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Aspects of Progression in Breast Carcinoma

from ductal carcinoma in situ to invasive cancer

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2012

ISSN 1651-6206
ISBN 978-91-554-8256-5
urn:nbn:se:uu:diva-166745

Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjölds väg 20, Uppsala. Saturday, February 25, 2012 at 13:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Zhou, W. 2012. Aspects of Progression in Breast Carcinoma: from ductal carcinoma *in situ* to invasive cancer. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 735. 38 pp. Uppsala. ISBN 978-91-554-8256-5.

In the past decades our knowledge concerning breast cancer progression from ductal carcinoma in situ (DCIS) to invasive cancer has grown rapidly. However, molecular factors driving the progression are still largely unknown.

In the first study, we investigated tumor evolution in breast cancer by analyzing *TP53* mutation status in tumors from various stages of the disease. Presence of the same *TP53* mutations in both DCIS and invasive components from the same tumor indicates same cellular origin. The role of mutant *TP53* in the progression of breast cancer is less clear and may vary between subtypes.

In the second study, we studied the prognosis of basal-like DCIS in a large population-based cohort. Basal-like DCIS was associated with about doubled but not statistically significant risk for local recurrence compared with the other molecular subtypes. Molecular subtype was a better prognostic parameter than histopathological grade.

In the third study, we studied markers in primary DCIS in relation to type of recurrence. Interestingly, recurrences after an ER-/HER2+, ER negative or EGFR positive primary DCIS were more often of the *in situ* type. The molecular subtype ER+/HER2+, FOXA1 positivity and FOXC1 positivity were risk factors for any recurrence.

In the fourth study, we proposed a histological classification system for a new entity: neoductgenesis. We also evaluated histologic criteria for neoductgenesis. According to our criteria, good agreements among pathologists were achieved. Neoductgenesis was related to more aggressive tumor biology and to mammographic features. The result indicates potential benefits for women earlier considered having pure DCIS but later diagnosed as breast carcinoma with neoductgenesis, suggesting a need to develop appropriate treatment regiments. Our findings have to be repeated and the relation to prognosis warrants further studies.

Keywords: progression, ductal carcinoma in situ, breast cancer

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ISSN 1651-6206
ISBN 978-91-554-8256-5
urn:nbn:se:uu:diva-166745 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-166745)

To my beloved family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Zhou W, Muggerud AA, Vu P, Due EU, Sørlie T, Børresen-Dale AL, Wärnberg F, Langerød A. Full sequencing of TP53 identifies identical mutations within in situ and invasive components in breast cancer suggesting clonal evolution. *Molecular oncology,* 2009;3:214-9.

II Zhou W, Jirstrom K, Johansson C, Amini RM, Blomqvist C,Agbaje O, Wärnberg F. Long-term survival of women with basal-like ductal carcinoma *in situ* of the breast: a population-based cohort study. *BMC cancer,* 2010;10:653.

III Zhou W, Johansson C, Jirström K, Ringberg A, Blomqvist C, Amini RM, Fjällskog ML, Wärnberg F. Tumor markers predicting type of recurrence after a primary ductal carcinoma *in situ*. *Under review*.

IV Zhou W, Tot T, Tabár L, Pinder S, Amini RM, Blomqvist C, Fjällskog ML, Christensson G, Abdsaleh S, Sollie T, Wärnberg F. Breast carcinoma with neoductgenesis: a new subgroup of breast cancer. *In manuscript*.

Contents

Abbreviations

DCIS	Ductal carcinoma *in situ*
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
CK5/6	Cytokeratins 5/6
EGFR	Epidermal growth factor receptor
FEA	Flat epithelial atypia
ADH	Atypical ductal hyperplasia
UDH	Usual epithelial ductal hyperplasia
IBC	Invasive breast cancer
PR	Progesterone receptor
GATA-3	Trans-acting T-cell-specific transcription factor 3
IHC	Immunohistochemistry
LCM	Laser capture microdissection
SISH	Silver-enhanced in situ hybridization
BCS	Breast-conserving surgery
OR	Odd ratio
HR	Hazard ratio
TN	Triple-negative

Introduction

1 Background

1.1 Microscopic anatomy of the breast and breast cancer definitions

The mammary glands are modified eccrine glands of the skin located on the anterior chest wall, the ductal and lobular units of which extend far into the adjacent subcutaneous fat. The gland itself is segmentally divided into 15 to 20 distinct glandular units, or lobes, each of which has a ductal orifice at the apex of the nipple. The glandular tissue of the breast is biochemically supported with estrogen; thus, when a women reaches menopause and her body estrogen levels decrease, the milk gland tissue then atrophies, withers, and disappears, resulting in a breast composed of adipose tissue, superficial fascia, suspensory ligaments and the skin envelope[1].

Breast cancer originates from breast tissue, most commonly from the inner lining of milk ducts or the lobules. Cancers arising from ducts are known as ductal carcinomas and comprise the majority of breast cancers; those originating from lobules are known as lobular carcinomas. In rare cases (less than 5%), breast cancer can arise in other areas of the breast[2].

1.2 Epidemiology

Breast cancer is the most common cancer in females worldwide and accounts for about 30% of all cancers in females in Sweden[3, 4]. In 2008, breast cancer caused 458,503 deaths worldwide (13.7% of cancer deaths in women)[5].Incidence rates of breast cancer are high in more developed countries, whereas rates in developing countries are low but increasing. Age is the most important determinant of breast cancer incidence[6]. The mean age of developing breast cancer in Sweden is about 60 years and only 5% of breast cancer cases are diagnosed in women younger than 40 years of age[4].

2 Classification of breast cancer

2.1 Histopathological classification

Non-invasive breast cancer is called carcinoma *in situ*, i.e. there is no invasion of the surrounding tissue. In contrast, invasive carcinoma does not confine itself to the initial tissue compartment and usually has the ability to metastasize.

2.2 Ductal carcinoma *in situ* of the breast (DCIS)

DCIS, also known as intraductal carcinoma, is the most common type of non-invasive breast cancer in women. It is characteristically contained within the epithelium, with the basement membrane intact, and without any signs of invasion[7]. Classification systems aim to categorize lesions reproducibly and facilitate prognostication and management decisions. Conventional histopathological types include: comedo, solid, cribriform and micro-papillary[8-10].

The increased use of screening mammography which began in the early 1980s resulted in a dramatic increase in the detection of DCIS, especially among women older than 50 years. Approximately 64,000 cases of DCIS are diagnosed annually in the United States, and DCIS accounts for 20% of incidental breast cancers[11]. It is estimated that 14% to 50% of DCIS lesions will progress to invasive cancer if left untreated[12]. However, the progression to invasive breast cancer is not completely understood and cannot be reliably predicted. Therefore, identifying modifiable risk factors associated with DCIS progression may prevent the development of some invasive cancers.

2.3 Invasive cancer

This is the largest group of malignant mammary tumors, comprising 75% to 80% of mammary carcinomas[2]. With invasive cancer, cancer cells start in a milk duct, break through the duct walls, and then invade fatty breast tissue. Invasive cancer can remain localized, or the cancer cells may enter the bloodstream or lymphatic system and metastasize. Invasive ductal carcinoma is the most common type of invasive breast cancer and accounts for 75% of all invasive breast cancers[13]. The prognosis of invasive breast cancer is strongly influenced by the stage of the disease, or to what extent the cancer has spread when it is first diagnosed. Staging takes into consideration size, local involvement, lymph node status and whether metastatic disease is present. The higher the stage at diagnosis, the poorer the prognosis.

2.4 Molecular classification

The development and use of microarray based technology, genomic and expression profiling, has led to the development of classification systems based on biology rather than morphology. The first molecular portraits of human breast tumors were published by Perou and colleagues, who characterized the variation in gene expression patterns by using RNA derived from 65 breast tumors (42 patients) with complementary DNA microarrays representing 8102 human genes[14].

Transcriptome analyses of human breast tumors have revealed remarkably robust molecular subtypes with distinctive gene signatures and clinical outcomes[14-17]. These intrinsic subtypes include luminal A and B, defined by the expression of genes in the luminal epithelial layer of the mammary gland, such as the estrogen receptor (ER) and its targets; human epidermal growth factor receptor 2 (HER2), characterized by high expression of the HER2 oncogene and neighboring genes on its 17q12–21 amplicon; basal-like, defined by the expression of genes characteristic of the outer or basally located epithelial layer of the mammary gland, such as cytokeratins 5/6 and the epidermal growth factor receptor (EGFR); and normal-like, which expresses adipose and other non-epithelial genes and have high basal-like and low luminal gene expression[14, 15]. Strikingly, these molecular subtypes are strongly associated with survival: luminal A tumors have the most favorable prognosis, normal-like tumors have an intermediate prognosis; luminal B, HER2-positive, and basal-like tumors are associated with the shortest relapse-free and overall survival[15-17].

2.5 Mammographic classification

Mammography is essential in the preoperative assessment of patients undergoing surgery for breast cancer. Preoperative mammographic features may also identify those patients who would be unsuitable for breast-conserving surgery[18]. Based on the mammographic appearance, Tabar *et al.*[19] classified breast cancer into six groups as 1) stellate without associated calcifications, 2) circular or oval-shaped mass without associated calcifications, 3) powdery calcifications with or without associated tumor mass on the mammogram, 4) casting-type calcifications with or without associated tumor mass, 5) crushed stone-like calcifications with or without associated tumor mass, and 6) others (e.g., galactographic findings, non-specific asymmetric density). Furthermore, some studies have shown that mammographic features were related to the prognosis of breast cancer[19-21].

2.6 Neoductgenesis

The theory of neoductgenesis has been proposed by László Tabárand[19]. The failure of the casting type calcifications to follow the orderly ductal pattern can lead to the conclusion that many of them are localized within tube-like/duct-like structures that have been formed by the disease itself, while some of the casting type calcifications may be localized within the pre-existing duct system as well. In this breast cancer subtype, the dominant feature appears to be the formation of new ducts or duct-like structures. This process was proposed and called "neoductgenesis". This theory helps us explain many seemingly contradictory features of this breast cancer subtype. Neoductgenesis represents abnormal branching of the ducts within a breast lobe resulting in an unnaturally large number of duct-like structures per square unit. It is a typical feature of some high-grade DCIS and is regularly associated with signs of altered epithelial – stromal interaction, such as periductal lymphocytic infiltration and remodeling of the specialized periductal stroma.

3 The progression of breast cancer

Epidemiological and morphological observations led to the formulation of several linear models of breast cancer initiation, transformation and progression. For the ductal subtype, two models have been proposed. The first 'ductal' model, put forth by Wellings and colleagues, recognizes flat epithelial atypia (FEA), atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS) as the non-obligate precursors of invasive and metastatic ductal carcinoma[22-24]. The second 'ductal' model, supported by epidemiological studies, proposed usual epithelial ductal hyperplasia (UDH) as an intermediate stage of progression between FEA and DCIS[25, 26].

Current thinking is that most invasive breast cancers (IBCs) evolve through a non-obligatory series of increasingly abnormal "stages" over long periods of time, probably decades in most cases. DCIS represents an advanced or late stage of premalignant tumor progression, and it is the direct precursor of most IBCs, which is supported by a great deal of indirect but compelling evidence[27, 28]. The major risk factors for developing IBC are the same for DCIS[29, 30]. Furthermore, DCIS diagnosed in the past, especially if not completely excised, is a strong risk factor for developing IBC in the future[31, 32]. DCIS and IBC share many identical genetic abnormalities, especially when they occur in the same breast[33]. Genetically engineered animal models of breast cancer progress from *in situ* to invasive disease[34, 35]. Progressions of noninvasive to invasive cancer occurs in other organs are easier to observe, such as skin and cervix, so there is ample biological precedence.

Some DCIS progress to IBC very rapidly, whereas others would not progress during a lifetime. Critical and poorly understood events in breast tumor progression that have dramatic impacts on clinical management and outcome including the transition of DCIS to invasive carcinoma and the metastatic spread of primary tumors to distant organs. Molecular studies revealed that myoepithelial cells associated with DCIS are not phenotypically normal; they have lost some of their differentiation markers and have up-regulated genes promoting angiogenesis and invasion[36, 37].It would be very useful to know the natural history of DCIS, including how it develops, whether it will progress to IBC, and when[12, 38, 39].

4 Prognostic factors

4.1 *TP53*

TP53 is a key tumor suppressor gene located on chromosome 17p with a potentially large clinical impact[40]. P53 is a multifunctional protein that is involved in the control of cell cycle progression, DNA integrity and cell survival; all of those are believed to be important in the development of abnormal cell proliferation[41]. Inactivation of *TP53* function is one of the most common genetic changes seen in human malignancies[42, 43].

TP53 mutations are present in a significant percentage of breast cancer ranging from 20 to 50%[44]. Previous studies have generally noted a poor prognosis for those patients with increased *TP53* expression[45].

4.2 HER2

Human epidermal growth factor receptor 2, known as HER2, is a member of the EGFR family. As other members of this family, HER2 is a transmembrane glycoprotein, which forms hetero-dimers with other members of the EGFR family leading to the activation of signaling pathways involved in cell growth, differentiation, survival, adhesion, and migration[46]. Overexpression and amplification of HER2 can be detected in about 15% of all primary breast cancers[47]. HER2 overexpression promotes the proliferation, motility, and survival rate of cancer cells and has been associated with resistance of cancers to therapeutic interventions including hormone therapy, radiation, and certain types of chemotherapy, which in turn lead to poor outcomes[48, 49].

In cases of HER2 positivity, breast cancer patients are more likely to suffer from relapse and tend to have a shorter overall survival[50-52].

4.3 Ki67

Ki67, the marker of proliferation, was first identified by Gerdes et al. in the 1980s[53]. Ki67 is a nuclear non-histone protein, and a cellular marker of proliferation[54]. During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The Ki-67 protein is present during G1, S, and G2 phases of cell cycle with a peak during mitosis and an absence in the G0 phase[55]. In DCIS about 40% of tumors express high levels of Ki67. Increased levels are associated with higher grade lesions, comedo necrosis, and the presence of microinvasion. Hence, it is not surprising that Ki67 is a predictor of recurrence in DCIS[56, 57]. A possible prognostic role for the proliferation marker Ki67 in breast cancer has been investigated in many studies.

Although the most recently published analysis of 15,790 cases from 43 studies reported an association of Ki67 positivity with shorter overall survival, Ki67 staining is still not recommended as a prognostic marker for routine purposes[58].

4.4 Hormone receptors (ER and PR)

ER and progesterone receptor (PR) expression is an independent prognostic factor in breast cancer. Patients with ER and/or PR positive tumors have a better survival than those with hormone receptor negative tumors, with a 5-year overall survival (all stages) of 83% in the ER+/PR+ group versus 69% in the double negatives[59]. High cellular expression of ER and PR predicts benefit from endocrine therapy in the adjuvant and metastatic setting[60]. Tumor hormone receptor status is, therefore, routinely assessed in breast cancer. It also becomes clear that hormone receptor status in a patient can change during the course of the disease and may differ intralesionally. For example, the ER status of metastatic disease is different from that of the primary tumor in about 20% of cases[61]. In addition, PR expression is lost in 40% of previously positive tumors when they metastasize.

4.5 EGFR

EGFR is a 53-amino acid, 170-kd transmembrane polypeptide of the erbB family. Like other members of the family, most in vitro studies have demonstrated that EGFR activation relies on co-receptor and ligand interaction for phosphorylation and thus activation of subcellular pathways[62]. The activation of these EGFR signaling pathways is known to increase proliferation, angiogenesis and decrease apoptosis. Such changes are consistent with a transformed cellular phenotype and enhanced mitogenesis with growth and survival advantages[63, 64]. Increased EGFR expression is therefore likely to be

a strong prognostic feature in multiple tumor types, and the inhibition of its cellular actions appears to produce substantial therapeutic benefits[65]. However, the roles that EGFR and its ligands play in breast cancer have been a subject of intensive study and controversy. Some retrospective immunohistochemistry studies have indicated that EGFR overexpression in primary tumors is an indicator of poor prognosis[66-69], whereas other studies have failed to establish such a link[70, 71].

4.6 CK 5/6

CKs are proteins of keratin-containing intermediate filaments found in the intra-cytoplasmic cytoskeleton of epithelial tissue. In the normal breast CK 5/6, a specific high–molecular weight CK, is expressed by myoepithelial cells and by stem cell epithelium that can self-renew and give rise to luminal epithelial cells[72]. Recent studies show that DCIS lack CK 5/6 expression. Hence, assessing CK5/6 expression by IHC might minimize the disagreement in the diagnosis of non-invasive proliferations when used in conjunction with morphological criteria[37, 73, 74].

4.7 FOXA1

FOXA1 is a member of the forkhead class of DNA-binding proteins[75]. It is normally expressed in the liver, pancreas, bladder, prostate, colon and lung, as well as in the mammary gland, and can bind to the promoters of more than 100 genes associated with metabolicprocesses, regulation of signaling pathways and cell cycle[76-78].

FOXA1 is now receiving considerable attention with respect to ER function because it interacts with cis-regulatory regions in heterochromatin and enhances the interaction of ER to its target genes[79, 80]. Recent studies have shown the requirement of FOXA1 for optimum expression of 50% of ER-regulated genes and estrogen-induced proliferation[76, 79, 81]. Thus, ER dependency of breast cancers for survival or proliferation may be related to the expression levels of FOXA1.

Also, FOXA1 has been suggested as a favourable prognostic factor in breast cancer, with potential relevance in the subclassification of luminal/ER-positive tumors[15]. FOXA1 and ERα have been suggested as potential participants involved in mammary tumors together with another gene, GATA-3, which regulates the lineage determination and differentiation of many cell types[82, 83].

4.8 GATA-3

The trans-acting T-cell-specific transcription factor GATA-3 is a protein that in humans is encoded by the GATA3 gene[84, 85]. The protein contains two GATA-type zinc fingers and is an important regulator of T cell development and plays an important role in endothelial cell biology. GATA-3 was shown to be required for the luminal A type of breast cancer, intertwined in pathways with ER[86, 87]

In the breast, GATA-3 plays a central role in luminal epithelia differentiation and subsequent formation of the ductal tree of differentiated epithelial cells, suggesting that this protein might be involved in breast tumorgenesis[88, 89].

In breast cancer cell lines and primary tumors, GATA3 expression was strongly correlated with ER expression[90]. Moreover, low or lack of GATA3 expression is associated with shorter survival, more malignant histological features, positive lymph nodes, increased tumor mass, lack of progesterone receptor expression, and overexpression of HER2, which is associated with aggressive forms of breast cancer independently[89, 91].

4.9 FOXC1

FOXC1 belongs to FOX. The specific function of this gene has not yet been determined; however, it has been shown to play a role in the regulation of embryonic and ocular development. Mutations in this gene cause various glaucoma phenotypes including primary congenital glaucoma, autosomal dominant iridogoniodysgenesis anomaly, and Axenfeld-Rieger anomaly[92]. Positive expression of FOXC1 was associated significantly with expression of basal cytokeratins. FOXC1 was thought to be a potentially significant diagnostic and this prognostic biomarker for basal-like breast carcinoma and may serve as a therapeutic target for this type of cancer[93].

Aims of the study

I To investigate whether *TP53* mutations are early events in the progression from DCIS to invasive breast cancer by exploring the status and timing of *TP53* mutations.

II To compare the prognosis of basal-like DCIS with other molecular subtypes in a large population cohort.

III To investigate the associations between several molecular markers and recurrence type (invasive vs. in situ) among primary DCIS patients.

IV To evaluate whether the diagnosis of neoductgenesis could be made in a reproducible way among pathologists and, if so, to study the correlations between neoductgenesis and mammographic features and some common immunohistochemistry (IHC) markers.

Materials and methods

1 Study subjects

1.1 SweDCIS Trial

The SweDCIS Trial accrued 1046 women from 1987 through 1999. The SweDCIS was a multicenter trial administered through the Regional Oncological Centers in six Swedish Health Care Regions. Inclusion criteria were a primary diagnosis of ductal carcinoma in situ of the breast occupying less than a quadrant of the breast, surgically treated with breast conservation, no prior history of cancer, no contraindication to radiotherapy and full informed consent. After a sector resection of the breast, women were randomized to postoperative radiotherapy of the breast or control only. A macroscopic lateral surgical margin of 1 cm was aimed at. Scarpas' fascia and the pectoral fascia were the ventral and dorsal borders. Microscopically free margins were not requested but achieved in 80% of all participants (11% had positive margins and 8.5% had unknown margins). The specification dose of radiotherapy was 50 Gy given in 25 fractions over 5 weeks or 54 Gy given in two series with a gap of 2 weeks. No women were lost to follow-up.

1.2 Paper I

The study cohort was a population-based cohort, including all 854 women who were diagnosed with either a pure DCIS, a pure invasive breast cancer (≤15 mm) or a mixed lesion (i.e., invasive carcinoma with an *in situ* component) between 1986 and 2004 in Uppland, Sweden. Of the 854 women, 258 had frozen tumor material prospectively preserved in the biobank at Uppsala University Hospital. In this study, we included 118 of the 258 women with sufficient tumor materials, which comprised all 32 with pure DCIS, all 38 with pure invasive breast cancer and a random sample of 48 with mixed lesions.

1.3 Paper II

We recruited all 458 women who were diagnosed with a primary DCIS between 1986 and 2004 in Uppland and Västmanland regions, Sweden.

1.4 Paper III

Patients were recruited from two different source populations. One was the same population-based cohort described in paper II. The other source was the SweDCIS Trial. We included all women from the study with a registered local recurrences (n=166) up to the December 31st, 2008.

1.5 Paper IV

Seventy-four women from three different source populations were included in this study. The criteria for inclusion were a diagnosis of DCIS, nuclear grade II or III, with or without an invasive component. Prospectively, we collected tumor tissue from 31 cases with pre-operatively diagnosed mammographic calcifications between 2005 and 2006 at Uppsala Academic Hospital and eligible according to histopathological criteria. To expand the cohort, 11 cases, diagnosed between 1996 and 2002, were selected from the bio-bank at Falun Hospital based on the original histopathological report. A further 32 cases were selected based on the histopathological criteria from the same cohort described in paper II.

2 Laser capture microdissection (LCM)

Microdissection was used to separate mixed-lesion samples (with both DCIS and invasive components). The samples were cryo-sectioned at both 4µm and 14µm thickness. The 4µm-thick section was stained by routine H&E staining to locate the corresponding areas to be microdissected in the consecutive 14 µm-thick sections. The 14 µm-thick sections were mounted on a slide pre-covered with a thin polyethylene membrane (PALM slide) and immediately stored at -80°C until microdissection.

Laser capture microdissection was performed on frozen sections using a Zeiss inverted microscope PALM Laser Micro-Beam System (Carl Zeiss, German). Cryosected sections were thawed for 30 seconds and immediately stained using 60µL of hematoxylin (mixed with RNasin) for 1 min, incubated in 60 µL of Zincfix for 30 seconds and followed by 30-second incubation steps in 75%, 95% and 100% ethanol, respectively. Slides were air-dried and kept desiccated to be dissected. Under light microscopic examination, we microdissected as far as possible 3,000 cells from different parts of the same component to obtain enough cells for DNA extraction. The *in situ* carcinoma cells and/or invasive carcinoma cells were captured into the collecting caps, preserved in 50 µL of Trizol and immediately stored at -80°C until DNA extraction.

3 Silver-enhanced in situ hybridization (SISH)

HER2 SISH was performed on an automated instrument, Ventana Benchmark (Ventana Medical Systems, Tucson, AZ) per the manufacturer' s protocols for the INFORM HER2 DNA probe and chromosome17 probes. Testing for the HER2 gene and chromosome 17 was performed on sequential sections. Both probes are labelled with dinitrophenol. Denature occurred on the instrument with enzyme digestion in protease 3for 8 minutes. The detection system used a multimer-labeled with goat antirabbit antibody horseradish peroxidaseas the linking step. Visualization occurred with the sequential addition of silver acetate as the source of ionic silver, hydroquinone, and hydrogen peroxide to give a black metallic silver precipitate at the probe site. Counterstaining was performed with hematoxylin II on the instrument. The time taken for the complete run was 6.5 hours. Both HER2 and chromosome 17 detections were performed on the same slide run. Gene amplification was assessed according to the American Society of Clinical Oncology/College of American Pathologists guideline and Australian HER2 Advisory Board criteria for single HER2 probe testing (diploid, 1 to 2.5 copies/nucleus; polysomy >2.5 to 4 copies/nucleus; equivocal, >4 to copies/nucleus; low-level amplification, >6 to 10 copies/nucleus; and high-level amplification >10 copies/nucleus) and for dual HER2/CHR17 probe testing (non-amplified ratio<1.8; equivocal ratio, 1.8 to 2.2; gene amplification, >2.2).

4 Statistic methods

All analyses were performed using The SAS System (SAS Institute, Cary, NC) and R software. Statistical significance threshold was set to 0.05. Pearson Chi-square ($\chi 2$) test and Fisher's exact test were performed to compare the distributions of baseline characteristics among different groups.

In paper II, Kaplan-Meier curves and Cox proportional hazard models were used to assess the association between molecular subgroups and progression of breast cancer.

In paper III, the association between baseline characteristics and type of recurrence were analyzed using Logistic regression models. In the multivariate models, we adjusted for age group, free margins and type of surgery.

In paper IV, a kappa value was used to evaluate the agreement of four independent pathologists' diagnosis. The correlations between neoductgenesis and mammographic features and IHC markers were analyzed using Logistic regression models.

Results

1 Paper I

A total of 19 *TP53* mutations (16.1%) were detected in 118 cases stratified into three distinct diagnosis groups (pure DCIS, pure invasive cancers, or mixed diagnosis). No significant difference was found between the three groups in terms of the position (codon, exon) of the *TP53* mutations or the location of missense mutations. Concerning the predicted effect of the mutation (missense, nonsense, frameshift, in-frame or splice) the pure DCISs harbored more missense mutations (4/5, 80%) than pure invasive cancers and mixed lesion combined (6/14, 43%), although this was not statistically significant (P=0.30). For the cases with mixed-lesion, the same *TP53* mutations detected in the bulk tumor, were seen both in the DCIS and in the invasive component. In invasive tumors (including pure invasive and mix-lesion tumors), strong association was found between *TP53* mutations and histopathological grade (P=0.007).

2 Paper II

Among the 392 women with available IHC information, 32 (8.2%) were classified as basal-like, 351 (89.5%) as luminal or HER2-positive, and 9 (2.3%) unclassified. During the follow-up period (median 122 months), 76 women had a local recurrence and 47 women developed invasive- or general recurrence.

In both the univariate and multivariate models, basal-like DCIS showed a higher risk of local recurrence compared with non basal-like DCIS; HR was 1.7 (95% CI: 0.8-3.8) and 1.8 (95% CI 0.8-4.2), respectively. For invasive- or general recurrence, basal-like DCIS also showed a higher risk compared with non basal-like DCIS; HR was 2.0 (95%CI: 0.8-5.0) and 1.9 (95% CI 0.7-5.1), respectively. Results did not differ substantially when we restricted the analyses in women with BCS only. We also compared different types of recurrences between triple-negative (TN) (ER-, PR- and HER2-negative) and non TN DCIS. TN tumors had a hazard ratio (HR) of 1.1 (0.4-2.9) for local recurrence and 1.6 (0.6-4.8) for invasive- or general recurrence when compared with non TN in the multivariate analyses (Table 1).

Table 1. Cox regression analyses by molecular subgroup, by immunohistochemistry

	All (n = 392)		Breast Conserving Surgery (n = 298)	
	Univariate HR (95% CI)	Adjusted* HR (95% CI)	Univariate HR (95% CI)	Adjusted* HR (95% CI)
	Event: local recurrence (n = 76)			
Gene-expression classification				
Non basal-like (n = 360)	1 (Reference)	1	1	1
Basal-like (n = 32)	1.7 (0.8-3.8)	1.9 (0.8-4.2)	1.6 (0.7-3.6)	1.8 (0.7-4.2)
Triple-negative classification				
Non TN (n = 377)	1 (Reference)	1	1	1
TN (n = 32)	1.1 (0.4-2.7)	1.1 (0.4-2.9)	1.0 (0.4-2.7)	1.0 (0.3-2.9)
Tumor grade				
I (n = 30)	1 (Reference)	1	1	1
II (n = 182)	1.2 (0.4-3.4)	1.4 (0.5-4.0)	1.3 (0.5-3.9)	1.4 (0.5-3.9)
III (n = 176)	1.0 (0.3-2.9)	1.3 (0.5-3.9)	1.4 (0.5-4.0)	1.4 (0.5-4.2)
Postoperation radiotherapy				
No (n = 252)	1 (Reference)	1	1	1
Yes (n = 140)	0.9 (0.5-1.5)	0.7 (0.4-1.3)	0.8 (0.4-1.4)	0.7 (0.4-1.4)
	Event: invasive- and general recurrence (n = 47)			
Gene-expression classification				
Non basal-like (n = 360)	1 (Reference)	1	1	1
Basal-like (n = 32)	2.0 (0.8-5.0)	1.9 (0.7-5.1)	2.2 (0.8-5.6)	2.3 (0.8-6.1)
Triple-negative classification				
Non TN (n = 377)	1 (Reference)	1	1	1
TN (n = 32)	1.4 (0.5-4.0)	1.6 (0.6-4.8)	1.7 (0.6-4.9)	2.1 (0.7-6.3)
Tumor grade				
I (n = 30)	1 (Reference)	1	1	1
II (n = 182)	1.0 (0.3-3.3)	1.1 (0.3-3.8)	1.1 (0.3-3.8)	1.1 (0.3-3.8)
III (n = 176)	0.7 (0.2-2.4)	1.1 (0.3-3.9)	0.9 (0.2-3.1)	1.0 (0.3-3.9)

3 Paper III

Of the 624 women in this study, 130 developed an *in situ* recurrence and 136 developed an invasive recurrence December 31st, 2008 (mean 95 months). The other 458 women with no recurrence from the population-based cohort were grouped as a reference.

Using the molecular subgroup ER+/HER2- as the reference, the ER+/HER2+ group was associated with higher risk of any local recurrence (OR 1.93, 95% CI 1.09 – 3.42), while other subtypes were not. Other molecular markers associated with higher risks for any local recurrence were

HER2 (OR 1.56, 95 CI 1.03 – 2.36), FOXA1 (OR 3.06, 95 CI 1.50 – 6.26) and FOXC1 (OR 2.94, 95 CI 1.71 – 5.03).

ER-positivity was associated with a higher risk of an invasive recurrence (OR 2.52, 95% CI 1.24 – 5.10) when compared with ER negative DCIS. With the molecular subgroup ER+/HER2- (luminal A) as the reference, the ER-/HER2+ group (HER2-positive) was associated with a lower risk of invasive recurrence (OR 0.24, 95% CI 0.09 – 0.62) while the other subtypes were not. HER2 positive and EGFR positive primary DCIS tumors were associated with an about halved risk of invasive recurrence (OR 0.48, 95% CI 0.26 – 0.90) and (OR 0.44, CI 0.22 – 0.88), respectively. Other molecular factors including PR, Cytokeratin 5/6, FOXA1, FOXC1, GATA-3, Ki67 and CD10 were not statistically significantly associated with this type of recurrence (Table 2).

Table 2. The associations between baseline clinical-, histopathologic- and molecular characteristics and the risks for recurrences being invasive- compared to *in situ* carcinoma, among women with primary DCISs (n=266).

Characteristics	Risk of a recurrence being invasive compared to *in situ*	
	Univariate*, OR (95% CI)	Multivariate†, OR (95% CI)
Mode of detection		
Screening	1.0	1.0
Clinically	1.72 (0.98 – 3.01)	1.80 (1.02 – 3.19)
Tumor size		
≤ 15mm	1.0	1.0
> 15mm or multifocal	0.55 (0.33 – 0.93)	0.54 (0.32 – 0.92)
Type of surgery		
Breast conserving surgery	1.0	-
Mastectomy	1.13 (0.29 – 4.42)	-
Postoperative radiotherapy		
No	1.0	1.0
Yes	1.32 (0.76 – 2.27)	1.41 (0.80 – 2.48)
Free margins		
No or doubtful	1.0	-
Yes	1.24 (0.69 – 2.22)	-
Nuclear grade		
I	1.0	1.0
II	0.75 (0.26 – 2.12)	0.70 (0.25 – 2.02)
III	0.53 (0.19 – 1.45)	0.49 (0.18 – 1.35)
Molecular subgroup		
ER+/HER2-	1.0	1.0
ER+/HER2+	0.84 (0.37 – 1.89)	0.83 (0.37 – 1.88)
ER-/HER2+	0.27 (0.11 – 0.68)	0.24 (0.09 – 0.62)
ER-/HER2-/CK5/6+ or EGFR+	0.54 (0.17 – 1.71)	0.52 (0.16 – 1.65)
Unknown	0.75 (0.42 – 1.34)	0.76 (0.42 – 1.35)

ER		
Negative	1.0	1.0
Positive	2.33 (1.17 – 4.65)	2.52 (1.24 – 5.10)
PR		
Negative	1.0	1.0
Positive	1.32 (0.73 – 2.38)	1.36 (0.75 – 2.47)
HER2		
Negative	1.0	1.0
Positive	0.50 (0.27 – 0.92)	0.48 (0.26 – 0.90)
EGFR		
Negative	1.0	1.0
Positive	0.45 (0.23 – 0.88)	0.44 (0.22 – 0.88)
Cytokeratin 5/6		
Negative	1.0	1.0
Positive	1.16 (0.32 – 4.24)	1.28 (0.34 – 4.84)
FOXA1		
Negative	1.0	1.0
Positive	0.36 (0.09 – 1.43)	0.33 (0.08 – 1.33)
FOXC1		
Negative	1.0	1.0
Positive	1.29 (0.58 – 2.85)	1.23 (0.53 – 2.85)
GATA-3		
Negative	1.0	1.0
Positive	2.08 (0.79 – 5.52)	1.98 (0.74 – 5.27)
Ki67		
Low	1.0	1.0
High	0.98 (0.50 – 1.94)	0.93 (0.46 – 1.85)
CD 10		
Negative	1.0	1.0
Positive	1.49 (0.67 – 3.31)	1.45 (0.65 – 3.27)

*****Adjustments for age group; †Adjustments for age group, free margin and type of surgery

4 Paper IV

Of all 74 cases, 68 could be classified as neoductgenesis or not by combining evaluation from pathologists and the Tn-C staining. Among these 68 cases, 37 were classified as neoductgenesis-positive. The carcinomas with neoductgenesis were more often of nuclear grade 3 (73.0 vs. 54.8%, *P*=0.1) and showed suspicious microcalcification on the mammogram significantly more often than in the group without neoductgenesis (73.0 vs. 48.4%, *P*=0.04, OR 2.9, 95% CI 1.05-7.92). Neoductgenesis was inversely correlated with ER-positivity (OR 0.21, 95% CI 0.05-0.84). The correlation between HER2 positivity and neoductgenesis was of borderline significance (OR 2.67, 0.92-7.76) (Table 3).

Table 3. Tumor and patient characteristics in breast carcinoma with neoductgenesis or not among 68 women with DCIS, grade 2-3, with or without an invasive component.

Characteristics	Breast carcinoma with neoductgenesis Yes (N=37) number (%)	No (N=31) number (%)	P-value
Age at diagnosis (n=68)			
≤ 55 years	16 (43.2)	11 (35.5)	0.5
> 55 years	21 (56.8)	20 (64.5)	
Nuclear Grade (n=68)			
II	10 (27.0)	14 (45.2)	0.1
III	27 (73.0)	17 (54.8)	
ER (n=62)			
Positive	22(62.9)	24 (88.9)	0.03
Negative	13 (37.1)	3 (11.1)	
PR (n=62)			
Positive	19 (54.3)	19 (70.4)	0.2
Negative	16 (45.7)	8 (29.6)	
HER2 (n=61)			
Positive	19 (54.3)	8 (30.8)	0.07
Negative	16 (45.7)	18 (69.2)	
Ki67 (n=61)			
High	14 (40.0)	7 (26.9)	0.29
Low	21 (60.0)	19 (73.1)	
Lymphocytic infiltration (n=56)			
No or mild	19 (57.6)	19 (82.6)	0.05
Intense	14 (42.4)	4 (17.4)	
Fibrosis-like thickening of the periductalstroma (n=42)			
No or little	10 (43.5)	19 (100.0)	-
Much	13 (56.5)	0 (0.0)	
Mammographic casting or crushed stone-like calcifications (n=68)			
Yes	27 (73.0)	15 (48.4)	0.04
No	10 (27.0)	16 (51.6)	

Discussion

1 Paper I

In the current study, in total 19 mutations were detected in the DCIS, pure invasive and mixed-lesion groups. The overall fraction of samples with mutated *TP53* (16.1%) is lower than that in the average breast cancer series (25.0%)[94] and may be due to the small sized lesions in our study. From the population-based cohort, only a subset of cases with sufficient frozen tissue was available for this study. The cases not available in this study are more of smaller size tumors, which may result in an overrepresentation of low-grade tumors. Moreover, *TP53* mutation is more observed in higher grade tumor[95, 96]. Thus, we might have overestimated the true proportion of *TP53* mutations. This overestimation was unlikely to be larger in DCIS than in the invasive cancer.

Although IHC has been used in a number of studies to detect the mutated p53 protein, about 30% of mutations detected by sequencing are missed using IHC[97]. In current study, we employed DNA sequencing to detect the mutation in the whole gene. By only sequencing exons 5–8, where mutations are mostly exist, 5–20% of the mutations could not be detected. The sequencing analysis detected 26% of the *TP53* mutations outside exons 5–8, pointing to the importance of analyzing the whole gene and not merely exons 5–8, as performed in most previous studies[95, 96, 98, 99].

The proportions of *TP53* mutations in DCIS and in the invasive group (pure invasive plus mixed lesion) were almost equal, suggesting that *TP53* mutation might be an early event occurring at or prior to the DCIS stage. Also, mix-lesions breast carcinoma both with *in situ* and invasive components were investigated synchronously. Interestingly, in those mixed-lesion samples, the same mutation found in the DCIS component was also observed in the adjacent invasive component. These findings suggested that *TP53* mutation might occur before invasion, at the DCIS or prior to the DCIS stage during the progression of breast cancer, which is in accordance with previous reports on *TP53* mutation. The same mutation detected in the two different components of the same tumor also indicated that the DCIS and invasive cells arose from the same tumor cell clone.

2 Paper II

An important finding in this study is that basal-like DCIS was suggested to a higher risk of local recurrence and a higher risk of developing invasive cancer compared with other DCIS. The risk almost doubled but was not statistically significant.

IHC criterions for defining genotype breast cancer were proposed by Nielsen et al[100]. Abd EL-Rehim[101] and Livasy[102] et al then verified and confirmed the criteria in invasive and DCIS tumors, respectively. Today, IHC is increasingly used as a surrogate for genetic profiling[103]. Moreover, these subgroups defined by IHC have distinguishing features closely associated with subtypes defined by gene expression profiling, including distinct clinical outcomes[104]. Basal-like DCIS, in our study, was defined as tumors that were ER-negative, HER2-negative, but positive for either CK 5/6 or EGFR.

Basal-like invasive breast cancer has been conformed to be associated with a poor prognosis in our study[105-110]. Our observations showed a doubled risk for local recurrence and invasive or general recurrences for basal-like DCIS. The higher risk was not statistically significant but, on the other hand, it was consistent in the univariate, multivariate and Kaplan-Meier analyses for all patients and for the subgroup of patients with breast-conserving surgery (BCS). However, the basal-like subgroup is small, which makes the statistical power low and a conclusive study would have to include a much larger number of patients.

3 Paper III

Our results indicate that ER-/HER2+, EGFR-expression and clinical detection are the three most important factors that predict higher risk of subsequent invasive breast cancer in patients with DCIS. Interestingly, new risk factors, FOXA1 and FOXC1, were shown to be related to increased risks of local recurrence.

In this study, we compared tumors that recurred as invasive cancer with those recurring as *in situ* tumors. We found that ER-positivity and low expression of HER2 and EGFR were strongly associated with a subsequent recurrence being invasive.

A certain combination of molecular markers (ER-/HER2+) showed a statistically significant association with a high risk of subsequent DCIS. The combination is however, only present in a small percentage (11.5%) of DCIS lesions. This finding is consistent with one previous nested case-control

study showing that ER- /HER2+ DCIS was associated with an increased risk of recurrent DCIS, but it was not associated with a risk of invasive recurrence[111].

Although FOXA1 is thought to be a significant marker of good prognosis in breast cancer[112-117], FOXA1 expression in our study showed a strong association with local recurrence in women with primary DCIS. It suggests that FOXA1 may play different roles in DCIS and in invasive breast cancer. FOXC1 protein expression, in previous studies, was found to be an independent prognostic biomarker in TN primary breast cancer[117]. In this study, we also found that FOXC1 expression was associated with a higher risk of local recurrence, independent of other conventional clinicopathological prognostic variables. However, the roles of FOXA1 and FOXC1 in DCIS should be evaluated in larger population-based studies.

EGFR is a potent stimulating factor of cell-growth-activating pathways and thus stimulates tumor growth when activated[118]. In our study, EGFR-positivity was a predictor of a higher risk of any local recurrence, but these recurrences were more often of the *in situ* type, similar to that observed with the HER2 positive tumors.

4 Paper IV

In this study, we studied a new entity of breast cancer: breast carcinoma with neoductgenesis. The group with neoductgenesis showed a picture of more aggressive tumor biology by the IHC markers used. Exploring a new entity of breast cancer was challenging. The cases included in this study were highly selected to guarantee that the cohort contained possible neoductgenesis cases and a substantial number of controls. The study was not designed to explore the progression the specific breast carcinoma.

The mammographic picture with malignant microcalcifications will raise the question already before surgery whether the patient has a lesion with neoductgenesis. Casting- and crossed stone-like calcifications did correlate with the histopathological picture of neoductgenesis. However, about half of the cases without neoductgenesis also showed malignant microcalcifications on the mammogram. We do not know whether surgical treatment will be influenced by the diagnosis of neoductgenesis and we do not know if, or in what way, adjuvant treatment might be beneficial.

The correlation with HER2 overexpression and ER negativity might suggest that breast carcinoma with neoductgenesis could be related to the HER2

positive molecular sub-group. As we included tumors with biobanked frozen tissue, we will be able to study the gene expression of these lesions. In invasive breast cancer, the HER2 positive subgroup has been shown to be related to a poor prognosis[119-121]. This is not very well studied in DCIS but we have seen indications of this being true also for DCIS[122]. We also found a slightly higher proportion of cases with high proliferation among the neoductgenesis cases. Proliferation has not been used as a prognostic marker for DCIS in the clinical setting in Sweden. In invasive breast cancer however, proliferation is used as one of the risk factors for deciding what type of adjuvant therapy that should be used[123, 124].

We developed reproducible histological criteria for a new subgroup of breast cancer: breast carcinoma with neoductgenesis. Neoductgenesis was related to more aggressive tumor biology and also related to mammographic features. Our findings have to be repeated and the relation to prognosis has to be studied. However, we can already predict a potential benefit for women previously considered to have a pure DCIS but now diagnosed as breast carcinoma with neoductgenesis and, we call attention to the need to develop appropriate treatment regimens.

Acknowledgements

I wish to express my deepest gratitude to everyone involved in my education, work and completion of the thesis. In particular I would like to thank:

Fredrik Wärnberg, my main supervisor, for admitting me to the doctoral program. Thank you for your emphatic and brilliant guidance and advice through the research process. I feel so lucky have had you as my supervisor who always is supportive and believe in my ability. Without you, there would not have been a thesis.

Marie-Louise Fjällskog, my supervisor, who long ago initiated all the work leading up to this thesis, for guiding me and always being positive despite drawbacks. It has been a great pleasure working with you.

Carl Blomqvist, my supervisor, for skilled analyses, for encouragement and for always being supportive and open-minded.

SiminTahmasebpoor for your assistances to picking up slides and blocks.

Christina Johnsson and Thomas Sollie, my co-authors, for helping me in scoring the TMA slides.

For all the wonderful and talented people I had the pleasure of meeting in Anne-Lise Børresen-Dale's research group at the Department of Genetics, Oslo University Hospital. Thank you all for being always encouraging, supporting and helping me during my stay in Oslo.

I am also grateful to my all-loving friends for always being there for me even in difficult times, for making life fun and joyful.

I would like to thank my parents Jianzhi and Meirong, for all their love, unconditional support and encouragement, and for being so proud of me throughout my life.

My beloved husband, Zongli, thank you for what you have done for me, I appreciate it more than you know. And my dear daughter, Tongtong, thank you for coming to my life and the happiness you brought to me.

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