dat er een subtiel verschil is tussen de signaalactiveerders en de stroomafwaartse effectors van de oncogene PI3K en RAS signaaltransductieroutes in borstepitheel en die in darmepitheel.

In **hoofdstuk 6** is de analyse beschreven van mutaties in het *BRCA1* gen in borstkankercellijnen. We hebben drie nieuwe humane *BRCA1* mutante cellijnen geïdentificeerd. Deze cellijnen lijken representatieve modellen die verder kunnen bijdragen aan het ontrafelen van de functie van BRCA1. Belangrijk is dat drie van de vier cellijnen met een mutatie in het *BRCA1* gen ook epigenetische inactivatie van *E-cadherine* hadden. Dit suggereert een associatie van *BRCA1* mutatie met *E-cadherine* methylatie.

In **hoofdstuk 7** rapporteren wij een grootschalige moleculaire karakterisatie van de borstkankercellijnen. Eiwit- en transcriptanalyses brachten aan het licht dat de cellijnen onderverdeeld kunnen worden in twee hoofdsubtypen van borstkanker, namelijk het luminale en het basale type borstkanker. Mutatieanalyse van 27 bekende kankergenen identificeerde 146 oncogene mutaties, waarvan 92 inactiverende mutaties in 12 tumorsuppressorgenen en 52 activerende mutaties in 12 oncogenen. Alle mutatiedata gecombineerd maakten twee genmutatiepatronen onder de borstkankercellijnen zichtbaar. Als eerste vonden we dat genen uit de p53, PI3K and RB signaaltransductieroutes vaak gemuteerd waren. Ten tweede identificeerden we verschillende mutatieprofielen die specifiek waren voor de basale en luminale typen van borstkanker. Het luminale mutatieprofiel was aanwezig in 21 van de 25 luminale borstkankercellijnen en omvatte zowel mutaties van *E-cadherine* en *MAP2K4*, als amplificatie van *ERBB2*, *CyclinD1* en *HDM2*. Het basale mutatieprofiel was aanwezig in 14 van de 15 basale borstkankercellijnen en omvatte *RB1*, *BRCA1*, *RAS* en *BRAF* genmutaties en *p16/p14ARF* deleties. De identificatie van twee subtypenspecifieke mutatieprofielen onder de borstkankercellijnen voegt een genetische dimensie toe aan de huidige, expressiegebaseerde indeling in luminale en basale typen van borstkanker.

Chapter 9

General Discussion

GENERAL DISCUSSION

In this thesis we have evaluated E-cadherin inactivation mechanisms by using human breast cancer cell lines as a model. We found that genetic inactivation of E-cadherin was biologically distinct from epigenetic inactivation and expression of E-cadherin's transcriptional repressors. Mutational inactivation of E-cadherin associated with a rounded cell morphology in breast cancer cell lines and with clinical breast cancers of the lobular subtype, whereas epigenetic silencing by promoter hypermethylation associated with a spindle cell morphology in cell lines and with clinical metaplastic breast cancers. Importantly, the two inactivation mechanisms of E-cadherin associated with vastly different transcriptional programs that we linked to luminal-type and basal-type breast cancers. Mutation analysis of 27 cancer genes then revealed that the two inactivation mechanisms also associated with distinct gene mutation profiles. The luminal mutation profile included mutation of *E-cadherin* and *MAP2K4* and amplification of *ERBB2*, *Cyclin D1* and *HDM2*. The basal mutation profile included hypermethylation of *E-cadherin*, mutation of *BRCA1*, *RB1*, and *RAS* pathway genes and deletion of *p16/p14ARF*.

Genetic versus epigenetic E-cadherin inactivation in breast cancer

The observation that genetic and epigenetic inactivation of *E-cadherin* involve distinct biological pathways explains recurrent controversies regarding both the functional role and prognostic value of E-cadherin. Whereas functional studies had suggested that inactivation of the *E-cadherin* tumor suppressor gene is involved in the invasion stage of carcinogenesis [1, 2], *E-cadherin* gene mutations were found to be already present in the premalignant carcinoma *in situ* stage of human breast carcinoma [3]. These studies initially appear contradictory. However, Frixen *et al.* and Vleminckx *et al.* had based their conclusions on experiments involving manipulation of an *E-cadherin* hypermethylated cell line and spindle-shaped v-*ras*-transformed MDCK cells, respectively. This suggests that epigenetic inactivation mechanisms are involved in the invasion steps of breast tumorigenesis, whereas mutation of *E-cadherin* is an earlier event in tumorigenesis. The association of the distinct modes of *E-cadherin* inactivation with their involvement at different time points in breast tumorigenesis thus strengthens our observed association with distinct biological pathways.

Recurrent discrepancies also exist among the prognostic value of E-cadherin as these studies are mostly based on loss of E-cadherin protein expression alone. However, the results of this thesis suggest that E-cadherin protein expression is not a good marker to assess the prognostic role of E-cadherin inactivation. First of all, loss of E-cadherin protein expression does not distinguish inactivation by mutation from inactivation by hypermethylation. Second, tumors that have hypermethylated *E-cadherin* genes without loss of E-cadherin protein expression will not correctly be assigned to the '*E-cadherin* hypermethylation' group. And third, tumors with in-frame mutations of *E-cadherin* will not correctly be assigned to the '*E-cadherin* mutant' group. Proper assessment of the prognostic value of E-cadherin would require analysis of both inactivation

modes separately and it is therefore necessary to also include the 4-protein signature of Nielsen *et al.*, (ERBB2, ER, CK5, EGFR) as well as our 3-protein spindle signature (CAV1, VIM, CALD1) to aid correct classification of the *E-cadherin* hypermethylated tumors ([4] and chapter 3). The correct classification of mutant *E-cadherin* breast tumors still poses a problem as tumors with in-frame mutations of *E-cadherin* can not be detected by immuno staining for E-cadherin and the two aforementioned protein signatures. Recently, p120ctn protein expression was shown to have diagnostic utility in discriminating lobular from ductal breast cancers [5]. In lobular breast cancers p120ctn was present in the cytoplasm only, whereas in ductal carcinomas p120ctn was present at the cell membrane. Immuno staining of our breast cancer cell lines for p120ctn showed that E-cadherin expressing cell lines with in-frame mutations had cytoplasmic staining, as did cell lines with truncating mutations of *E-cadherin* and E-cadherin protein loss (unpublished results), thereby making the correct classification of *E-cadherin* mutated cell lines possible. The ability of p120ctn to identify not only truncating *E-cadherin* mutants, but also in-frame *E-cadherin* mutants makes p120ctn a better protein marker for the classification of lobular breast cancer than E-cadherin. Therefore, pathologists in doubt of classifying a breast carcinoma as either lobular or ductal should strongly consider using p120ctn as a routine marker in the classification of lobular breast cancer.

The identification of distinct modes of E-cadherin inactivation challenges the paradigm that mutation and hypermethylation of a tumor suppressor gene are two means to the same end. Hypermethylation of *E-cadherin*, but not mutation of *E-cadherin*, associated with expression of the transcriptional repressors of E-cadherin and with a spindle cell morphology (chapter 3). Expression of the transcriptional repressors and a spindle cell morphology has been associated with epithelial to mesenchymal transition (EMT) and loss of E-cadherin is regarded as one of the hallmarks of EMT [6]. EMT involves the transdifferentiation of epithelial cells that express E-cadherin to cells with a mesenchymal phenotype that no longer express E-cadherin. Recently, induction of EMT by ectopic expression of Snail or Twist in immortalized human mammary epithelial cells was shown to generate cells with properties of stem cells, including acquisition of a mesenchymal phenotype, expression of stem cell markers and an increased ability to form mammospheres [7]. Our results that *E-cadherin* hypermethylation associated with expression of the transcriptional repressors and with a spindle cell morphology would therefore imply that EMT is associated with hypermethylation of the *E-cadherin* promoter, but not mutation of the *E-cadherin* gene. Moreover, we found that loss of E-cadherin through epigenetic mechanisms was not causal for the spindle cell morphology, nor was E-cadherin protein expression lost in all *E-cadherin* hypermethylated spindle cell lines (chapter 3). It is likely that inactivation of *E-cadherin* by hypermethylation is only a secondary effect of the EMT process, that may rather reflect the differentiation state of the cell than an oncogenic event that drives tumorigenesis. Importantly, our results question whether loss of E-cadherin expression is a hallmark of EMT.

The differential β-catenin protein expression pattern of genetically and epigenetically-inactivated *E-cadherin* breast cancer cell lines also is of interest. β-Catenin protein expression,

but not transcript expression, was absent or decreased in all *E-cadherin* mutant cell lines that had deleted E-cadherin's β-catenin binding domain (chapter 3). Most likely, failure of β-catenin proteins to interact with E-cadherin results in phosphorylation and ubiquitylation of the β-catenin pool and subsequent degradation by the APC/GSK3β destruction complex. Consistent with this notion, we observed restoration of β-catenin protein expression in SK-BR-3 cells upon reconstitution of E-cadherin's β-catenin binding domain. In contrast, β-catenin proteins were expressed in all breast cancer cell lines with epigenetic silencing of *E-cadherin* (chapter 3). Indeed, it recently was shown that several aspects of EMT required β-catenin expression, and that this β-catenin pool was largely unphosphorylated [8]. It appears that β-catenin has a dual role in cell adhesion and in EMT, similar to E-cadherin. The recent identification of frequent mutations in the Wnt pathway members β-*catenin*, *APC* and *WISP3* among metaplastic breast cancers — the pathological breast cancer subtype that we have associated with EMT — may provide a genetic basis for β-catenin's role in EMT [9]. Interestingly, we have found no evidence for canonical Wnt pathway activation in the spindle breast cancer cell lines [10]. However, it is still possible that non-canonical Wnt signaling is involved in EMT through expression regulation of E-cadherin's repressor Snail by GSK3β [11, 12].

Genetic inactivation of *E-cadherin* has been observed only in lobular breast cancers and has been shown to be causal for this subtype of breast cancer (chapter 3 and [13-15]). This implies that mutation of *E-cadherin* evokes a cell morphology change which we had expected to involve downstream signaling. However, we could not measure the biological effect of *E-cadherin* gene mutation by gene expression analysis of *E-cadherin* wild-type epithelial cell lines versus *E-cadherin* mutant rounded cell lines (chapter 4). This suggests that either gene expression changes upon mutation of *E-cadherin* are very small, the downstream pathway affects only protein expression, kinase activity and/or cellular localization, or the tumor suppressive effect of *E-cadherin* mutations is solely based on loss of cell-cell adhesion. Alternatively, genes in a putative downstream signaling pathway of E-cadherin could be mutated in the wild-type *E-cadherin* cell lines with an epithelial phenotype. In this scenario, mutation of this particular gene (or genes) would have no impact on cell morphology as it signals downstream of the cytoskeleton in the E-cadherin pathway. If this premise is accurate, gene expression profiling of a mutant *E-cadherin* breast cancer cell line versus its isogenic cell line reconstituted with wild-type *E-cadherin* cDNA should reveal differential gene expression. Importantly, it would render the E-cadherin pathway far more important in breast cancer than currently thought.

Mutation profiles among cancer signaling pathways

According the pathway theory [16], mutations of genes from the same signaling pathway are expected to occur in a mutually exclusive fashion because the tumorigenic clone would not acquire a selective survival advantage upon mutation of a second gene in a pathway. In chapter 4 we have identified mutations of α-*catenin* in four breast cancer cell lines, suggesting that α-*catenin* is a new tumor suppressor gene. Strikingly, all cell lines with the rounded cell

morphology had mutations in either α-*catenin* or *E-cadherin*. This mutually exclusive mutation pattern was consistent with our observation that *E-cadherin* gene mutations were causal for the rounded cell morphology of breast cancer cell lines (chapter 3). However, one α-*catenin* mutant (MDA-MB-157) did not have a rounded cell morphology but a spindle cell morphology and had loss of E-cadherin through promoter hypermethylation. Thus, MDA-MB-157 has inactivated both E-cadherin pathways: the E-cadherin mutation pathway by α-*catenin* gene mutation and the E-cadherin epigenetic pathway in association with *E-cadherin* promoter hypermethylation. The dominant morphological phenotype of the E-cadherin epigenetic pathway was consistent with E-cadherin reconstitution experiments that we had performed, where the spindle cell morphology of the *E-cadherin* hypermethylated cell line MDA-MB-231 was not affected by reconstitution with wild-type *E-cadherin* cDNA (chapter 3). The expression of E-cadherin proteins in some of the *E-cadherin* hypermethylated spindle cell lines also suggested that inactivation of *E-cadherin* by hypermethylation is secondary to the spindle cell morphology and the distinct differentiation program that goes along with this genotype. The presence of an α-*catenin* mutation in a cell line with spindle cell morphology and hypermethylation of *E-cadherin* thus only further substantiates our finding that genetic inactivation and epigenetic inactivation of the E-cadherin pathway are biologically distinct.

Mutual exclusiveness of gene mutations among different cancer signaling pathways may also reflect functional cross talk between these pathways. For example, we found that *RAS* and *BRAF* mutations were mutually exclusive with *PTEN* and *PIK3CA* mutations in breast cancer cell lines (chapter 5). However, in melanomas and colorectal cancers, mutations of the RAS and PI3K pathways were not mutually exclusive. In melanomas, mutations of *PTEN* coincided with *BRAF* mutations, but not *NRAS* mutations [17], whereas *PIK3CA* mutations coincided with *KRAS* or *BRAF* mutations in colorectal cancers [18]. Together, these observations suggest that RAS and BRAF signal through the PI3K pathway in breast cancers but not in melanomas and colorectal cancers. It thus seems that there is a distinction in upstream activators and downstream effectors of the PI3K signaling pathway that is inherent to the different tissues.

Mutual exclusiveness of gene mutations in genes from the same signaling pathway not necessarily implies similar biological effects. Consistent with the pathway theory, we found that mutations of the RB pathway genes *RB1*, *p16* and *Cyclin D1* were mutually exclusive (chapter 7). However, we observed a duality among cell lines with mutational activation of the RB pathway by *RB1* or *Cyclin D1* mutations. *RB1* inactivation was found in ER-negative cell lines while *Cyclin D1* amplification was found in ER-positive cell lines. Importantly, this association with ER status had also been observed in primary breast cancers [19, 20]. The preference of these two RB pathway genes for either ER-negative or ER-positive breast cancers suggests that there is a fine functional distinction between mutation of each gene. This has not only been observed for different genes within a signaling pathway, but also for distinct inactivation mechanisms within a single gene. For example, *E-cadherin* mutation and hypermethylation, although occurring mutually exclusive, associate with distinct subtypes of breast cancer, implying distinct

biological pathways (chapter 3). Similarly, *p16* mutation appears to be mutual exclusive with mutation of *Cyclin D1* and *RB1*, whereas hypermethylation of *p16* concurrently occurs in *Cyclin D1* mutants (chapter 7). Importantly, the pathway theory does not hold true if mutual exclusive mutation patterns associate with distinct biological pathways.

Concurrent occurrence of mutations in genes from the same biological pathway also may imply distinct biological functions of the genes. By mutational analysis of the p53 pathway members, we found that mutations of *CHEK2* and *p53* were not present in the same breast cancer cell lines, although they often occurred concurrent with mutations of *p14ARF*, *c-MYC* or *HDM2* (chapter 7). These results suggested that *p14ARF*, *c-MYC* or *HDM2* mutations have a distinct or additional biological function from *p53* or *CHEK2* mutations. This is supported by the finding that triple knock-out mice lacking functional p53, HDM2 and p14ARF proteins developed multiple tumors at a greater frequency than mice lacking functional p53 and HDM2 or p53 alone [21]. It is possible that mutation of multiple genes within the same pathway has an additive effect in carcinogenesis. In this respect, it is of interest that all nine *p53/c-MYC* and *p53/p14ARF* double mutants had *p53* missense mutations. One could envision that *p53* missense mutations are less deleterious than truncating or deletion mutations and that the concurrent occurrence of mutations amongst genes from the p53 pathway reflects an additive functional effect instead of distinct biological functions of the genes. Similarly, mutations of *p53* are more frequently observed among *BRCA1* and *BRCA2* mutant tumors. Interestingly, the functional effects of *p53* mutations observed in *BRCA1* and *BRCA2* mutant tumors tend to be distinct from those observed in sporadic cases, suggesting an additive effect of *BRCA1* and *BRCA2* mutations on a *p53* mutation [22]. Indeed, all four *BRCA1* mutant breast cancer cell lines also had mutations of the *p53* gene (chapter 6). We also found double mutants among the PI3K, RAS and RB pathways that harbor a strong oncogenic mutation together with a less oncogenic mutation, likely as a result from either heterozygosity or the functional effect of the mutation (chapters 5 and 7). We observed double mutants having two mutations in one single gene, as well as double mutants harboring mutations in two different genes from the same tumorigenic pathway. Of course, the assumption then is that the less oncogenic mutation arose first in the tumor. Concurrent occurrence of mutations in genes within the same signaling pathway may thus not necessarily imply distinct functions of these genes.

Thus, the pathway theory of mutual exclusive mutation patterns as a result of similar biological effects may not always hold true. Genes that function in the same tumorigenic pathway may have (slightly) different biological effects when these genes specifically associate with distinct cancer subtypes. This does not only apply to different genes from a signaling pathway but also to different inactivation mechanisms within a single gene. In addition, concurrent occurrence of mutations in two genes from a signaling pathway may not always imply distinct functions of these genes. Large scale cancer gene analysis as the one conducted in chapter 7 of this thesis may therefore contribute to the unraveling of molecular mechanisms of tumorigenesis.

Mutation profiles among luminal and basal-type breast cancers

ER expression has for years been the major classifier among breast cancers and breast cancer patients are treated according their tumor's ER status. In concordance, ER also was a major discriminator in gene expression profiling [23-31]. ER-positive tumors were mainly luminal tumors, whereas ER-negative tumors were mostly of the normal-like, basal-like or ERBB2+ intrinsic subtypes. However, the subdivision among ER was not perfect, with a minority of ER-positive tumors being of the "ER-negative" intrinsic subtypes, and vice versa. Our Pearson correlation based on the gene expression profiles of the breast cancer cell lines identified two clusters of cell lines that had an immense difference in their differentiation programs, largely associating with ER status. Additional characterizations revealed that the two clusters resembled normal luminal and basal epithelial cells. Importantly, mutations of *E-cadherin* were present in luminal-type breast cancer cell lines, whereas hypermethylation of *E-cadherin* associated with basal-type breast cancers. To be precise, all *E-cadherin* hypermethylated cell lines with loss of E-cadherin protein expression were of the normal-like intrinsic subtype, whereas those of the basal-like intrinsic subtype had retained expression of E-cadherin proteins. Apart from their differential E-cadherin protein expression, we could also distinguish normal-like from basal-like cell lines by their loss of expression of both luminal and basal cytokeratins. However, expression of vimentin, N-cadherin and the epithelial growth factor receptor (EGFR) was found in both basal-like and normal-like cell lines. In addition, we identified a so-called basal mutation profile for both normal-like and basal-like cell lines, involving deletion of *p16* and *p14ARF* and mutation of *RB1*, *BRCA1*, *RAS* and *BRAF*. Together, these results suggested that breast cancers of the basal-like and normal-like intrinsic subtypes constitute two ends of a spectrum of basal-type breast cancers. We also identified a luminal mutation profile for luminal and ERBB2+ intrinsic subtype breast cancer cell lines. The luminal mutation profile involved mutation of *E-cadherin* and *MAP2K4*, and amplification of *ERBB2*, *Cyclin D1* and *HDM2*. Thus, the ERBB2+ and luminal intrinsic subtypes of breast cancer also appear to constitute two ends of a spectrum, with amplification of *HDM2* and mutation of *MAP2K4* being the discriminatory events. We therefore propose that there exist two major subtypes of breast cancer: the luminal and basal-types, and that these are subdivided in the luminal and ERBB2+ intrinsic subtypes and the basal-like and normal-like intrinsic subtypes, respectively.

The identification of two distinct mutation profiles provides a genetic basis for luminal and basal-type breast cancers and aids our understanding of breast tumorigenesis. Importantly, the mutation profiles suggest three plausible scenarios for breast tumorigenesis. In the first "transdifferentiation" scenario, all breast cancers arise from a luminal lineage-restricted progenitor cell, initially all as luminal-type breast cancer. Breast cancers of the basal-like intrinsic subtype arise from luminal-type breast cancers by an EMT-like transdifferentiation. Normal-like breast cancers then represent cancers with fully completed EMT, defined by loss of E-cadherin expression and loss of luminal and basal cytokeratin expression. We found that mutations of *p53*, *PIK3CA* and *PTEN* are not associated with a particular breast cancer subtype and they

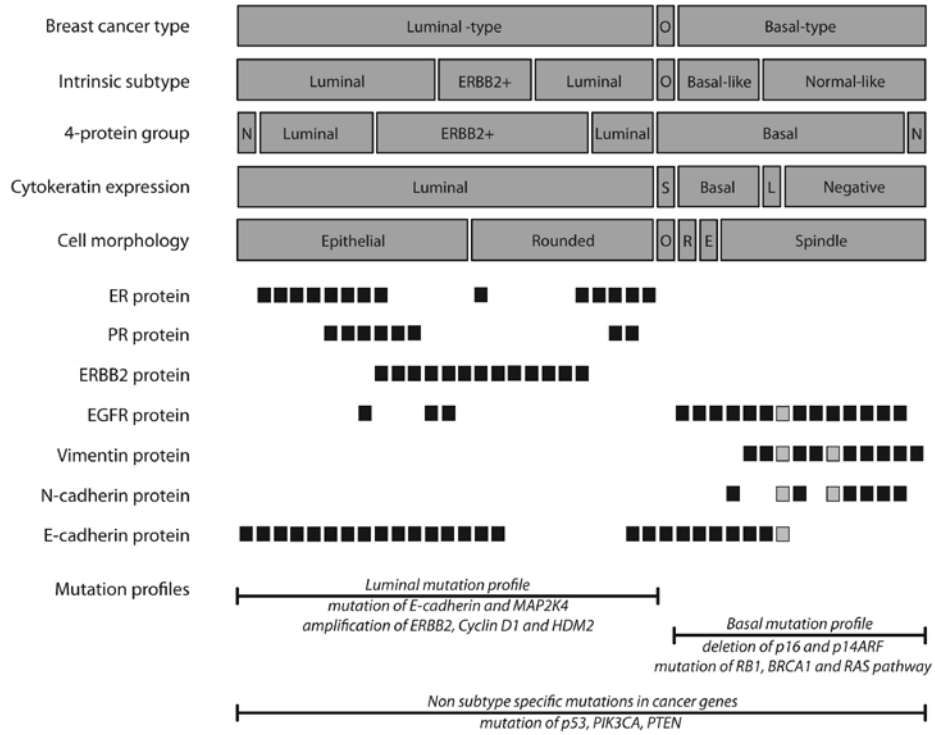

Figure 9.1 Schematic representation of the molecular characterization of 41 breast cancer cell lines. Breast cancer cell lines were classified according breast cancer type, intrinsic subtype, 4-protein group, cytokeratin expression and cell morphology. O, other; N, negative; S, stem cell-like; L, luminal; R, rounded; E, epithelial. Protein expression of ER, PR, ERBB2, EGFR, Vimentin, N-cadherin and E-cadherin is indicated by black boxes. Gray boxes represent cell lines that have not been tested but are anticipated to express a protein.

would therefore represent early events in breast tumorigenesis, whereas mutations of subtype-specific genes necessarily occur after the tumor becomes dedicated either to remain luminal or to transdifferentiate to the basal-type. Alternatively, subtype-specific mutations may be causal in determining whether the tumor remains luminal-type or becomes basal-type. EMT would then be driven by mutations of genes from the basal mutation profile, implying that these mutations regulate the activation and inactivation of EMT-associated pathways. In the second "distinct cell of origin" scenario, luminal and basal-type breast cancers arise from different cell lineages: luminal-type breast cancers arise from normal luminal epithelial cells or from a lineage-restricted luminal progenitor cell in the breast, whereas basal-like intrinsic subtype breast cancers arise from normal basal epithelial cells or from a lineage-restricted basal progenitor cell in the breast. Normal-like intrinsic subtype breast cancers then again are dedifferentiated basal-like breast cancers. In the "distinct cell of origin" scenario, the subtype-specific mutations may reflect necessities for malignant transformation in each of the different cell lineages. In contrast, mutations of *p53*, *PTEN* and *PIK3CA* would be necessary for tumorigenesis in all cell

lineages. In the third "common progenitor cell" scenario, both luminal and basal-type breast cancers arise directly from a single cell, being either a bi-potential progenitor cell or a stem cell in the breast. In this scenario, subtype-specific mutations would be causal in determining whether the tumor becomes luminal-type or basal-type. In contrast, mutations of *p53*, *PTEN* and *PIK3CA* would represent driving events all breast cancers.

Experimental evidence for any of these three scenarios for human breast tumorigenesis is limited. We have shown that metaplastic breast cancers are of the basal-type (chapter 3), even though they frequently also contain a luminal component. Importantly, the distinct components within metaplastic breast tumors have been shown to be clonally related by gene mutation analysis [32-34]. The coexistence of both luminal and basal components within a single breast tumor seems to argue against the "distinct cell of origin" and "common progenitor cell" scenarios and to favor the "transdifferentiation" scenario. However, the rarity of metaplastic breast cancers (about 1% of all breast cancers) does not seem to support transdifferentiation as a general mechanism. In this respect, it should also be realized that it is possible in the "common progenitor cell" scenario that the tumorigenic clone undergoes expansion before committing to luminal or basal-type breast cancer. A recent SAGE profiling study of clinical breast cancers is noteworthy because it showed that both CD24+ and CD44+ components may be present within single breast tumors [35]. Since the CD44 versus CD24 SAGE signature was highly reminiscent to our 1144-gene spindle cell signature, it might be inferred that CD24+ cells are luminal-type cells and CD44+ cells are basal-type. Shipitsin *et al*. thus have provided evidence that luminal and basal components also coexist in other breast cancer subtypes than metaplastic breast cancer, favoring the "transdifferentiation" scenario. However, they also determined that the CD24+ and CD44+ components were genetically identical in some breast tumors but that in other breast tumors the CD24+ component contained additional genetic aberrations, suggesting that these cells had undergone further clonal evolution and rendering the "transdifferentiation" scenario less likely. However, it had not been specified what proportion of breast tumors contained both CD24+ and CD44+ components, nor what proportion of breast tumors had genetically distinct components [35]. To distinguish between the "transdifferentiation" and "common progenitor cell" scenarios, further research should thus focus on determining how often luminal and basal components coexist within breast tumors and whether or not coexistence is restricted to particular pathological subtypes of breast cancer. Either way, based on these data, the "distinct cell of origin" scenario seems an unlikely mechanism in breast tumorigenesis.

In order to distinguish between the "transdifferentiation" and "common progenitor cell" scenarios, it would be helpful to gain insight in the timing of the mutations in the subtype-specific genes as well as the non-subtype-specific genes. For example, if mutations in basal subtype-specific genes prove to be early events in breast tumorigenesis, it would argue against the "transdifferentiation" scenario. The cell of origin of the luminal and basal-types of breast cancer could be revealed through genetic manipulation of the distinct cell lineages in normal breast epithelium, by introducing mutant oncogenes or siRNA-silencing of wild-type tumor suppressor

genes from the basal and luminal mutation profiles. However, such experiments might require further characterization of the different cell types present in normal breast epithelium and their cellular hierarchy. Similarly, introducing mutant oncogenes or siRNA-silencing of wild-type tumor suppressor genes of the basal and luminal mutation profiles in either luminal or basal-type breast cancer cell lines, respectively, may reveal whether the "transdifferentation" scenario is involved in breast tumorigenesis. Such experiments may also provide clues as to which of the genes from the basal mutation profile would be causally involved in the transdifferentiation process and whether the ability to transdifferentiate is a property of all or only a subset of luminal-type breast cancer cell lines.

Even without complete understanding of how breast cancers evolve, the mutation profiles identified in this thesis may already allow further refinement of current molecular breast cancer classification and aid the development of new treatment modalities that target the here identified potential drug targets. However, our mutation profiles include only a proportion of the genes mutated in breast cancer and extension of the number of subtype-specific cancer genes, for example with those genes found in recent whole genome screens [36, 37], may draw a more complete landscape of the breast cancer genome. But most importantly, the here identified mutation profiles will need to be confirmed in uncultured, clinical breast cancers.

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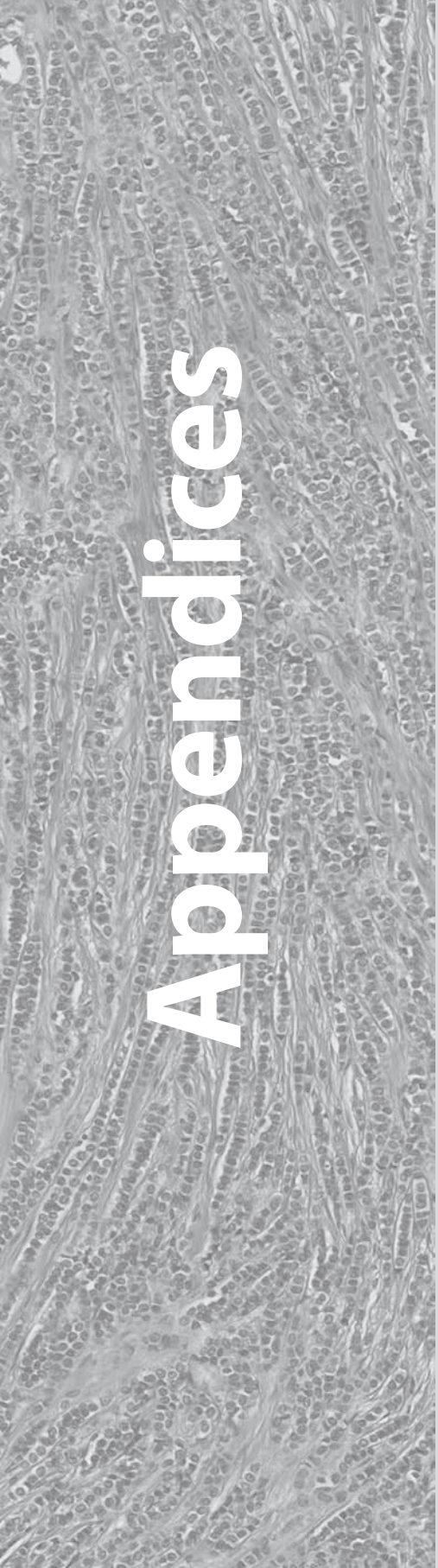

Appendices

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DANKWOORD

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Lieve Grace, jouw steun, betrokkenheid, vertrouwen en liefde geven mij de kracht om dingen te doen die voor mij in de eerste instantie te hoog gegrepen lijken. Wij vullen elkaar precies goed aan en samen zijn wij een geweldig "team". Ik ben zo dankbaar dat ik jou in mijn leven mag hebben. Op naar het volgende hoogtepunt in ons leven.

LIST OF PUBLICATIONS

Publications related to this thesis

α-*Catenin* is a putative new tumor suppressor gene.
Antoinette Hollestelle, Fons Elstrodt, Mieke Timmermans, Anieta Sieuwerts, Justine K. Peeters, Jan G.M. Klijn, Peter J. van der Spek, John A. Foekens, Michael A. den Bakker, and Mieke Schutte.
In preparation

Distinct gene mutation profiles among luminal and basal type breast cancer cell lines.
Antoinette Hollestelle*, Jord H.A. Nagel*, Marcel Smid, Suzanne Lam, Fons Elstrodt, Marijke Wasielewski, Ser Sue Ng, Pim J. French, Justine K. Peeters, Marieke J. Roozendaal, Muhammad Riaz, Ellen C. Zwarthoff, Amina Teunisse, Peter J. van der Spek, Jan G.M. Klijn, Stephen P. Ethier, Hans Clevers, Aart G. Jochemsen, Michael A. den Bakker, John A. Foekens, John W.M. Martens, and Mieke Schutte.
Submitted for publication

Epigenetic silencing and mutational inactivation of *E-cadherin* associate with distinct breast cancer subtypes.
Antoinette Hollestelle, Justine K. Peeters, Marcel Smid, Leon C. Verhoog, Pieter J. Westenend, Mieke Timmermans, Alan Chan, Jan G.M. Klijn, Peter J. van der Spek, John A. Foekens, Michael A. den Bakker, and Mieke Schutte.
Submitted for publication

Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines.
Antoinette Hollestelle, Fons Elstrodt, Jord H.A. Nagel, Wouter W. Kallemeijn, and Mieke Schutte.
Mol Cancer Res 2007; 5(2): 195-201

BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants.
Fons Elstrodt, Antoinette Hollestelle, Jord H.A. Nagel, Michael Gorin, Marijke Wasielewski, Ans van den Ouweland, Sofia D. Merajver, Stephen P. Ethier, and Mieke Schutte.
Cancer Res 2006; 66(1): 41-45

Other publications

Low-risk susceptibility alleles in 40 human breast cancer cell lines.
Muhammad Riaz, Fons Elstrodt, Antoinette Hollestelle, Jan G.M. Klijn, Mieke Schutte.
Submitted for publication

Exon expression arrays as a tool to identify new cancer genes.
Mieke Schutte, Fons Elstrodt, Linda B.C. Bralten, Jord H.A. Nagel, Elza Duijm, Antoinette Hollestelle, Maartje Vuerhard, Marijke Wasielewski, Justine K. Peeters, Peter van der Spek, Peter A. Sillevis Smitt, Pim J. French.
PloS ONE 2008; 3(8): e3007

Deleterious *CHEK2* 1100delC and L303X mutants identified among 38 human breast cancer cell lines.
Marijke Wasielewski, Pejman Hanifi-Moghaddam, Antoinette Hollestelle, Sofia D. Merajver, Ans van den Ouwenland, Jan G.M. Klijn, Stephen P. Ethier, and Mieke Schutte
Breast Cancer Res Treat 2008; DOI 10.1007/310549-008-9942-3

The *CHEK2* 1100delC mutation identifies families with a hereditary breast and colorectal cancer phenotype.
Hanne Meijers-Heijboer, Juul Wijnen, Hans Vasen, Marijke Wasielewski, Anja Wagner, Antoinette Hollestelle, Fons Elstrodt, Renate van den Bos, Anja de Snoo, Grace Tjon A Fat, Cecile Brekelmans, Shantie Jagmohan, Patrick Franken, Paul Verkuijlen, Ans van den Ouwenland, Pamela Chapman, Carli Tops, Gabriela Moslein, John Burn, Henry Lynch, Jan Klijn, Riccardo Fodde, and Mieke Schutte.
Am J Hum Genet 2003; 72(5): 1308-1314

Low penetrance susceptibility to breast cancer due to *CHEK2* 1100delC in noncarriers of *BRCA1* or *BRCA2* mutations
Hanne Meijers-Heijboer, Ans van den Ouwenland, Jan G.M. Klijn, Marijke Wasielewski, Anja de Snoo, Rogier Oldenburg, Antoinette Hollestelle, Mark Houben, Ellen Crepin, Monique van Veghel-Plandsoen, Fons Elstrodt, Cornelia van Duijn, Carina Bartels, Carel Meijers, Mieke Schutte, Lesley McGuffog, Deborah Thompson, Douglas F. Easton, Nayanta Sodha, Sheila Seal, Rita Barfoot, Jon Mangion, Jenny Chang-Claude, Diana Eccles, Rosalind Eeles, D. Gareth Evans, Richard Houlston, Victoria Murday, Steven Narod, Tamara Peretz, Julian Peto, Catherine Phelan, Hong Xiang Zhang, Csilla Szabo, Peter Devilee, David Goldgar, P. Andrew Futreal, Katherine L. Nathanson, Barbara L. Weber, Nazneen Rahman, and Michael R. Stratton.
Nat Genet 2002; 31(1): 55-59

Mutant *E-cadherin* breast cancer cells do not display constitutive Wnt signaling
Marc van de Wetering, Nick Barker, I. Clara Harkes, Marcel van der Heyden, Nicolette J. Dijk, Antoinette Hollestelle, Jan G.M. Klijn, Hans Clevers, and Mieke Schutte.
Cancer Res 2001; 61(1): 278-284

Book chapters

Representational difference analysis as a tool in the search for new tumor suppressor genes.
Antoinette Hollestelle and Mieke Schutte.
In: Methods in Molecular Medicine, Pancreatic Cancer Methods and Protocols. Humana Press, Totowa, New Jersey, 2004

PHD PORTFOLIO

A summary of PhD training and teaching activities

Name PhD student:	Antoinette Hollestelle
Erasmus MC department:	Medical Oncology
Research school:	Postgraduate school Molecular Medicine
PhD period:	February 2005-December 2008
Promotor:	Prof.Dr. J.G.M. Klijn
Supervisor:	Dr. M. Schutte

1. PhD TRAINING

	Year	Workload
General academic skills		
-Biomedical English writing and communication	2006	3.0 ECTS
Research skills		
-Microsoft Access	2002	0.9 ECTS
-Classical methods for data analysis	2005	5.7 ECTS
-Risk and quality management in laboratories	2005	0.3 ECTS
In-depth courses		
-Molecular medicine	2000	1.9 ECTS
-Oncogenesis and tumor biology	2003	1.5 ECTS
-From development to disease	2003	0.9 ECTS
-Cellular responses to DNA damage and cancer risk	2004	1.5 ECTS
-Analysis of microarray gene expression data	2004	0.6 ECTS
-SNPs and human diseases	2005	1.5 ECTS
-Prognostic research	2005	1.4 ECTS
-Clinical trials and drug risk assessment	2005	1.4 ECTS
-In vivo imaging: from cell to organism	2005	1.5 ECTS
-Comparative gene analysis: from yeast to man	2006	1.5 ECTS

Presentations

-Annual oral presentation at the JNI Scientific Lab meetings	2004-2008	1.5 ECTS
-Oral presentation at the annual Tumor Cell Biology meeting of the Dutch Cancer Society in Lunteren	2005	0.3 ECTS
-Poster presentation at the annual Molecular Medicine Day in Rotterdam	2005	0.3 ECTS
-Poster presentation at the annual meeting of the American Association for Cancer Research (AACR) in Los Angeles, CA, USA	2007	0.3 ECTS

International conferences

-Annual meeting of the American Association for Cancer Research (AACR) in Los Angeles, CA, USA	2007	1.5 ECTS

Seminars and workshops

-Monthly JNI Oncology Lectures	2005-2008	0.9 ECTS
-Annual Tumor Cell Biology meeting of the Dutch Cancer Society in Lunteren	2005	0.6 ECTS
-Annual Molecular Medicine Day in Rotterdam	2005	0.3 ECTS
-Master class with Prof.dr. Mary-Claire King	2006	0.3 ECTS

2. TEACHING ACTIVITIES

	Year	Workload
Supervising practicals and bachelor's thesis		
-Internship of HLO student Wouter Kallemeijn May 2005–February 2006	2005-2006	10.8 ECTS

COLOR FIGURES

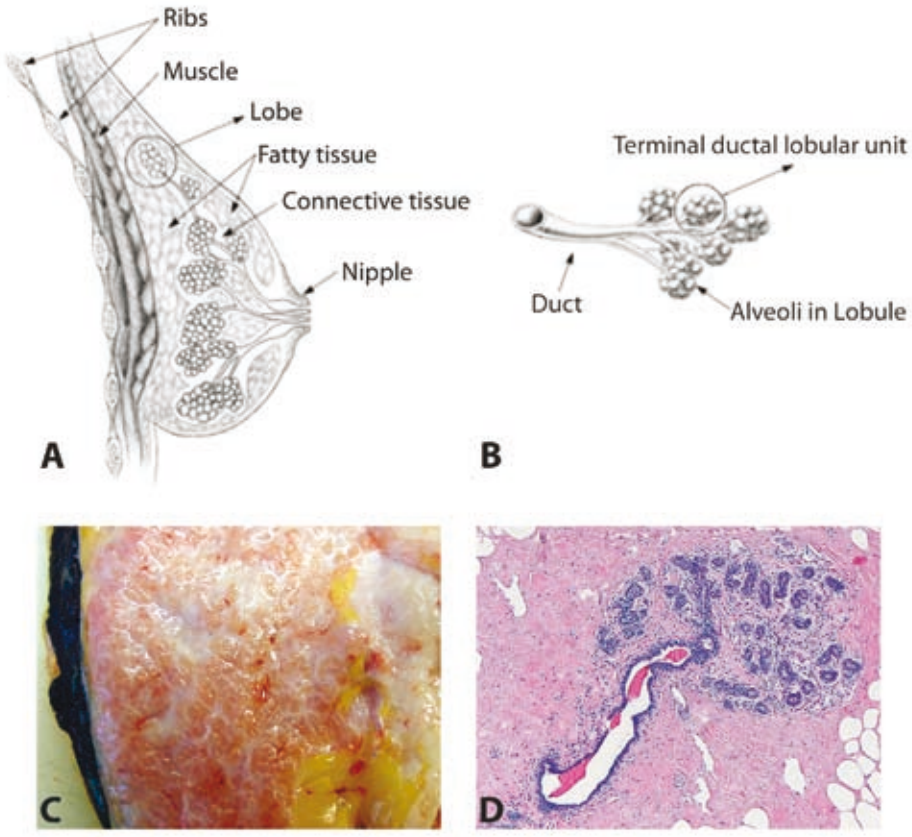

Figure 1.1 (A), schematic representation of the anatomy of the breast; (B), schematic representation of a breast lobe; (C), macroscopic view of the normal breast parenchyma; (D), microscopic view of a normal terminal ductular lobular unit of the breast. (A) and (B) were adapted from http://www.blogsforcompanies.com/TTimages/dcis_in_situ.jpg.

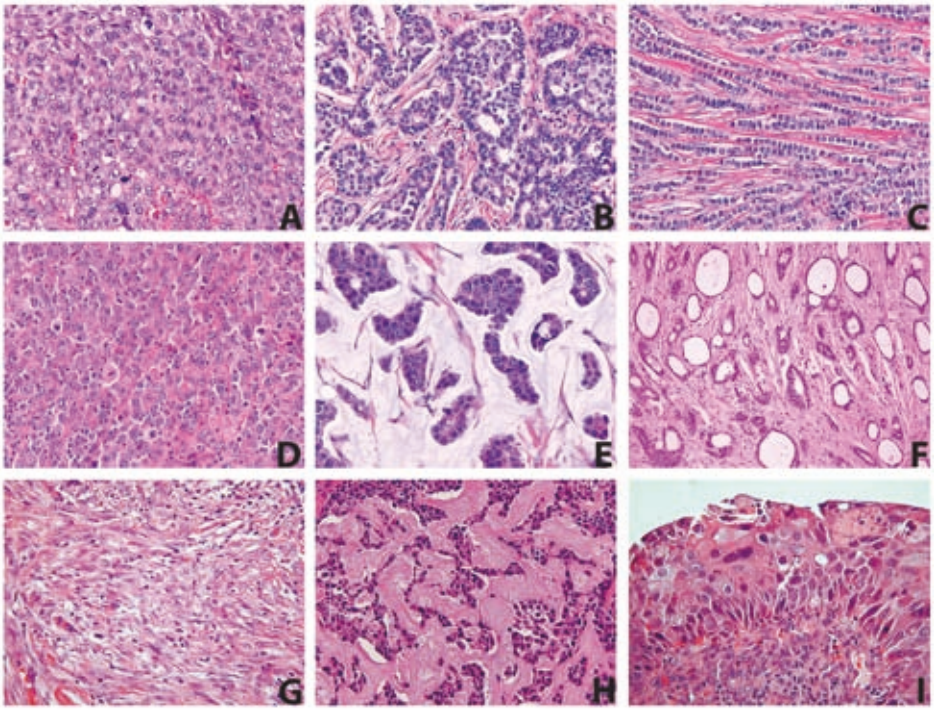

Figure 1.2 Microscopic views of histopathological subtypes of breast cancer. (A) and (B), ductal carcinoma of high and low grade, respectively; (C), lobular carcinoma with strings of cells called "Indian files"; (D), medullary carcinoma; (E), mucinous carcinoma; (F), tubular carcinoma; (G) through (I), metaplastic carcinoma of the breast with spindle, matrix-producing and squamous differentiation, respectively.

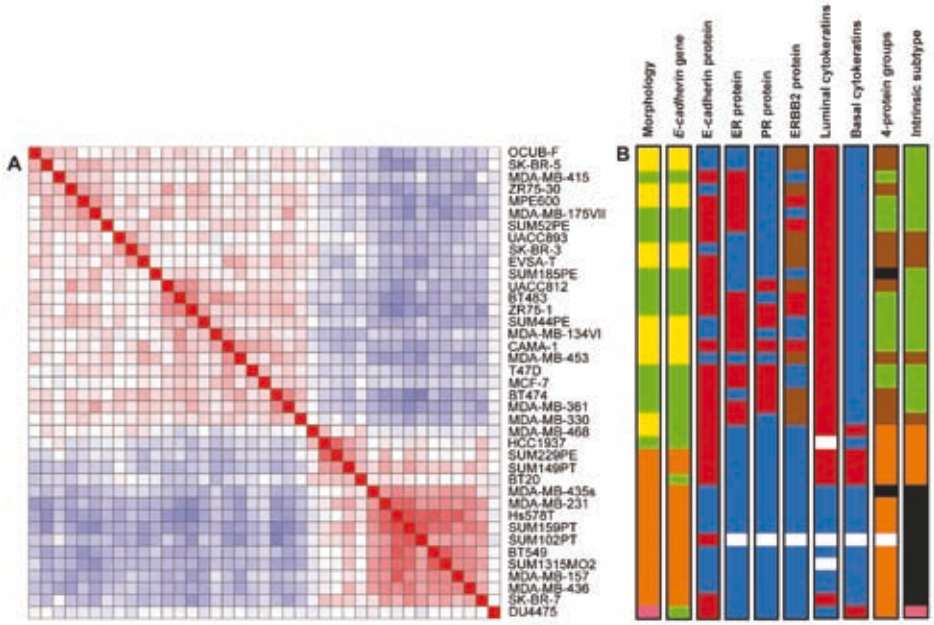

Figure 3.4 Pearson correlation and expression of breast cancer associated proteins in human breast cancer cell lines. (A), Pearson correlation plot based on the log2GM <-2 and >2 subset (5490 probe sets). The Pearson correlation coefficient algorithm positions samples according their overall similarity in gene expression, where red indicates high overall similarity (positive correlation coefficient) and blue indicates low similarity (negative correlation coefficient). (B), Various characterizations of the cell lines indicated that the upper cluster in the Pearson correlation plot contains the epithelial and rounded cell lines intermingled whereas the lower cluster contains all spindle cell lines and a single epithelial cell line and a single rounded cell line (HCC1937 and MDA-MB-468). One cell line was atypical as it did not belong to either of the two clusters (DU4475). The lower cluster included two subgroups that by the intrinsic gene set classified as basal-like and normal-like intrinsic subtypes, where all E-cadherin-negative spindle cell lines classified as normal-like. The lower cluster classified as basal breast cancers by the 4-protein signature of ERBB2, ER, CK5 and EGFR. Color coding morphology column: green, epithelial morphology; yellow, rounded cell morphology; orange, spindle cell morphology; pink, other cell morphology. E-cadherin gene column: green, wild-type E-cadherin gene; yellow, mutant E-cadherin gene; orange, methylated E-cadherin gene. E-cadherin protein, ER protein, PR protein, ERBB2 protein, luminal cytokeratins and basal cytokeratins columns: red, protein expression; blue, no protein expression; brown, protein overexpression. 4-protein groups column: green, luminal group; brown, ERBB2+ group; black, negative group; orange, basal-like group. Intrinsic subtypes column: green, luminal subtype; brown, ERBB2+ subtype; orange, basal-like subtype; black, normal-like subtype; pink, not of any subtype.

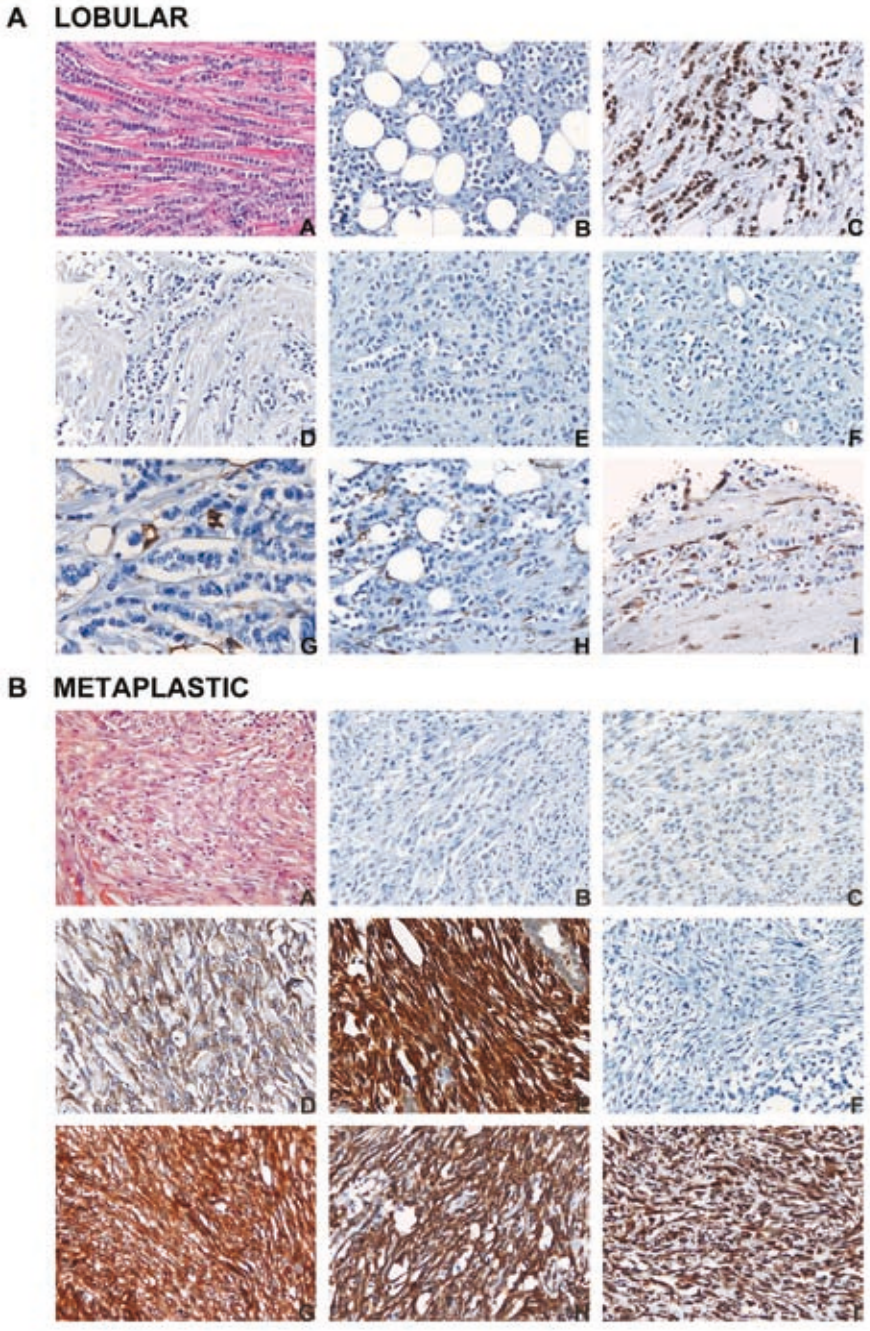

Figure 3.6 Protein expression in clinical breast cancers. Examples of immunohistochemical analysis of (A), lobular breast cancers and (B,) metaplastic breast cancers. Microscopic views: (A), HE-staining; (B), ERBB2; (C), ER; (D), EGFR; (E), CK5; (F), E-cadherin; (G), Caveolin-1; (H), Caldesmon; (I), Vimentin.

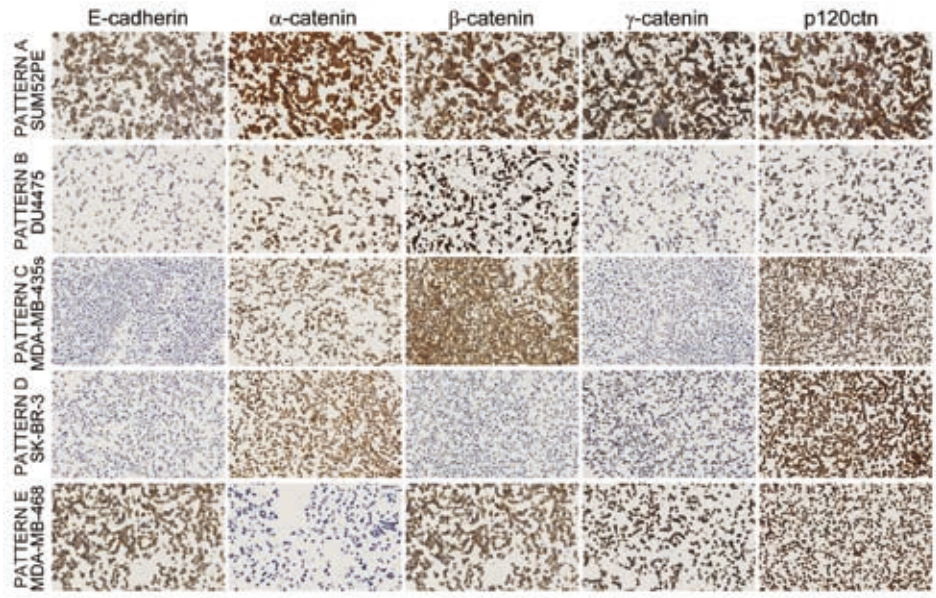

Figure 4.1 Expression analysis of the E-cadherin/catenin protein complex in 41 human breast cancer cell lines. Pattern A entails expression of E-cadherin and all four catenins at apparently normal levels. This pattern is represented by SUM52PE and included fifteen cell lines with wild-type *E-cadherin* genes, five cell lines with (partially) methylated *E-cadherin* genes, and all three cell lines with mutant *E-cadherin* genes that caused in-frame deletions in the encoded proteins. Pattern B is identified for DU4475 only, which had increased β-catenin protein expression, decreased E-cadherin and γ-catenin protein expression and apparently normal α-catenin and p120ctn protein expression. Pattern C entails loss of E-cadherin protein expression, mostly decreased γ-catenin protein expression and mostly normal α-catenin, β-catenin and p120ctn protein expression. Pattern C is represented by MDA-MB-435s and included eight cell lines that had a methylated *E-cadherin* gene promoter. Pattern D entails decreased or absent E-cadherin, β-catenin and γ-catenin protein expression and mostly normal α-catenin and p120ctn protein expression. This pattern is represented by SK-BR-3 and included seven cell lines that had a mutant *E-cadherin* gene that caused a premature termination in the encoded proteins. Pattern E is represented by MDA-MB-468 and included both MDA-MB-468 and MDA-MB-330, which had wild-type *E-cadherin* genes and apparently normal expression levels for all proteins, except for complete absence of α-catenin proteins.

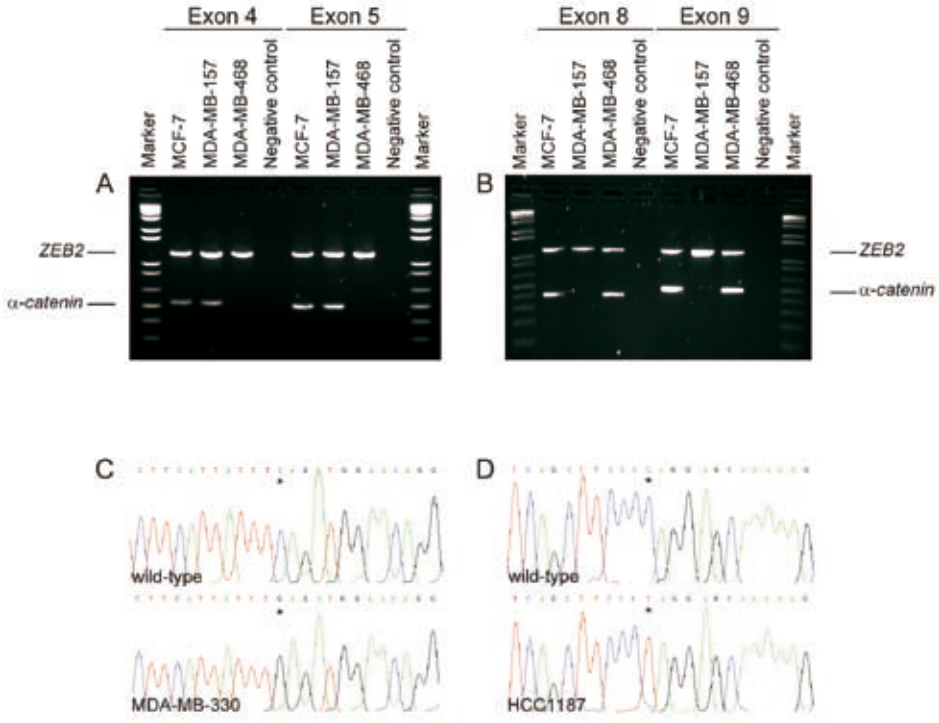

Figure 4.2 Identification of α-*catenin* gene mutations among 55 breast cancer cell lines. (A), homozygous deletion of exons 4 and 5 of α-*catenin* in cell line MDA-MB-468 and (B), homozygous deletion of exons 8 and 9 of α-*catenin* in cell line MDA-MB-157. Duplex PCR amplification products are shown for three breast cancer cell lines, using primers specific for *ZEB2* and α-*catenin* (upper and lower fragments, respectively). Negative control, template negative control; Marker, size marker 1 kb+ DNA ladder (Invitrogen). (C), α-*catenin* nonsense mutation identified in MDA-MB-330 and (D), α-*catenin* nonsense mutation identified in HCC1187. Mutations were identified by PCR amplification and sequencing of genomic DNA (lower electropherograms). The wild-type α-*catenin* gene sequence is shown for comparison (top electropherograms).

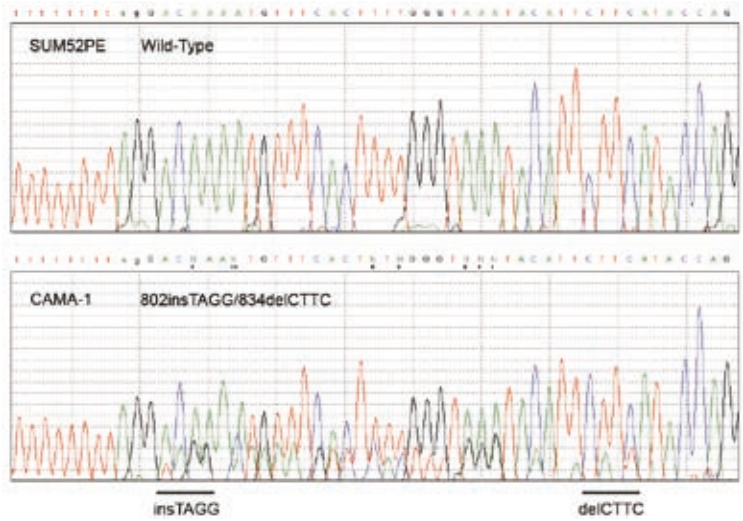

Figure 5.1 Identification of the *PTEN* 802insTAGG/834delCTTC mutation in cell line CAMA-1 by PCR amplification and sequencing of genomic DNA (bottom electropherogram). The wild-type *PTEN* gene sequence is shown for comparison (top electropherogram).

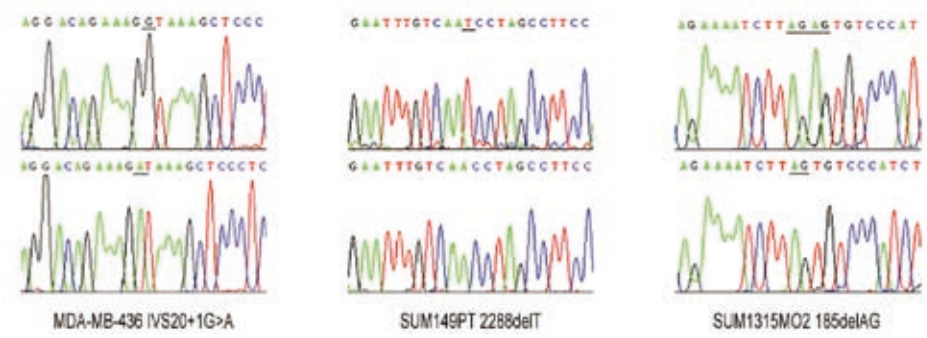

Figure 6.1 Identification of three new *BRCA1* mutant breast cancer cell lines by PCR amplification and direct sequencing. Top, electropherograms displaying the wild-type sequence. Bottom, electropherograms displaying the mutations.

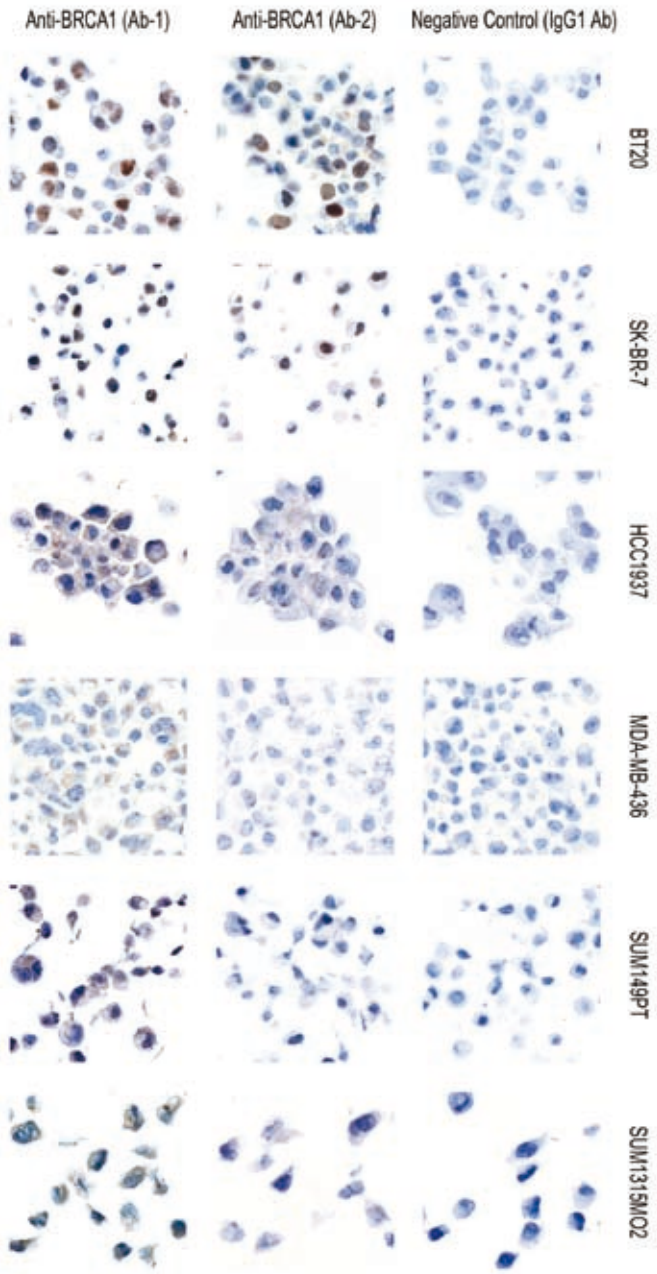

Figure 6.2 BRCA1 immunocytochemistry in *BRCA1* mutant and wild-type breast cancer cell lines. In contrast with the two wild-type cell lines (BT20 and SK-BR-7), none of the four *BRCA1* mutants had nuclear BRCA1 staining with either of the two anti-BRCA1 monoclonal antibodies Ab-1 and Ab-2. There is some cytoplasmic staining of unclear significance in all samples with Ab-1, which is not observed with more diluted Ab-1 antibodies nor with Ab-2 (see also Supplementary Data). The negative control antibody is an IgG1 isotype-matched antibody. Magnification 40X.

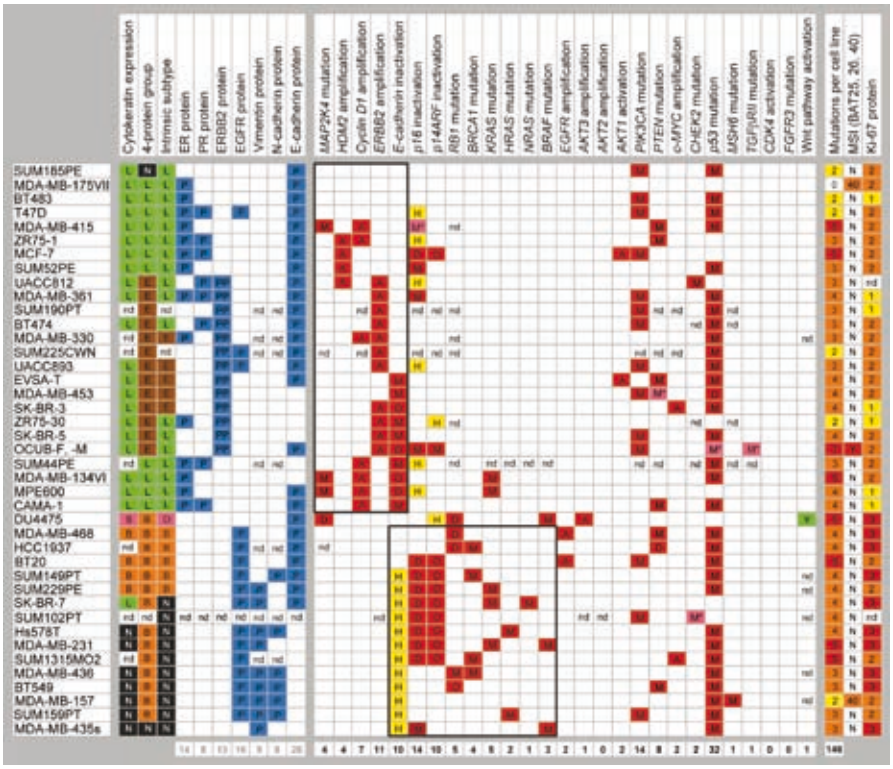

Figure 7.1 Molecular characterization of 41 human breast cancer cell lines. (Left panel) Classification of the cell lines by expression analyses based on cytokeratin proteins, the 4-protein signature of ERBB2, ER, CK5 and EGFR, the intrinsic gene set, and several proteins relevant for breast cancer. Classification by cytokeratin expression: luminal (L), CK8/18+ and/or CK19+, CK5-; basal (B), CK8/18+ and/or CK19+, CK5+; null (N), CK8/18 low, CK19-, CK5-; stem cell (S), CK8/18 low, CK19-, CK5+. Classification by the 4-protein signature: ERBB2 overexpression (E); luminal (L), ERBB2-, ER+; basal (B), ERBB2/ER-, CK5+ and/or EGFR+; negative (N), ERBB2/ER/CK5/EGFR-. Classification by intrinsic gene expression (see also Figure 7.2): ERBB2+ (E); luminal (L); basal-like (B); normal-like (N); other subtype (O). For individual proteins, expression (P) and overexpression (PP) is indicated in blue and absence of expression in white. The protein expression profiles confirmed the remarkably concordant classification by histological criteria or by intrinsic gene expression, and suggested that basal/basal-like and null/negative/normal-like cell lines represent two related subtypes of basal-type breast cancers. (Middle panel) Cancer gene mutation analysis of the cell lines. Genes are indicated at the top and the number of oncogenic mutations identified in each gene at the bottom. Oncogenic mutations (M), sizeable deletions (D) and amplifications (A) are in red; heterozygous oncogenic mutations in tumor suppressor genes (M*) are in pink; and wild-type genes are in white. Promoter hypermethylation (H) is in yellow and constitutive Wnt pathway activation (Y) is in green. nd, not determined. The observed dichotomy among the breast cancer cell lines by protein and gene expression analyses was further supported by the two distinct gene mutation profiles among luminal-type and basal-type breast cancer cell lines (areas with black borders). (Right panel) Number of oncogenic mutations identified in each cell line, microsatellite instability (MSI) and Ki-67 proliferation analysis. MSI: no (N); yes (Y), MSI with BAT 25, 26 and 40; (40), MSI with BAT 40 only. Ki-67 immunohistochemistry: 1, less than 33% of cells positive; 2, 33-66% of cells positive; 3, more than 66% of cells positive. There was a correlation of proliferation rate with breast cancer type (χ^2 $P<0.001$), which was not associated with the number of mutations identified per cell line.

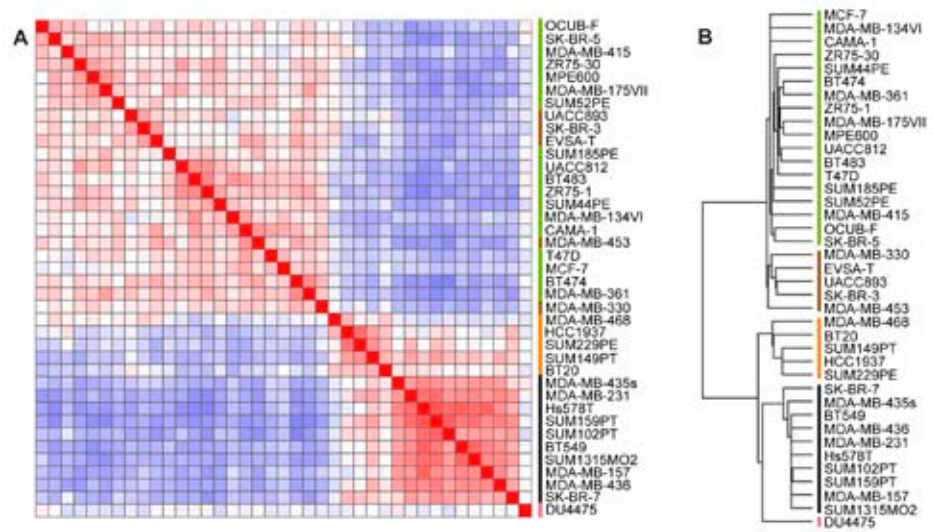

Figure 7.2 Global gene expression and intrinsic subtypes of breast cancer cell lines. (A), Pearson correlation plot of 39 cell lines based on the subset of 5,490 log2GM <-2 and >2 differentially expressed probe sets by Affymetrix U133A microarrays. The Pearson correlation coefficient algorithm positions samples according their overall similarity in gene expression, where red indicates high overall similarity and blue indicates low similarity. Two main clusters of 23 and 15 cell lines were apparent, whereas the DU4475 cell line did not belong to either cluster. (B), Dendrogram of hierarchical clustering of 39 cell lines based on the intrinsic gene set and U133A microarray data. The luminal and ERBB2+ intrinsic subtypes coincided with the major cluster of cell lines in Figure 1A, and the basal-like and normal-like subtypes coincided with the minor cluster of cell lines. Cell line DU4475 did not classify for any of the intrinsic breast cancer subtypes. Color coding of intrinsic subtypes (see also Figure 7.1): green, luminal; brown, ERBB2+; orange, basal-like; black, normal-like; pink, not of any subtype.