Ductal Carcinoma in Situ: The Journey to Invasive Breast Cancer

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Charlane Doebar

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UITNODIGING

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Ductal Carcinoma in Situ: The Journey to Invasive Breast Cancer

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DUCTAL CARCINOMA IN SITU: THE JOURNEY TO INVASIVE BREAST CANCER

Ductaal carcinoma in situ: de weg naar invasief borstkanker

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Ductaal carcinoma in situ: de weg naar invasief borstkanker

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DUCTAL CARCINOMA IN SITU: THE JOURNEY TO INVASIVE BREAST CANCER

Ductaal carcinoma in situ: de weg naar invasief borstkanker

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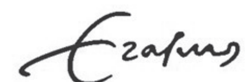

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CHAPTER

1

General Introduction and Outline of the Thesis

DUCTAL CARCINOMA IN SITU

Breast cancer is the most common cancer in women worldwide.[1] In the Netherlands, 1 out of 7 women are diagnosed with breast cancer during her life and this incidence is still rising.[2] Although the prognosis improved over the last decades, it is still the deadliest cancer in women worldwide.[1] Ductal carcinoma in situ (DCIS) is regarded as a non-obligate precursor of invasive breast cancer (IBC).[3] DCIS is characterized by an intraductal proliferation of neoplastic epithelial cells within the mammary ductal-lobular system (Figure 1).[4,5] There is an intact myoepithelial cell layer and basement membrane without invasion in the surrounding stromal tissue. Risk factors for DCIS are similar as described for IBC such as older age, family history of breast cancer, null parity or an older age at first pregnancy.[6-8] The detection rate of DCIS has rapidly increased over the last decades as a result of the increased use and improved resolution of mammographic screening.[9] Currently, DCIS accounts for approximately 15-30% of all breast carcinomas detected in the well-screened population.

Male breast cancer is rare, representing <1% of all breast cancer cases worldwide.[10] Despite its low prevalence, male breast cancer is associated with a worse outcome than female breast cancer patients due to a more advanced stage at presentation.[10-12] In this group of patients, breast precursor lesions are poorly described and understood. Improved understanding of carcinogenesis in the male breast could therefore result in earlier detection of these lesions.

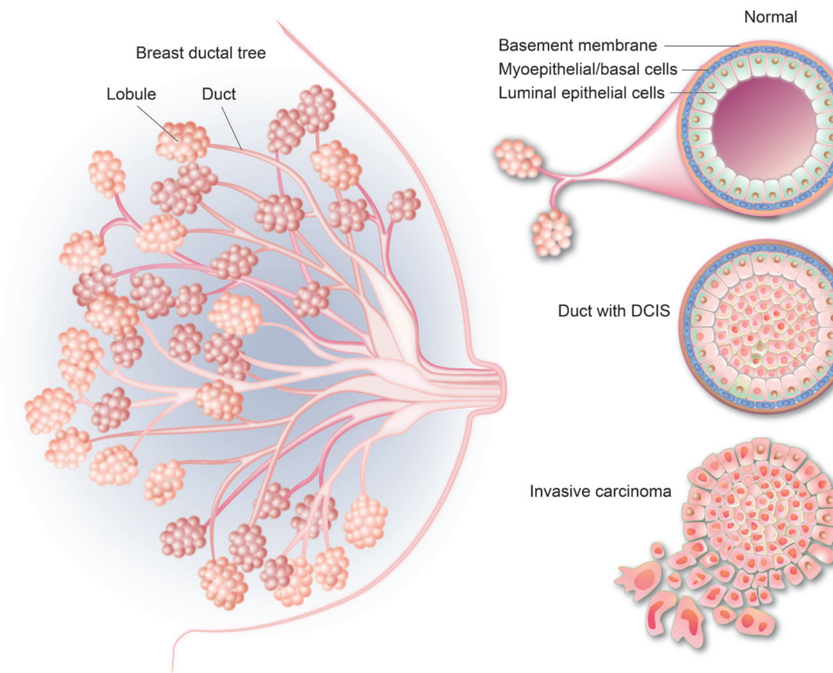

Figure 1. Neoplastic cells initially arise and grow inside milk ducts. Normal breast ducts with luminal and myoepithelial cells, surrounded by a basement membrane. DCIS shows intraluminal growth of neoplastic cells with an intact myoepithelial layer and basement membrane. Invasive carcinoma is characterized by neoplastic cells in the surrounding stroma with no myoepithelial layer and basement membrane.

DCIS subtypes

Based on nuclear grade, DCIS can be categorized as low, intermediate or high grade.[13] Various growth patterns are recognized, based on the arrangement of the intraductal neoplastic cells, i.e. solid, cribriform, micropapillary and papillary. DCIS is a heterogeneous disease and can also be classified into several subtypes that are originally described for IBC.[14-17] Originally, these subtypes are based on gene expression patterns, but each subtype has an immunohistochemical surrogate based on ER, PR, HER2, and Ki-67 index[18] :luminal (ER+ with or without HER2+), basal (triple negative (ER-, PR- and HER2-) and HER2-driven (ER-, HER2+). These subtypes are strongly associated with clinical outcome and therapeutic decisions. There is a strong correlation between histological grade and molecular subtype: ER+/HER2- tumors are more likely to be low grade whereas ER-/HER2+ and triple negative tumors are more likely to be high grade.[19,20] In addition, comparative genomic hybridization (CGH) studies reported a strong correlation between specific copy number alterations

and histological tumor grade, in which high grade lesions demonstrated complex genomic alterations.

Biological behavior of DCIS

DCIS is regarded as a precursor of IBC, as mentioned above.[3] This is supported by remarkable similarities between DCIS and adjacent paired IBC at the morphological and molecular level.[3,21,22] Molecular concordance was initially demonstrated by CGH and gene expression studies. In line with this, known breast cancer mutations, including TP53 and PIK3CA, were also observed at the in situ stage by in-depth sequence studies.[23,24] Currently, it is generally accepted that there are multiple parallel pathways of breast carcinogenesis, rather than a linear progression from low grade to high grade: low grade carcinomas arise from low grade DCIS and have a good prognosis while high grade carcinomas arise from high grade DCIS, which is associated with a worse prognosis.[25-27] However, data is limited regarding the treatment-naive behavior of DCIS, since most patients are treated. Untreated, a subset of DCIS cases, estimated around 40-50%, will progress to invasive disease, while others remain indolent, although exact numbers are unknown.[28-30]

Several studies reported a different distribution of DCIS subtypes in series including patients with pure DCIS (without an invasive component) as compared to studies restricted to IBC.[21] In studies restricted to pure DCIS cases, the prevalence of HER2+ DCIS is higher compared to IBC studies (15-49% versus 5-14% respectively).[21,31,32] In contrast, the frequency of triple negative pure DCIS is low (6-8%) compared to IBC studies (11-13%).[21,31,33-35] With respect to ER+/HER2- cases, reported frequencies do not differ between pure DCIS series and IBC series.[20,21,31,33,34] Furthermore, HER2+ IBC has a relative high frequency of an extensive adjacent DCIS component, compared to other IBC subtypes.[35,36] Overall, these data imply a different biological behavior according to DCIS subtypes. This is supported by clinical studies reporting that DCIS subtype is an independent predictor for ipsilateral recurrence.[16,37]

Molecular evolution of DCIS

Gene expression and CGH analysis of DCIS and synchronous IBC have been performed to identify changes that are specific to each stage (DCIS and IBC) of breast cancer progression.[3,38] However, these analyses could not differentiate between both components due to remarkable similar signatures between DCIS and paired IBC. With the development of novel molecular techniques, genomic in-depth studies of synchronous DCIS and IBC reported intra-tumoral genetic heterogeneity and genetic differences between the in situ and the invasive component.[24,39] Some authors proposed the Darwinian evolution model, also named evolutionary bottleneck model, as an explanation for this phenomenon.[3,40,41] According to this hypothesis (Figure 2A), only distinct subclones with specific genetic changes are selected during the transition from DCIS to invasive disease, which leads to differences in the prevalence of specific mutations between the DCIS and the paired IBC component. In contrast to this model, a multiclonal evolution theory has been proposed, which assumes that multiple subclones in DCIS co-migrate during the transition from DCIS to IBC (Figure 2B).[3,38] Additional in-depth sequence techniques as next generation sequencing (NGS) of synchronous DCIS and IBC might provide additional information regarding the prevalence of subclones, and therefore unravel the clonal evolution of breast cancer at the in situ stage. This technique also allows a more detailed overview of specific genomic alterations that might play an important role in the transition from DCIS to IBC.[42] However, previous genomic in-depth studies of synchronous DCIS and IBC faced several limitations, including the lack of fresh-frozen samples, low tumor volume and intra-tumoral genetic heterogeneity.[38] Consequently, the underlying genomic mechanism of DCIS progression to IBC remains unclear. Beside genetic alterations, micro-environmental changes seem to contribute to invasive progression of DCIS.[43,44] This is based on gene expression analysis that reported substantial stromal changes in the transition from pre-invasive to invasive growth. This was observed in various cell types, including fibroblasts, myoepithelial cells and inflammation cells.[45] In addition, based on the absence of known mutations in myoepithelial cells and fibroblasts surrounding DCIS and IBC, it is suggested that epigenetic changes (methylation and microRNAs) play an important role in these events.[19]

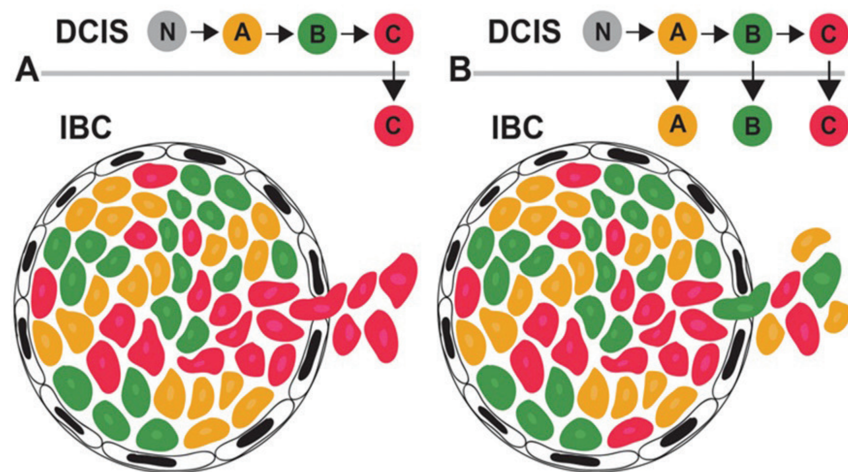

Figure 2. Evolutionary models of DCIS progression: according to the evolutionary bottleneck theory (A), there is an accumulation of subclones, of which only a single subclone is selected to become invasive. In contrast, according to the multiclonal invasion theory (B), there are several combinations of genetic aberrations that result in progression. In this model, all DCIS clones become invasive.

Risk stratification to optimize individualized therapy

Since we cannot accurately predict which cases of DCIS are likely to progress to IBC, clinicians are faced with a major challenge when treating patients with DCIS. Ideally, DCIS with a high malignant potential would be subjected to aggressive therapy whereas indolent DCIS would benefit from an active surveillance (Figure 3). However, since lesions with a high malignant potential are largely indistinguishable from lesions with a low malignant potential, the majority of patients currently undergo relatively extensive local treatment (wide local excision followed by radiation or mastectomy).[46] This leads to overtreatment for a substantial number of patients with DCIS. Therefore, there is a high need for improved risk stratification for DCIS patients. For this purpose, reliable markers are needed to discriminate DCIS cases with a low invasive potential from cases with a high invasive potential. Currently, although several studies reported risk factors for DCIS progression, none of them are considered reliable enough to safely select patients that can avoid adjuvant therapy.[19] Some progress has been made with gene expression assays including Oncotype DX, in order to predict local recurrence risk (invasive or DCIS) of DCIS.[47] This test allows some risk prediction, but its clinical implementation is still limited.

Recent data indicate that apolipoprotein B mRNA editing catalytic polypeptide-like 3B (APOBEC3B) is a key molecular driver inducing mutations in several type of cancers, including breast cancer. Its overexpression and aberrant activation result in increased mutation load and poor clinical outcome in IBC.[48,49] Several studies showed promising results for APOBEC enzymes as a strong candidate for targeted intervention.[49,50] No data is available regarding expression levels of this enzyme at the pre-invasive stage. Increased knowledge with respect to the role of APOBEC3B during breast carcinogenesis could contribute to the development of targeted treated at a very early non-invasive stage of breast cancer.

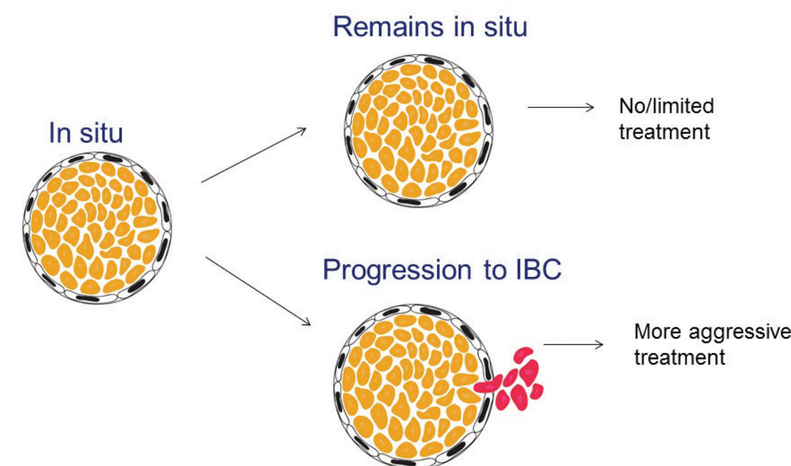

Figure 3. Desired stratification model of DCIS treatment: no/limited treatment for patients with an indolent variant of DCIS and a more aggressive approach restricted to patients with high-risk DCIS.

OUTLINE

Since DCIS is a heterogeneous disease, a better understanding of its behavior is needed to achieve personal treatment strategies in order to avoid over- or undertreatment. This thesis aims to unravel the underlying mechanisms of the transition from DCIS to IBC and to identify potential biomarkers to discriminate DCIS with a low invasive potential from DCIS with a high invasive potential. Further, we studied characteristics of precursor lesions in a large cohort of males with breast cancer in order to increase our understanding of carcinogenesis in this group of patients.

Patients with pure DCIS have no metastatic potential and therefore nodal staging is not necessary. However, a substantial proportion of patients with a pre-operative needle biopsy diagnosis of DCIS shows upgrading to IBC after surgical excision, and consequently, a potential indication for lymph node staging.[51-53] This leads to the clinical dilemma whether or not to conduct a sentinel node biopsy (SNB) in patients with a pre-operative diagnose of DCIS. **Chapter 2** describes novel predictors of invasion in patients with a needle-biopsy diagnosis of DCIS. These predictors could select patients that may benefit from a SNB.

Chapter 3 is a large national cohort study that describes the presence and extent of a DCIS component according to IBC subtypes. These data provide information regarding differences in biological behavior of DCIS subtypes including their clinical relevance (surgical margin status, which is important to achieve optimal local control). **Chapter 4** preludes further on the biological behavior of DCIS. For this study, we hypothesized that patients with extensive involvement of the breast with pure DCIS (without invasion) have a limited invasive potential. On the other hand, we hypothesized that patients with a limited amount of DCIS adjacent to an invasive component have a high invasive potential. In this chapter, we report several differences between these two groups at the transcriptional level by using robust quantitative real-time polymerase chain reaction (RT-PCR). These differences might point towards important molecular alterations of DCIS progression. In **chapter 5**, we describe the levels of APOBEC3B expression in the DCIS component and paired IBC at the mRNA level by quantitative RT-PCR assays. Upregulation of APOBEC3B in the pre-invasive stage might in the future allow early targeted treatment by inhibition of APOBEC3B.

With the development of technologies as NGS, a great opportunity has been created to answer questions regarding the clonal evolution of neoplastic cells during the transition from DCIS to IBC. **Chapter 6** provides results of whole exome and targeted sequencing on a subset of synchronous DCIS and paired IBC lesions.

Chapters 7, **8** and **9** provide results of cancer precursor lesions of the male breast. Data regarding these lesions is sparse and mainly based on case reports or small single center studies. **Chapter 7** provides information regarding the presence of breast cancer precursor lesions in a large series of male breast cancer. For this evaluation we used data from The International Male Breast Cancer Program, a worldwide collaborative effort to collect male breast cancer cases. In addition, this chapter presents NGS data on a subset of male breast cancer and early precursor lesions, which could determine their clonal relation. **Chapter 8** elaborates further on this question by using copy number profiling in synchronous male DCIS and IBC and on a subset of pure DCIS lesions of the male breast. **Chapter 9** discusses the results of this thesis and provides an overview of ongoing research and future prospects. **Chapter 10** includes the appendices.

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CHAPTER

2

Ductal Carcinoma in Situ Diagnosed by Breast Needle Biopsy: Predictors of Invasion in the Excision Specimen

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Breast 2016;27:15-21

ABSTRACT

Background

A substantial proportion of women with a pre-operative diagnosis of pure ductal carcinoma in situ (DCIS) has a final diagnosis of invasive breast cancer (IBC) after surgical excision and, consequently, a potential indication for lymph node staging. The aim of our study was to identify novel predictors of invasion in patients with a needle-biopsy diagnosis of DCIS that would help us to select patients that may benefit from a sentinel node biopsy (SNB).

Patients and Methods

We included 155 patients with a needle-biopsy diagnosis of DCIS between 2000 and 2014, which was followed by surgical excision. Several pre-operative clinical, radiological and pathological features were assessed and correlated with the presence of invasion in the excision specimen. Features that were significantly associated with upstaging in the univariable analysis were combined to calculate upstaging risks.

Results

Overall, 22% (34/155) of the patients were upstaged to IBC. The following risk factors were significantly associated with upstaging: palpability, age ≤40 years, mammographic mass lesion, moderate to severe periductal inflammation and periductal loss of decorin expression. The upstaging-risk correlated with the number of risk factors present: e.g. 9% for patients without risk factors, 29% for patients with 1 risk factor, 37% for patients with 2 risk factors and 54% for patients with ≥3 risk factors.

Conclusion

The identified risk factors may be helpful to predict the upstaging-risk for patients with a needle-biopsy diagnosis of pure DCIS, which facilitates the performance of a selective SNB for high-risk patients and avoid this procedure in low-risk patients.

INTRODUCTION

Ductal carcinoma in situ (DCIS) is regarded as a non-obligate precursor of invasive breast cancer (IBC).[1] The detection rate of DCIS has dramatically increased over the last decades as a result of breast screening and improved resolution of mammography.[2] Currently, DCIS accounts for approximately 20% of all breast carcinomas detected in the well-screened population.[3,4] Pure DCIS has no metastatic potential and therefore lymph node staging is not indicated. However, a substantial proportion (13-48%) of patients with an initial needle-biopsy diagnosis of pure DCIS is upstaged to IBC after final breast surgery.[5-8] These patients with a final diagnosis of IBC may benefit from lymph node staging. So, if we could pre-operatively identify patients with a high risk of upstaging after surgery, we would be able to select patients that may benefit from a sentinel node biopsy (SNB) in the same session as the breast surgery.

So far, several risk factors have been reported to be associated with IBC in the final surgical specimen. These predictive factors included clinical features (age, size of lesion and palpable mass), radiological features (number and type of biopsies, density on mammography, signal intensity curves and large size on MRI) and histopathological features (high grade DCIS, Her2 positive DCIS, comedonecrosis, and solid growth pattern).[9-21] However, as the interpretation of these predictive features in daily practice is equivocal, it results in inadequate prediction of invasion, and consequently, a suboptimal use of nodal staging in patients with a biopsy-diagnosis of pure DCIS. This obviously results in increased costs and complications without a clinical benefit.[22-23]

Several studies reported that the microenvironment of DCIS might play an important role in the progression of DCIS to IBC.[24-32] The role of inflammation in DCIS progression has not been elucidated yet. Angiogenesis increases with the malignant transformation of benign ducts to DCIS and IBC.[29,30] Therefore, microvessel density in needle biopsies with DCIS could be a predictive factor for upstaging. Decorin is a protein of the extracellular matrix and is a potent inhibitor of tumor cell proliferation .[31] A reduced expression of periductal decorin expression in the excision specimen of patients with DCIS has been reported to be associated with an increased risk for ipsilateral locoregional recurrence, either in situ or invasive.[32] However, data is limited regarding the significance of stromal changes in needle biopsies with pure DCIS. We hypothesize that analysis of the microenvironment in needle biopsies with pure DCIS could identify better predictors of upstaging after final excision. Therefore, the aim of our study is to combine several known pre-operative clinical and radiological features

with novel pathological features in order to build a prediction model for upstaging, which may facilitate the selective use of a SNB for high-risk patients only.

PATIENT AND METHODS

Patients

In this retrospective study, patients with a breast needle-biopsy diagnosis of pure DCIS were consecutively selected from the histopathology files of the Erasmus MC Cancer Institute. These patients were diagnosed and treated between 2000 and 2014. Only cases with a subsequent surgical excision within 3 months after diagnosis were included. The following exclusion criteria were applied: history of ipsilateral BC or suspicion of invasion on needle biopsy. Patient characteristics included age, palpability of the lesion and history of mantle field radiation for non-Hodgkin disease.

Radiology

In all patients a mammography was performed before breast needle biopsy, with or without ultrasound and/or MRI. The following imaging features were documented: type of image guidance (stereotactic, ultrasound or MRI), number of biopsies, type of biopsy (vacuum-assisted biopsy (VAB) or automated core biopsy (ACB)) and needle size. The number and size of biopsies were categorized in 2 groups: low number (≤ 10) vs. high (>10) and small size (14- and 18- gauge) vs. large size (10- gauge) respectively.

The pre-operative mammogram and/or ultrasound and/or MRI were reviewed by a dedicated breast radiologist (CM) regarding the presence, type and size of a lesion. Lesions were categorized according to the BIRADS classification.[33] Imaging features for mammography included breast composition as defined by the ACR reporting system.[33] Type of mammographic lesion was categorized as microcalcifications only or mass (± microcalcifications). The enhanced MRI features included type of enhancement (mass vs. non-mass) and late enhancement-time curves. Late enhancement-time curves were recorded as type 1 (slow and persisting curve), type 2 (curve with plateau), and type 3 (curve with washout).

Pathology

Needle biopsy samples were reviewed by two pathologists (CvD and CD), blinded for outcome. Several histologic features of DCIS were reported, including the predominant growth pattern, grade[34], presence or absence of comedonecrosis, microcalcifications and lobular cancerization. Stromal changes included the intensity

of peri-tumoral inflammation (minimal to mild or moderate to severe) and stromal architecture (sclerotic or myxoid). Inflammation intensity was scored according to the method previously described for IBC, as illustrated in Figure 1 of the study published by Lee et al.[35] Examples of a case with minimal inflammation and a case with moderate to severe inflammation are provided in Figure 1 (A and B). Myxoid stroma was defined as “loose”, pale-to-lightly basophilic peritumoral stroma.[36] In case of mixed patterns, the predominant pattern was recorded.

Oestrogenreceptor and Progesteronreceptor were considered positive when at least 10% of the tumor cell nuclei were positive, according to Dutch guidelines for scoring of IBC.[37] Her2 expression was scored according to international guidelines.[38] The proliferation index of DCIS was estimated by the percentage of Ki-67- positive tumor cells. The cut-off value for high proliferative index was a percentage of 20%, according to the St. Gallen criteria for IBC.[39] The number of CD31 positive microvessels were counted with a 40-time magnification in 5 HPF of periductal lesional stroma.[40] Microvessel density was arbitrarily categorized in 2 groups: low density (≤75 vessels per 5 HPF) and high density (>75 vessels per 5 HPF). The intensity of periductal stromal decorin expression was scored semiquantitatively, using normal stroma outside the DCIS area as an internal reference, as either normal expression (comparable to normal stroma), slightly decreased expression (weak loss of intensity as compared to normal stroma) or highly decreased expression (strong loss of intensity as compared to normal stroma). Figure 1 (C and D) shows an example of normal and highly decreased decorin expression.

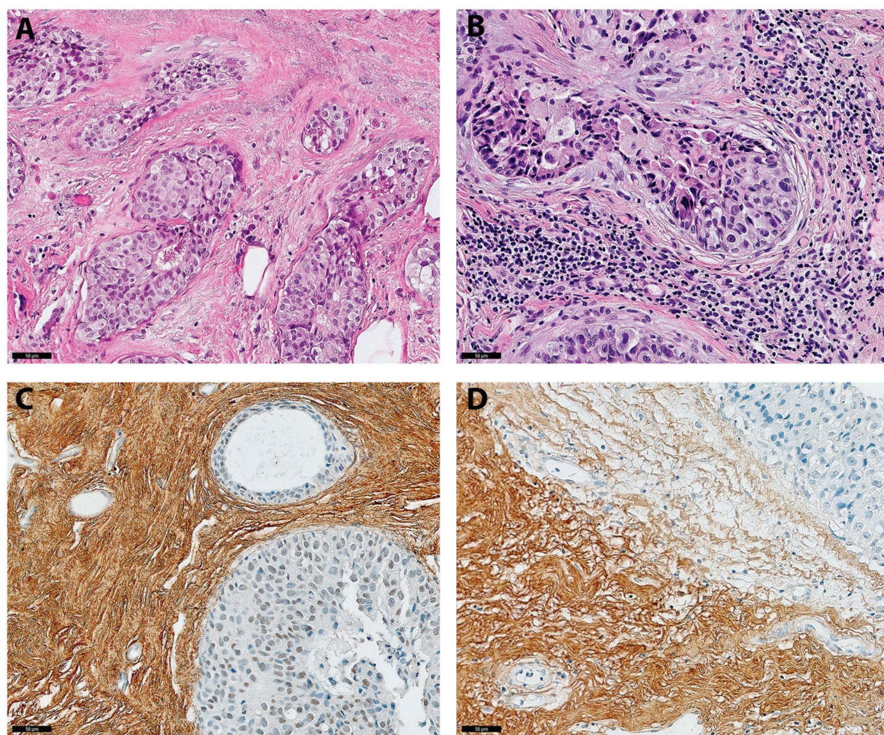

Fig 1. Microscopy (magnification 200x) showing Hematoxylin & Eosin (A, B) and immunohistochemical staining of decorin (C, D). Minimal inflammation (A) in comparison with moderate to severe inflammation (B) in peritumoral stroma. Normal staining of periductal decorin (C) in comparison with loss of periductal decorin (D).

Surgical excision

Several features of the final surgical excision were recorded including type of surgery (BCS or mastectomy, presence/absence of lymph nodes), number of sampled tissue blocks and final diagnosis (pure DCIS or IBC). The extent of DCIS and, if present, the invasive component was recorded. Nodal status was recorded for those patients that underwent nodal staging.

Statistics

Statistical analysis was performed using SPSS version 21.0. Two groups of patients were created: patients with invasion and patients without invasion in the subsequent excision. Chi-square tests were used to analyze the correlation between categorical variables and presence of invasion in the surgical excision. All variables that were associated with upstaging in the univariable analysis were tested as potential

predictors in a multivariable logistic regression analysis. P-values < 0.05 were considered to be statistically significant.

RESULTS

A total number of 155 biopsies, derived from 153 patients (two patients had bilateral lesions) with a pre-operative diagnose of DCIS were included. DCIS was confirmed in 121 (78%) women after surgery. The remaining 34 cases (22%) were upstaged to IBC.

Clinical factors associated with upstaging

In the univariable analysis the following clinical features were significantly associated with IBC at final diagnosis: age ≤ 40 years (P= 0.013) and the presence of a palpable mass (P= 0.017). Table 1. provides an overview of all clinical variables analyzed in this study population.

Table 1. Comparison of clinical features of patients with a final diagnosis of DCIS versus patients upstaged to IBC.

Variables		DCIS (n=121)	IBC (n=34)	Univariable P-value
Age	Median	54 (range 28-84)	52 (range 26-74)	
	≤40 years	12 (10%)	9 (27%)	0.013*
	>40 years	109 (90%)	25 (73%)	
Palpability	No	98 (87%)	22 (69%)	0.017*
	Yes	15 (13%)	10 (31%)	
	Unknown	11	2	
Post mantle field radiation	No	114 (94%)	33 (97%)	0.510
	Yes	7 (6%)	1 (3%)	

*P <0.05

Radiological factors associated with upstaging

All radiologic features assessed in this study are listed in Table 2. In univariable analysis, the presence of a mass lesion on mammography was the only feature that was significantly associated with upstaging (P= 0.009). The following features showed a trend with upstaging, but were not statistically significant: a smaller needle biopsy size (P= 0.062), BIRADS classification 4 or 5 based on ultrasound (P= 0.057), mass enhancement (P= 0.060) and type 3 late enhancement on MRI (P= 0.060).

Table 2. Comparison of pre-operative radiological features of patients with a final diagnosis of DCIS versus patients upstaged to IBC.

Variables		DCIS (n=121)	IBC (n=34)	Univariable P-value
Type of biopsy	ACD	39 (32%)	15 (44%)	0.199
	VAB	82 (68%)	19 (56%)	
Size of CNB	14-,18-gauge	31 (33%)	15 (52%)	0.062
	10-gauge	64 (67%)	14 (48%)	
	Unknown	26	5	
Number of cores	≤ 10	89 (82%)	25 (86%)	0.627
	> 10	19 (18%)	4 (14%)	
	Unknown	13	5	
Mammography				
Performed	No	28 (23%)	10 (29%)	
	Yes	93 (77%)	24 (71%)	
Lesion	No	6 (7%)	4 (17%)	0.111
	Yes	87 (93%)	20 (83%)	
Size (mm)	Median	16 (range 2-110)	16 (range 5-121)	
	≤ 20mm	49 (56%)	13 (62%)	0.642
	> 20mm	38 (44%)	8 (38%)	
Breast composition (ACR)	1	6 (7%)	2 (8%)	0.842
	2	36 (395)	8 (33%)	
	3	31 (33%)	7 (29%)	
	4	20 (21%)	7 (29%)	
BIRADS	1+2	6 (7%)	3 (13%)	0.611
	3	4 (4%)	1 (4%)	
	4+5	83 (89%)	20 (83%)	
Type of lesion	Microcalcification only	73 (92%)	15 (71%)	0.009**
	Mass (± microcalcifications)	6 (8%)	6 (29%)	
	Unknown	14	3	
Ultrasound				
Performed	No	71 (59%)	17 (50%)	
	Yes	50 (41%)	17 (50%)	
Lesion	No	32 (64%)	7 (41%)	0.100
	Yes	18 (36%)	10 (59%)	
Size (mm)	Median	10 (range 3-50)	11 (range 5-29)	
	≤ 10mm	9 (60%)	3 (33%)	0.206
	> 10mm	6 (40%)	6 (67%)	
BIRADS	1+2	32 (64%)	7 (41%)	0.057
	3	11 (22%)	3 (18%)	
	4+5	7 (14%)	7 (41%)	
MRI				
Performed	No	57(47%)	31 (27%)	
	Yes	64 (53%)	13 (73%)	
Size (mm)	Median	39 (range 7-113)	23 (range 8-79)	
	≤ 20mm	14 (36%)	3 (27%)	0.594
	> 20mm	25 (64%)	8 (73%)	

Table 2. Continued.

Variables		DCIS (n=121)	IBC (n=34)	Univariable P-value
Suspicious enhancement	No	16 (28%)	2 (15%)	0.345
	Yes	41 (72%)	11 (85%)	
	Unknown	7	1	
Type of enhancement	Mass	8 (20%)	3 (27%)	0.060
	Non-mass	30 (75%)	5 (46%)	
	Combination	2 (5%)	3 (27%)	
	Unknown	24	5	
Late enhancement				
Type 1 curve	Yes	32 (60%)	10 (83%)	0.133
Type 2 curve	Yes	29 (55%)	9 (75%)	0.198
Type 3 curve	Yes	24 (45%)	9 (75%)	0.060
BIRADS	1+2	21 (37%)	2 (17%)	0.185
	3	3 (5%)	0 (0%)	
	4+5	33 (58%)	11 (85%)	
	Missing cases	7	1	

**P <0.01

Pathological factors associated with upstaging

The following pathologic features were significantly associated with upstaging to IBC: moderate to severe periductal inflammation (P= 0.040) and periductal loss of decorin expression (P= 0.028).

None of the other morphological DCIS features was associated with upstaging to IBC. ER, PR and Her2 expression, either analyzed separately or combined with Ki-67 as immunohistochemical surrogates for molecular subtypes, did not significantly correlate with upstaging. Table 3 provides an overview of all pathological variables analyzed in this study.

Table 3. Comparison of pathological features on needle biopsies of patients with a final diagnosis of DCIS versus patients upstaged to IBC.

Variables		DCIS (n=121)	IBC (n=34)	Univariable P-value
Nuclear grade	Low	12 (10%)	2 (6%)	0.754
	Intermediate	53 (45%)	16 (50%)	
	High	53 (45%)	14 (44%)	
	Missing cases	3	4	
Growth pattern	Micropapillary	3 (2%)	1 (3%)	0.158
	Papillary	2 (2%)	3 (10%)	
	Cribriform	41 (35%)	9 (28%)	
	Solid	72 (61%)	19 (59%)	
	Missing cases	3	2	
Comedonecrosis	No	42 (36%)	17 (53%)	0.072
	Yes	76 (64%)	15 (47%)	
	Missing cases	2	2	
Microcalcification	No	32(27%)	11 (34%)	0.421
	Yes	86(73%)	21 (66%)	
	Missing cases	2	2	
Cancerization of lobules	No	80 (68%)	22 (69%)	0.918
	Yes	38 (32%)	10 (31%)	
	Missing cases	2	2	
Inflammation	Minimal to mild	82 (70%)	16 (50%)	0.040*
	Moderate to severe	36 (30%)	16 (50%)	
	Missing cases	3	2	
Stromal architecture	Sclerotic	102 (86%)	26 (81%)	0.462
	Myxoid	16 (14%)	6 (19%)	
	Missing cases	3	2	
Immunohistochemistry				
ER	Negative	15 (14%)	7 (25%)	0.149
	Positive	94 (86%)	21 (75%)	
	Missing cases	12	6	
PR	Negative	26 (24%)	9 (32%)	0.415
	Positive	80 (76%)	19 (68%)	
	Missing cases	15	6	
HER2	Negative	77 (72%)	19 (68%)	0.670
	Positive	30 (28%)	9 (32%)	
	Missing cases	19	6	
Proliferation index (Ki-67)	Low	78 (76%)	18 (67%)	0.299
	High	24 (24%)	9 (33%)	
	Missing cases	19	7	
Microvessel density (CD31)	Low (≤75 vessels/5 HPF)	48 (47%)	16 (59%)	0.260
	High (>75 vessels/5 HPF)	54 (53%)	11 (41%)	
	Missing cases	19	7	
Decorin expression	Normal to slightly decreased	97 (92%)	21 (78%)	0.028*
	Highly decreased	8 (8%)	6 (22%)	
	Missing cases	16	7	

*P <0.05

Prediction of upstaging by combining risk factors

A multivariable adjustment, including all variables that were significant in the univariable analyses, was performed to identify independent risk factors for upstaging to IBC. The presence of a palpable mass (OR 4.26 [1.14; 15.94], P= 0.030) remained the only significant independent risk factor for upstaging.

The probability of upstaging was further estimated by combining risk factors that showed a significant correlation with upstaging in the univariable analysis. The probability of invasion was more likely with an increasing number of risk factors present. In patients without any risk factors (n=75), the probability of upstaging was 9% as compared to 29%, 37% and 54% for patients with one (n=50), two (n=19), or three or more risk factors (n=11) respectively. In-depth analyses of combined risk factors (e.g. palpability AND inflammation or mass lesion AND young age) were not informative due to small group sizes.

Final excision specimen, correlation with imaging and treatment

The majority of patients (56%) underwent BCS, the remaining patients underwent mastectomy. The median number of sampled tissue blocks/specimen was 21 (range 4-58 blocks). The median pathological DCIS size was 29 mm (range 0.5-170 mm).

A total number of 34 cases (22%) were upstaged to IBC, including micro-invasion in 4 patients and multifocal IBC in 4 patients. The median size of the invasive component was 5.0 mm (range 0.9- 29 mm).

A SNB was performed in 57% (88/155) of all cases, either pre-operatively (n=10), concurrent with the breast surgery (n=64) or as a secondary procedure after breast surgery (n=14). The SN showed presence of tumor cells in 15% (13/88), including isolated tumor cells (ITC) in 61% (8/13), micrometastasis in 8% (1/13) and macrometastasis in 31% (4/13). SNB was performed in the majority (74%; 25/34) of patients upstaged to IBC. Of these cases, a secondary SNB was performed in 28% (7/25) due to upstaging after breast surgery. A total number of 7 patients upstaged to IBC underwent an axillary lymph node dissection (ALND), including four patients with a previous positive SNB containing either micrometastasis (n=1) or macrometastasis (n=3). Of these 4 cases, only one patient with a macrometastasis in the SNB had additional nodal involvement in the ALND. The remaining 3 patients underwent an ALND without a prior SNB due to upstaging after surgery, of which 2 patients had nodal involvement. In the remaining (6/34), neither a SNB nor an ALND was performed. All of these six patients had a tumor diameter of less than 0.3 cm, including micro-invasive

disease in two patients. No recurrent disease was reported after long-term follow up (follow-up range of 6-15 years).

In the group of 63 patients with a final diagnosis of pure DCIS that underwent a SNB, 8 patients had ITC and one patient had a pre-operative SNB with a macrometastasis. This patient underwent a subsequent ALND, which showed no additional lymph nodes involved.

DISCUSSION

A substantial proportion of patients with a pre-operative diagnosis of DCIS are upstaged to IBC after surgery. Several previous studies reported predictive factors for upstaging after a biopsy-diagnosis of DCIS, but the application of these results in daily practice is still suboptimal. This results in a large number of SNBs that are performed in DCIS-patients without clinical benefit and, on the other hand, in a second surgical procedure for upstaged patients who did not undergo a SNB during the first surgical excision.

In our series, the underestimation rate for IBC was 22%, which is in line with underestimation rates reported in literature.[5-10] Palpability, age ≤ 40, mass lesion on mammography, periductal inflammation and loss of decorin expression were significantly associated with IBC at final excision. In our study, about half of the patients had no risk factors for upstaging and consequently, since the risk for upstaging is only 9% for these patients, nodal staging could be avoided for those patients undergoing BCS. In patients with one or more risk factors on the other hand, primary nodal staging by a SNB could be considered. Our study also supports the lack of indication for nodal staging for patients with a final diagnosis of pure DCIS, since only one patient had a macrometastasis and the remaining patients had only ITCs without local recurrences.

In literature, multiple studies reported that palpability is a strong clinical risk factor for upstaging[17,18], which is consistent with our findings. The presence of a mammographic mass lesion (with or without associated microcalcifications) is also known to be associated with invasive disease.[41-43] In our study, however, there was no significant difference in upstaging-risk according to type and size of needle biopsies, which is in contrast with some previous studies.[10,18] This could partly be explained by study size, since we reported a trend towards increased upstaging risk after taking smaller biopsies.

Histologically, we observed a significant correlation between periductal inflammation and upstaging. In patients with IBC and DCIS, there is growing interest in the role of inflammation in relation to prognosis and therapy-response.[44,45] The exact role of inflammation in the progression of DCIS remains to be elucidated, but our data suggest that inflammation might play an important role. Besides, we observed a significant correlation between loss of periductal decorin expression and upstaging. Although this is the first study assessing decorin expression in this context, this result is in line with a previous study that showed loss of decorin in DCIS-associated stroma as compared to normal glandular tissue.[31] Similar results have been reported in colorectal tumors, with a lower expression of stromal decorin in adenomas as compared to normal and hyperplastic tissue.[46] The lowest decorin expression was detected in adenocarcinomas, which supports the hypothesis that downregulation of decorin expression is associated with carcinogenesis. Further research is warranted regarding the potential function of decorin in cancer progression.

In conclusion, we reported several risk factors, including a novel pathological factor regarding stromal changes, associated with upstaging to IBC after a biopsy-diagnosis of pure DCIS. Besides, the risk of upstaging increased with the number of risk factors present in a patient. These factors may be helpful to predict the upstaging-risk for individual patients, which facilitates the performance of a selective SNB for high-risk patients and avoid this procedure in low-risk patients.

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CHAPTER

3

Extent of Ductal Carcinoma in Situ According to Breast Cancer Subtypes: A Population Based Cohort Study

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ABSTRACT

Ductal carcinoma in situ (DCIS) is a precursor of invasive breast carcinoma (IBC). The DCIS component is often more extensive than the invasive component, which affects local control. The aim of our study was to analyze features of DCIS within different IBC subtypes, which may contribute to the optimization of personalized approaches for patients with IBC. Patients with IBC reported according to the synoptic reporting module in the Netherlands between 2009 and 2015 were included. Data extraction included characteristics of the invasive component and, if present, several features of the DCIS component. Resection margin status analyses were restricted to patients undergoing breast-conserving surgery (BCS). Differences between subtypes were tested by a Chi-square test, spearman's Rho test or a one-way ANOVA test. Overall, 36.937 cases of IBC were included. About half of the IBCs (n = 16.014; 43.4%) were associated with DCIS. Her2+ IBC (irrespective of ER status) was associated with a higher prevalence of adjacent DCIS, a larger extent of DCIS and a higher rate of irradicality of the DCIS component as compared to ER+/Her2- and triple-negative subtypes ($P < 0.0001$ for all variables). The prevalence of DCIS in triple-negative IBC on the other hand was lowest. In this large population-based cohort study, we showed significant differences between the prevalence and extent of DCIS according to IBC subtypes, which is also reflected in the resection margin status in patients treated with BCS. Our data provide important information regarding the optimization of local therapy according to IBC subtypes.

INTRODUCTION

Invasive breast cancer (IBC) is a heterogeneous disease which can be categorized into several histologic or intrinsic subtypes that differ in their biological behavior and clinical outcome.[1–3] Intrinsic subtypes are most precisely categorized based on multigene expression assays, although each subtype has an immunohistochemical surrogate based on ER, PR, Her2, and Ki-67 index.[4–7] Ductal carcinoma in situ (DCIS) is seen as a nonobligate precursor of invasive ductal carcinoma (IDC). In the last decades, the detection rate of DCIS increased markedly in the age group of 50–75 years, as a result of the increased use and improved resolution of mammographic mass screening.[8,9] Synchronous DCIS and adjacent IDC show a high degree of concordance regarding morphology and genetic profiles.[10–15] The concordance of receptor expression of ER, PR, and Her2 in DCIS and coexisting IDC is high, with 92% for ER, 93–97% for PR, and about 98–10 % for Her2.[10–12]

Data regarding the process of progression of DCIS to IBC is limited. Several studies reported frequencies of pure DCIS subtypes based on immunohistochemical surrogates originally described for IBC.[5,13,14,16] In these pure DCIS studies, the distribution of subtypes differs from studies including IBC. In pure DCIS studies, frequencies of Her2-positive subtypes are higher as compared to reported frequencies in IBC; about 15–32% of pure DCIS cases are Her2 positive, while this frequency is lower in IBC, about 6–14%.[5, 13, 14, 16–18] Reported frequencies of triple-negative pure DCIS on the other hand are lower than reported frequencies in IBC, 6–8 % in pure DCIS versus 11–13% in IBC.[5,13,14,16,17] Regarding the Luminal A and Luminal B subgroups, the reported frequencies for pure DCIS and IBC are overlapping (38–63% in pure DCIS versus 38–73% in IBC for luminal A and 7–28% in pure DCIS versus 5–26% in IBC for luminal B).[5,13,14,16,17,19] Based on these prevalences, a mathematical, hypothetical model has been built, suggesting different speeds of progression according to breast cancer subtypes.[20] This model suggests that Her2+ DCIS has the slowest progression to IBC, while triple-negative DCIS has the fastest progression.

Since the last decades, the proportion of patients undergoing mastectomy decreased and the majority of patients with localized DCIS are treated with breast-conserving surgery (BCS), followed by breast irradiation.[21] Overall, the local recurrence rate (LRR) for patients with DCIS treated with BCS followed by breast irradiation is about 10–17% within the first 15 years after treatment, of which 50% concerns IBC.[22–24] Recent studies reported that DCIS subtype was an independent predictor for ipsilateral recurrence after treatment by breast surgery alone (BCS or

mastectomy) or breast surgery followed by breast irradiation.[25–28] The overall LRR in patients with pure DCIS was the highest in Her2-positive and luminal B subgroups (10–48% and 25–42% recurring within 10 years of follow-up, respectively) and the lowest in the luminal A subgroup (9–21%).[25–27] Regarding triple-negative DCIS, no firm conclusion could be drawn from the reported LRRs due to limited numbers of patients. Nevertheless, based on LRRs per subtype, Her2-positive DCIS seems to have an increased risk for LR after breast surgery as compared to Her2-negative DCIS. In line with this, the highest LRR was also observed for Her2-positive IBC following breast surgery and irradiation (LRR of 8–21% within 10 years of follow-up), as compared to Luminal A and Luminal B type IBC (LRR 1–8% and 2–10% respectively).[29–31] These data suggest that adjustment of current treatment guidelines according to breast cancer subtypes, e.g., aggressive local therapy restricted to patients with a high LRR, could result in reduction of complications and costs for low risk patients.

Subtyping of DCIS has the potential to study progression-related features and to identify patients at high risk for LR. However, in daily practice, pure DCIS cases are not routinely analyzed for ER, PR and Her2 status, which limits the opportunity for large-scale retrospective studies. Patients with IBC on the other hand are routinely studied for ER, PR, and Her2 status. This provides the opportunity to indirectly assess adjacent DCIS features, which, as mentioned above, share receptor expression pattern in the vast majority of cases. The aim of this study was to analyze features of DCIS within different IBC subtypes, including the resection margin status in patients treated with BCS, which may contribute to the optimization of personalized approaches for patients with IBC.

PATIENTS AND METHODS

Data acquisition

In the Netherlands, all pathology reports are archived in the Dutch Pathology Registry (PALGA).[32] Since 2009, synoptic reporting modules for reporting several common tumor types including breast cancer became available. In these modules, the parameters are captured in numerous variables instead of free text fields. This offers the unique opportunity to analyze all reports created with the module simultaneously.

Patient and tumor characteristics

For this study, we included all patients with IBC reported according to the protocol module in the Netherlands between January 1, 2009 and September 1, 2015 (n = 36.937 cases). Patients with missing ER, PR, and/or Her2 status; pure DCIS; and patients with IBC after previous treatment (irradical resection, neoadjuvant therapy) were excluded.

Patients with bilateral IBC were included as two cases. In case of multiple IBCs in one breast, the largest IBC was included for analysis of tumor characteristics, except for resection margin status, which was assessed for all tumors. Clinicopathologic characteristics included age, type of surgical procedure (BCS or mastectomy), tumor size (≤2 cm, >2 to ≤ 5 cm or > 5 cm), histological type (according to WHO), grade (according to the modified Bloom and Richardson grading system),[33] ER status, PR status, Her2 status, presence of angioinvasion, presence of DCIS, and nodal status. ER status and PR status were defined as positive in case more than 10 % of the cancer cells that showed nuclear staining, irrespective of density, according to the Dutch Guideline for breast cancer treatment.[34] Her2 status was scored according to the international guidelines.[35]

Based on immunohistochemistry, tumors were divided according to the surrogate definitions of intrinsic subtypes as reported in the St Gallen International Expert Consensus 2013.[36] Low PR expression was defined as ≥20 %.[37] However, the absence of information regarding Ki-67 indexes in our dataset limited the ability to differentiate between Luminal A and Luminal B (Her2-) subtypes, so based on the available information, our cases were subtyped according to the following 5 categories:

1. ER+/PR high/Her2-,
2. ER+/PR- or low/Her2-,
3. ER+/Her2+,
4. ER-/PR-/Her2+, and
5. ER-/PR-/Her2-

In case DCIS was present, the following features were documented: relation to the invasive component (restricted to invasive component or not), diameter, nuclear grade, and presence of microcalcifications.[38] The overall resection margin status was reported, as well as the margin for both the invasive component and the DCIS component as either free, focally irradical, or more than focally irradical, according to the Dutch Guideline for Breast Cancer Treatment.[34] Focally irradical is defined as tumor (either invasive or DCIS) reaching the ink in a small area (≤4 mm). In case the tumor (either invasive or DCIS) reaches the ink in a larger area or multiple smaller areas, it is defined as more than focally irradical. This distinction has important clinical consequences in the Netherlands, since patients with a focally positive resection margin of IBC or adjacent DCIS do not undergo second surgery (since radiation with a boost dose results in adequate local control), while patients with a more than focally positive resection margin undergo reexcision, according to the Dutch Guideline for Breast Cancer Treatment 2002.[39] However, these definitions are not applied in most other European and North American countries.[40] Therefore, in this study, we use the term irradicality to describe either focally or more than focally irradical resection margins.

Statistical Analysis

Differences between IBC subtypes were tested by means of a chi-square test (categorical variables) or a one-way ANOVA (continuous variables). Missing values are included in the tables but excluded in the analyses. Furthermore, the correlation between grade of the invasive component and the DCIS component was tested with chi-square. The correlation between the extent of the DCIS-component and resection margin status of the DCIS component was tested with a spearman's correlation coefficient. All analyses were performed with SAS Enterprise Guide 7.1.

RESULTS

Baseline characteristics

Overall, we included 36.937 consecutive cases of IBC reported between January 1, 2009 and September 1, 2015. The median age of our patient cohort was 62 years (range 18–100). The majority of patients (60.4%) underwent BCS. Table 1 provides an overview of clinicopathologic data of all patients. About half of the IBCs (n = 16.014; 43.4%) were associated with DCIS, either restricted within or outside the invasive component (45.3 and 54.7%, respectively). Table 2 provides details of all patients with IBC and adjacent DCIS. Overall, there was a strong correlation between grade of the DCIS component and grade of the invasive component ($p<0.0001$, Chi-square test). Both the extent of DCIS and DCIS extending beyond the invasive component correlated with irradicality of the DCIS component (spearman's rho = 0.3, $p<0.0001$ and $p<0.0001$, Chi-square test, respectively). The frequency of multiple IBCs was significantly higher in IBC cases with adjacent DCIS (10.2%) as compared to IBC cases without adjacent DCIS (7.4%) ($p<0.0001$, Chi-square test).

Table 1. Baseline characteristics of all patients with IBC (n=36937).

Characteristic	N	(%)
Age at diagnosis, years, mean, median (range)	Mean: 61.0 Median: 62.0 (18-100)	
Type of surgery		
Breast-conserving surgery	22328	60.45
Mastectomy	14609	39.55
Histologic tumor type		
Ductal	29630	80.22
Lobular	4703	12.73
Other	2604	7.05
Tumor size		
≤ 2 cm	24359	65.95
> 2 cm-≤ 5 cm	11117	30.10
> 5 cm	1461	3.96
Tumor grade		
1	8622	27.13
2	14894	46.86
3	8266	26.01
Missing	5155	
ER status		
Positive	31662	85.72
Negative	5275	14.28
PR status		
Positive	25400	68.77
Negative	11487	31.10
Her2 status		
Positive	4140	11.21
Negative	32797	88.79
Multiple invasive tumors		
Yes	2650	8.63
No	28051	91.37
Missing	6236	
Angio-invasion		
Yes	3715	14.03
No	22773	85.97
Missing	10449	
Presence of DCIS component		
Yes	16014	43.35
No	20923	56.65
Overall resection margin status (invasive component and/ or DCIS component) *		
Free	18552	83.09
Focally irradical	2286	10.24

Table 1. Continued.

Characteristic	N	(%)
More than focally irradical	1490	6.67
Resection margin status of invasive component only *		
Free	19755	88.48
Focally irradical	1621	7.26
More than focally irradical	**952**	**4.26**
Nodal status		
Negative	11428	60.22
Positive	7550	39.78
Missing	17959	

*Analysis restricted to patients with BCS (n=22328)

Table 2. DCIS-characteristics of all patients with IBC and adjacent DCIS (n=16014).

Characteristic	N	(%)
DCIS grade		
1	2598	16.33
2	7896	49.64
3	5414	34.03
Missing	106	
DCIS restricted to invasive component		
Yes	4452	45.29
No	5377	54.71
Missing	6185	
Diameter of DCIS, cm, mean, median (range)	**Mean: 2.08** **Median: 1.50 (0-20)**	
Presence of DCIS-associated microcalcifications Unknown		
Yes	4400	49.97
No	4406	50.03
Missing	7208	
Resection margin status of DCIS component only *		
Free	8323	83.67
Focally irradical	1168	11.74
More than focally irradical	456	4.58
Missing	34	

*Analysis restricted to patients with BCS (n=9981)

Clinicopathologic features according to breast cancer subtypes

Based on immunohistochemical stainings, IBCs were categorized in the following 5 categories: ER+/PR high/Her2- (n=21315; 57.7%), ER+/PR- or low/Her2- (n=7541; 20.4%), ER+/Her2+ (n=2806; 7.6%), ER-/PR-/Her2+ (n=1334; 3.6%) or ER-/PR-/Her2- (n=3941; 10.7%) . Table 3 provides an overview of patient and tumor characteristics according to different IBC subtypes.

Overall, regarding the invasive component, the ER-/Her2+ and triple-negative subgroups showed the most aggressive biological features. The ER+/Her2- subgroups showed the most favorable biological features while the ER+/Her2+ subgroup showed intermediate results. Regarding the ER+/Her2- subgroups, the presence of a high PR expression was associated with more favorable tumor characteristics as compared to those cases with absence or low PR expression.

In general, patients with Her2+ (irrespective of ER status) and triple-negative IBC were younger as compared to patients with ER+/Her2- IBC (P<0.0001). Besides, median tumor size of these subtypes was larger (P<0.0001), which was in line with the higher proportion of patients undergoing a mastectomy (P<0.0001). Histologically, these tumors were more often of ductal type (p<0.0001) and of higher grade (P<0.0001). The frequency of angioinvasion and nodal involvement was the highest in the ER-/Her2+ subgroup (P<0.0001).

Table 3. Clinicopathologic characteristics according to different subtypes of IBC (n=36937).

Characteristic	ER+, PR high, Her2- (n=21315)		ER+, PR- or low Her2- (n=7541)		ER+, Any PR, Her2+ (n=2806)		ER- , PR-, Her2+ (n=1334)		ER-, PR-, Her2- (n=3941)		P-value
Age at diagnosis, years, mean, median and range	mean: 61.1 median: 62 range: 18-99		mean: 63.4 median: 64 range: 21-97		mean: 57.6 median: 57 range: 19-100		mean: 59.3 median: 59 range: 24-97		mean: 59.0; median: 59 range: 22-98		<0.0001
Type of surgery, no (%)											
Breast-conserving surgery	13507	63.37	4476	59.36	1514	53.96	599	44.90	2232	56.64	<0.0001
Mastectomy	7808	36.63	3065	40.64	1292	46.04	735	55.10	1709	43.36	
Tumor type, no (%)											
Ductal	16695	78.33	5753	76.29	2548	90.81	1255	94.08	3379	85.74	<0.0001
Lobular	3130	14.68	1319	17.49	147	5.24	20	1.50	87	2.21	
Other	1490	6.99	469	6.22	111	3.96	59	4.42	475	12.05	
Tumor size, no (%)											
≤ 2 cm	14849	69.66	4931	65.39	1719	61.26	729	54.65	2131	54.07	<0.0001
> 2 cm-≤ 5 cm	5771	27.07	2267	30.06	964	34.35	528	39.58	1587	40.27	
> 5 cm	695	3.26	343	4.55	123	4.38	77	5.77	223	5.66	
Tumor grade, no (%)											
1	6513	35.17	1786	27.41	203	8.63	29	2.62	91	2.77	<0.0001
2	9559	51.62	3379	51.86	1018	43.28	273	24.66	665	20.21	
3	2445	13.20	1351	20.73	1131	48.09	805	72.72	2534	77.02	
Missing	2798		1025		454		227		651		
Multiple invasive tumors, no (%)											
Yes	1624	9.02	510	8.25	216	9.45	106	9.97	194	5.97	<0.0001
No	16296	90.94	5671	91.75	2070	90.55	957	90.03	3057	94.03	
Missing	3395		1360		520		271		690		
Angio-invasion, no (%)											
Yes	1734	11.20	727	13.46	427	21.72	267	29.28	560	20.52	<0.0001
No	13747	88.80	4673	86.54	1539	78.28	645	70.72	2169	79.48	
Missing	5834		2141		840		422		1212		
Overall resection margin status (invasive and/or DCIS component)*											
Free	11243	83.24	3676	82.13	1205	79.59	459	76.63	1969	88.22	<0.0001
Focally irradical	1366	10.11	492	10.99	178	11.76	92	15.36	158	7.08	
More than focally irradical	898	6.65	308	6.88	131	8.65	48	8.01	105	4.70	
Resection margin status of invasive component*, no (%)											
Free	11879	87.95	3910	87.35	1344	88.77	540	90.15	2082	93.28	<0.0001
Focally irradical	1028	7.61	356	7.95	112	7.40	41	6.84	84	3.76	
More than focally irradical	600	4.44	210	4.69	58	3.83	18	3.01	66	2.96	
Nodal status, no (%)											
Negative	6717	62.17	2293	59.19	774	53.20	359	47.61	1285	61.45	<0.0001
Positive	4087	37.83	1581	40.81	681	46.80	395	52.39	806	38.55	
Missing	10511		3667		1351		580		1850		

*Analysis restricted to patients with BCS (n=22328)

There was a strong correlation between the presence of DCIS and breast cancer subtype (P<0.0001). Table 4 provides an overview of all DCIS-characteristics according to the different subtypes of IBC. DCIS was most often present adjacent to IBCs with overexpression of Her2 (irrespective of ER status) with a frequency of 59.1% in the ER+/Her2+ subgroup and 57.4% in the ER-/Her2+ subgroup. The frequency of a DCIS component was the lowest in the triple negative subgroup (34.1%).

Besides a higher prevalence of DCIS in the Her2+ groups, DCIS was more often located outside the invasive component and the DCIS component was more extensive (P<0.0001 for all variables). DCIS-associated microcalcifications were most often seen adjacent to Her2+ IBC, while the frequency was the lowest in the triple-negative group (p<0.0001).

Analysis of resection margin status was restricted to patients treated with BCS. Overall, the frequency of irradicality (of either the invasive or the DCIS component) was the highest in the Her2+ subgroups and the lowest in the triple negative subgroup (P<0.0001). Analysis of irradicality of the invasive component separately showed the highest frequency of irradicality in the ER+/Her2- subgroups and the lowest in the triple-negative subgroup (P<0.0001). Analysis of irradicality of the DCIS component however showed another distribution as compared to the irradicality of the invasive component; the frequency of irradicality of the DCIS component was the highest in the Her2+ subgroups (P<0.0001).

Table 4. DCIS-characteristics according to different subtypes of IBC (n=16014).

Characteristic	ER+, PR high, Her2- (n=21315)		ER+, PR- or low Her2- (n=7541)		ER+, Any PR, Her2+ (n=2806)		ER-, PR-, Her2+ (n=1334)		ER-, PR-, Her2- (n=3941)		P-value
Presence of DCIS, no (%)											
Yes	9168	43.01	3078	40.82	1658	59.09	766	57.42	1344	34.10	<0.0001
No	12147	56.99	4463	59.18	1148	40.91	568	42.58	2597	65.90	
DCIS grade, no (%)											
1	1983	21.77	512	16.72	61	3.70	4	0.53	38	2.86	
2	5310	58.32	1613	52.66	572	34.73	106	13.93	294	21.11	<0.0001
3	1813	19.91	938	30.62	1014	61.57	651	85.55	998	75.04	
Missing	61		15		11		5		14		
Presence of DCIS-associated microcalcifications, no (%)											
Yes	2464	48.21	876	52.02	512	59.26	240	60.91	308	40.90	<0.0001
No	2647	51.79	808	47.98	352	40.74	154	39.09	445	59.10	
Missing	4057		1394		794		372		591		
DCIS restricted to invasive component, no (%)											
Yes	2774	48.52	823	43.82	383	39.24	127	29.13	345	41.97	<0.0001
No	2943	51.48	1055	56.18	593	60.76	309	70.87	477	58.03	
Missing	3451		1200		682		330		522		
Diameter of DCIS, cm, mean, median and range	mean: 1.9; median: 1.4 range: 0-20		mean: 1.9; median: 1.4 range: 0-19		mean: 2.6; median: 2.0 range: 0-20		mean: 3.2; median: 2.3 range: 0-20		mean: 2.1; median: 1.5 range: 0-15		<0.0001
Resection margin status of DCIS component*											
Free	5103	85.33	1622	83.44	707	78.82	251	71.10	640	82.79	
Focally irradical	640	10.70	245	12.60	127	14.16	73	20.68	83	10.74	<0.0001
More than focally irradical	237	3.96	77	3.96	63	7.02	29	8.22	50	6.47	
Missing	22		5		2		0		5		

*Analysis restricted to patients with BCS and presence of DCIS

3

DISCUSSION

Our national registration system for pathology reporting provided a unique opportunity for this large-scale population- wide cohort study describing the presence and extent of DCIS according to breast cancer subtypes, in relation to other clinicopathologic features.

In our study, we showed substantial differences between immunohistochemical breast cancer subtypes regarding age, type of surgery, histology, tumor grade, and tumor size, which is consistent with literature.[41,42] Briefly, Her2+ and triple-negative tumors are associated with younger age, larger size, and higher grade compared to luminal subtypes. However, on the other side of the spectrum, ER+/Her2- IBC showed the most favorable tumor characteristics, especially in the case of a high PR expression. This is in line with recent work of Prat et al. in which they concluded that the addition of a PR expression of more than 20 % adds prognostic value within the current immunohistochemical-based luminal A definition by improving the identification of IBCs with a good prognosis.[37] The ER+/Her2+ group seems to be an intermediate subgroup.

Regarding DCIS, we showed that Her2+ IBC is associated with a higher prevalence of adjacent DCIS and a larger extent of DCIS as compared to other IBC subtypes. In line with this, we reported a relatively high rate of irradicality of the DCIS component in Her2+ IBC. These findings are consistent with previous studies reporting a relatively high rate of Her2 positivity in pure DCIS cases, presence of extensive DCIS adjacent to Her2+ IBC, and a high LRR after BCS for Her2+ IBC.[13,29,30,42,43] Since the risk of an irradical resection is higher for IBCs that are associated with an extensive DCIS component as compared to those with a limited in situ component,[41,44] it seems likely that the DCIS component adjacent to Her2+ IBC is responsible for the high LRR. Therefore, preoperative knowledge regarding the extent of DCIS according to breast cancer subtypes may result in adjustment of local therapy and consequently local control. This may reduce undertreatment in those patients with a large DCIS component, including fewer secondary surgeries and local recurrences. On the other hand, it may result in less overtreatment in those patients with a low prevalence and/ or limited extent of DCIS, e.g., by reduction of excision volume which affects cosmetic outcome. In recent years, there is an increased number of pathology laboratories performing the ER, PR, and Her2 status on preoperative needle biopsies on a routine basis, mainly as a result of the increased use of neoadjuvant treatment, which provides a better understanding of tumor growth patterns preoperatively. The presence of

DCIS-associated microcalcifications adjacent to the majority of Her2+ IBCs, as shown in this study, may provide important preoperative information regarding imaging by mammography. Besides, since the DCIS component adjacent to Her2+ IBCs is mainly of high grade, a preoperative MRI could be beneficial for these patients, particularly for those without microcalcifications, since this imaging technique is considered to be the most sensitive modality in detecting the presence and extent of intermediate- and high-grade DCIS.[45,46]

According to our knowledge, our study includes the largest series of patients ever published regarding the presence and extent of DCIS adjacent to breast cancer subtypes, thanks to our national protocolled registration of breast cancer pathology reports. However, our study also has several weaknesses including the missing data regarding receptor expression of the DCIS component. However, since several studies reported a very high concordance (90–100%) of ER, PR, and Her2 expression between DCIS and adjacent IBC, it is highly unlikely that this has affected our results. The second limitation is the lack of information regarding proliferation, because Ki-67 is not routinely performed in our pathology laboratories. This limited an accurate categorization of luminal A versus luminal B subtypes, which is partly based on a low versus a high Ki-67 index. A third limitation of our study is the lack of clinical follow-up regarding local control. In this study, we used data from 2009 (in this year we started registering according to standard pathology protocols) until 2015, resulting in inadequate follow-up time.

In conclusion, in this large population-based cohort study, we showed significant differences between the prevalence and extent of DCIS according to breast cancer subtypes. Her2+ IBC was associated with the highest prevalence and extent of DCIS, while on the other side of the spectrum, triple-negative IBC had the lowest prevalence of DCIS of all IBC subtypes. Since the extent of DCIS was also reflected in the resection margin status in patients treated with BCS, these data provide important information regarding the optimization of local therapy.

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CHAPTER

4

Gene Expression Differences Between Ductal Carcinoma in Situ With and Without Progression to Invasive Breast Cancer

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ABSTRACT

To understand the molecular alterations driving the progression of ductal carcinoma in situ (DCIS), we compared patients with pure DCIS and patients with DCIS and synchronous invasive breast cancer (IBC). Twelve patients with extensive pure DCIS were included as a representation of indolent lesions with limited invasive capacity. These cases were matched with 12 patients with a limited DCIS component and IBC, representing lesions with a high invasive potential. Matching included age and surrogate DCIS subtypes. Gene expression profiling was performed on DCIS cells to identify transcriptional differences between these two groups. The identified genes were validated by immunohistochemistry. Nine genes showed significantly different expression. Most of these genes were highly expressed in DCIS samples with IBC, including PLAU ($P = 0.002$), COL1A1 ($P = 0.006$), KRT81 ($P = 0.009$), S100A7 ($P = 0.015$), SCGB1D2 ($P = 0.023$), KRT18 ($P = 0.029$), and NOTCH3 ($P = 0.044$), whereas EGFR and CXCL14 showed a higher expression in cases with pure DCIS ($P = 0.015$ and $P = 0.028$, respectively). This difference was only significant for SCGB1D2 ($P = 0.009$). Hierarchical clustering revealed distinct clustering of patients with and without invasion. Patients with pure DCIS have a different gene expression pattern as compared to patients with DCIS and synchronous IBC. These genes may pinpoint to driver pathway(s) that play an important role in DCIS progression.

INTRODUCTION

Ductal carcinoma in situ (DCIS) is a nonobligate precursor lesion of invasive breast cancer (IBC).[1] In the past decades, the detection rate of DCIS increased dramatically as a result of the increased use and improved resolution of mammographic screening.[2] Nowadays, DCIS accounts for 15% to 30% of all new breast cancer cases detected in a well-screened population.[1,2]

The mechanism behind progression of DCIS to IBC remains to be elucidated. In daily practice, most patients with pure DCIS are treated with local resection with or without radiation. Therefore, data are limited regarding the biological behavior of DCIS. Only a few small retrospective studies reported on the frequency of progression of untreated patients with a biopsy diagnosis of pure DCIS.[3,4] In these series, approximately 40% to 50% of cases progressed to IBC after a follow-up of 20 to 30 years, whereas the other cases remained indolent.

There is much debate regarding the optimal treatment of DCIS. Because DCIS is a noninvasive disease, current local treatment protocols result in overtreatment for many patients, which is associated with increased costs and morbidity without clinical benefit. On the other hand, a substantial proportion of DCIS cases progress to IBC and, obviously, these patients may benefit from prevention and early treatment.

Paired comparative genomic assays have widely been performed on cases with DCIS and synchronous IBC, showing a high genomic resemblance.[5-7] However, comparative genomic assays of pure DCIS versus DCIS with progression to IBC are sparse and partly biased by the inclusion of different DCIS subtypes.[8-10] Recent studies reported differences in the behavior of DCIS according to DCIS subtypes, based on immunohistochemistry or gene expression patterns.[11-17] These reported features allow some recurrence risk prediction, but they are not widely used to select individual patients who can avoid adjuvant therapy.[15] The identification of novel genetic alterations and molecular pathways underlying the transformation from DCIS to IBC may help to establish biomarkers that have the potential to distinguish low-risk patients who do not require aggressive treatment and high-risk patients who are likely to progress to IBC.

In daily practice, a proportion of patients presents with extensive involvement of the breast with DCIS without any signs of invasion. Although one cannot exclude that these cases would progress over time, the DCIS growth pattern suggests that these cases have a limited invasive potential. On the other hand, other patients have a limited amount of DCIS adjacent to an invasive component or multiple foci of invasion,

suggesting a high invasive potential. These differences in biological behavior of DCIS imply different alterations at the molecular level. On the basis of these observations, we attempted to identify molecular differences at the transcriptional level with robust quantitative RT-PCR assays. For this purpose, we compared breast tissues of patients with extensive DCIS (representing a group with limited invasive potential) with breast tissues of patients who presented with a limited DCIS component and synchronous IBC (as a surrogate for a DCIS subtype with a high invasive potential). On the basis of these data, we aimed to increase our understanding regarding molecular alterations driving DCIS progression and, consequently, facilitate the identification of novel, potential therapeutic targets.

METHODS

Patients

In this retrospective study, two groups of patients were selected from the histopathology files of the Erasmus MC Cancer Institute. The first group included patients with extensive pure DCIS, which was defined as DCIS with a diameter of ≥ 5 cm, to represent a group of DCIS with a biologically indolent behavior with limited invasive capacity. The second group included patients with a limited amount of DCIS (defined as DCIS with a diameter of ≤ 1 cm) with adjacent IBC. This latter group was selected as a representation of a biologically aggressive type of DCIS with high invasive capacity. Patients from the first group were matched with patients from the second group to correct for potential confounders. Matching included age (categorized from 30-40 years, 40-50 years, 50-60 years and 70-80 years) and surrogate DCIS subtypes, as described below. Patients with a history of breast cancer, ipsilateral breast irradiation or a BRCA mutation were excluded.

We used coded leftover patient material in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.federa.org/codes-conduct, last accessed January 10, 2017). According to institutional and national guidelines, no informed consent was needed for this study.

Pathologic evaluation

Formalin-fixed-paraffin-embedded (FFPE), hematoxylin and eosin (H&E) stained whole sections of excision specimens were collected and reviewed by two pathologists (C.H.D, S.C.D). Cases of pure DCIS were extensively sampled according the national Dutch guidelines with a minimum of 10 tissue blocks of the lesion (Dutch

national guideline breast cancer 2012, https://richtlijnendatabase.nl/en/richtlijn/breast_cancer/pathology/criteria_for_dcis.html, last accessed January 10, 2017). Histopathological features of DCIS included grade according The Dutch Guidelines Database[18] and surrogate subtyping based on immunohistochemistry, as originally described for IBC.[19] According to these criteria, DCIS was categorized as luminal A [estrogen receptor (ER)+, progesterone receptor (PR) high, Her2-, Ki-67 low], luminal B Her2- (ER+, Her2-, PR-, or low and/or Ki-67 high), luminal B Her2+ (ER+, Her2+, any PR, any Ki-67), or nonluminal Her2 positive (ER-, PR-, Her2+). A cutoff of 20% Ki-67 (MIB-1; Dako, Glostrup, Denmark) positive cells was used to distinguish cases with a low versus high proliferative index. Low progesterone (PR 1E2; Ventana, Tucson, AZ) expression was defined as ≤20%.[20]

Immunohistochemical evaluation was performed on FFPE whole slides (4 mm thick) using the Ventana Benchmark Ultra automatic stainer. ER (ER SP1; Ventana) was considered positive when at least 10% of the DCIS cells were positive, irrespective of intensity (https://www.gov.uk/government/ collections/breast-screening-professional-guidance, last accessed January 10, 2017). Immunohistochemical HER2 expression (Her2 4B5; Ventana) was scored on all cases, according to international guidelines.[21] Equivocal cases were evaluated by silver in situ hybridization.

We also stained for P53 (BP53-11; Ventana), which was considered aberrant in case of a confluent negative staining or a strong diffuse positive staining. An intermediate expression of any intensity was considered to be normal.[22]

RNA extraction, cDNA synthesis, Pre-Amplification and gene expression evaluation (RT-qPCR)

Areas composed of at least 50% DCIS cells were microdissected from 10 to 15 hematoxylin and eosinestained sections (6 mm thick) of FFPE tissue. Microdissection was performed with a sterile needle under a stereomicroscope (Zeiss, Oberkochen, Germany). On the basis of this method, contamination of other cell types (myoepithelial cells, stroma, lymphocytes) cannot completely be avoided. However, the estimated tumor cell percentage in our series was high (75% to 90% in the group of patients with pure DCIS and 70% to 85% in the group of patients with DCIS and synchronous IBC). RNA was extracted from these cells using the Qiagen (Hamburg, Germany) AllPrep DNA/RNA FFPE Kit, according to the manufacturer's instructions. Concentrations were measured with a Nanodrop 1000 system (Thermo Fisher Scientific, Waltham, MA). cDNA was generated from a total of 100 ng RNA for 30 minutes at 48°C with RevertAid H minus (Thermo Fisher Scientific). Gene-specific preamplification was performed

for 96 genes (93 tumor-specific genes and 3 reference genes, including GUSB, HMBS, and HPRT1), using the TaqMan PreAmp Master mix (ThermoFisher Scientific) for 15 cycles. This was followed by TaqMan probeebased real-time PCRs, according to the manufacturer's instructions, in an MX3000P Real-Time PCR System (Agilent, Santa Clara, CA).

These 93 tumor-specific target genes were selected based on their reported involvement in tumorigenesis and/or mutagenesis.[23] Gene expression levels were quantified relative to the average expression of GUSB, HMBS, and HPRT1 using the 2^(average Cq reference genes - Cq target gene) method. Samples with an average reference gene expression of Cq >25 were considered to be of insufficient RNA quality and were excluded from further analysis. In one of our previous studies, we compared the expression levels of 55 of our 93-gene panel between paired freshly frozen and FFPE samples and reported high levels of concordance (data not shown).[24]

Immunohistochemistry and Gene Function

Genes with a significantly different expression level between pure DCIS and DCIS with synchronous IBC were validated by immunohistochemistry. Antibodies and scoring methods are described below.

We used the DAVID Gene Functional Classification tool to evaluate the gene function of the differently expressed genes according to Gene Ontology (GO).[25,26]

Statistical Analysis

We used a paired samples t-test (IBM SPSS statistics 23) to compare the expression levels of the 93 genes in matched pure DCIS and DCIS with synchronous IBC.

To evaluate whether pure DCIS cases could be distinguished from cases with DCIS and synchronous IBC, a DCIS index-score was calculated to evaluate the impact of significant differences between the matched samples. For this DCIS index-score, the paired-samples t-test was used in the following equation:

SUM_{1-9} (t-test value gene X_1 * ΔCq of gene transcript X_1 + t-test value gene X_2 * ΔCq of gene transcript X_2 + ….t-test value gene X_9 * ΔCq of gene transcript X_9).

The X^2 test was used to analyze immunohistochemical differences between pure DCIS lesions and DCIS with synchronous IBC. $P < 0.05$ was considered to be statistically significant.

RESULTS

Patients

In total, 24 patients were included, divided into two matched groups of 12 patients each. The overall median age was 56 years (range, 31 to 80 years). The median age in the group of patients with pure DCIS was 55 years (range, 31 to 76 years); in the group with an adjacent invasive component, it was 58 years (range, 32 to 80 years). The median follow-up of patients with pure DCIS was 37 months (range, 24 to 76 months). No invasive recurrences or distant metastases were reported. In the pure DCIS group, the median DCIS size was 7 cm (range, 5 to 13 cm). The DCIS lesions of both groups were graded as grade 2 or grade 3. On the basis of immunohistochemical subtyping, nine matched pairs were categorized as luminal A subtype and three matched pairs as luminal B subtype. None of the cases showed an aberrant P53-staining pattern. **Table 1** provides an overview of the clinicopathological features of the 12 matched pairs.

Table 1. Clinicopathological features of patients with pure DCIS and matched patients with DCIS and synchronous IBC (n=12 pairs).

Matched pairs	Pure DCIS or DCIS and synchronous IBC	Age	Histological grade DCIS	ER	PR	HER2	Ki67	DCIS subtype
1	Pure DCIS	56	3	+	-	-	Low	Luminal B
	DCIS+IBC	51	2	+	+	-	High	Luminal B
2	Pure DCIS	56	2	+	+	-	Low	Luminal A
	DCIS+IBC	58	2	+	+	-	Low	Luminal A
3	Pure DCIS	61	2	+	+	-	Low	Luminal A
	DCIS+IBC	67	2	+	+	-	Low	Luminal A
4	Pure DCIS	76	2	+	+	-	Low	Luminal A
	DCIS+IBC	77	2	+	+	-	Low	Luminal A
5	Pure DCIS	51	2	+	+	-	Low	Luminal A
	DCIS+IBC	59	2	+	+	-	Low	Luminal A
6	Pure DCIS	71	2	+	+	-	Low	Luminal A
	DCIS+IBC	80	2	+	+	-	Low	Luminal A
7	Pure DCIS	53	2	+	+	-	Low	Luminal A
	DCIS+IBC	51	2	+	+	-	Low	Luminal A
8	Pure DCIS	54	2	+	+	-	Low	Luminal A
	DCIS+IBC	58	2	+	+	-	Low	Luminal A
9	Pure DCIS	53	3	+	-	+	Low	Luminal B
	DCIS+IBC	51	2	+	+	+	Low	Luminal B
10	Pure DCIS	57	3	+	-	-	Low	Luminal B
	DCIS+IBC	59	2	+	-	-	Low	Luminal B
11	Pure DCIS	31	2	+	+	-	Low	Luminal A
	DCIS+IBC	32	3	+	+	-	Low	Luminal A
12	Pure DCIS	49	2	+	+	-	Low	Luminal A
	DCIS+IBC	42	2	+	+	-	Low	Luminal A

Gene expression profiles of matched cases with pure DCIS and DCIS with synchronous IBC

On the basis of the 93 selected genes, 9 showed a significant different expression between patients with pure DCIS and patients with DCIS and synchronous IBC. In total, 4 of 93 genes (AURKA, CD133, MAGEA3, and SNAPC2) were not expressed at all. **Supplemental Table S1** provides an overview of the remaining 89 genes. The differently expressed genes included COL1A1, CXCL14, EGFR, KRT81, KRT18, NOTCH3, PLAU, S100A7, and SCGB1D2 (**Table 2**). Most of these nine genes were significantly highly expressed in DCIS samples with synchronous IBC as compared to pure DCIS cases: PLAU (P = 0.002), COL1A1 (P = 0.006),=KRT81 (P = 0.009), S100A7 (P = 0.015), SCGB1D2 (P = 0.023), KRT18 (P = 0.029), and NOTCH3 (P = 0.044). The remaining two genes, EGFR and CXCL14, showed a significantly higher expression in cases with pure DCIS as compared to cases with DCIS and synchronous IBC (P = 0.015 and P = 0.028, respectively).

Supplemental Table 1. Analysis of 89 tumor specific-genes in twelve paired samples.

	Paired differences							
	Mean	**Std. Deviation**	**Std. error Mean**	**Lower**	**Upper**	**T**	**Df**	**P-value (2-tailed)**
PLAU	1.178	1.021	0.295	0.530	1.827	3.998	11	0.002*
COL1A1	2.164	2.179	0.629	0.779	3.549	3.44	11	0.006*
KRT81	2.347	2.564	0.740	0.718	3.976	3.171	11	0.009*
EGFR	-1.076	1.289	0.372	-1.895	-0.257	-2.891	11	0.015*
S100A7	2.918	3.519	1.016	0.682	5.154	2.873	11	0.015*
SCGB1D2	3.228	4.224	1.219	0.544	5.911	2.647	11	0.023*
CXCL14	-1.482	2.026	0.585	-2.769	-0.195	-2.534	11	0.028*
KRT18	0.499	0.689	0.199	0.062	0.937	2.511	11	0.029*
NOTCH3	0.868	1.318	0.380	0.030	1.705	2.280	11	0.044*
CEA CAM5	2.552	4.682	1.352	-0.423	5.527	1.888	11	0.086
SCGB2A2	2.168	4.139	1.195	-0.462	4.797	1.814	11	0.097
MUC1 EMA	1.053	2.013	0.581	-0.225	2.332	1.813	11	0.097
MYL3	-0.078	0.149	0.043	-0.172	0.017	-1.797	11	0.100
TFF1	1.892	3.674	1.061	-0.443	4.226	1.784	11	0.102
IL17BR3	-0.734	1.435	0.414	-1.646	0.177	-1.773	11	0.104
DUSP4	1.750	3.430	0.990	-0.429	3.929	1.767	11	0.105
FOXA1	0.395	0.775	0.224	-0.098	0.888	1.765	11	0.105
IGFBP5	1.560	3.113	0.899	-0.418	3.538	1.736	11	0.110
DTX3	0.449	0.964	0.278	-0.163	1.061	1.615	11	0.135
FEN1	0.247	0.530	0.153	-0.090	0.583	1.614	11	0.135
AGR2	-1.474	3.314	0.957	-3.580	0.632	-1.541	11	0.152
TM4SF13	-0.668	1.513	0.437	-1.629	0.294	-1.528	11	0.155
COL2A1	-0.954	2.177	0.629	-2.338	0.429	-1.518	11	0.157
TWIS	-1.018	2.355	0.680	-2.515	0.478	-1.498	11	0.162
CD44	0.421	1.006	0.290	-0.218	1.060	1.449	11	0.175
PLOD2	0.522	1.300	0.375	-0.304	1.347	1.391	11	0.192
CAV1	-0.918	2.407	0.695	-2.447	0.611	-1.322	11	0.213
CD24	0.743	2.081	0.601	-0.579	2.064	1.236	11	0.242
CCNE2	0.553	1.602	0.463	-0.465	1.570	1.195	11	0.257
SEPP1	0.466	1.409	0.407	-0.429	1.361	1.146	11	0.276
KIF11	0.468	1.431	0.413	-0.442	1.377	1.131	11	0.282

Supplemental Table 1. Continued.

	Paired differences							
	Mean	**Std. Deviation**	**Std. error Mean**	**Lower**	**Upper**	**T**	**Df**	**P-value (2-tailed)**
PKP3	0.625	1.962	0.566	-0.622	1.872	1.103	11	0.293
KRT17	-0.419	1.328	0.383	-1.263	0.425	-1.093	11	0.298
PTRF	-0.578	1.833	0.529	-1.743	0.586	-1.093	11	0.298
PTPRK	-0.374	1.189	0.343	-1.130	0.381	-1.090	11	0.299
CTTN EMS1	0.363	1.176	0.339	-0.385	1.110	1.068	11	0.308
CD29	0.245	0.798	0.230	-0.262	0.752	1.063	11	0.310
CD45	0.364	1.218	0.352	-0.410	1.138	1.036	11	0.323
ERBB2	0.690	2.327	0.672	-0.788	2.168	1.027	11	0.326
GALGT	0.369	1.265	0.365	-0.434	1.173	1.011	11	0.334
CDH5	-0.625	2.218	0.640	-2.034	0.784	-0.976	11	0.350
SBEM	-1.680	6.068	1.752	-5.536	2.176	-0.959	11	0.358
CLDN3	-0.433	1.569	0.453	-1.430	0.564	-0.957	11	0.359
ERBB3	0.216	0.783	0.226	-0.282	0.713	0.955	11	0.360
FGFR3	-0.758	2.969	0.857	-2.644	1.129	-0.884	11	0.396
MET	-0.502	2.064	0.596	-1.813	0.810	-0.842	11	0.418
MKI67	0.189	0.826	0.238	-0.335	0.714	0.794	11	0.444
MELK	0.397	1.756	0.507	-0.719	1.512	0.783	11	0.450
VWF	-0.469	2.097	0.605	-1.802	0.863	-0.775	11	0.455
IGFBP4	0.366	1.674	0.483	-0.698	1.429	0.757	11	0.465
MCAM	-0.443	2.112	0.610	-1.784	0.899	-0.726	11	0.483
FGFR4	0.553	2.685	0.775	-1.154	2.259	0.713	11	0.491
GATA3	0.253	1.290	0.373	-0.567	1.073	0.68	11	0.511
S100A16	0.328	1.696	0.489	-0.749	1.406	0.671	11	0.516
LAD1	-0.284	1.518	0.438	-1.248	0.680	-0.649	11	0.530
LOXL2	0.258	1.431	0.413	-0.652	1.167	0.623	11	0.546
ITGA6	-0.246	1.395	0.403	-1.132	0.640	-0.611	11	0.554
KRT7	-0.494	2.811	0.811	-2.280	1.292	-0.609	11	0.555
IGFBP3	0.480	2.825	0.816	-1.315	2.275	0.589	11	0.568
PSMD10	0.280	1.737	0.501	-0.824	1.384	0.558	11	0.588
TOX3	0.389	2.541	0.734	-1.225	2.004	0.531	11	0.606
IGFBP2	-0.285	1.903	0.549	-1.494	0.924	-0.519	11	0.614
FGFR2	0.241	1.669	0.482	-0.819	1.301	0.500	11	0.627
TOP2A	0.265	2.182	0.630	-1.122	1.652	0.421	11	0.682
ESR1	-0.216	1.802	0.520	-1.361	0.929	-0.415	11	0.686
EPCAM	-0.123	1.174	0.339	-0.869	0.623	-0.364	11	0.723
MSMB	-0.204	1.949	0.563	-1.442	1.034	-0.363	11	0.724
CDH1	-0.159	1.596	0.461	-1.173	0.855	-0.345	11	0.736
CCNE1	0.139	1.470	0.424	-0.795	1.073	0.328	11	0.749
PIP	0.457	4.904	1.416	-2.659	3.573	0.323	11	0.753
TIMP3	0.176	1.933	0.558	-1.052	1.404	0.315	11	0.759
DTL	0.137	1.585	0.458	-0.870	1.144	0.299	11	0.771
BST1	0.150	1.763	0.509	-0.970	1.270	0.295	11	0.774
PLK1	-0.170	2.289	0.661	-1.624	1.284	-0.257	11	0.802
FKBP10	-0.111	1.608	0.464	-1.133	0.911	-0.239	11	0.816
NME1	0.058	0.898	0.259	-0.512	0.629	0.225	11	0.826
SELE	-0.173	2.672	0.771	-1.871	1.525	-0.225	11	0.826
TFF3	-0.261	4.616	1.332	-3.194	2.672	-0.196	11	0.848
CCND1	0.095	1.700	0.491	-0.985	1.175	0.194	11	0.850
CRABP2	-0.236	4.267	1.232	-2.947	2.475	-0.191	11	0.852
KRT19	0.057	1.326	0.383	-0.786	0.899	0.148	11	0.885
CCNB1	0.055	1.326	0.383	-0.788	0.898	0.144	11	0.888
VEGFR2	-0.093	2.516	0.726	-1.692	1.505	-0.129	11	0.90
ERBB4	0.092	2.711	0.783	-1.631	1.814	0.117	11	0.909
KPNA2	-0.041	1.370	0.396	-0.911	0.830	-0.103	11	0.920
SPDEF	-0.013	0.464	0.134	-0.308	0.283	-0.093	11	0.927
EEF1A2	-0.045	1.887	0.545	-1.244	1.154	-0.083	11	0.936
SMA	0.074	3.626	1.047	-2.230	2.378	0.071	11	0.945
CEP55	-0.013	1.500	0.433	-0.967	0.940	-0.031	11	0.976

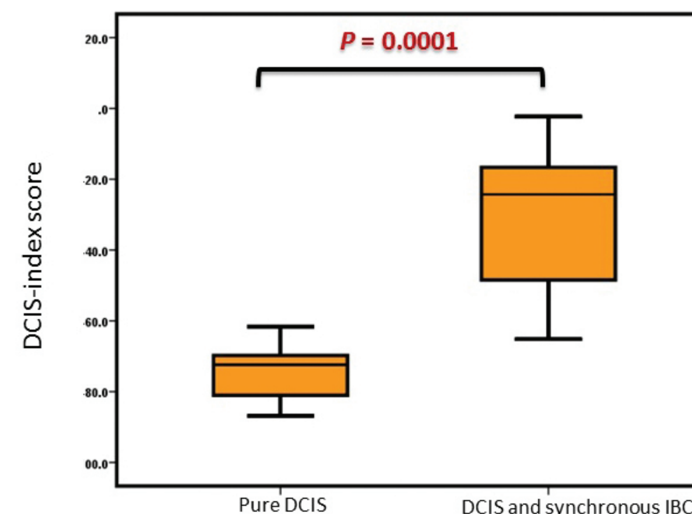
*Based on T-test. genes that are significantly differed (P < 0.05)

Table 2. Overview of genes with a significantly different expression between pure DCIS and DCIS with synchronous IBC.

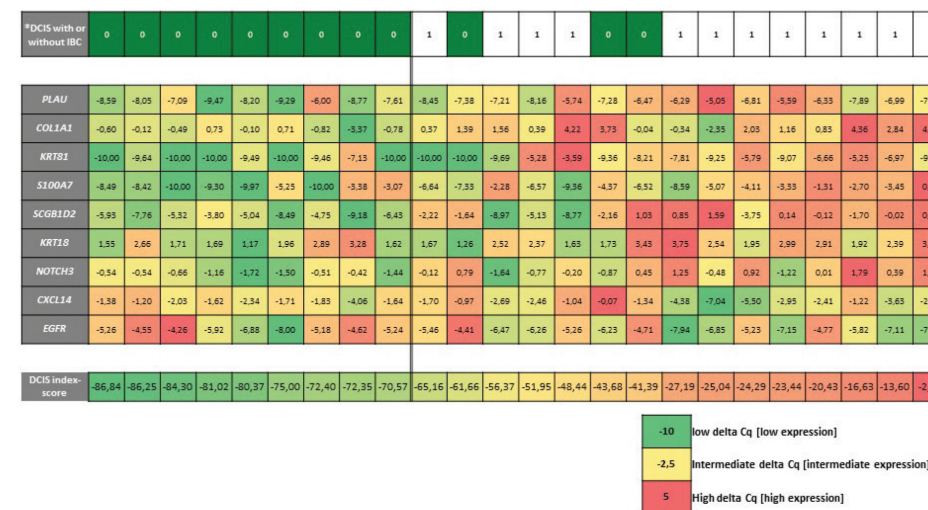
Gene	Gene Name	Paired differences						
		Mean	Std. Deviation	Std. error Mean	Lower	Upper	T	P-value (2-tailed)
PLAU	Plasminogen activator, urokinase	1.178	1.020	0.294	0.529	1.827	3.998	0.002
COL1A1	Collagen, type I, alpha 1	2.164	2.179	0.629	0.779	3.548	3.440	0.006
KRT81	Keratin 81	2.346	2.563	0.740	0.717	3.975	3.171	0.009
EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	-1.075	1.289	0.372	-1.894	-0.256	-2.891	0.015
S100A7	S100 calcium binding protein A7	2.918	3.519	1.015	0.682	5.154	2.873	0.015
SCGB1D2	Secretoglobin, family 1D, member 2	3.227	4.224	1.219	0.543	5.911	2.647	0.023
CXCL14	Chemokine (C-X-C motif) ligand 14	-1.481	2.025	0.584	-2.768	-0.194	-2.534	0.028
KRT18	Keratin 18; keratin 18 pseudogene 26; keratin 18 pseudogene 19	0.499	0.688	0.198	0.061	0.936	2.511	0.029
NOTCH3	Notch homolog 3 (Drosophila)	0.867	1.317	0.380	0.030	1.704	2.280	0.044

On the basis of these nine genes with a significantly different expression between both groups, a DCIS index score was calculated (**Figure 1**). The DCIS index value ranged from -2.31 to -86.84. The optimal cutoff value to discriminate pure DCIS samples from DCIS samples with synchronous IBC was -65.16 (**Figure 1**A). Supervised hierarchical clustering analysis based on these nine genes separated the most pure DCIS lesions from DCIS lesions with synchronous IBC (**Figure 1**B). However, three samples of pure DCIS clustered within the group of DCIS cases with synchronous IBC as a result of a high DCIS index score. Notably, two of these three samples were PR negative and/or HER2 positive and, therefore, categorized as luminal B.

Figure 1. DCIS-index scores of differently expressed genes between matched pairs of patients.



A. Box-plot with DCIS index-scores that significantly differentiate patients with pure DCIS from patients with DCIS and synchronous IBC.



*DCIS with or without IBC	0	0	0	0	0	0	0	0	0	1	0	1
PLAU	-8,59	-8,05	-7,09	-9,47	-8,20	-9,29	-6,00	-8,77	-7,61	-8,45	-7,38	-7,21
COL1A1	-0,60	-0,12	-0,49	0,73	-0,10	0,71	-0,82	-3,37	-0,78	0,37	1,39	1,56
KRT81	-10,00	-9,64	-10,00	-10,00	-9,49	-10,00	-9,46	-7,13	-10,00	-10,00	-10,00	-9,69
S100A7	-8,49	-8,42	-10,00	-9,30	-9,97	-5,25	-10,00	-3,38	-3,07	-6,64	-7,33	-2,28
SCGB1D2	-5,93	-7,76	-5,32	-3,80	-5,04	-8,49	-4,75	-9,18	-6,43	-2,22	-1,64	-8,97
KRT18	1,55	2,66	1,71	1,69	1,17	1,96	2,89	3,28	1,62	1,67	1,26	2,52
NOTCH3	-0,54	-0,54	-0,66	-1,16	-1,72	-1,50	-0,51	-0,42	-1,44	-0,12	0,79	-1,64
CXCL14	-1,38	-1,20	-2,03	-1,62	-2,34	-1,71	-1,83	-4,06	-1,64	-1,70	-0,97	-2,69
EGFR	-5,26	-4,55	-4,26	-5,92	-6,88	-8,00	-5,18	-4,62	-5,24	-5,46	-4,41	-6,47
DCIS index-score	-86,84	-86,25	-84,30	-81,02	-80,37	-75,00	-72,40	-72,35	-70,57	-65,16	-61,66	-56,37

*DCIS with or without IBC	1	1	0	0	1	1	1	1	1	1	1	1
PLAU	-8,16	-5,74	-7,28	-6,47	-6,29	-5,05	-6,81	-5,59	-6,33	-7,89	-6,99	-7,34
COL1A1	0,39	4,22	3,73	-0,04	-0,34	-2,35	2,03	1,16	0,83	4,36	2,84	4,16
KRT81	-5,28	-3,59	-9,36	-8,21	-7,81	-9,25	-5,79	-9,07	-6,66	-5,25	-6,97	-9,44
S100A7	-6,57	-9,36	-4,37	-6,52	-8,59	-5,07	-4,11	-3,33	-1,31	-2,70	-3,45	0,44
SCGB1D2	-5,13	-8,77	-2,16	1,03	0,85	1,59	-3,75	0,14	-0,12	-1,70	-0,02	0,89
KRT18	2,37	1,63	1,73	3,43	3,75	2,54	1,95	2,99	2,91	1,92	2,39	3,47
NOTCH3	-0,77	-0,20	-0,87	0,45	1,25	-0,48	0,92	-1,22	0,01	1,79	0,39	1,28
CXCL14	-2,46	-1,04	-0,07	-1,34	-4,38	-7,04	-5,50	-2,95	-2,41	-1,22	-3,63	-2,59
EGFR	-6,26	-5,26	-6,23	-4,71	-7,94	-6,85	-5,23	-7,15	-4,77	-5,82	-7,11	-7,21
DCIS index-score	-51,95	-48,44	-43,68	-41,39	-27,19	-25,04	-24,29	-23,44	-20,43	-16,63	-13,60	-2,31

B. Supervised-clustering analysis demonstrated distinct clustering of patients with pure DCIS and patients with DCIS and synchronous IBC.
In this figure, samples are ranked from low to high according to their individual DCIS-index score. In addition, the Cq-values of each gene (horizontal) are shown for each individual sample (vertical).
*DCIS with or without IBC; 0= without IBC, 1= with IBC.

Immunohistochemistry

The identified nine genes with a significantly different gene expression between pure DCIS and DCIS with synchronous IBC were evaluated by immunohistochemistry. **Figure 2** provides an overview of the immunohistochemical staining pattern. For each antibody, a representative case is shown.

As described above, seven of these nine genes showed a higher gene expression in cases with DCIS and synchronous IBC. Immunohistochemically, CK81 (catalog number H00003887-M01; Abnova, Taipei, Taiwan) and NOTCH3 (catalog number ab 23426; Abcam, Cambridge, UK) were only expressed in the myoepithelial cells, whereas the luminal cells were negative in both groups (**Figure 2**, A and B). COL1A1 (catalog number NB600-408; Novus Biologicals, Littleton, CO) showed periductal stromal staining (**Figure 2**C) in six cases, but no significant difference (P = 0.317) was observed between both groups.

SCGB1D2 (lipophilin B; catalog number NBP1-81304; Novus Biologicals) was positive in the cytoplasm of neoplastic DCIS cells (**Figure 2**D) and was scored dichotomous (negative/weak or moderate/strong). There was a significantly higher expression in cases with DCIS and synchronous IBC as compared to the pure DCIS cases (P = 0.009), which was in line with the gene expression pattern of SCGB1D2.

S100A7 (catalog number NB100-56559; Novus Biologicals) was positive in the nucleus of the neoplastic DCIS cells (**Figure 2**E) and was also scored dichotomous (negative/ weak or moderate/strong). Although no significantly different expression was seen between both groups, there was a trend toward a higher expression in those cases with DCIS and synchronous IBC as compared to the pure DCIS cases (P = 0.150).

CK18 (catalog number HPA001605; Sigma-Aldrich, Darmstadt, Germany) and urokinase plasminogen activator (clone 150; Grünenthal, Stolberg, Germany) were expressed cytoplasmatically in the neoplastic DCIS cells (**Figure 2**, F and G). No difference was seen between both groups (P = 0.48 and P = 0.572, respectively).

Epidermal growth factor receptor (EGFR) and CXCL14 showed a higher gene expression in the group of patients with pure DCIS. Immunohistochemically, EGFR (3C6 790- 2988; Ventana) was only expressed in myoepithelial cells (**Figure 2**H). None of the cases showed expression in the luminal cells. Immunohistochemical evaluation of CXCL14 was not feasible because of a non-specific staining pattern.

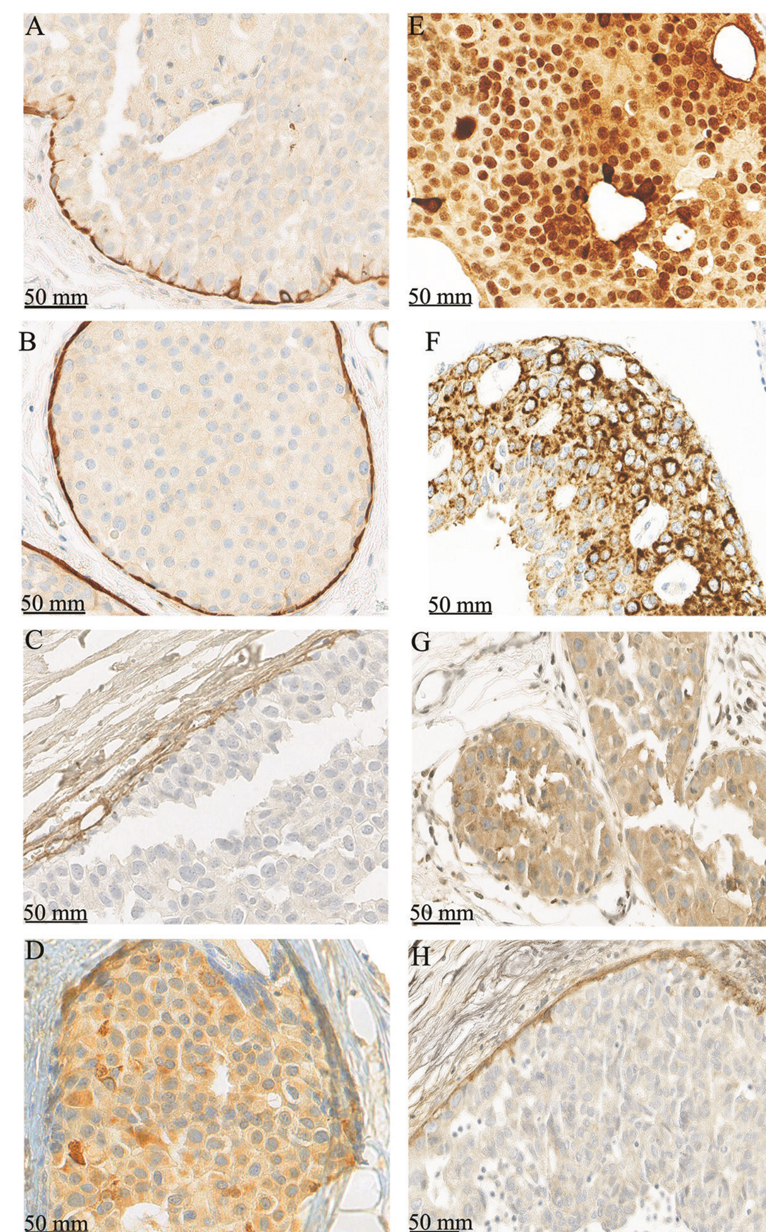


Figure 2. Staining pattern for each antibody.
CK81 (A) and NOTCH3 (B) were positive in the myoepithelial cells and negative in the neoplastic DCIS cells. COL1A1 (C) showed periductal stromal staining. SCGB1D2 (D) was positive in the cytoplasm of neoplastic DCIS cells and S100A7 (E) showed nuclear staining. Both CK18 (F) and UPA (G) were positive in the cytoplasm of neoplastic DCIS cells. EGFR (H) was positive in the myoepithelial cells and negative in the neoplastic DCIS cells.
Scale= 50 mm.

Functional annotation of differently expressed genes according to Gene Ontology

According to Gene Ontology, a total of five genes (of the nine differently expressed genes) were annotated. These genes are involved in several biological processes, including signal transduction (EGFR, CXCL14, and PLAU), chemotaxis (CXCL14 and PLAU), angiogenesis (S100A7 and PLAU), cellular response to epidermal growth factor stimulus (EGFR and COL1A1), positive regulation of extracellular signal regulated kinase 1 and 2 cascade (EFGR and S100A7), response to hyperoxia (COL1A1 and PLAU), and cellular response to amino acid stimulus (EGFR and COL1A1). None of these functions were statistically significantly enriched ($P > 0.05$) compared to the functions annotated to the total list of 93 measured genes.

DISCUSSION

To achieve optimal individualized treatment for patients with DCIS, it is necessary to unravel the molecular events that contribute to DCIS progression. In this study, we identified significantly different gene expression profiles between patients with extensive pure DCIS (representing a group with a biologically indolent behavior) and patients with a limited amount of DCIS and synchronous IBC (representing a group with a biologically aggressive behavior). Most of these differently expressed genes (7/9) showed a higher expression in the DCIS group with synchronous IBC, including PLAU, COL1A1, KRT81, S100A7, SCGB1D2, KRT18, and NOTCH3. At the protein level, this could only be confirmed for SCGB1D2. The remaining two genes, EGFR and CXCL14, were upregulated in pure DCIS lesions at the transcriptional level. These findings are in line with previous studies that reported differences between pure DCIS lesions and DCIS with synchronous IBC based on gene copy number changes and whole exome sequencing.[10,27]

On the basis of these identified genes, supervised hierarchical cluster analysis showed distinct clustering for patients with pure DCIS (characterized by lower expression levels) and patients with DCIS and synchronous IBC (characterized by higher expression levels). However, three patients with pure DCIS clustered within the group of DCIS cases with synchronous IBC. Two of these three cases were classified as luminal B, which might explain a gene expression profile that is more similar to DCIS with synchronous IBC.[15,28]

The progression of in situ to invasive carcinoma is a multistep process that includes several biological processes (ie, regulation of transcription, cell adhesion,

immune response, chemotaxis, apoptosis, and cell proliferation).[8,29,30] According to Gene Ontology, the differently expressed genes in our study are involved in several of these processes, mainly signal transduction. This suggests that signal transduction might play an important role in the progression of DCIS. In our series, COL1A1 and NOTCH3 were up-regulated in cases with DCIS with synchronous IBC as compared to pure DCIS cases. This is in line with previous studies that reported that these genes have an important role in cell adhesion and migration.[31,32] Furthermore, NOTCH3 plays an important role in cell growth by the inhibition of apoptosis and induction of cell proliferation, although the exact mechanism in breast cancer remains unknown.[32] Another gene with an important role in cell growth is CXCL14, although in contrast to NOTCH3, overexpression of this gene inhibits cell proliferation and invasion.[33] In line with this, a recent study reported that this gene is a negative regulator of growth and metastases in breast cancer. This anticancer effect correlates with the upregulation of CXCL14 in pure DCIS samples in our study, where it might have contributed to the indolent behavior.[33]

Several previous studies reported that disruption of the myoepithelial cell layer is one of the critical events in DCIS progression.8,34 This is in line with the results of our study, in which several of the up-regulated genes (EGFR,COL1A1, KRT81, and NOTCH3) are involved in the myoepithelial cell layer.[29,34,35] Our finding that up-regulation of these genes was reported in both groups suggests that they could have both proinvasive and anti-invasive effects. Besides, it makes it unlikely that this finding was influenced by the amount of myoepithelial cells in the analyses. EGFR has been described as a specific marker in myoepithelial cells of the breast.[35] COL1A1, together with other members of the collagenase family, was recently characterized as a myoepithelial-type gene in ER-positive breast cancer.[29] Regarding KRT81 and NOTCH3, we could not find such data in the literature, although we identified this protein in the myoepithelial compartment by immunohistochemistry.

The strength of our study is that we matched for surrogate DCIS subtypes. This was assumed to be more reliable than matching on grade, which is known to be a subjective feature. Ideally, matching should also be based on other factors (including grade, PR status, and Ki-67 index) because these factors could affect biological behavior, but this was not feasible. Our study also has several other limitations, in particular the sample size, and as such, our work should be considered as a hypothesis-generating study. In addition, the presence of an invasive component cannot be ruled out in those cases classified as pure DCIS. However, because these

specimens were examined extensively, it is unlikely that a large invasive component was missed.

The immunohistochemical staining provided important information regarding the localization of the expression (neoplastic cells, myoepithelial cells, or periductal stroma), but the ability to detect different expression levels between pure DCIS and DCIS with synchronous IBC was limited by group size. Besides, we only evaluated a subset of luminal-like cases. Although this is the most common subtype, our data cannot be extrapolated to other DCIS subtypes (eg, ER-negative/HER2-positive cases and triple-negative cases). Furthermore, because we evaluated only a selected number of tumor-specific target genes, it is likely that some cancer genes and functional pathways have been missed.

In conclusion, we reported distinct gene expression profiles in cases with pure DCIS and cases with DCIS and synchronous IBC. If these results can be validated in independent and larger cohorts, these differently expressed genes could be used to predict progression in individual patients diagnosed with DCIS to facilitate individualized treatment. Besides, these genes may pinpoint potentially targetable driver pathway(s) that play an important role in the progression of DCIS to IBC, which could ultimately result in the prevention of progression.

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CHAPTER

5

APOBEC3B Gene Expression in Ductal Carcinoma in Situ and Synchronous Invasive Breast Cancer

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ABSTRACT

The underlying mechanism of the progression of ductal carcinoma in situ (DCIS), a non-obligate precursor of invasive breast cancer (IBC), has yet to be elucidated. In IBC, Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-Like 3B (APOBEC3B) is upregulated in a substantial proportion of cases and is associated with higher mutational load and poor prognosis. However, APOBEC3B expression has never been studied in DCIS. We performed mRNA expression analysis of *APOBEC3B* in synchronous DCIS and IBC and surrounding normal cells. RNA was obtained from 53 patients. The tumors were categorized based on estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Her2) and phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA) mutation status. *APOBEC3B* mRNA levels were measured by RT-qPCR. The expression levels of paired DCIS and adjacent IBC were compared, including subgroup analyses. The normal cells expressed the lowest levels of *APOBEC3B*. No differences in expression were found between DCIS and IBC. Subgroup analysis showed that *APOBEC3B* was the highest in the ER subgroups of DCIS and IBC. While there was no difference in *APOBEC3B* between wild-type versus mutated PIK3CA DCIS, *APOBEC3B* was higher in wild-type versus PIK3CA-mutated IBC. In summary, our data show that *APOBEC3B* is already upregulated in DCIS. This suggests that APOBEC3B could already play a role in early carcinogenesis. Since APOBEC3B is a gain-of-function mutagenic enzyme, patients could benefit from the therapeutic targeting of APOBEC3B in the early non-invasive stage of breast cancer.

INTRODUCTION

Ductal carcinoma in situ (DCIS) is a non-obligate precursor of invasive breast cancer (IBC).[1] This is supported by previous studies that reported a high genomic concordance of synchronous DCIS and IBC.[2–4] However, despite molecular similarities, recent in-depth genetic studies also reported specific mutations that were either restricted to the in situ or the invasive component.[3,5] Increased insight in the molecular changes during DCIS progression has the potential to reveal novel, potentially targetable drivers of progression.

A major role of Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-Like 3B (APOBEC3B) has been reported in breast cancer and several other cancers.[6–9] This enzyme is a member of the APOBEC family of deaminases and is involved in DNA cytosine deaminase activity, which has diverse biological functions, including activities in the innate immune system by restricting virus replication.[10] The upregulation of APOBEC3B is correlated with increased C-to-T transitions and increased mutational load, including known driver mutations in PIK3CA and tumor protein 53 (TP53).[10–12] *APOBEC3B* mRNA is upregulated in a substantial proportion of IBC cases and an association with poor clinical outcome has been reported in Estrogen receptor (ER)-positive subtypes.[13] In addition, we recently reported higher mRNA levels of *APOBEC3B* in breast cancer metastasis as compared to the corresponding primary tumor, which implied that breast cancer progression is associated with the upregulation of APOBEC3B.[14]

In this study we investigated *APOBEC3B* mRNA expression levels in synchronous DCIS and IBC and correlated the expression with PIK3CA mutation status in order to increase our understanding regarding the expression levels of this enzyme during progression from the in situ to the invasive stage. We believe this could improve breast cancer care in the future since APOBEC3B is a gain-of-function mutagenic enzyme, so patients could potentially be treated with small molecules at a very early, non-invasive stage.

RESULTS

General Clinicopathological Data

In total, 53 patients were included. Table 1 provides an overview of the clinicopathological data of all patients. The overall median age was 53 years (range 28–102 years). The majority of DCIS and IBC samples were high grade (62.3 and 54.7%, respectively). There was no difference in grade between DCIS and adjacent IBC (Fisher Exact Probability Test $p = 0.92$). Based on immunohistochemical staining, IBCs were categorized into the following five breast cancer subtype categories: ER+/PR high/Her2− (n = 13), ER+/PR− or low/Her2− (n = 12), ER+/any PR/Her2+ (n = 11), ER−/PR−/Her2+ (n = 8), or ER−/PR−/ Her2− (n = 9).

Table 1. Clinicopathological features of patients with ductal carcinoma in situ (DCIS) and adjacent invasive breast cancer (IBC) (n = 53).

Characteristic	*n*	(%)
Age at diagnosis	53	
years, median (range)	(28–102)	
Type of surgery		
Breast-conserving surgery	24	45.3
Mastectomy	29	54.7
Grade DCIS		
1	1	49.1
2	19	39.6
3	33	7.5
Grade IBC		
1	1	49.1
2	21	39.6
3	31	7.5
Tumor size		
≤ 2 cm	28	49.1
> 2–5 cm	21	39.6
> 5 cm	4	7.5
Missing	0	3.8
Subtypes based on immunohistochemistry		
ER+/PR high/Her2−	13	24.5
ER+/PR− or low/Her2−	12	22.6
ER+/any PR/Her2+	11	20.8
ER−/PR−/Her2+	8	15.1
ER−/PR−/Her2−	9	17.0

APOBEC3B Expression in Synchronous Normal, DCIS and IBC Cells

Both the Kruskal-Wallis Test and the Median Test indicated that there was a significant difference ($p < 0.001$) in APOBEC3B mRNA levels between the normal controls, DCIS and IBC. APOBEC3B mRNA was lower expressed in the normal mammary epithelial tissue adjacent DCIS and IBC (unpaired Mann-Whitney U Test and paired Wilcoxon Signed Ranks Test $p < 0.001$) (Figure 1). There was no statistically significant difference in APOBEC3B mRNA expression between DCIS and IBC (unpaired Mann–Whitney U Test $p = 0.065$ (Figure 1), Wilcoxon Signed Ranks Test $p = 0.082$). (Figure 2).

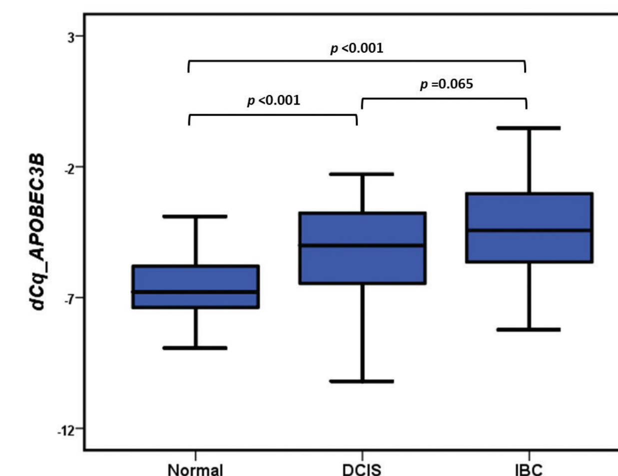

Figure 1. Boxplots of APOBEC3B mRNA expression levels in paired normal, DCIS and IBC (n = 53). Differences between normal, DCIS and IBC were analyzed by the Mann-Whitney U test.

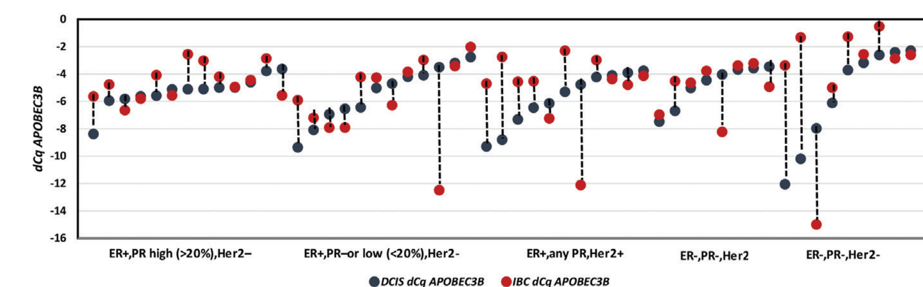

Figure 2. APOBEC3B expression levels in paired DCIS and IBC (n = 53). Wilcoxon Signed Ranks Test $p = 0.082$.

APOBEC3B mRNA Subgroup Analysis

Previous studies reported elevated *APOBEC3B* mRNA levels in breast cancers with otherwise aggressive characteristics, including high histological grade and lack of estrogen expression.[7,13,15] For both DCIS and IBC, there was no correlation between APOBEC3B expression levels and tumor diameter (Spearman Rank Correlation Test $p > 0.05$) or histological grade (Kruskal-Wallis Test $p > 0.05$). Our breast cancer subtype analysis showed that the expression of APOBEC3B was the highest in the ER− subgroup (Mann–Whitney *U* Test, $p = 0.037$) (Figure 3).

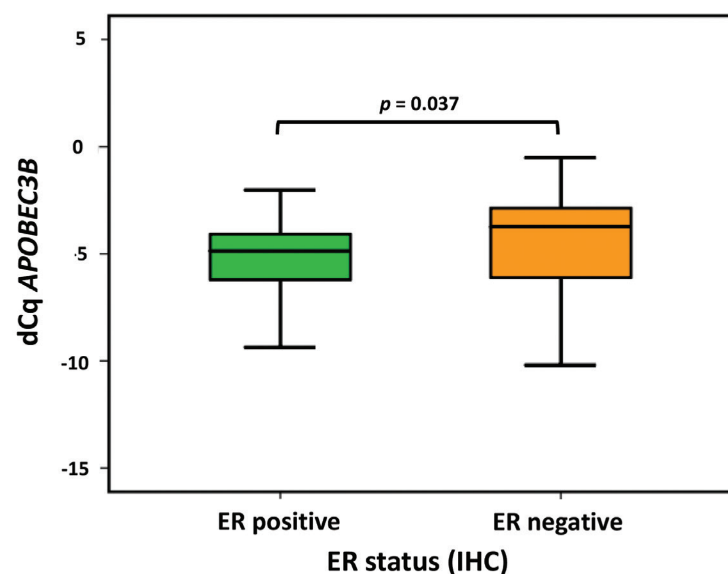

Figure 3. Boxplots of Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-Like 3B (APOBEC3B) mRNA expression levels according to ER status. The difference between ER+ and ER− cases was analyzed by the Mann-Whitney *U* test.

APOBEC3B Expression in Epithelial Versus Inflammatory Cells

Based on the positive correlation between APOBEC3B and marker for epithelial content (EPCAM) mRNA levels (Spearman Rank Correlation test, $p = 0.005$ for DCIS, $p = 0.001$ for IBC), APOBEC3B mRNA was mostly expressed by epithelial cells. Of note, there was no significant difference in the levels of EPCAM mRNA between DCIS and synchronous IBC (Wilcoxon Signed Ranks Test, $p = 0.18$).

Since inflammatory cells also express APOBEC3B,[16] we investigated whether the number of inflammatory cells could have biased our results by comparing Protein Tyrosine Phosphatase Receptor Type C (PTPRC, gene for the common leukocyte antigen CD45) mRNA levels from DCIS and IBC. There was no correlation between APOBEC3B and PTPRC mRNA levels (Spearman Rank Correlation test, $p = 0.18$ for DCIS and $p = 0.29$ for IBC). However, IBC expressed slightly higher levels of PTPRC when compared with DCIS (Wilcoxon Signed Ranks Test, $p = 0.023$).

APOBEC3B Expression and PIK3CA Mutation Status

In a recently published study,[17] we detected a PIK3CA somatic hotspot mutation in 24.7% (18 out of 73) patients. For these 18 PIK3CA-positive patients, a significantly higher PIK3CA variant allele frequency (VAF) was detected in the DCIS component (45.8%) when compared with the synchronous IBC component (31.7%) ($p = 0.007$). For the $n = 14$ PIK3CA mutation-positive patients (26.4%) included in the current study, a significantly higher PIK3CA VAF was also detected in the DCIS component (52.3%) when compared with the synchronous IBC component (37.2%) ($p = 0.027$). The correlation of PIK3CA VAF with APOBEC3B showed a negative Spearman Rank correlation in IBC (rs = −0.33, $p = 0.001$, $n = 53$). For the DCIS cases, there was no such correlation (rs = 0.02, $p = 0.89$, $n = 53$). Analyzing these data irrespective of the degree of the PIK3CA VAF levels revealed that for the 53 patients analyzed in this study, APOBEC3B mRNA levels in IBC were significantly lower in the eight patients with exon 9 (G to A)-mutated PIK3CA when compared with the $n = 39$ wild-type PIK3CA cases (Mann-Whitney *U* test $p = 0.017$). No such difference was observed for the DCIS cases ($p = 0.28$) (Figure 4). Albeit not statistically significant, APOBEC3B mRNA levels were higher overall in the $n = 39$ PIK3CA wild-type IBC samples when compared with the PIK3CA wild-type DCIS samples (Mean ± SEM: −4.54 ± 0.36 for IBC versus −5.38 ± 0.35 for DCIS) and lower in the $n = 8$ G-to-A PIK3CA-mutated IBC samples when compared with G-to-A PIK3CA-mutated DCIS samples (Mean ± SEM: −6.52 ± 1.66 for IBC versus −6.14 ± 0.74 for DCIS). Although the majority of samples with a PIK3CA mutation were ER+, there was no significant interaction effect between ER status and the absence or presence of the two types of tested PIK3CA mutations ($p = 0.46$ for DCIS and $p = 0.20$ for IBC).

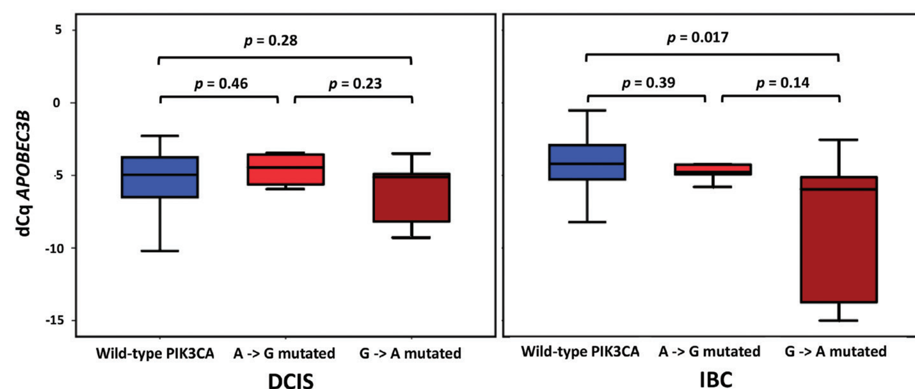

Figure 4. Boxplots of APOBEC3B mRNA expression levels according PIK3CA mutation status. The differences between wild-type (blue boxes) and mutated (red boxes) PIK3CA cases were analyzed by the Mann-Whitney *U* test.

MATERIALS AND METHODS

Patient Materials

Fifty-three patients with synchronous DCIS and IBC were enrolled. We used coded leftover patient material in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.federa.org/codes-conduct). This article is approved by the Medical Ethics Committee of the Erasmus MC (approval number MEC 02.953). According to national guidelines, no informed consent was needed for this study.

Formalin-fixed-paraffin-embedded (FFPE) hematoxylin and eosin (H&E)-stained whole sections of excision specimens were collected and reviewed by two pathologists (Carolien H. M. van Deurzen and Shusma C. Doebar). Histopathological features included the grade of IBC ,[22] IBC diameter, ER, PR and Her2 status, and grade of DCIS.[23] Tumors were divided into subtypes based on immunohistochemistry (ER, PR and Her2), including the following 5 categories: ER+/PR high/Her2−; ER+/PR− or low/Her2−; ER+/any PR/Her2+; ER−/PR−/Her2+; ER−/PR−/Her2−. ER was considered positive when at least 10% of the tumor cells were positive, irrespective of intensity, according to national guidelines (https://richtlijnendatabase.nl). Low PR was defined as ≤ 20%.[24] Immunohistochemical HER2 expression was scored according to international guidelines.[25] Equivocal cases were evaluated by silver in situ hybridization.

RT-qPCR

RNA was extracted from tissue areas composed of at least 50% IBC or DCIS cells and analyzed by RT-qPCR as described before.[14,17] In brief, these cells were obtained by microdissection from FFPE tissue, which was performed with a sterile needle under a stereomicroscope. RNA was extracted from these cells using the Qiagen (Hamburg, Germany) AllPrep DNA/RNA FFPE Kit according the manufacturer's instructions. RNA concentrations were measured with a Nanodrop 2000 system. cDNA was generated from 50 ng/μL cDNA and was generated for 30 min at 48 °C with the RevertAid H minus kit (Thermo Fisher Scientific, Breda, The Netherlands) and gene-specific pre-amplified with Taqman PreAmp Master mix (Thermo Fisher Scientific) for 15 cycles, followed by Taqman probe based real-time PCR according to the manufacturer's instructions in a MX3000P Real-Time PCR System (Agilent, Amsterdam, The Netherlands). The following intron-spanning gene expression assays (all from Thermo Fisher Scientific) were evaluated: *APOBEC3B*, assay ID: hs00358981_m1; *EPCAM,* hs00158980_m1, and *PTPRC,* hs00236304_m1. Messenger RNA levels were quantified relative to the average expression of 2 reference genes (*GUSB,* hs9999908_m1 and *HMBS,* hs00609297_m1) using the delta Cq (average Cq reference genes −Cq target gene) method. According GeNorm and NormFinder, the average of these two reference genes was the most stable expressed across our samples (M-value = 0.59, SD = 0.29). Also, when taking the different groups into account, the inter and intra variation was the lowest for the average of our 2 reference genes (SD = 0.19 for the NormFinder analysis across the control, DCIS and IBC groups and SD = 0.24 for the NormFinder analysis across the ER/PR/Her2 groups). Samples with an average reference gene expression of Cq > 25 were considered to be of insufficient RNA quality and excluded from further analysis, together with their paired samples. A serially diluted RNA pool of FFPE breast tumor samples was included in each experiment to evaluate the linear amplification and efficiencies for all genes included in the panel and absence of amplification in the absence of reverse transcriptase. All gene transcripts were equally efficient amplified (range 94–106%) and were negative in the absence of reverse transcriptase. A summary of the performance of our assays on these serially diluted samples is shown in Supplementary Table S1.

PIK3CA Mutation Status

PIK3CA mutation status and VAFs were measured as described before.[17] In brief, DNA was extracted from the same micro-dissected FFPE tissues used for RNA extraction using the Qiagen (Hamburg, Germany) AllPrep DNA/RNA FFPE Kit. The SNaPshot

Multiplex System for SNP Genotyping (Thermo Fisher Scientific) was used to identify samples positive for PIK3CA hotspot mutations in exon 9 and exon 20. Next, we used digital PCR (dPCR) to validate the SNaPshot results and quantify the relative number of PIK3CA-mutated copies (of E542K, E545K in exon 9 and H1047R and H1047L in exon 20) in both the DCIS and IBC component of those patients with a PIK3CA mutation identified by SNaPshot analysis.

Statistical Analyses

GeNorm and NormFinder,[26,27] present in GenEx qPCR data analysis software (version 6.1, MultiD, Götenborg, Sweden), were used to assess the stability of our reference genes. SPSS version 24 was used for the statistical analyses. Because our APOBEC3B mRNA data were not normally distributed (skewness -1.01 ± 0.33 and -1.75 ± 0.33, kurtosis 0.80 ± 0.64 and 3.60 ± 0.64 for DCIS and IBC, respectively), we only used non-parametric tests. The Wilcoxon Signed Ranks Test was used to compare levels in paired DCIS and IBC and unpaired analyses were performed using the Wilcoxon or Mann-Whitney *U* Test or the Fisher Exact Probability Test for contingency tabled data. Continuous variables were analyzed by the Spearman Rank Correlation test. *p*-values ≤ 0.05 were considered statistically significant.

DISCUSSION

APOBEC3B has been identified as an important factor in the evolution of breast cancer.[8] In a recently published pan-tissue, pan-cancer analysis of RNA-seq data specific to the seven APOBEC3 genes in 8951 tumors, 786 cancer cell lines and 6119 normal tissues, *APOBEC3B* consistently demonstrated its association with proliferative cells and processes, in contrast to other APOBEC3s, especially *APOBEC3G* and *APOBEC3H*, which were revealed as more immune cell related.[9] Our current data showed that *APOBEC3B* mRNA is already upregulated in the in situ stage of breast cancer, which is in line with the high genomic resemblances between DCIS and IBC.[18] In a study we performed earlier, we observed higher mRNA levels of *APOBEC3B* in breast cancer metastasis as compared to the corresponding primary tumor,[14] supporting our hypothesis that, already starting from DCIS, breast cancer progression is associated with deregulated expression of *APOBEC3B*.

Tumors with upregulated *APOBEC3B* demonstrate a higher mutational load, which could explain the aggressive behavior of these tumors.[7,12] Two hotspot G-to-A mutations in exon 9 of the—often mutated in breast cancer—PIK3CA gene (E542K and E545K) are

thought to be generated by *APOBEC3B* induced C-to-T (G-to-A) transitions.[11] Whether *APOBEC3B* is still needed once the mutations are present needs further investigation. In the study of Kosumi et al., *APOBEC3B* expression in esophageal squamous cell carcinoma was significantly correlated with PIK3CA mutations in exon 9.[10] However, no correlation was found between *APOBEC3B* expression and PIK3CA mutations status in a Japanese breast cancer cohort.[15] Although PIK3CA mutations are known to be more prevalent in ER+ cases,[19] and thus might have been a confounder in our analysis, we found no significant difference in the distribution of wild-type and mutated PIK3CA in ER+ and ER− cases. In our cohort, *APOBEC3B* levels were decreased in specifically the G-to-A PIK3CA-mutated IBC samples when compared with wild-type PIK3CA IBC tumors. In the synchronous DCIS counterpart, however, there was no difference in *APOBEC3B* levels between mutated and PIK3CA wild-type tumors. This might suggest that, in contrast to DCIS, the invasive tumors no longer need *APOBEC3B* to proliferate and metastasize. Previous studies reported elevated *APOBEC3B* mRNA levels in breast cancers with otherwise aggressive characteristics, including high histological grade and lack of estrogen expression.[7,13,15] This is consistent with our subgroup analysis, which showed higher *APOBEC3B* levels in synchronous DCIS and IBC of ER− tumors as compared to ER+ tumors. However, in our study, no significant correlations were found between *APOBEC3B* levels and histological grade and/or tumor diameter. This could be due to the fact that the majority of our samples were high grade.

This is the first study evaluating *APOBEC3B* levels within DCIS and co-existing IBC, including different breast cancer subtypes. However, our study has several limitations, such as the relatively small size of our cohort and the analysis of a limited mRNA panel only, with the main focus on *APOBEC3B*. Since upregulated *APOBEC3B* is associated with higher mutational load, evaluation of the mutational status of additional markers besides PIK3CA will be interesting. Another limitation is that *APOBEC3B* is also expressed by inflammatory cells, which could have influenced our data because we performed manual microdissection, and thus contamination with inflammatory cells was not completely avoidable. Although IBC expressed slightly higher levels of *PTPRC* (the gene for leukocyte antigen CD45) than DCIS, there was no correlation between *APOBEC3B* and *PTPRC* mRNA levels. Based on this analysis, it seems unlikely that the number of inflammatory cells biased our data.

Increased insight in molecular mechanisms that contribute to DCIS progression will improve the development of a personalized treatment strategy for patients with DCIS. APOBEC3B could be a potential therapeutic target since it is non-essential,

but it has an active enzymatic activity that may be inhibited.[7] Patients with DCIS could therefore benefit from such therapeutic molecules by inhibiting tumor evolution. Concept inhibitors have already been developed for the related enzyme APOBEC3G.[20,21] Additional clinical and pharmaceutical assays are necessary to develop and explore the potential benefit of APOBEC3B inhibitors.

CONCLUSIONS

In conclusion, our results indicate that *APOBEC3B* mRNA is similarly upregulated in DCIS and IBC, but declines in PIK3CA-mutated IBC, which suggests that APOBEC3B plays a role in the early stages of breast carcinogenesis. Since APOBEC3B is a gain-of-function mutagenic enzyme, it could be a candidate for therapeutic targeting in an early, non-invasive stage of breast cancer.

Supplementary Materials: The following are available online at *http://www.mdpi.com/2072-6694/11/8/1062/s1*, Table S1: Performance of the Taqman mRNA assays used in this study.

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CHAPTER

6

Progression of Ductal Carcinoma In Situ to Invasive Breast Cancer: Comparative Genomic Sequencing

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ABSTRACT

Several models have been described as potential mechanisms for the progression of ductal carcinoma in situ (DCIS) to invasive breast cancer (IBC). The aim of our study was to increase our understanding of DCIS progression by using massive parallel sequencing of synchronous DCIS and IBC. We included patients with synchronous DCIS and IBC (n = 4). Initially, IBC and normal tissue were subjected to whole exome sequencing. Subsequently, targeted sequencing was performed to validate those tumor-specific variants identified by whole exome sequencing. Finally, we analyzed whether those specific variants of the invasive component were also present in the DCIS component. There was a high genomic concordance between synchronous DCIS and IBC (52 out of 92 mutations were present in both components). However, the remaining mutations (40 out of 92) were restricted to the invasive component. The proportion of tumor cells with these mutations was higher in the invasive component compared to the DCIS component in a subset of patients. Our findings support the theory that the progression from DCIS to IBC could be driven by the selection of subclones with specific genetic aberrations. This knowledge improves our understanding of DCIS progression, which may lead to the identification of potential markers of progression and novel therapeutic targets in order to develop a more personalized treatment of patients with DCIS.

Ductal carcinoma in situ (DCIS) is a non-obligate precursor of invasive breast cancer (IBC). However, no reliable biomarkers or clinical tests are available to predict which DCIS cases are most likely to progress. In-depth genetic studies of DCIS and synchronous IBC reported intra-tumoral genetic heterogeneity and genetic differences between DCIS and synchronous IBC.[1,2] Based on these findings, an evolutionary bottleneck selection model has been proposed.[3,4] According to this theory, distinct subclones with specific genetic changes are selected during the transition from DCIS to invasive disease. This leads to differences in the prevalence of specific mutations between the neoplastic DCIS cells and invasive counterpart.[3,5,6] In contrast to this model, a multiclonal evolution theory has been proposed, which assumes that multiple subclones in DCIS co-migrate during the transition from DCIS to IBC.[4,7] To increase our understanding of DCIS progression, we performed massive parallel sequencing of synchronous DCIS and IBC. We reported overlapping mutations between synchronous DCIS and IBC combined with the presence of invasive-specific mutations, which support the theory that the progression from DCIS to IBC could be driven by the selection of subclones with specific genetic aberrations.

We examined the exomes of four patients diagnosed with Estrogen receptor (ER) positive, Human Epidermal Growth Factor Receptor 2 (HER2) negative DCIS and synchronous IBC after surgical excision. All cases had an invasive ductal carcinoma that was graded in each case as grade 3. Regarding the in situ component, the DCIS grade was in all cases concordant with the grade of the invasive carcinoma. The proportion of DCIS in each case was ranging from 2cm to 5cm.

Figure 1 provides an overview of the workflow. Initially, fresh frozen (FF) tissue of IBC and normal cells were subjected to whole exome sequencing. The sequence reads were aligned to the human genome build 19 (hg19) using BW.[8] For each sample, at least four gigabases of sequences were aligned to the genome with an average coverage of at least 120x for IBC and at least 70x for normal tissue samples. Subsequently, the aligned reads were processed using the Indel Realigner, Mark Duplicates and PHRED Recalibration tools from the Genome Analysis Toolkit (GATK)[9] to remove systematic biases and to recalibrate the PHRED quality scores in the alignments. Genetic variants were called using the Unified Genotyper Tool from GATK.

Based on the selected invasive tumor-specific variants, a specific custom-made panel was designed per patient. This custom-made cancer panel was performed

on the Ion Torrent Personal Genome Machine (PGM) in order to validate whether those tumor-specific variants identified by whole exome sequencing could also be detected by targeted sequencing, using the same FF DNA samples, to ensure an accurate concordance between these two platforms. Subsequently, IBC-specific variants verified by Ion Torrent PGM were validated in DNA of formalin fixed paraffin embedded (FFPE) tissue of IBC, using a minimal genomic DNA input of 10 ng. In the final step we validated only those IBC-specific variants verified in both FF tissue and FFPE tissue of IBC on DNA extracted from FFPE tissue of DCIS.

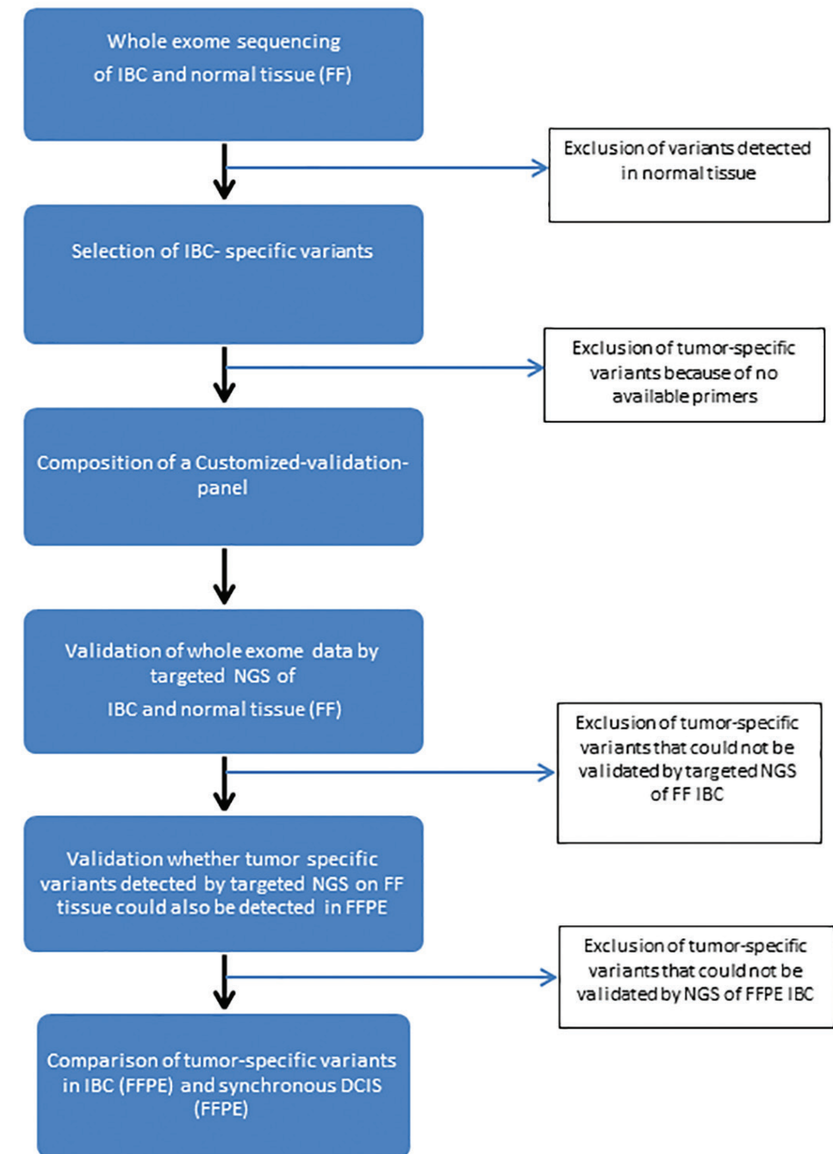

Figure 1. Schematic overview of the DNA-sequencing process from whole exome sequencing to targeted NGS.

Library and template preparations were performed consecutively with the AmpliSeq Library Kit 2.0-384 LV and the Ion PGM Hi-Q Chef Kit. Templates were sequenced using the Ion PGM Hi-Q Chef Kit on an Ion 318v2 chip. Sequence information was analyzed with Variant Caller v4.4.2.1 (Life Technologies Carlsbad, CA, USA) and variants were annotated in a local Galaxy pipeline using ANNOVAR.[10] Variants were called when the position was covered at least 100 times. Nonsynonymous somatic point mutations, insertions and deletions that change the protein amino acids sequence and splice site alterations were selected. Variants found in at least 5% of the called reads and ≥ 10 variant-reads were considered reliable.

Based on whole-exome sequencing of the four IBC samples, a total number of 792 tumor-specific variants were identified. Out of these 792 tumor-specific variants, primers were available for 585 variants. In total, 433 out of 585 tumor-specific variants could not be verified as a tumor-specific variant at the (ion-torrent) validation stage in FF tissue of IBC. Out of the remaining 152 tumor-specific variants, 60 variants could not be validated in FFPE tissue of IBC. These variants were excluded for further analysis.

This resulted in a total number of 92 tumor-specific variants that remained for targeted validation in the DCIS component. Within each patient, a proportion of tumor-specific variants overlapped between the DCIS component and the invasive counterpart (in total 52 out of 92). In patient 1, all tumor-specific variants (17 out of 17) that were identified in IBC were also detected in the DCIS component. In the remaining 3 patients, the number of tumor-specific variants detected in the DCIS component was lower compared to the number detected in the invasive component.

We also compared the frequencies of tumor-specific variants between DCIS and adjacent IBC, as shown in Figure 2. In patient 1, the frequency of tumor-specific variants was higher in the invasive component as compared to the DCIS component, which could not be explained by a difference in tumor cell percentage. This trend was also seen in patient 2, although less tumor-specific variants were detected as compared to patient 1. In patient 3, there was no consistent pattern with respect to differences in the distribution of tumor-specific variant percentages between the two components. For patient 4, there were only four overlapping tumor-specific variants between the invasive component and the in-situ component. This patient showed a higher frequency of tumor-specific variants in DCIS as compared to IBC for 3 out of the 4 mutations.

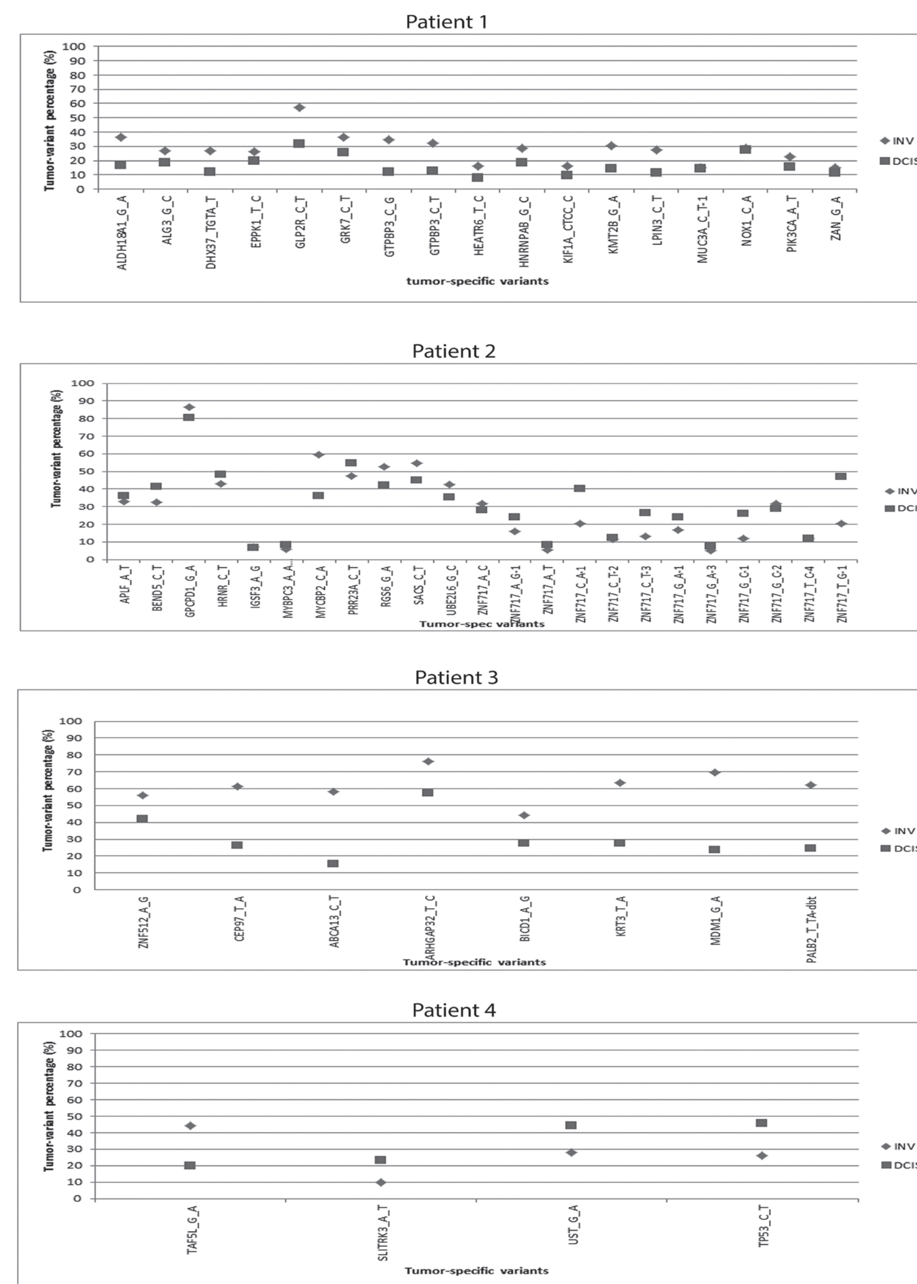

Figure 2. Differences in tumor specific-variant percentages between DCIS (square) and adjacent IBC (rhomb) in all 4 patients.

6

Taken together, our analyses confirmed a high genomic resemblance between synchronous DCIS and IBC; more than half (52 out of 92) of the mutations identified in the invasive component were also detected in the adjacent in situ component. However, a proportion of mutations (40 out of 92) identified in IBC were not detected in the adjacent DCIS component. In addition, in a subset of patients the frequencies of the mutations seemed to be higher in the invasive component as compared to DCIS, which could not be explained by the tumor cell percentages in the analyses.

It is important to note that these findings are based on a small number of patients and should be considered as hypothesis generating only. Besides, our study has several other limitations. First of all, we used two different platforms of massive parallel sequencing. At the validation stage major differences were observed between these two platforms, due to unreadable sequence regions by ion-torrent, an insufficient number of reads and false-positive tumor-specific variants (validated in normal tissue by ion-torrent sequencing). The latter might be the result of a lower sequence depth of whole exome sequencing as compared to ion-torrent sequencing. In addition, a substantial proportion of variants detected in FF tissue of IBC using Ion-torrent sequencing, could not be detected in FFPE tissue of the same tumor, which could be due to tumor heterogeneity. Another limitation of this study is the lack of whole exome data for DCIS, due to lack of available FF tissue of DCIS. Therefore, we could only perform a one-way evaluation of genetic alterations between synchronous DCIS and IBC; genetic alterations restricted to the DCIS component could not be evaluated. At last, we only included ER positive/ HER2 negative breast cancer.

In conclusion, we reported overlapping mutations between synchronous DCIS and IBC (with differences regarding the frequencies of mutations between both components), combined with the presence of invasive-specific mutations, which supports the theory that DCIS progression could be driven by the selection of subclones. This knowledge might facilitate future studies regarding potential progression markers and novel therapeutic targets in order to establish a more effective personalized treatment for patients with DCIS.

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CHAPTER

7

Male Breast Cancer Precursor Lesions: Analysis of the EORTC 10085/TBCRC/BIG/NABG International Male Breast Cancer Program

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ABSTRACT

In men, data regarding breast cancer carcinogenesis are limited. The aim of our study was to describe the presence of precursor lesions adjacent to invasive male breast cancer, in order to increase our understanding of carcinogenesis in these patients. Central pathology review was performed for 1328 male breast cancer patients, registered in the retrospective joint analysis of the International Male Breast Cancer Program, which included the presence and type of breast cancer precursor lesions. In a subset, invasive breast cancer was compared with the adjacent precursor lesion by immunohistochemistry (n=83) or targeted next generation sequencing (n=7). Additionally, we correlated the presence of ductal carcinoma in situ with outcome. A substantial proportion (46.2%) of patients with invasive breast cancer also had an adjacent precursor lesion, mainly ductal carcinoma in situ (97.9%). The presence of lobular carcinoma in situ and columnar cell-like lesions were very low (< 1%). In the subset of invasive breast cancer cases with adjacent ductal carcinoma in situ (n=83), a complete concordance was observed between the estrogen receptor, progesterone receptor, and HER2 status of both components. Next generation sequencing on a subset of cases with invasive breast cancer and adjacent ductal carcinoma in situ (n=4) showed identical genomic aberrations, including PIK3CA, GATA3, TP53, and MAP2K4 mutations. Next generation sequencing on a subset of cases with invasive breast cancer and an adjacent columnar cell-like lesion showed genomic concordance in two out of three patients. A multivariate Cox model for survival showed a trend that the presence of ductal carcinoma in situ was associated with a better overall survival, in particular in the Luminal B HER2+ subgroup. In conclusion, ductal carcinoma in situ is the most commonly observed precursor lesion in male breast cancer and its presence seems to be associated with a better outcome, in particular in Luminal B HER2+ cases. The rate of lobular carcinoma in situ and columnar cell-like lesions adjacent to male breast cancer is very low, but our findings support the role of columnar cell-like lesions as a precursor of male breast cancer.

INTRODUCTION

Male breast cancer is rare, with an estimated incidence of approximately 1.1 per 100,000 a year, representing less than 1% of all breast cancer cases reported worldwide.[1] Male breast cancer seems to resemble hormone receptor-positive postmenopausal female breast cancer, although there is a later age of onset, a more advanced stage at presentation, and consequently an overall worse prognosis.[1–3] Furthermore, there appears to be a markedly lower prevalence of invasive lobular carcinomas in men (1–2%) as compared with women (15%).[4]

In women, terminal ductal lobular units of the breast are regarded as the origin of invasive breast cancer.[5] Ductal carcinoma in situ and lobular carcinoma in situ are seen as precursor lesions of invasive ductal carcinoma and invasive lobular carcinoma, respectively.[6] Besides carcinoma in situ, columnar cell lesions are regarded as precursor lesions of (low-grade estrogen receptor positive) female breast cancer.[7] Pure ductal carcinoma in situ accounts for up to 15–30% of all breast cancers detected in women nowadays and it is detected adjacent to invasive breast cancer in a substantial proportion of patients.[8,9]

In women, coexisting ductal carcinoma in situ has been reported to be associated with lower biological aggressiveness in luminal type breast cancer as compared with pure luminal breast cancer without coexisting ductal carcinoma in situ.[10,11]

Obviously, the anatomy of male breasts is different as compared with female breasts since male breasts tissues mainly consist of ducts without the formation of lobules. Based on this difference, one could hypothesize a different pattern of carcinogenesis in men as compared with women. In men, pure ductal carcinoma in situ accounts for about 10% of all breast cancers detected and we could find no published data regarding the frequency of carcinoma in situ adjacent to invasive breast cancer.[12,13]

Besides, there are also no published data regarding the biological significance of coexisting ductal carcinoma in situ in male breast cancer, which are estrogen receptor (ER) positive/HER2 negative breast cancers in the vast majority of cases. In literature, there is no consensus regarding the existence of columnar cell-like lesions in males.[7,14] Verschuur- Maes et al.[7] found no convincing columnar cell-like lesions at the periphery of 89 male breast cancer cases, but identified Keratine 5 clonally negative ducts, which might indicate that these lesions are breast cancer precursor lesions. In line with this, Ni et al.[14] reported the presence of ducts with a columnar cell-like morphology in a small subset of male breast cancer cases. However, both studies

were based only on morphology supplemented with immunohistochemistry, lacking additional molecular analyses to evaluate genomic aberrations in these potential male breast cancer precursor lesions.

In this study, we report the presence of various breast cancer precursor lesions in the largest male breast cancer series ever published, supplemented with next generation sequencing on a selected number of cases. Furthermore, we correlated the presence of these lesions with other clinicopathologic features and outcome, in order to increase our understanding of carcinogenesis in this population, which may facilitate future studies regarding prevention and early diagnosis.

MATERIALS AND METHODS

Patients

The International Male Breast Cancer Program is a worldwide collaborative effort, coordinated by the European Organization for Research and Treatment of Cancer (study number 10085), with the help of Translational Breast Cancer Research Consortium in the USA, and run under the Breast International Group and North American Breast Cancer Group networks. It is composed of three parts, where part 1 was a retrospective joint analysis of all male breast cancer cases treated in the participating centers for a period of 20 years (1990–2010). In this part 1, 1822 male breast cancer cases were enrolled in 23 centers from nine countries. A subgroup of this initial population was selected based on eligibility for this male breast cancer program (22 excluded) and availability of a tumor tissue block for central pathology review (446 excluded) for which the precursor lesion status could be assessed (26 excluded). Therefore, the present analysis population consists of 1328 patients. Patient and tumor characteristics studied include age, stage, tumor size, and nodal status.

In this study we adhered to the Declaration of Helsinki and the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl). Since this was a retrospective study with coded patient identification without risks, no informed consent was needed.

Pathologic evaluation

One representative formalin-fixed-paraffin-embedded, hematoxylin and eosin-stained tumor tissue block was selected for central pathology review (performed by CvD or PvD). Tumor characteristics were evaluated, including histological type (according to the WHO), grade (according to the modified Bloom and Richardson grading system),[15] and presence of a precursor lesion. The precursor lesions were categorized as columnar cell-like lesions (with or without atypia), atypical lobular neoplasia/lobular carcinoma in situ, atypical ductal hyperplasia or ductal carcinoma in situ. In cases where ductal carcinoma in situ was present, nuclear grade was recorded.[16]

ER, Progesterone receptor (PR), Ki67, and HER2 expression were assessed on a Tissue Micro Array in a different central lab. ER and PR were reported as Allred scores, using a cutoff point of 42 as positive. HER2 status was reported as per the ASCO-CAP guideline.[17] Immunohistochemistry-based surrogate intrinsic breast cancer subtypes were defined according to the 2013 St Gallen consensus guidelines (referred to as surrogate breast cancer subtypes).[18] A subset of 83 cases with invasive breast cancer and adjacent ductal carcinoma in situ was selected for additional immunostaining with ER, PR, and HER2 on whole sections. These cases were selected based on the presence of sufficient ductal carcinoma in situ for additional immunostaining.

Molecular analysis: microdissection, DNA extraction and Next Generation Sequencing

We selected four cases of male breast cancer with a sufficient amount of adjacent ductal carcinoma in situ and three cases with invasive breast cancer and an adjacent lesion resembling columnar cell-like lesions. These cases with a columnar cell-like lesion were selected based on the availability of a tissue block. Additional immunohistochemistry was performed on these three cases with a columnar cell-like lesion, using antibodies against Keratine 5 and ER. Microdissection was performed manually with a sterile scalpel under a stereomicroscope (Zeiss, Oberkochen, Germany). Normal tissue, columnar cell-like lesions, ductal carcinoma in situ, and invasive breast cancer cells were dissected from 10 to 15 hematoxylin-stained sections (6 µm) of formalin-fixed-paraffin-embedded tissue blocks. The percentage of the dissected tumor cells of invasive breast cancer and ductal carcinoma in situ was approximately 80–90%. Of all isolated lesions, DNA was extracted using a lysis buffer (Promega Benelux, Leiden, The Netherlands) with proteinase K and 5% Chelex 100 resin. We started by analyzing DNA extracted from the invasive breast cancer regions. Next generation sequencing was performed on the Ion Torrent Personal Genome Machine with a broad breast cancer-related panel. Genes listed in this panel included 37 breast cancer-related genes and 9 hotspot-regions as described for female breast cancer, that is, PIK3CA, TP53, AKT1, GATA3, and MAP3K119–21 (details of genes listed in this breast cancer panel are available in Supplementary Table S1).

The minimal DNA input was 10 ng per primer pool. In brief, library and template preparations were performed consecutively with the Ampli- Seq Library Kit 2.0-384 LV and the Ion PGM Hi-Q Chef Kit. Templates were sequenced using the Ion PGM Hi-Q Chef Kit on an Ion 318v2 chip. Sequence information was analyzed with Variant Caller v4.4.2.1 (Life Technologies, Carlsbad, CA, USA) and variants were annotated in a local Galaxy pipeline using ANNOVAR.[22–24] Variants were called when the position was covered at least 100 times. Nonsynonymous somatic point mutations, insertions, and deletions that change the protein amino acid sequence and splice site alterations were selected. Variants found in at least 25% of the called reads were considered reliable. Non-reproducible sequence artifacts due to cytosine deamination, G>A, or C>T mutations, were excluded when not listed in the COSMIC database (http://cancer. sanger. ac.uk/cosmic). To find genomic resemblances between breast cancer and the adjacent columnar cell-like lesion and/or ductal carcinoma in situ, we started with next generation sequencing analyses of the invasive component. Based on the selected invasive tumor-specific variants, a specific custom-made panel was designed per patient, which was used for targeted analyses in the adjacent columnar cell-like lesion and/or adjacent ductal carcinoma in situ component. Furthermore, the originally reported variants of the invasive component were validated with this custom-made panel.

Supplementary table S1. Details of genes listed in the breast cancer panel.

Type	Name	Chromosome	Chr. start	Chr. end	Number Amplicons	Total Bases	Covered Bases	Missed Bases
GENOME REGION	AKT1_ex17	chr14	105246500	105246625	1	126	126	0
GENOME REGION	BRAF_V600	chr7	140453099	140453224	1	126	126	0
GENOME REGION	CTNNB1_ex3	chr3	41266061	41266175	1	115	115	0
GENOME REGION	EGFR_ex18	chr7	55241596	55241779	1	184	184	0
GENOME REGION	EGFR_21	chr7	55259397	55259581	1	185	185	0
GENOME REGION	KRAS_ codon1213	chr12	25398183	25398310	1	128	128	0
GENOME REGION	KRAS_codon61	chr12	25380240	25380323	1	84	84	0
GENOME REGION	PIK3CA_ codon538	chr3	178936059	178936176	2	118	118	0
GENOME REGION	PIK3CA_ codon1020	chr3	178952001	178952103	1	103	103	0
GENE	AKAP9	chr7	.	.	119	12438	11913	525
GENE	APC	chr5	.	.	62	8873	8841	32
GENE	ARID1A	chr1	.	.	47	7330	7181	149

Supplementary table S1. Continued.

Type	Name	Chromosome	Chr. start	Chr. end	Number Amplicons	Total Bases	Covered Bases	Missed Bases
GENE	ATM	chr11	.	.	108	9853	9365	488
GENE	BRCA1	chr17	.	.	46	6286	6209	77
GENE	CDH1	chr16	.	.	22	2825	2825	0
GENE	CDK12	chr17	.	.	34	5324	5267	57
GENE	CHEK2	chr22	.	.	19	1926	1777	149
GENE	CREBBP	chr16	.	.	57	7670	7662	8
GENE	ERBB2	chr17	.	.	39	4211	4169	42
GENE	FBXW7	chr4	.	.	25	2675	2675	0
GENE	GATA3	chr10	.	.	10	1544	1534	10
GENE	JAK1	chr1	.	.	29	3729	3719	10
GENE	KIT	chr4	.	.	27	3355	3355	0
GENE	LRP2	chr2	.	.	123	14837	14743	94
GENE	MAP2K4	chr17	.	.	13	1321	1306	15
GENE	MAP3K1	chr5	.	.	42	4759	4508	251
GENE	MED12	chrX	.	.	55	7029	6813	216
GENE	MLH1	chr3	.	.	23	2581	2578	3
GENE	MLL	chr11	.	.	83	12440	12149	291
GENE	MLL2	chr12	.	.	108	17208	16865	343
GENE	MLL3	chr7	.	.	135	15385	15237	148
GENE	MLLT3	chr9	.	.	16	1828	1828	0
GENE	KAT6B	chr10	.	.	45	6398	6384	14
GENE	NCOA3	chr20	.	.	38	5314	5184	130
GENE	NCOR1	chr17	.	.	76	8748	8490	258
GENE	NCOR2	chr12	.	.	71	8438	8401	37
GENE	NF1	chr17	.	.	104	9310	9191	119
GENE	PDE4DIP	chr1	.	.	84	9820	9603	217
GENE	PIK3R1	chr5	.	.	25	2524	2520	4
GENE	PPP2R1A	chr19	.	.	18	1935	1935	0
GENE	PTCH1	chr9	.	.	36	4962	4927	35
GENE	PTEN	chr10	.	.	15	1311	1311	0
GENE	RB1	chr13	.	.	41	3084	2874	210
GENE	RNF213	chr17	.	.	114	16821	16622	199
GENE	RUNX1	chr21	.	.	15	1852	1852	0
GENE	RYR1	chr19	.	.	150	16283	16020	263
GENE	SMAD4	chr18	.	.	19	1780	1758	22
GENE	TP53	chr17	.	.	14	1569	1510	59

Statistics

The association between the presence of ductal carcinoma in situ and lobular carcinoma in situ with histological type of the tumor was assessed, as was the association between the presence of ductal carcinoma in situ and M stage, HER2 status, breast cancer subtype, and nodal status (for patients who were free of metastases at diagnosis (M0 patients)). Also, the relationship between grade of the ductal carcinoma in situ component vs grade of the adjacent invasive breast cancer was explored. For all the aforementioned contingency tables, Fisher exact tests for association were performed. The relationship between the presence of ductal carcinoma in situ and outcome, as measured by relapse-free survival for M0 patients and overall survival, was investigated. Subgroup analyses were added for the three breast cancer subtypes with a prevalence of at least 50 patients: Luminal A, Luminal B (HER2 negative), and Luminal B (HER2 positive). A multivariate model for overall survival was fitted to assess the effect of the presence of ductal carcinoma in situ when adjusting for the baseline factors included in Table 3. Patients with missing information on one of the aforementioned factors, or with a different breast cancer subtype than the ones mentioned above were excluded from the analysis. Relapse-free survival was defined as the time from diagnosis until one of the following events: local recurrence, distant relapse, or death due to any cause. Overall survival constitutes the time interval from diagnosis until death due to any cause. Patients without an event of interest for the above end points are censored at their last follow-up date. Patients with missing data on (any of) the events of interest for relapse-free survival or overall survival are excluded from the analyses on that end point. Outcome data are analyzed per the Kaplan–Meier method, reported P-values correspond to the logrank test, and the hazard ratio was estimated from the Cox proportional hazards model (95% confidence intervals are per Wald test). The reported analyses should be considered exploratory. No multiple testing adjustments were implemented.

RESULTS

General Patient and Treatment Characteristics

We collected a total number of 1328 primary male breast cancers. Median age was 67 years. The majority of patients were treated with a mastectomy (60.1%). A small subset of patients underwent either breast-conserving surgery (2.6%) or no surgery (0.6%). The remaining cases (36.6%) missed data regarding breast surgery. About half of the patients with known data regarding adjuvant radiotherapy received radiation

(29.9% with radiation vs 29.7% without radiation). The majority of patients (43.2%) did not receive chemotherapy (only 16.6% of the patients did receive chemotherapy and 40.2% of the patients had missing data). In contrast, the majority of patients (43.9%) received endocrine therapy (14.5% did not receive endocrine therapy and remaining data were missing). The majority of Her2-positive patients received Trastuzumab from 2006 onwards (43.3% vs 16.7% who did not receive Trastuzumab, remaining data were missing).

Patients with Precursor Lesions

Out of 1328 cases, 613 (46.2%) had a precursor lesion adjacent to the invasive component. In the remaining 715 cases (53.8%), no precursor lesion was detected within the selected tissue block. The majority of precursor lesions consisted of ductal carcinoma in situ (97.9%), mainly grade 2 (64%). The observed frequency of lobular carcinoma in situ, atypical ductal hyperplasia, and columnar cell-like lesions was very low (<1%). Table 1 provides an overview of subtypes of precursor lesions. A total of 13 patients had a combination of precursor lesions. The majority of these cases (11 out of 13) had a combination of ductal carcinoma in situ with a columnar cell-like lesion, one patient had atypical ductal hyperplasia with a columnar cell-like lesion and one case had a combination of lobular carcinoma in situ with ductal carcinoma in situ grade 1.

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Table 1. Overview of subtypes of BC precursor lesions.

		Total (N=613) N (%)
DCIS, all grades		**599 (97.7)**
	DCIS grade 1	83 (13.9)
	DCIS grade 2	384 (64.1)
	DCIS grade 3	132 (22)
ADH		**6 (1,0)**
LCIS		**5 (0.8)**
Other		**2 (0.3)**
Missing		**1 (0.2)**

Presence of Precursor Lesions According to Other Clinicopathological Features

Table 2 provides an overview of patient and tumor characteristics by the presence of a precursor lesion. The majority of breast cancers were classified as invasive ductal carcinoma (84.6%), mainly grade 2 (49.8%). The prevalence of invasive lobular carcinoma was low (1.4%). Most carcinomas were classified by immunohistochemistry as luminal-like subtype, either luminal A (35.9%) or luminal B (49.3%). There was no significant association between surrogate breast cancer subtype or HER2 status and the presence of a precursor lesion (P = 0.14 and 0.31 respectively). More detailed patient and tumor characteristics were presented before.[25]

Table 2. Patient and tumor characteristics according to the presence of a precursor lesion.

	Presence of precursor lesion		
	No (N=715) N (%)	**Yes (N=613) N (%)**	**Total (N=1328) N (%)**
Age at diagnosis			
≤ 50	57 (8.0)	74 (12.1)	131 (9.9)
51 - 65	211 (29.5)	212 (34.6)	423 (31.9)
66 - 75	237 (33.1)	172 (28.1)	409 (30.8)
> 75	210 (29.4)	155 (25.3)	365 (27.5)
Median	68.9	66.3	67.8
T status			
T1	270 (37.8)	306 (49.9)	576 (43.4)
T2	256 (35.8)	199 (32.5)	455 (34.3)
T3	15 (2.1)	7 (1.1)	22 (1.7)
T4	115 (16.1)	52 (8.5)	167 (12.6)
Missing	59 (8.3)	49 (8.0)	108 (8.1)
Histological type			
Ductal	595 (83.2)	528 (86.1)	1123 (84.6)
Lobular	11 (1.5)	7 (1.1)	18 (1.4)
Mixed	36 (5.0)	36 (5.9)	72 (5.4)
Other	73 (10.2)	42 (6.9)	115 (8.7)
Missing	0 (0.0)	2 (0.3)	2 (0.2)
Grade of invasive BC			
1	161 (22.5)	131 (21.4)	292 (22.0)
2	356 (49.8)	305 (49.8)	661 (49.8)
3	193 (27.0)	166 (27.1)	359 (27.0)
Missing	5 (0.7)	11 (1.8)	16 (1.2)
Molecular subtypes *			
Luminal A	268 (37.5)	209 (34.1)	477 (35.9)
Luminal B (HER2 negative)	323 (45.2)	270 (44.0)	593 (44.7)
Luminal B (HER2 positive)	26 (3.6)	36 (5.9)	62 (4.7)
HER2 positive (non-luminal)	1 (0.1)	1 (0.2)	2 (0.2)
Basal	9 (1.3)	4 (0.7)	13 (1.0)
Not classified (ER-, PgR+)	2 (0.3)	0 (0.0)	2 (0.2)
Missing	86 (12.0)	93 (15.2)	179 (13.5)
LN status (pN, but cN reported if pN is missing)			
N-	356 (49.8)	321 (52.4)	677 (51.0)
N+	222 (31.0)	177 (28.9)	399 (30.0)
Missing	137 (19.2)	115 (18.8)	252 (19.0)

***Subtypes according to St. Gallen consensus 2013, Ki67 high: %pos cells ≥ 20%, ER/PgR positive: allred > 2, PgR low: allred < 5

Comparison of Ductal Carcinoma In Situ and Lobular Carcinoma In Situ with Adjacent Invasive Breast Cancer

We observed a significant correlation between the presence of ductal carcinoma in situ and the histology of the invasive breast cancer ($P = 0.02$). The prevalence of ductal carcinoma in situ adjacent to invasive ductal carcinoma was the highest (46.6%), as compared with lobular or other subtypes (27.8% and 36.8% respectively). Similarly, there was a significant correlation between the presence of lobular carcinoma in situ and histologic breast cancer subtype ($P < 0.01$). Although the prevalence of lobular carcinoma in situ was low (n = 5), it was mainly seen adjacent to invasive lobular carcinoma (3 out of 5). The remaining two cases with lobular carcinoma in situ were associated with a mixed ductal and lobular carcinoma. In cases with invasive breast cancer and adjacent ductal carcinoma in situ, there was a positive correlation between nuclear grade of ductal carcinoma in situ and nuclear grade of invasive breast cancer, where grade was frequently similar in both components (Trend test for association $P < 0.01$). In line with this, there was a strong correlation of ER, PR, and HER2 status between ductal carcinoma in situ and the adjacent invasive breast cancer where tested. Regarding ER, the majority of cases (82 out of 83 cases) were positive for ER in both the ductal carcinoma in situ and the invasive component. One case was negative in both components. PR status was positive in both components in 81 out of 83 patients. The remaining two cases were negative in both components. Regarding HER2 status, no discrepancies were detected between ductal carcinoma in situ and the adjacent invasive component. The majority of cases (78 out of 83) were not overexpressed in both components; the remaining cases (n = 5) were overexpressed in both components. For four patients, we performed targeted next generation sequencing of invasive ductal carcinoma and adjacent ductal carcinoma in situ. The results of these analyses are presented in Table 3. In three out of four patients, well-known breast cancer mutations noted in the COSMIC database were found in both the ductal carcinoma in situ component and the adjacent invasive component, which supports the hypothesis that ductal carcinoma in situ is indeed a precursor lesion of male breast cancer. In one out of these four cases, no specific somatic mutation was found in either the invasive or the in situ component within this focused panel of genes.

Table 3. Non-silent somatic mutations in 4 patients with invasive BC and adjacent DCIS.

	Invasive BC-DCIS	Pathogenic somatic variants	Percentage of variant reads with PGM (variant/total reads)	Type of mutation
Patient 1	Invasive BC	**GATA3**: NM_001002295: c.925 - delCA	29% (310/1079)	Splicing
	DCIS		26% (319/1242)	
Patient 2	Invasive BC	**MAP2K4**:NM_003010: exon8:c.880G>A: p.G294R,	59% (691/1163)	Missense
	DCIS		42% (203/483)	
	Invasive BC	**PIK3CA**:NM_006218: exon10:c.1633G>A: p.E545K	36% (675/1862)	Missense
	DCIS		42% (386/915)	
Patient 3	Invasive BC	**GATA3**:NM_001002295: exon6:c.1298_1304del: p.P433fs	25% (435/1720)	Indel
	DCIS		41% (319/779)	
	Invasive BC	**TP53**:NM_000546: exon8:c.916C>T: p.R306X	33% (443/1350)	Stopgain
	DCIS		70% (71/102)	
	Invasive BC	**PIK3CA**:NM_006218: exon10:c.1624G>A: p.E542K	44% (824/1853)	Missense
	DCIS		10% (28/281)	
Patient 4	Invasive BC	No pathogenic variants validated	-	-
	DCIS			

Presence of Columnar Cell-Like Lesions

In 13 patients, a lesion resembling columnar cell-like lesions in women was detected adjacent to invasive breast cancer. The majority of these cases (11 out of 13) also had adjacent ductal carcinoma in situ. These columnar cell-like lesions mainly consisted of dilated ducts with apical snouting and cytonuclear atypia. Notably, the cytonuclear aspects resembled the cellular aspects of the adjacent invasive component (Figure 1). However, a convincing morphologic architecture of these lesions, as seen in women, is missing. Next generation sequencing was performed for three of these cases. The selection was based on availability of tissue. In two out of three cases, we found similar mutations in the columnar cell-like lesion and the adjacent invasive component (Figure 2).

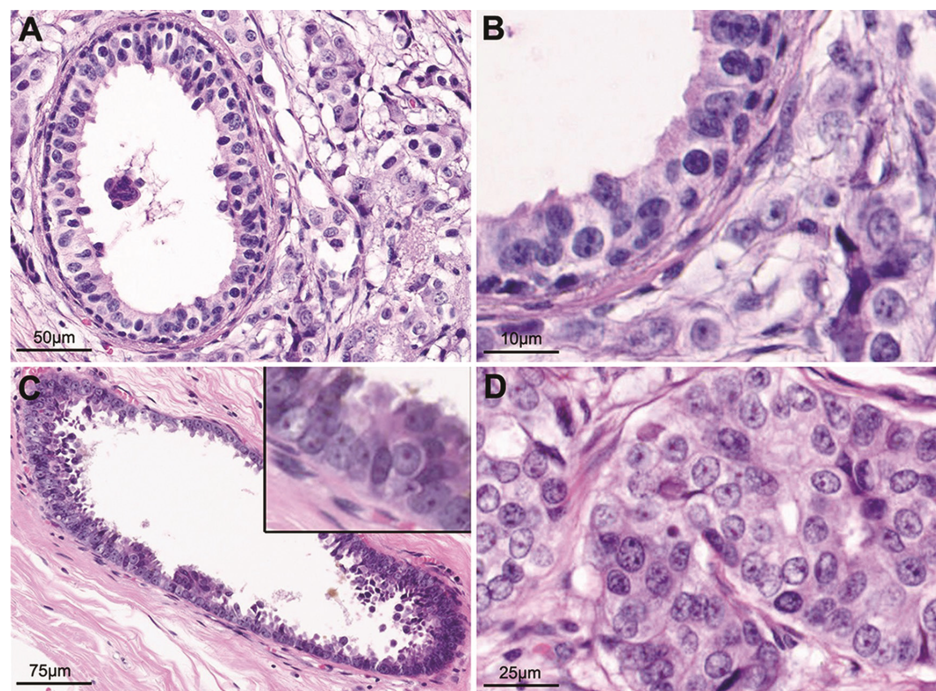

Figure 1. Two cases with distended ducts lined by myoepithelial cells and an inner layer of columnar cells with apical snouting, rounded nuclei and prominent nucleoli (A and C), interpreted as a columnar cell-like lesion, and an adjacent invasive component with similar cytonuclear features (B and D respectively) (original magnification x 40).

In case 1 (Figure 2A–F), the invasive breast cancer was associated with both a ductal carcinoma in situ component and a columnar cell-like lesion. These three components showed similar mutations, including a PIK3CA and a GATA3 mutation. Case 2 (Figure 2G–L) showed a PIK3CA mutation in both the invasive breast cancer and the adjacent columnar cell-like lesion. In the remaining case, we identified a TP53 mutation in the invasive component, which could not be found in the adjacent columnar cell-like lesion. These findings are in line with the overlapping morphology and support the hypothesis that columnar cell-like lesions are a putative precursor lesion of male breast cancer.

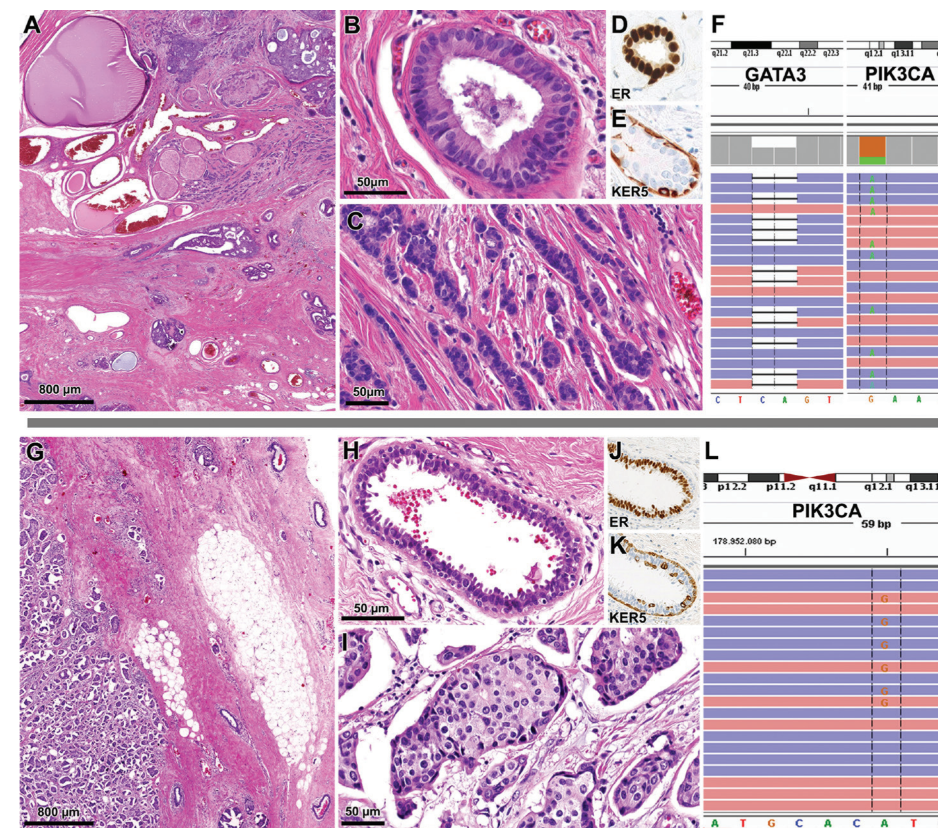

Figure 2. Two cases (A-F: case 1, G-L: case 2) with a CCL-like lesion and adjacent invasive BC. H&E staining of the CCL-like lesions adjacent to invasive BC in **A** and **G** (original magnification 20x). A detailed H&E staining of CLL-like ducts (**B** and **H**) and an adjacent invasive component with similar cytonuclear features (**C** and **I**) (original magnification 40x). The luminal columnar cells show strong nuclear staining with ER (**D** and **J**) while only a few cells are positive for CK5 (**E** and **K**). NGS showed identical mutations in both the CCL-like lesion and the adjacent invasive component (**F:** GATA3 deletion mutation and a PIK3CA missense mutation, **L**: PIK3CA missense mutation).

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Association Between the Presence of Ductal Carcinoma In Situ and Clinical Outcome

There was no significant association between the presence of ductal carcinoma in situ and metastatic or nodal status (P = 0.17 and P = 0.41, respectively). Relapse-free survival for M0 patients with ductal carcinoma in situ vs patients without ductal carcinoma in situ was not statistically different (HR = 0.84; 95% CI 0.65, 1.08; P = 0.18). Subgroup analyses for Luminal A, Luminal B HER2 − , and Luminal B HER2+ did also not indicate an effect for ductal carcinoma in situ vs no ductal carcinoma in situ (Luminal A: HR= 0.81, 95% CI 0.52–1.24, P = 0.33; Luminal B HER2 − : HR = 0.96, 95% CI 0.67–1.37, P = 0.80; Luminal B HER2+: HR = 0.43, 95% CI 0.09– 2.07, P = 0.29). For overall

survival, however, there was a difference between patients with ductal carcinoma in situ as compared with patients without ductal carcinoma in situ (HR = 0.74, 95% CI 0.63– 0.87, P < 0.01; Figure 3a). Subgroup analyses showed that this effect is mainly driven by the Luminal A cases (HR = 0.64, 95% CI 0.49–0.84, P < 0.01; Figure 3b) and Luminal B HER2+ patients (HR = 0.34, 95% CI 0.15-0.79, Po0.01; Figure 2d) and was not seen in the Luminal B HER2 − cases (HR = 0.91, 95% CI 0.72–1.16, P = 0.44; Figure 2c).

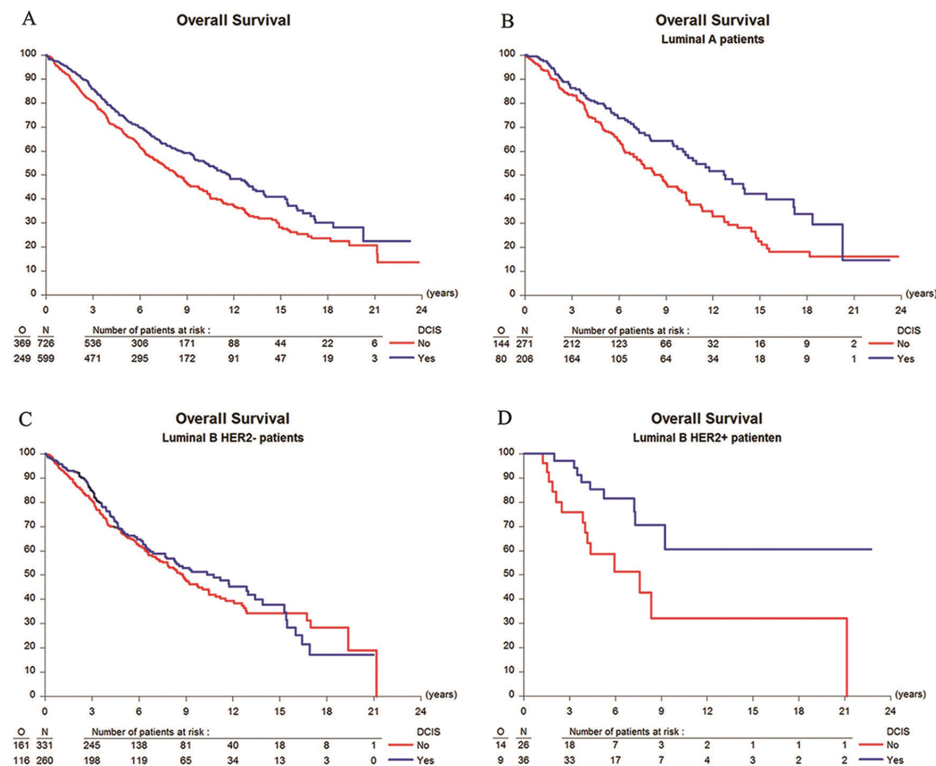

Figure 3. Kaplan-Meier curves for Overall Survival of all M0 patients with DCIS versus patients without DCIS (figure A) and subgroup analyses for Luminal A (figure B), Luminal B HER2- (figure C) and luminal B HER2+ (figure D) cases.

A multivariate Cox model for overall survival was fitted including potential confounding covariates (Table 4). After adjusting for these factors in this model, there was a trend that the presence of ductal carcinoma in situ was associated with a better overall survival, in the Luminal A but in particular in the Luminal B HER2+ subgroup.

Table 4. Multivariate Cox model for Overall Survival (N=749) for Luminal A-like and Luminal B-like cases.

Multivariate Cox Model for Overall Survival					
Parameter		**Hazard Ratio**	**95% Hazard Ratio Confidence Limits**		**p-value**
Age at diagnosis					<.0001
	≤ 40 (reference)	1			
	41 - 50	0.94	0.26	3.37	
	51 - 65	1.38	0.43	4.45	
	66 - 75	2.35	0.73	7.56	
	> 75	4.70	1.47	15.12	
M status					<.0001
	M0 (reference)	1			
	M1	3.58	2.22	5.77	
LN status					0.118
	Negative (reference)	1			
	Positive	1.22	0.95	1.56	
T status					<.0001
	T1 (reference)	1			
	T2	1.67	1.28	2.18	
	T3	2.44	1.19	5.02	
	T4	2.19	1.55	3.09	
BC subtype					0.052
	Luminal A (reference)	1			
	Luminal B (HER2 negative)	1.18	0.84	1.66	
	Luminal B (HER2 positive)	0.28	0.09	0.93	
DCIS by BC subtype					0.062
	DCIS (yes vs no) lum A	0.85	0.58	1.24	
	DCIS (yes vs no) lum B HER2-	1.17	0.85	1.61	
	DCIS (yes vs no) lum B HER2+	0.21	0.06	0.77	
Histological type					0.14
	Invasive ductal (reference)	1			
	invasive lobular	1.64	0.75	3.58	
	Other	0.76	0.52	1.11	
Grade of Invasive BC					0.54
	1 (reference)	1			
	2	1.14	0.83	1.57	
	3	1.24	0.85	1.81	

DISCUSSION

In our series, a substantial proportion (46%) of patients with invasive breast cancer also had adjacent ductal carcinoma in situ. Although we cannot draw conclusions regarding the exact frequency of ductal carcinoma in situ adjacent to male breast cancer (since we only received one block/ patient), we can conclude that ductal carcinoma in situ is present in a large proportion of male breast cancer. There was a strong positive correlation between nuclear grade, ER, PR, and HER2 status of ductal carcinoma in situ and the adjacent invasive breast cancer. In line with this, molecular analysis confirmed similarities on the genomic level, including identical PIK3CA, GATA3, TP53, and MAP2K4 mutations in both components. These data are supportive but not definitive evidence that ductal carcinoma in situ represents a precursor lesion of male breast cancer.

The frequency of lobular carcinoma in situ in our series was very low (< 1%), which is in line with the very low incidence of invasive lobular carcinoma previously reported in male breast cancer patients. No classic columnar cell-like lesions were reported in this large series of male breast cancer patients, which is in line with a previous smaller series.[7] However, we reported a few cases with columnar cell-like lesions adjacent to invasive breast cancer, including dilated, twisted ducts with apical snouts, and morphological resemblance with the adjacent invasive component. These ducts lacked the classical morphology of female columnar cell-like lesions, including rounded ducts with intraluminal calcifications, which limits the ability to recognize these lesions. Therefore, since distinct morphological criteria to define columnar cell-like lesions in male are lacking, the incidence remains unknown. In our series, we reported several identical genomic alterations, including PIK3CA and GATA3 mutations, in two out of three patients with a columnar cell-like lesion and an adjacent invasive component.

A limitation of this study is that next generation sequencing analysis was performed on only a small subset of cases with a columnar cell-like lesion, due to the low detection rate and the lack of available tissue blocks to perform additional analyses. Regarding ductal carcinoma in situ, there was not such a restriction regarding availability of tissue, but performing next generation sequencing on more samples would not have changed the conclusion that ductal carcinoma in situ is indeed a precursor of male breast cancer. Another limitation is that we only sequenced a panel of selected tumor-specific variants and, therefore, we were not able to evaluate the full spectrum of mutational events. A larger panel could have

identified additional genes. However, the goal of this part of the study was to support the morphological finding of resemblance of the columnar cell-like lesions and the adjacent invasive component by providing additional information on the genetic level, rather than providing an overview of all mutations present in these lesions.

In women, ductal carcinoma in situ is more often detected adjacent to ER, PR, and/or HER2 positive invasive breast cancer. In this male breast cancer series, there was no significant association between the presence of ductal carcinoma in situ and surrogate breast cancer subtype. A potential explanation for this difference is the different distribution of breast cancer surrogate subtypes in men as compared with women, including a low frequency of HER2+ and triple negative cases.

In the literature, no data exist regarding the association between the presence of ductal carcinoma in situ and outcome of male breast cancer. In our series, Luminal A and Luminal B HER2+ patients with an adjacent ductal carcinoma in situ component were observed to have a better overall survival compared with those without a ductal carcinoma in situ component, also after adjustment for potential confounders, which suggests that coexisting ductal carcinoma in situ could represent an earlier or biologically less aggressive form of disease. However, the observed associations between the presence of ductal carcinoma in situ with clinical outcome in this study should be interpreted cautiously since the treatments these patients received were not highly standardized and not controlled by protocols. Therefore, the reported analysis is informative and hypothesis generating but cannot be considered a classical prognostic factor analysis.

In conclusion, this is the first and largest study describing the presence and significance of breast cancer precursor lesions in male breast cancer, supplemented with next generation sequencing. Ductal carcinoma in situ seems to be the most common precursor lesion in male breast cancer, as in female patients. The frequency of lobular carcinoma in situ was very low, which is in line with the low frequency of lobular carcinomas in male patients. Based on our data, no definite conclusion can be drawn regarding the prevalence of columnar cell-like lesions in men, but the morphological and genetic overlap between columnar cell-like lesions and adjacent invasive breast cancer suggest a possible causal relationship between these lesions.

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CHAPTER

8

Copy Number Profiling of Oncogenes in Ductal Carcinoma in Situ of the Male Breast

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ABSTRACT

Characterizing male breast cancer (BC) and unraveling male breast carcinogenesis is challenging because of the rarity of this disease. We investigated copy number status of 22 BC-related genes in 18 cases of pure ductal carcinoma in situ (DCIS) and in 49 cases of invasive carcinoma (IC) with adjacent DCIS (DCIS-AIC) in males using multiplex ligation-dependent probe amplification (MLPA). Results were compared to female BC and correlated with survival. Overall, copy number ratio and aberration frequency including all 22 genes showed no significant difference between the 3 groups. Individual unpaired analysis revealed a significantly higher MTDH copy number ratio in IC compared to DCIS-AIC and pure DCIS (P = 0.009 and P = 0.038, respectively). ADAM9 showed a significantly lower copy number aberration frequency in male BC, compared to female BC (P = 0.020). In DCIS-AIC, MTDH, CPD, CDC6 and TOP2A showed a lower frequency of copy number increase in males compared to females (P < 0.001 for all 4 genes). In IC, CPD gain and CCNE1 gain were independent predictors of poor overall survival. In conclusion, male DCIS and IC showed a similar copy number profile for 21 out of 22 interrogated BC-related genes, illustrating their clonal relation and the genetically advanced state of male DCIS. MTDH showed a higher copy number ratio in IC compared to adjacent and pure DCIS and may therefore play a role in male breast carcinogenesis. Differences were detected between male and female DCIS for 4 genes pointing to differences in breast carcinogenesis between the sexes.

INTRODUCTION

Breast carcinogenesis is a multi-step process involving accumulation of DNA alterations and epigenetic changes. An important event during cancer development is oncogene amplification. Several genes have been described to be frequently amplified in female breast cancer (BC), of which the best-known example is the human epidermal growth factor receptor 2 (HER2). HER2 is amplified in 10–20% of female BC and is correlated to overall survival, time to relapse and response to trastuzumab, a humanized monoclonal anti-HER2 antibody (Slamon *et al*. 1987, Hudis 2007, Moelans *et al*. 2009). Other oncogenes that have been described to have clinical implications in female BC include the estrogen receptor (ESR1), epidermal growth factor receptor 1 (EGFR), MYC, topoisomerase IIa (TOP2A), fibroblast growth factor receptor 1 (FGFR1), cyclin E (CCNE1) and cyclin D1 (CCND1) (Holst et al. 2007, Rodriguez-Pinilla *et al*. 2007, Turner *et al*. 2010, Holm *et al*. 2012, Masuda *et al*. 2012, Almeida *et al*. 2014, Lundgren *et al*. 2015).

Invasive ductal type cancers (IDC) of the breast are thought to arise from ductal carcinoma in situ (DCIS) via parallel breast cancer progression pathways in which low-grade DCIS progresses to low-grade IDC and high-grade DCIS to high-grade IDC. These parallel pathways have been postulated to have distinct genomic aberrations (Hwang *et al*. 2004, Moelans *et al*. 2010*a*, Burger *et al*. 2013). Progression through grade is a phenomenon that has been rarely observed in BC (Schymik *et al*. 2012).

The final step in breast carcinogenesis, where the basement membrane of the ducts is breeched and the malignant epithelial cells infiltrate the surrounding stroma, is poorly understood. Several female BC studies have shown similar levels of gene amplification in DCIS and adjacent IC, indicating that these genes play an early role in breast carcinogenesis, but not in the progression from DCIS to invasive carcinoma (Aubele *et al*. 2000, Burkhardt *et al*. 2010, Moelans *et al*. 2010a). Furthermore, not all patients diagnosed with pure DCIS show progression to IC when left untreated. A previous study showed progression from low-grade DCIS to IC in 11/28 cases, the remaining cases showing an indolent course (Sanders *et al*. 2005). Therefore, unraveling the drivers that control the progression of DCIS to IC has proved to be challenging in female BC, let alone in male BC, where the rarity of the disease hampers thorough investigation. This knowledge is however needed to understand the biological course of male DCIS, to predict patients' outcome and to optimize DCIS treatment strategies. In this study, we compare pure DCIS, DCIS adjacent to IC (DCIS-

AIC) and IC, as differences at molecular level have been described between these two types of DCIS in females, using RT-PCR (Doebar *et al*. 2017).

Male BC is a rare disease, accounting for approximately 1% of all BC (Siegel *et al*. 2015). Pure DCIS represents approximately 5% (range 1–17%) of all cancers in the male breast (Pappo *et al*. 2005). In female BC, the diagnosis of pure DCIS is made in approximately 20% of all BC, and this difference in DCIS frequency between male and female BC can perhaps be explained by the participation of women in BC screening programs (Leonard & Swain 2004).

There are many similarities but also important differences between male and female BC. There are differences in distribution of histologic subtypes as well as molecular subtypes; men tend to be older at the time of diagnosis and have more advanced disease at presentation compared to women (Giordano *et al*. 2004, Ge *et al*. 2009, Anderson *et al*. 2010, Kornegoor *et al*. 2012b). Also, there is some evidence suggesting differences in gene amplification frequencies (Kornegoor *et al*. 2012a). In a previous male BC study, gain of CCND1 and EGFR was more frequent in male BC compared to female BC, and amplification of TRAF4 and EMSY was more often observed in female BC in comparison to male BC (Kornegoor *et al*. 2012a). In the present study, we used multiplex ligation-dependent probe amplification (MLPA) to investigate DNA copy number changes of 22 breast cancer-related genes in a group of male IC with adjacent DCIS and in a group of male pure DCIS. We correlated these copy number aberrations with clinicopathologic features and 10-year survival data and compared our results to a previous female BC study using a similar MLPA kit (Moelans *et al*. 2010*a*).

MATERIALS AND METHODS

Patient material

Patients with DCIS and adjacent IC or pure DCIS were enrolled from a previously selected large male BC cohort (Cardoso *et al*. 2015, Vermeulen *et al*. 2017). A subgroup of this initial population was selected based on availability of a tumor tissue block for central pathology review and sufficient tissue for DNA isolation. This resulted in a total of 51 cases with IC and adjacent DCIS and 20 cases of pure DCIS. Patient and tumor characteristics including age at diagnosis and 10-year overall survival status (defined as death due to any cause) were recorded. Data concerning BRCA1/2 testing was not available. Hematoxylin and eosin (H&E) slides were reviewed by an experienced pathologist to confirm the diagnosis and to type and grade the IC according to the World Health Organization and modified Bloom and Richardson score (Elston & Ellis 1991). DCIS was graded according to the classification by Holland and coworkers (1994). ER, PgR and HER2 were evaluated using immunohistochemistry and scored according to the Allred score (Allred *et al*. 1998) and ASCO-CAP guidelines (Wolff *et al*. 2013). The areas of interest (pure DCIS, DCIS-AIC and IC) were dissected either manually with a sterile scalpel when big enough or by laser capture microdissection using a Zeiss PALM MD3 laser microdissection system, from 5 sections (4 µm) of formalin-fixed paraffin-embedded (FFPE) tissue blocks. Laser capture microdissection was done in cases with only small areas of DCIS or with abundant inflammatory cells surrounding the area of interest. The DNA was extracted by overnight incubation in proteinase K (10 mg/mL; Roche) at 56°C, followed by boiling for 10 min and centrifugation. Normal male breast tissue was taken along as control. Results from a previous female BC study comparing DCIS and adjacent IC (N = 39) using a similar MLPA kit were used to compare copy number status in female and male BC (Moelans et al. 2010a).

Clinicopathological data are shown in Table 1. Hormone receptor status showed a high concordance (100%) between DCIS and adjacent IC.

Table 1. Clinicopathological data of all male breast cancer cases (invasive carcinoma (IC), male pure ductal carcinoma *in situ* (pure DCIS) and DCIS adjacent to invasive carcinoma).

	Invasive carcinoma	Adjacent DCIS	*Pure* DCIS
Age (years)			
Mean (range)	63.2 (37-85)	63.2 (37-85)	62.3 (37-76)
Histologic subtype IC			
Ductal type carcinoma	46 (90.2 %)		
Mucinous carcinoma	1 (2 %)		
Micropapillary carcinoma	1 (2 %)		
Encapsulated papillary carcinoma	1 (2 %)		
Mixed type			
Ductal/micropapillary	1 (2%)		
Ductal/mucinous	1 (2%)		
Grade			
1	14 (27.5 %)	11 (21.6 %)	3 (15 %)
2	22 (43.1 %)	32 (62.7 %)	16 (80 %)
3	15 (29.4 %)	8 (15.7 %)	1 (5 %)
ER			
Positive	51 (100 %)	51 (100 %)	20 (100 %)
Negative	0 (0 %)	0 (0 %)	0 (0%)
PR			
Positive	49 (96.1 %)	49 (96.1 %)	20 (100 %)
Negative	2 (3.9 %)	2 (3.9 %)	0 (0%)
HER2			
Positive	2 (3.9 %)	2 (3.9 %)	1 (5.3 %)
Negative	49 (96.1 %)	49 (96.1 %)	18 (94.7 %)
Missing	0	0	1

Multiplex Ligation-dependentProbe Amplification (MLPA)

MLPA analysis was performed on all isolated DNA using the P078-C1 kit (MRC Holland, Amsterdam, The Netherlands), containing 41 probes targeting 22 breast cancer-related genes (Supplementary Table 1, see section on supplementary data given at the end of this article). MLPA was performed according to the manufacturer's instructions (MRC Holland), using an ABI 9700 PCR machine (Applied Biosystems). All tests were done in duplicate, and each MLPA run included 7 negative reference samples (3 healthy blood samples, 3 normal male breast FFPE samples and 1 normal female breast FFPE sample). The PCR products were separated by capillary electrophoresis on a 3730 DNA analyzer (Applied Biosystems). Gene copy numbers were analyzed using GeneScan analysis (Applied Biosystems) and Coffalyser. net software (MRC-Holland). For genes targeted by more than one probe, the mean of all probe ratios was calculated. Four of the 12 reference probes showed above average copy number variations and were

excluded from further analyses (NRAP located at 10q25.3, TGIF1 located at 18p11.31, CETN3 located at 05q14.3 and SNCA located at 04q22.1).

Cut-off values were set as described previously with a copy number ratio of <0.7 for gene loss, 1.3–2.0 for copy number gain and >2.0 for amplification (Moelans *et al.* 2010*a*, Kornegoor *et al.* 2012*a*). Values between 0.7 and 1.3 were considered copy number neutral.

Statistics

Statistical calculations were done using SPSS, version 21.0. The Kruskal–Wallis test was used to compare the overall copy number ratio including all 22 genes between the 3 groups and to compare copy number ratios between the 3 groups for the 22 individual genes. After dichotomization, the chi-square test was used to compare the frequency of gains, amplifications or losses between groups. Mean copy number aberration frequency, for gains, amplifications and losses, including all genes was analyzed using the Kruskal– Wallis test. Individual genes in pure DCIS and DCIS-AIC were compared using Mann–Whitney test for copy number ratio and chi-square for dichotomized results. For paired data (IC and DCIS-AIC) the Wilcoxon signed-rank test was used to compare mean copy number ratio, and McNemar's test was used to compare copy number aberration frequency for the 22 individual genes. The overall copy number ratios between low/ intermediate-grade and high-grade DCIS, as well as between low/intermediate-grade and high-grade IC were compared by Mann–Whitney test. Dichotomized data per grade category were evaluated by chi-square. P values less than 0.05 were considered significant and correction for multiple comparisons was done using the Holm–Bonferroni method. Survival data were available for all IC and DCIS-AIC cases with a median follow-up of 8.1 years (range 0.86–19.56 years). For univariate survival analysis, Kaplan– Meier curves were plotted and analyzed with the log-rank test. Multivariate survival analysis was done with Cox regression (backward LR) and included age, mitosis and grade. Finally, unsupervised hierarchical clustering (Euclidian distance method) of copy number ratios was performed using the statistical program R (www.r-project.org).

RESULTS

Copy number ratio and aberration frequencies in DCIS and invasive carcinoma

One case of invasive carcinoma, one case of DCIS-AIC and two cases of pure DCIS had an insufficient DNA yield and were excluded from further analysis, leaving 49 cases of DCIS with adjacent IC and 18 cases of pure DCIS suitable for copy number analysis. Supplementary Table 2 shows raw MLPA copy number data.

Table 2 summarizes copy number status for all 22 analyzed genes in each subgroup and Fig. 1 illustrates the copy number aberration frequency for each studied gene. The frequencies of losses, gains and amplifications were similar between the three groups (P = 0.167, P = 0.132 and P = 0.361, respectively). Copy number gain/amplification (cut-off >1.3) was most frequently observed for ZNF703, CCND1 and MYC, but none of these genes showed a significant difference between the groups.

Table 2. Frequencies of losses, gains and amplifications in 22 genes for male pure ductal carcinoma *in situ* (pure DCIS), DCIS adjacent to invasive carcinoma (DCIS-AIC) and invasive carcinoma (IC) including the p-value for gain/amplification (copy number ratio >1.3), amplification (copy number ratio >2.0), and the average copy number aberration frequency for all 22 genes.

		Frequencies (%)										
		pure DCIS (N=18)			DCIS-AIC (N=49)			IC (N=49)				
Gene	**Chromosome**	**Loss (<0.7)**	**Gain (1.3-2.0)**	**Amplification (>2.0)**	**Loss (<0.7)**	**Gain (1.3-2.0)**	**Amplification (>2.0)**	**Loss (<0.7)**	**Gain (1.3-2.0)**	**Amplification (>2.0)**	**p-value (gain/ amplification, >1,3) Chisquare**	**p-value (amplification, >2,0) Chisquare**
ESR1	6q25.1	0	0	0	1 (2%)	5 (10%)	0	0	5 (10%)	0	0.362	.
EGFR	7p11.2	0	2 (11%)	1 (6%)	0	5 (10%)	1 (2%)	0	8 (16%)	1 (2%)	0.697	0.701
ZNF703	8p11.23	0	11 (61%)	5 (28%)	0	22 (45%)	12 (24%)	1 (2%)	25 (51%)	10 (20%)	0.267	0.395
FGFR1	8p11.22	0	2 (11%)	1 (6%)	1 (2%)	11 (22%)	7 (14%)	1 (2%)	13 (27%)	7 (14%)	0.162	0.443
ADAM9	8p11.22	5 (28%)	0	1 (6%)	8 (16%)	7 (14%)	3 (6%)	9 (18%)	5 (10%)	4 (8%)	0.426	0.901
IKBKB	8p11.21	0	4 (22%)	0	0	7 (14%)	2 (4%)	0	15 (31%)	1 (2%)	0.252	0.682
PRMD14	8p13.3	0	4 (22%)	0	0	7 (14%)	1 (2%)	3 (6%)	12 (24%)	1 (2%)	0.375	0.817
MTDH	8q22.1	0	3 (17%)	2 (11%)	0	9 (18%)	1 (2%)	0	22 (45%)	1 (2%)	**0.018**	0.237
MYC	8q24.21	0	4 (22%)	2 (11%)	0	17 (35%)	3 (6%)	3 (6%)	14 (29%)	9 (18%)	0.429	0.137
CCND1	11q13.3	0	7 (39%)	1 (6%)	0	18 (37%)	8 (16%)	0	24 (49%)	9 (18%)	0.166	0.241
C11ORF30	11q13.5	0	1 (6%)	0	2 (4%)	3 (6%)	1 (2%)	5 (10%)	3 (6%)	1 (2%)	0.897	0.814
CDH1	16q22.1	0	4 (22%)	0	1 (2%)	9 (18%)	1 (2%)	2 (4%)	8 (16%)	2 (4%)	0.992	0.618
CPD	17q11.2	1 (6%)	0	0	7 (14%)	0	0	5 (10%)	4 (8%)	0	0.061	.
MED1	17q12	0	2 (11%)	1 (6%)	0	11 (22%)	2 (4%)	1 (2%)	10 (20%)	7 (14%)	0.295	0.148
ERBB2	17q12	0	5 (27%)	1 (6%)	0	7 (14%)	2 (4%)	0	9 (18%)	3 (6%)	0.423	0.857
CDC6	17q21.2	0	1 (6%)	0	1 (2%)	2 (4%)	1 (2%)	6 (12%)	4 (8%)	2 (4%)	0.376	0.532
TOP2A	17q21.2	0	0	0	1 (2%)	2 (4%)	2 (4%)	2 (4%)	5 (10%)	3 (6%)	0.109	0.483
MAPT	17q21.31	0	2 (11%)	0	1 (2%)	4 (8%)	0	1 (2%)	6 (12%)	1 (2%)	0.628	0.486
PPM1D	17q23.2	0	1 (6%)	0	1 (2%)	3 (6%)	2 (4%)	1 (2%)	4 (8%)	2 (4%)	0.715	0.672
BIRC5	17q25.3	0	3 (17%)	0	0	8 (16%)	0	1 (2%)	9 (18%)	2 (4%)	0.682	0.231
CCNE1	19q12	0	0	0	1 (2%)	1 (2%)	0	1 (2%)	3 (6%)	0	0.366	.
AURKA	20q13.2	1 (6%)	0	0	2 (4%)	8 (16%)	1 (2%)	3 (6%)	8 (16%)	2 (4%)	0.166	0.566
Total		7	56	15	27	166	50	45	216	68	***0.133***	***0.012***

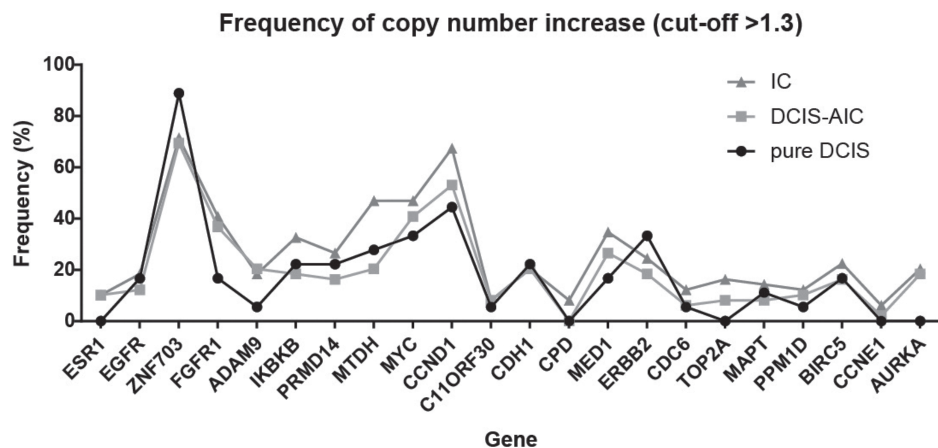

Figure 1. Frequencies of gain/amplification by MLPA for all 22 analyzed genes in male pure ductal carcinoma *in situ* (pure DCIS), DCIS adjacent to invasive carcinoma (DCIS-AIC) and invasive carcinoma(IC).

Overall, the copy number ratio including all 22 genes showed no significant difference between pure DCIS, DCIS-AIC and IC. At the individual gene level, MTDH showed a significantly higher copy number ratio in IC as compared to DCIS-AIC and pure DCIS (P = 0.009 and P = 0.038, respectively). Using a cut-off of >1.3, MTDH showed a significantly higher aberration frequency in IC (46.9%) as compared to DCIS-AIC (20.4%) (P = 0.005).

The copy number ratio for PRDM14, C11ORF30 and FGFR1 was higher in DCIS-AIC compared to pure DCIS (P = 0.007, P = 0.027 and P = 0.042, respectively). However, these genes lost their significance after dichotomization.

No significant differences were found when comparing copy number aberration frequency (gain and amplification) with histologic subtype in IC, although these results should be interpreted with caution due to small sample sizes.

Paired comparison of DCIS-AIC and adjacent IC showed a high concordance of copy number status for all interrogated genes, with no significant differences present. The highest concordance rates were seen for the genes CCNE1 (95.9%) and CDC6 (93.9%). Copy number ratio was significantly higher in IC compared to the paired DCIS-AIC for MTDH (P < 0.001), MYC (P = 0.039), CPD (P = 0.015), TOP2A (P = 0.043) and PPM1D (P = 0.036). Figure 2 shows the median copy number ratio for the 22 analyzed genes and Fig. 3 the copy number ratio for MTDH, MYC, CPD, TOP2A and PPM1D in paired IC and DCIS-AIC.

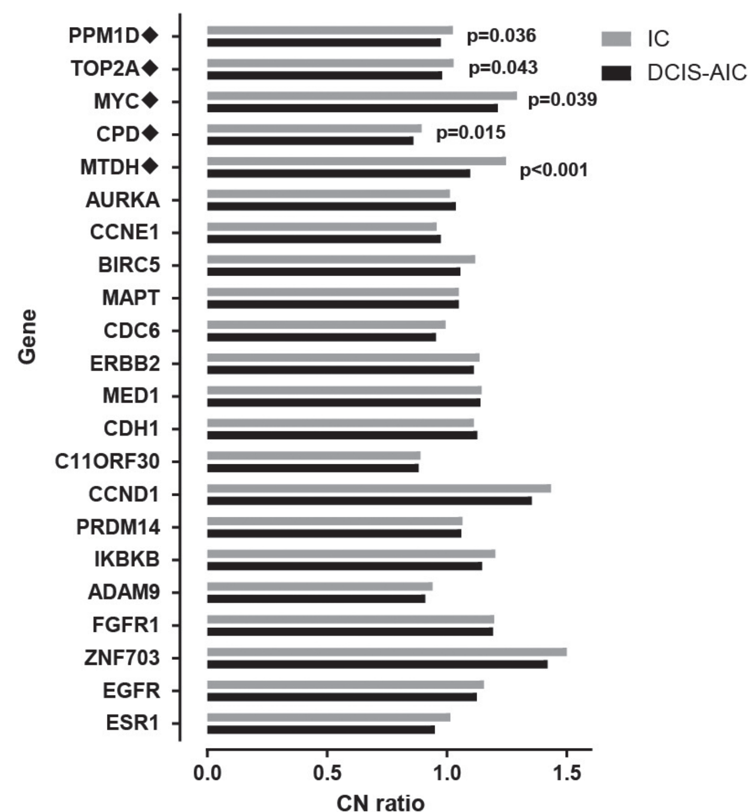

Figure 2. Median copy number ratio for all 22 analyzed genes in male invasive carcinoma (IC) and adjacent ductal carcinoma *in situ* (DCIS-AIC). Genes with a diamond show a significantly higher copy number ratio in IC.

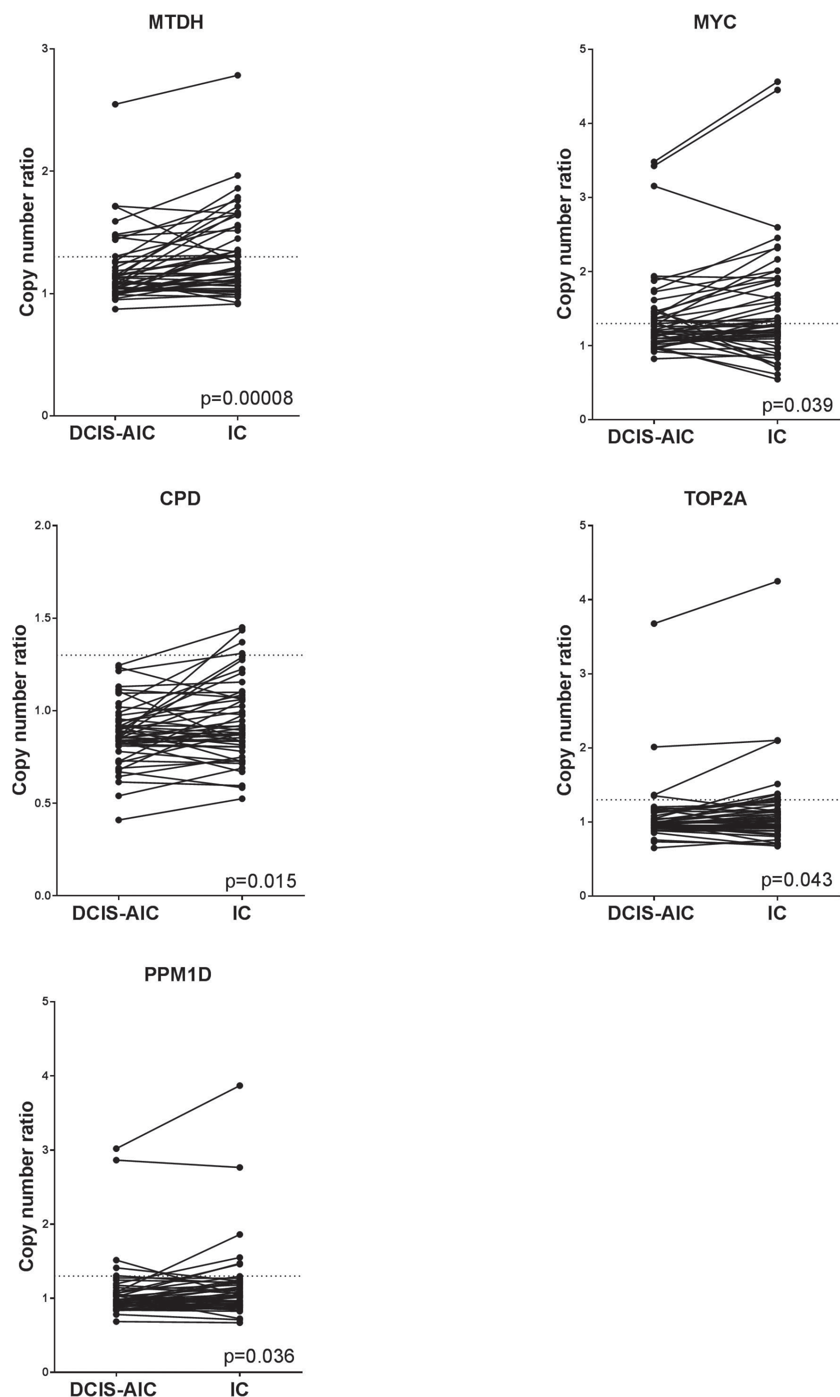

Figure 3. Copy number ratios for *MTDH*, *MYC*, *CPD*, *TOP2A* and *PPM1D* in male invasive carcinoma (IC) and adjacent ductal carcinoma *in situ* (DCIS-AIC).

Correlation between copy number and grade in DCIS-AIC and invasive carcinoma

Copy number ratios and aberration frequencies were compared for DCIS-AIC and IC between low/ intermediate-grade and high-grade lesions. The mean copy number ratio was 1.17 ± 0.22 vs 1.32 ± 0.25 for low/ intermediate-grade vs high-grade DCIS-AIC (P = 0.165), and 1.15 ± 0.16 vs 1.42 ± 0.44 for low/intermediate grade vs high grade IC (P = 0.040). The average number of gains/ amplifications in the 22 analyzed genes was 3.7 vs 8.4 for low/intermediate-grade vs high-grade DCIS-AIC (P = 0.019) and 4.8 vs 8.3 for low/intermediate-grade vs high-grade IC (P = 0.037).

DCIS-AIC showed a significantly higher copy number ratio in high-grade lesions for the genes ESR1 (P = 0.047), PPM1D (P = 0.004), BIRC5 (P = 0.002) and CCNE1 (P = 0.005). After dichotomization (cut-off >1.3), these differences remained significant (P < 0.001, P = 0.002, P = 0.040 and P = 0.014, respectively). In addition, PRDM14 (P = 0.040), CDC6 (P = 0.003), TOP2A (P = 0.018) and AURKA (P = 0.006) showed a significantly higher copy number aberration frequency in high-grade DCIS-AIC lesions. Only MTDH showed a significantly higher frequency of amplification in high-grade DCIS-AIC (P = 0.007).

IC showed a significantly higher copy number ratio in high-grade lesions for the genes EGFR (P = 0.005) and CCND1 (P = 0.005). Dichotomized data (cut-off >1.3) showed a significantly higher aberration frequency for ESR1 (P = 0.007), EGFR (P = 0.047), C11ORF30 (P = 0.001), CDC6 (P = 0.022) and PPM1D (P = 0.020) in high-grade lesions. ADAM9 (P = 0.029), MYC (P = 0.031), CCND1 (P = 0.005), CDH1 (P = 0.029), CDC6 (P = 0.013), TOP2A (P = 0.004) and PPM1D (P = 0.012) showed significant amplification more often in high-grade lesions.

After correction for multiple comparisons, only BIRC5 in DCIS-AIC remained significant with regard to copy number ratio difference (1.068 in low/intermediate grade vs 1.353 in high grade). For the dichotomized data, C11ORF30 in IC (17.1% in low/intermediate grade vs 35.7% in high grade) and ESR1 in DCIS-AIC (2.4% vs 57.1%) remained significant.

Comparison of DCIS-AIC and IC copy number status between male and female breast cancer

Results from a previous female BC study including 39 patients (IC and adjacent DCIS) were used to compare copy number status between female and male BC (Moelans *et al*. 2010a). This previous study used a prior version of the MLPA kit used here. Twenty genes were similar in both MLPA kits, with some differences in the probes used for the genes and were used for analysis. In IC, ADAM9 showed a significantly lower copy number aberration frequency (cut-off >1.3) in male BC (22.5%) compared to female BC (56.4%) (P = 0.020). In DCIS, MTDH, CPD, CDC6 and TOP2A showed a lower frequency of copy number increase in male compared to female BC (P < 0.001 for all 4 genes) (Fig. 4). The frequencies of amplifications (cut-off >2.0) and losses were similar between female and male BC.

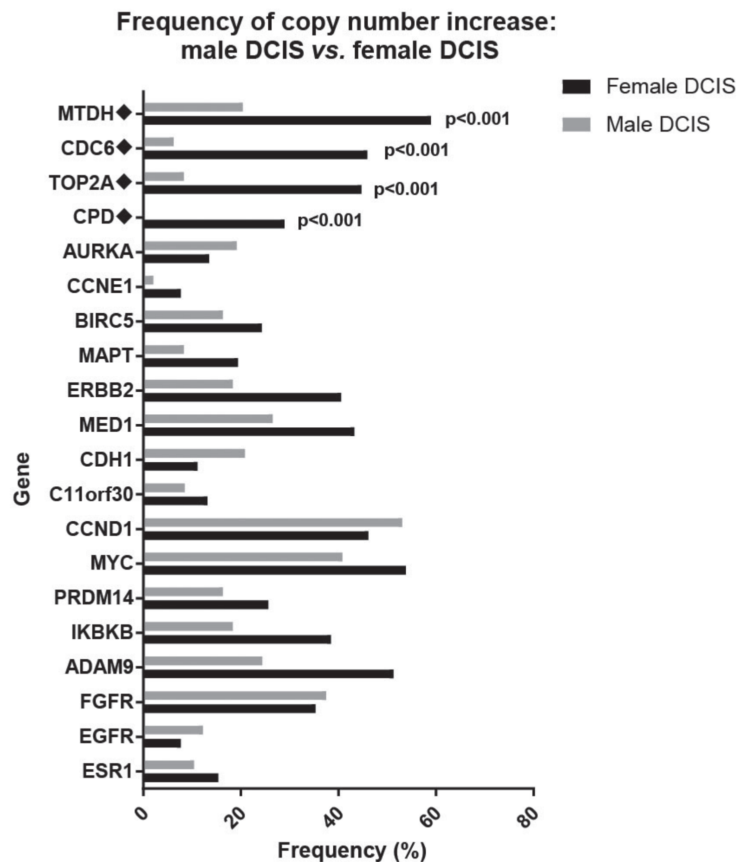

Figure 4. Frequency of copy number increase (cut-off >1.3) in female and male ductal carcinoma *in situ* (DCIS). Genes with a diamond show a significantly higher frequency of copy number gain in female BC.

In addition, we compared copy number aberration frequencies of 21/22 interrogated genes (EMSY data not available) with a large public female breast cancer cohort (METABRIC, www.cbioportal.org, (Curtis *et al*. 2012, Pereira *et al*. 2016)). Supplementary Table 3 shows a high amplification frequency similarity for all genes except for PRDM14 and MTDH, which both showed a difference of at least 10% in amplification frequency, with a higher amplification percentage in the METABRIC population (N = 2173).

Supplemental table 3.

Gene	N	Missing	Total	METABRIC gain (%)	MALE BC gain (%)	METABRIC amplification(%)	MALE BC amplification(%)
ESR1	2509	336	2173	6,44	10,0	2,30	0,0
EGFR	2509	336	2173	14,13	16,0	2,39	2,0
ZNF703	2509	336	2173	12,10	51,0	14,27	20,0
FGFR1	2509	336	2173	12,47	27,0	13,12	14,0
ADAM9	2509	336	2173	12,33	10,0	11,23	8,0
IKBKB	2509	336	2173	15,14	31,0	10,26	2,0
PRDM14	2509	336	2173	21,58	24,0	15,74	2,0
MTDH	2509	336	2173	22,46	45,0	19,24	2,0
MYC	2509	336	2173	22,09	29,0	25,49	18,0
CCND1	2509	336	2173	11,18	49,0	16,29	18,0
EMSY	2509	2509	0		6,0		2,0
CDH1	2509	336	2173	2,95	16,0	0,18	4,0
CPD	2509	336	2173	8,10	8,0	2,76	0,0
MED1	2509	336	2173	8,51	20,0	11,92	14,0
ERBB2	2509	336	2173	8,42	18,0	15,74	6,0
CDC6	2509	336	2173	8,65	8,0	6,53	4,0
TOP2A	2509	336	2173	8,74	10,0	4,92	6,0
MAPT	2509	336	2173	8,70	12,0	1,10	2,0
PPM1D	2509	336	2173	14,17	8,0	10,08	4,0
BIRC5	2509	336	2173	16,57	18,0	5,71	4,0
CCNE1	2509	336	2173	7,87	6,0	2,81	0,0
AURKA	2509	336	2173	20,66	16,0	6,35	4,0

Correlation between copy number alterations and survival

CPD and CCNE1 gain (no amplifications were observed) in IC were predictors of poor 10-year overall survival (P = 0.050 and P = 0.001) and remained independent prognosticators when grade, mitoses and age were included in multivariable analysis (P = 0.017 (HR 5.1) and P = 0.003 (HR 6.9)). Kaplan–Meier curves are presented in Fig. 5. None of the other interrogated genes were associated with survival.

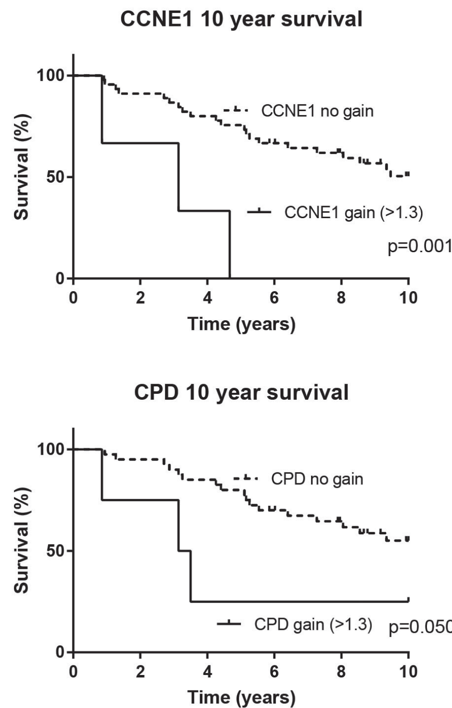

Figure 5. K Kaplan-Meier 10-year overall survival plots for *CCNE1* gain and *CPD* gain.

Cluster analysis of all male pure DCIS, DCIS-AIC and IC lesions

Unsupervised hierarchical cluster analysis of all pure DCIS, DCIS-AIC and IC showed 2 main clusters that differed significantly according to grade (grade 1/2 vs grade 3) with more high-grade lesions in cluster B (n = 29) compared to cluster A (n = 20) (P = 0.001) (Fig. 6). In addition, all genes showed a higher copy number ratio in cluster B. Of the 49 paired DCIS-AIC and IC samples in the cluster analysis, 40 samples (81.6%) were in the same cluster, and of these, 17 pairs (34.7%) clustered closely together indicating that these adjacent in situ and invasive components share many genetic alterations.

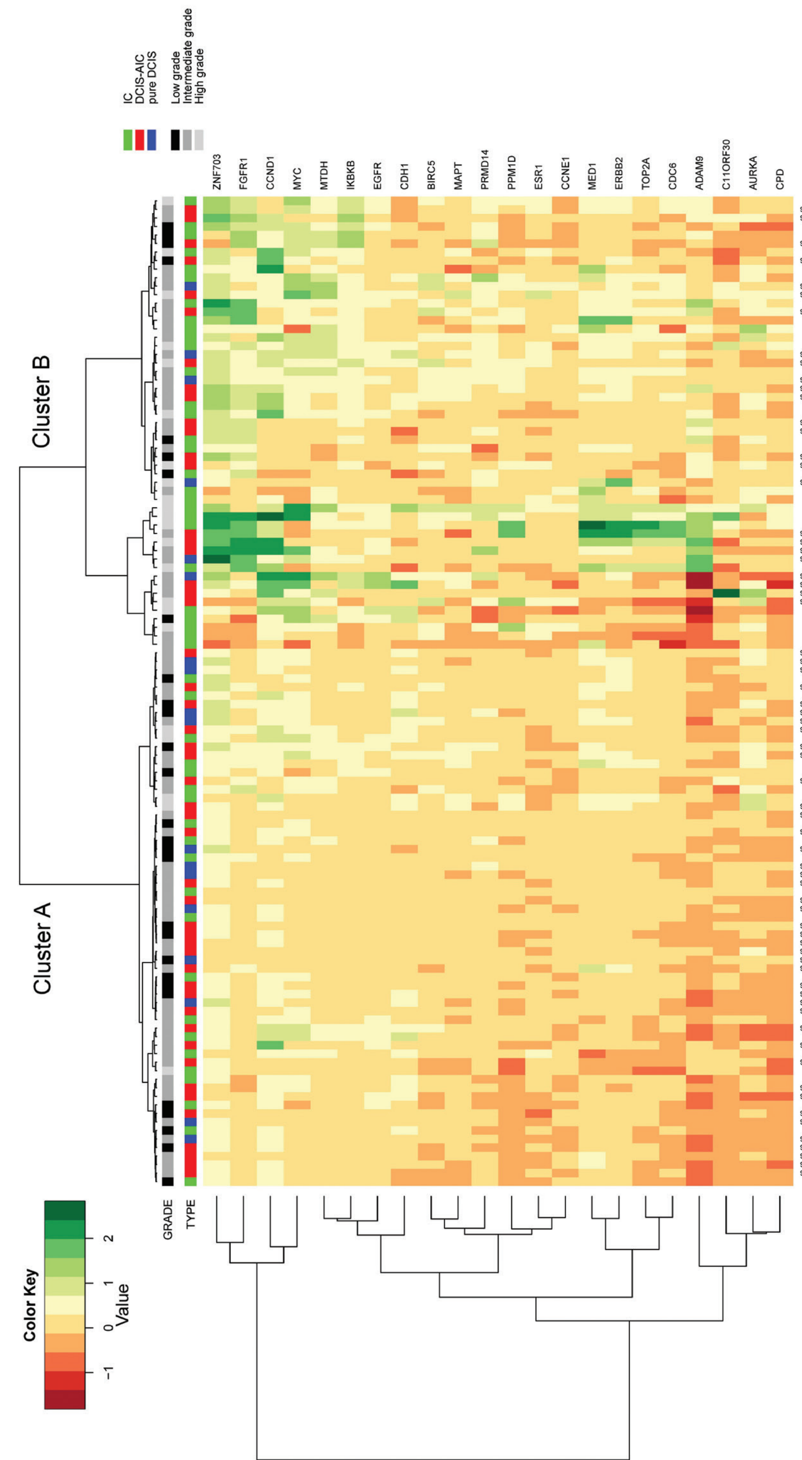

Figure 6. Unsupervised hierarchical cluster analysis of 22 genes in male breast cancer lesions, including pure ductal carcinoma in situ (DCIS), DCIS adjacent to invasive carcinoma (DCIS-AIC) and invasive carcinoma (IC).

8

DISCUSSION

To discover drivers that may control the progression of DCIS to IC and to establish the precursor role of DCIS in male breast carcinogenesis, we studied copy number status of 22 breast cancer-related genes in IC, DCIS-AIC and pure DCIS of the male breast by MLPA. Overall, in line with previous studies on their female counterparts, there were only few copy number differences between male DCIS and IC (Aubele *et al*. 2000, Burkhardt *et al*. 2010, Moelans *et al.* 2010*a*). Copy number ratios were similar in pure DCIS, DCIS-AIC and IC for most of the studied genes, indicating that copy number gain of the majority of these genes does not seem to play a significant role in the transition from male DCIS to IC. This finding is in line with a previous copy number and gene expression study in female BC (Moelans *et al.* 2010a). There was however one gene, MTDH, that showed a significantly higher copy number ratio and frequency of gain in IC as compared to DCIS-AIC. This implies that gain of MTDH could play a role in the progression of DCIS to IC. In a previous MLPA-based male BC study, MTDH showed gain/amplification in 46% of the IC samples, similar to our results (Kornegoor *et al.* 2012*a*). MTDH is located on chromosome 8 and encodes Metadherin, a transmembrane protein that plays a key role in the activation of several signaling pathways including PI3K/Akt, NFκβ, Wnt/βcatenin and the MAPK pathways (Shi & Wang 2015). These pathways play a role in cell proliferation, apoptosis, invasion, angiogenesis and metastasis. Metadherin is frequently overexpressed in female BC and overexpression correlates with advanced clinical stage, distant metastasis and an aggressive phenotype (Tokunaga *et al.* 2014). Moelans and coworkers compared MTDH copy number in 39 paired cases of female DCIS-AIC and IC but found no significant differences in copy number ratio, suggesting that this event may be specific for male breast carcinogenesis (Moelans *et al.* 2010*a*).

Interestingly, almost all of the analyzed genes showed copy number changes in DCIS, indicating that copy number gain is a relatively early event in male breast carcinogenesis. Paired analysis of IC and DCIS-AIC samples showed a high concordance of gain/amplification status between individual patients, supported by cluster analysis. This confirms the clonal relation between male DCIS and IC, as has also been accepted in female breast carcinogenesis (Moelans *et al*. 2010*a*). CCND1, a cell cycle regulatory protein, showed a high copy number aberration frequency in all three groups with 49% CCND1 gain and 18% CCND1 amplification in IC. CCND1 amplification is more frequent in ER-positive and PR tumors, so these high frequencies can be explained by the high rate of ER positivity (all cases being ER positive) and PR

positivity (96% of DCIS-AIC/IC cases and 100% of pure DCIS cases being positive) in our male BC cohort (Reis-Filho *et al.* 2006).

Several genes showed a higher aberration frequency in high-grade lesions compared to low-grade lesions (ESR1, PPM1D, BIRC5, CCNE1, PRDM14, CDC6, TOP2A and AURKA for DCIS-AIC and ESR1, EGFR, C11ORF30, CDC6 and PPM1D for IC). Also, the average copy number ratio was higher in high grade IC compared to low/ intermediate-grade IC. After correction for multiple comparisons, BIRC5 copy number ratio and ESR1 gain in DCIS-AIC and C11ORF30 gain in IC were significantly higher/more frequent in high-grade lesions. Although the sample sizes of high-grade DCIS-AIC and high-grade IC were small (n = 7 and n = 14, respectively), this does suggest that tumors with a higher copy number gain have a tendency to have higher histological grade, as previously demonstrated in male BC (Kornegoor *et al.* 2012a). BIRC5 codes for the protein Survivin, a regulatory protein involved in cell proliferation and apoptosis. It has been extensively studied in female BC where an increased expression of Survivin was correlated with a higher risk of recurrence and with a decreased overall survival rate (Davis *et al.* 2007, Li *et al.* 2014). ESR1 codes for estrogen receptor alpha, a transcription factor located on chromosome 6q25 and an important therapeutic target in female BC with tamoxifen being the standard endocrine therapy for ER-positive breast cancers (Holst *et al.* 2007). In a previous study using MLPA, ESR1 amplification and gain were shown in 2% and 6% of 135 female breast tumors, respectively (Moelans *et al.* 2011). C11ORF30 (also known as EMSY) is a transcription regulatory protein that can compromise BRCA2 function in sporadic breast cancer and ovarian cancer (Hughes-Davies *et al.* 2003). In female BC, it has been associated with a reduced overall survival in ER-positive patients (Kirkegaard *et al.* 2008).

Upon comparison of our findings with female BC, a high concordance was evident, especially for IC. For DCIS, 4 genes (MTDH, CPD, CDC6 and TOP2A) showed a higher frequency of gain in female BC, although no differences in amplification frequency were observed. Copy number aberration frequencies for 21 genes were also compared with a large female breast cancer cohort (METABRIC, www.cbioportal.org, (Curtis *et al.* 2012, Pereira *et al.* 2016)), showing a high amplification frequency similarity.

Two of the 22 studied genes showed a correlation with overall survival. CCNE1 and CPD gain were both indicative of a decreased 10-year overall survival; however, the number of cases showing gain of these genes (n = 3 and n = 4, respectively) were

small and none of the cases showed amplification. Also, treatment regimens and lymph node status were not known so could not be included in the survival analysis. Therefore, results should be interpreted with caution. High levels of Cyclin E have been described to have prognostic value in female breast cancer, especially as a predictor of endocrine therapy failure (Keyomarsi *et al*. 2002, Span *et al.* 2003).

CPD has been investigated in breast cancer cell lines (MCF-7 cells), where prolactin/17β-estradiol-induced cell surface CPD increased intracellular NO production, which increased the survival and inhibited apoptosis (Abdelmagid & Too 2008).

Although a limitation of this study is the relatively small study population, it should be noted that male BC is rare, male DCIS is even rarer, and our DCIS samples have been extracted from a large cohort study and were enriched for tumor cells by scalpel or laser microdissection. We used MLPA for copy number analysis, a multiplex PCR-based method that simultaneously assesses relative copy numbers of a variety of genes in a quantitative way. The major advantage of this technique is that it requires only minimal amounts of small DNA fragments, which makes it very suitable to study small lesions in paraffin-embedded tissue, such as DCIS (Moelans et al. 2009). The MLPA kit used was pre-designed by the manufacturer and contains 22 cancer-related genes that often show copy number aberrations in female BC (Moelans et al. 2010a,b). Although there are some genetic differences between male and female BC, we expected the bigger part of these genes to play a role in male breast carcinogenesis as well (Moelans et al. 2010b). We did not include PIK3CA, TP53 and GATA3, possible important genomic drivers in female BC and described to be frequently mutated in female BC (2012). In this study, we only focused on copy number variations and not on specific mutations. In conclusion, this MLPA-based study showed a similar copy number status for 21 out of 22 studied breast cancer-related genes in male DCIS and IC, illustrating the clonal relation between male DCIS and adjacent IC, and the genetically advanced state of male DCIS. MTDH showed a higher copy number ratio and aberration frequency in IC compared to DCIS and could therefore play a role in the transition of male DCIS to IC.

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CHAPTER

9

Summary and Future Perspectives

This thesis describes several clinical and molecular aspects of DCIS and provides potential progression markers that could contribute to the development of personalized treatment strategies in patients with DCIS.

Chapter 1 provides an introduction on several histological, molecular and clinical DCIS characteristics, including an outline of this thesis. **Chapter 2** describes a study aiming to find novel markers for upstaging to IBC in the surgical resection specimen, after having a diagnosis of pure DCIS based on needle biopsy, since this could aid in the selection of patients for nodal staging. According to current Dutch guidelines, several risk factors for upstaging have been proposed (i.e. palpable mass, young age (<55 years), enhancement on MRI, high grade DCIS and tumor size > 25 mm),[1] but these factors are suboptimal in daily clinical practice. This results in the use of a sentinel node biopsy (SNB) in patients with DCIS without clinical benefit and, in addition, secondary nodal staging for patients who did not undergo a SNB during the initial surgical procedure. In our study, we analyzed micro-environmental changes and epithelial characteristics of DCIS in needle-biopsies with DCIS only. Overall, the upstaging rate to IBC in our cohort was 22%. We reported several factors that were significantly associated with upstaging to invasive cancer in the final excision: palpability of the lesion, young age (≤ 40 years), mass lesion on imaging, peri-ductal changes such as inflammation and loss of Decorin expression. The upstaging-risk correlated with the number of risk factors present: i.e. 9% for patients without risk factors, 29% for patients with 1 risk factor, 37% for patients with 2 risk factors and 54% for patients with ≥3 risk factors. Therefore, these factors may be helpful to select patients with a high upstaging-risk for nodal staging during the initial surgical procedure. Primary nodal staging could be avoided in low-risk patients undergoing breast-conserving surgery (BCS).

In **chapter 3**, the presence and extent of DCIS was evaluated within breast cancer subtypes in a large national population-based cohort of women treated for IBC. Substantial differences in clinical and pathological characteristics of DCIS were found within the breast cancer subtypes, including the resection margin status for patients treated with BCS. HER2+ IBC subtypes showed a higher prevalence and a larger extension of adjacent DCIS. In addition, this subgroup showed a higher rate of irradicality of the DCIS component as compared to HER2- IBC subtypes. This finding is consistent with previous studies reporting higher local recurrence rates after BCS of patients with HER2+ IBC, which might be due to an extensive DCIS

component.[2-4] Pre-operative knowledge regarding the extent of DCIS according to breast cancer subtype could support clinicians in adjusting local therapy control. In **chapter 4**, the biological behavior of DCIS was further evaluated by gene expression profiling. For this study, we hypothesized that extensive DCIS without any sign of invasion represents an indolent lesion with a limited invasive potential, harboring less molecular alterations, in comparison to small DCIS lesions adjacent to IBC, representing lesions with a high invasive potential. Our gene expression analysis indeed identified significant differences in the in situ component between patients with extensive pure DCIS versus patient with a limited DCIS component adjacent to IBC. Most of these differently expressed genes showed a higher expression in the DCIS component adjacent to IBC, including genes involved in several biological processes: signal transduction, chemotaxis, angiogenesis, cellular response to epidermal growth factor stimulus, positive regulation of extracellular signal regulated kinase 1 and 2 cascade, response to hyperoxia and cellular response to amino acid stimulus. Based on these identified genes, it was possible to create a distinct gene expression pattern that discriminates DCIS lesions with invasion from pure DCIS lesions. Our findings were in line with previous studies that reported differences between pure DCIS lesions and DCIS with synchronous IBC based on gene copy number changes and whole sequencing.[10,27] However, validation of these results in an independent larger cohort is required to validated whether these differently expressed genes could be used to predict progression in individual patients diagnosed with DCIS. This might improve individualized treatment strategies for these patients, in which the identified genes (involved in potentially targetable driver pathway(s) that play an important role in the progression of DCIS to IBC) might be inhibited in an early stage of breast cancer, which could ultimately result in the prevention of progression. **Chapter 5** elaborates on gene expression differences between DCIS and paired IBC by evaluating APOBEC3B levels. Additionally, we correlated APOBEC3B levels with PIK3CA mutational status. Upregulated APOBEC3B plays an important role in several human cancers, which is associated with a high mutational load, including PICK3CA and TP53 mutations.[5] This enzyme is non-essential (not expressed in normal tissue) and therefore upregulation could be inhibited by small molecules. In our analysis, APOBEC3B mRNA levels were upregulated in both DCIS and adjacent IBC. No difference was measured in APOBEC3B between wild-type versus mutated PIK3CA DCIS, while APOBEC3B was higher in wild-type versus PIK3CA-mutated IBC. This might imply that once the invasive stage has been reached, PIK3CA is less important for further progression. Higher APOBEC3B levels were found in synchronous DCIS and IBC in ER- tumors as

compared to ER+ tumors. This is in line with previous studies that found upregulated APOBEC3B levels in breast cancer with aggressive characters.[6,7-9] This chapter suggests that APOBEC3B could be a potential therapeutic target in patients with DCIS, although the development of inhibitors for related enzymes is still in progress and additional research is necessary to develop and explore the potential benefit of APOBEC3B inhibitors. In **chapter 6** we described the results of in depth- sequencing of synchronous DCIS and IBC to find answers regarding the mechanism of DCIS progression. We found overlapping mutations between synchronous DCIS and IBC, which was consistent with previous molecular studies that found similar alterations between these two components.[10,11] However, a proportion of mutations was only found in the invasive component, which implies that DCIS progression could be driven by the selection of subclones.[10,12] In other words, DCIS evolution might follow a Darwinian evolution; distinct subclones with specific genetic changes are selected during invasion. This leads to differences in the prevalence of specific mutations between the neoplastic DCIS cells and the invasive counterpart. This knowledge might facilitate future studies in the search of potential markers that play an important role in the transition from DCIS to IBC.

In **chapter 7** we evaluated the presence, characteristics and prognostic relevance of pre-cursor lesions adjacent to invasive cancer of the male breast in a large international cohort, supplemented with NGS data. In this cohort, male breast cancer showed a high prevalence of adjacent DCIS (46% of cases) while the frequency of other pre-cursor lesions (lobular carcinoma in situ, atypical ductal hyperplasia, and columnar cell-like lesions) was very low (< 1%). DCIS and adjacent invasive cancer showed a strong positive correlation for nuclear grade (p= <0.001) and a high concordance in receptor status of ER, PR and HER2. These strong similarities between synchronous DCIS and IBC, which is already known in female breast cancer, imply that DCIS is indeed a precursor lesion of male breast cancer. This was further supported by molecular analysis in a small subset of cases (n=4) that confirmed resemblances on the genomic level of well-known breast cancer mutations in both components (including identical PIK3CA, GATA3, TP53, and MAP2K4 mutations). In literature, the presence of columnar cell-like lesions in the male breast, as described in female breast, is controversial. In male, distinct morphological criteria to define columnar cell-like lesions are lacking and therefore the incidence remains unknown since the ability to recognize these lesions is limited. In our series, we recognized ducts with overlapping features as described for female columnar cell lesions. In a small subset of those lesions, NGS showed identical genomic alterations (including PIK3CA and GATA3 mutations) between the columnar cell-like lesion and adjacent invasive component. These similarities on the genomic level suggest a possible causal relationship between these lesions and support the role of columnar cell-like lesions as a precursor of male breast cancer. Our analysis regarding the presence of DCIS and clinical outcome showed that Luminal A and Luminal B HER2+ patients with an adjacent DCIS component had a better overall survival compared with those without an adjacent DCIS component. This suggests that IBC with coexisting DCIS of the luminal A and B subtype could represent a biologically less aggressive form of disease. In **chapter 8,** a subset of male breast cancer cases extracted from the same male breast cancer series as described in the previous chapter was further evaluated. For this analysis, male breast cancer, DCIS adjacent to MBC and pure male DCIS lesions were subjected to multiplex ligation-dependent probe amplification (MLPA) to investigate DNA copy number changes of 22 breast cancer-related genes. These results were compared to a previous analysis of copy number changes in female breast cancer using a similar MLPA kit. In addition, copy number aberrations were correlated with clinicopathologic features and 10-year survival data. This analysis demonstrated a similar copy number status of 21 out of 22 genes in MBC, synchronous DCIS adjacent to male breast cancer and pure male DCIS. This finding supports the clonal relation between male breast cancer and adjacent DCIS. Furthermore, it demonstrates the genetically advanced state of male DCIS; copy number changes are early events in male breast carcinogenesis. However, one gene, MTHD, involved in the activation of several signaling pathways including PI3K/Akt, NFκβ, Wnt/βcatenin and the MAPK pathways,[13] showed a higher copy number ratio in the invasive component as compared to synchronous DCIS. In literature, this gene is also expressed in female breast cancer where it is associated with aggressive tumor behavior,[14] suggesting that this gene might play an important role in the carcinogenesis of male breast cancer. Some genes showed a higher frequency of copy number aberrations in high-grade lesions as compared to low-grade lesions, including BIRC5 and ESR1 for DCIS adjacent to male breast cancer and C11ORF30 for male breast cancer. Although sample sizes were small, this suggests that a high frequency of copy number aberrations is seen in lesions of higher grade. Two out of the 22 studied genes, CCNE1 and CPD gain, were associated with a decreased 10-year overall survival. However, the numbers of cases with gain of these genes were small and should be interpreted with caution. The results of Chapter 7 and 8 contribute to a better understanding of early events in male breast carcinogenesis.

PERSPECTIVES

Over the past years, DCIS and its progression to IBC has been an emerging field of research. There is a general consensus that current treatment guidelines for patients with DCIS are not specific enough, leading to overtreatment of a substantial proportion of patients. On the other hand, IBC remains the most common (1 out of 8 women) and deadliest cancer in women worldwide, despite major global efforts to treat this disease. Therefore, the urgency is high to invest in research programs focusing on early therapeutic/preventive strategies, since only early intervention has the potential to substantially reduce breast cancer mortality and morbidity by decreasing the incidence. In this thesis, we demonstrated that DCIS is a heterogeneous disease including several subtypes with a different biological behavior and different underlying genetic changes. These features involve changes of the neoplastic DCIS cells, but also DCIS-associated micro-environmental changes, including stromal and inflammatory cells. This could be relevant since recent developments of immune therapies are rapidly progressing.

Many publications reported identical genomic profiles between DCIS and adjacent IBC. This finding makes it challenging to filter specific genetic changes that are related to invasive progression. However, novel molecular approaches such as in-depth sequencing could enable us to identify predictive markers. This technique has already extensively been used to study IBC, which dramatically increased our knowledge regarding its mutational landscape and subclonal evolution. However, in-depth analysis of DCIS by NGS is still relatively sparse since several limitations were encountered such as insufficient input material and lack of fresh frozen tissue. In the last few years, innovative improvement increased the possibility to perform certain massive parallel sequence techniques (i.e. whole exome sequencing) on paraffin-embedded tissue blocks. In this thesis, we reported preliminary data of intra-tumor heterogeneity of synchronous DCIS and IBC; specific genetic aberrations are restricted to the invasive component, which might be responsible for progression to invasive cancer. However, we are still in the beginning of this process, which will ultimately lead towards a more individual treatment for patients with DCIS.

There is a strong need for a risk stratification tool that distinguishes indolent DCIS from potentially hazardous DCIS. To develop and validate such a predictive tool, a strong collaborative approach between medical centers is necessary to obtain large cohorts of patients (including clinical, radiological and pathological data). Besides, it requires a multidisciplinary approach, including i.e. clinicians, pathologists, molecular biologists and bio-informaticians. Current histopathological grading of DCIS based on nuclear atypia is suboptimal due to inter-observer variability, resulting in suboptimal reproducibility. Since DCIS is a heterogeneous lesion and the full molecular portrait is not yet known, there should also be an emphasis on its molecular characterization, including the DCIS-associated microenvironment. For this matter, it is necessary to perform molecular assays on tissue blocks with sufficient DCIS and a high purity of DCIS and surrounding cells. This could contribute to an accurate interpretation of molecular data in which a full molecular portrait of DCIS can be obtained, which could be related to clinical outcome such as progression to IBC or DCIS recurrence. Ultimately, in the future, specific genetic signatures could be used to develop a targeted inhibition therapy contributing to the prevention of progression to IBC. Currently, prospective trials are ongoing in which patients with low and intermediate grade DCIS are subjected to active surveillance. Although this could provide important information regarding the biological behavior of low-grade DCIS based on histopathologic grading, there is a large need to have more robust and reproducible progression markers.

Collaborative efforts should lead to a multidisciplinary predictive stratification tool in which clinical and pathological parameters are integrated with molecular approaches. The clinical implementation of this algorithm could contribute to the selection of patients who can benefit from less invasive treatment.

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CHAPTER

10

Samenvatting
PhD Portfolio
Dankwoord
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List of Publications

SAMENVATTING

Dit proefschrift richt zich op klinische en moleculaire kenmerken van DCIS en beschrijft potentiele markers voor progressie die kunnen bijdragen aan de ontwikkeling van een meer gerichte behandelstrategie voor patiënten met DCIS.

Hoofdstuk 1 omvat een introductie over histologische, moleculaire en klinische karakteristieken van DCIS, inclusief de inhoudsopgave van dit proefschrift. **Hoofdstuk 2** richt zich op het vinden van nieuwe markers die de aanwezigheid van invasief borstkanker kunnen voorspellen in het mamma resectiepreparaat bij een preoperatieve naaldbiopt diagnose van puur DCIS. Dit kan in deze groep patiënten namelijk bijdragen aan een betere selectie voor lymfklierstagering. De huidige nationale richtlijn beschrijft verschillende risicofactoren voor opwaardering naar invasief borstkanker (palpabele laesie, jonge leeftijd (< 55 jaar), versterkte opname van signaal op MRI, hooggradig DCIS en een diameter > 25 mm).[1] Echter, in de dagelijkse praktijk is de toepassing van deze risicofactoren suboptimaal. Dit resulteert in een inadequate voorspelling van borstkanker, hetgeen leidt tot een overbodige schildwachtklier procedure bij patiënten met puur DCIS. Tegelijktijdig worden patiënten met een preoperatieve diagnose van puur DCIS, waarbij na de operatie toch een invasieve component gevonden wordt, onderworpen aan secundaire chirurgie om alsnog de lymfklieren te stageren. In dit hoofdstuk beschrijven we verschillende karakteristieken van zowel DCIS cellen als peri-ductale omgevingsfactoren in naaldbiopten met de diagnose van puur DCIS. In deze studie had 22% van de patiënten met een preoperatieve naaldbiopt diagnose van DCIS uiteindelijk toch invasief borstkanker in het resectiepreparaat. Verschillende risicofactoren waren significant geassocieerd met invasief borstkanker: palpabele laesie, jonge leeftijd (< 40 jaar), massa op beeldvorming, peri-ductale veranderingen zoals inflammatie en verlies van Decorine expressie. Het risico voor invasief borstkanker na chirurgie correleerde met het aantal aanwezige risicofactoren: 9% voor patiënten zonder risicofactoren, 29% voor patiënten met 1 risicofactor, 37% voor patiënten met 2 risicofactoren en 54% voor patiënten met ≥3 risicofactoren. Deze risicofactoren kunnen dus bijdragen aan een betere preoperatieve selectie van patiënten met een verhoogd risico voor invasief borstkanker, waarbij lymfklierstagering overwogen kan worden gedurende initiële chirurgie. Dit in tegenstelling tot patiënten met een laag risico voor invasief borstkanker waarbij primaire lymfklierstagering juist achterwege gelaten kan worden bij borst sparende chirurgie.

In **hoofdstuk 3** hebben we de uitgebreidheid van de DCIS component onderzocht bij verschillende borstkanker subtypes in een groot nationaal cohort, gebruik makend van een retrospectief landelijk cohort van patiënten die zijn geopereerd i.v.m. borstkanker. Er waren significante verschillen tussen de DCIS karakteristieken binnen de borstkanker subtypes. HER2 positieve borstkanker was vaker geassocieerd met een aanwezige DCIS component en deze DCIS component had een grotere omvang vergeleken met de overige borstkanker subtypes. Deze subgroep toonde ook een hoger percentage van een irradicaal gereseceerde DCIS component in vergelijking met HER2 negatieve subtypes. Deze bevindingen zijn consistent met voorgaande studies die een hoger lokaal recidief percentage rapporteerden na borst sparende chirurgie bij patiënten met een HER2 positieve borstkanker, waar een uitgebreide DCIS component de oorzaak zou kunnen zijn.[2-4] Preoperatieve kennis omtrent de uitgebreidheid van de DCIS component bij verschillende types invasief borstkanker kan clinici ondersteunen bij de optimalisatie van lokale behandeling. In **hoofdstuk 4** wordt het biologische gedrag van DCIS verder onderzocht door gebruik te maken van genexpressie profielen. Onze hypothese was dat uitgebreide DCIS zonder invasie een indolente laesie vertegenwoordigt en waarschijnlijk minder onderliggende moleculaire afwijkingen heeft vergeleken met kleine DCIS laesies aangrenzend aan invasief borstkanker, dat een hoge potentie heeft op invasieve groei en waarschijnlijk meer onderliggende moleculaire afwijkingen. Deze hypothese werd ondersteund door onze genexpressie analyse waarbij significante verschillen werden gevonden tussen patiënten met een uitgebreide DCIS (zonder invasie) versus patiënten met een kleine DCIS component naast invasief borstkanker. De meeste van deze genen toonden een hogere expressie in DCIS aangrenzend aan invasief borstkanker. Deze genen zijn betrokken bij diverse biologische processen als signaal transductie, chemotaxie, angiogenese, cellulaire reactie op epidermale groei factor stimulus, positieve regulatie van extracellulaire signaal gereguleerde kinase 1 en 2 cascade, respons op hyperoxie en cellulaire respons op aminozuur stimulus. Op basis van de geïdentificeerde genen was het mogelijk een genexpressie patroon te genereren die puur DCIS laesies onderscheidt van DCIS met invasief borstkanker. Onze resultaten kwamen overeen met eerdere moleculaire studies (gen copy number en whole exome sequence data) welke verschillen rapporteerden tussen puur DCIS en synchroon DCIS met invasief borstkanker.[10,27] Echter, validatie van onze studie resultaten is nodig om te verifiëren of de geïdentificeerde genen geschikt zijn voor predictie op progressie naar invasief borstkanker in individuele patiënten met DCIS. Dit zou voor deze patiënten kunnen bijdragen aan verbetering van een meer geïndividualiseerde

behandelstrategie, waar een gerichte inhibitie van de geïdentificeerde genen in een vroeg stadium van borstkanker zou kunnen plaatsvinden, hetgeen uiteindelijk resulteert in preventie van progressie. In **hoofdstuk 5** onderzochten we verschillen tussen DCIS en synchroon borstkanker aangaande APOBEC3B genexpressie. Deze levels werden ook gecorreleerd met PIK3CA mutatie status. Verhoogde APOBEC3B expressie speelt een belangrijke rol bij verschillende soorten kanker, waar het is geassocieerd met proliferatie, inclusief PIK3CA en TP53 mutaties.[5] Dit enzym is niet-essentieel (komt niet tot expressie in normaal weefsel), waardoor het mogelijk is om verhoogde APOBEC3B levels te remmen met kleine moleculen. In onze analyse detecteerden we verhoogde APOBEC3B mRNA levels in zowel DCIS als synchroon invasief borstkanker. Er werden geen verschillen gemeten in APOBEC3B levels tussen wild-type versus gemuteerde PIK3CA DCIS. Dit in tegenstelling tot de aangrenzende invasieve component waar APOBEC3B hoger tot expressie kwam in wild-type PIK3CA in vergelijking met gemuteerde PIK3CA. Dit impliceert dat, wanneer het invasieve stadium is bereikt, PIK3CA mogelijk een minder belangrijke rol speelt bij tumorprogressie. Hogere APOBEC3B levels werden gemeten in synchroon DCIS en invasief borstkanker met een ER negatieve receptorstatus vergeleken met ER positieve tumoren. Deze bevinding werd ook beschreven in eerdere studies waar verhoogde APOBEC3B levels in borstkanker zijn geassocieerd met agressieve kenmerken.[6,7-9] De bevindingen beschreven in dit hoofdstuk suggereren dat APOBEC3B als een potentieel therapeutisch doelwit kan fungeren in patiënten met DCIS, echter dergelijke enzymatische therapeutische interventie is nog steeds in ontwikkeling. Additioneel onderzoek is nodig voor verdere ontwikkeling en exploratie van het potentiele voordeel van APOBEC3B blokkers.

Hoofdstuk 6 beschrijft de resultaten van diepgaande sequentie analyse in synchroon DCIS en invasief borstkanker met als doel antwoorden te vinden naar het onderliggende mechanisme van DCIS progressie. Onze analyse toonde overlappende mutaties tussen synchroon DCIS en invasief borstkanker, hetgeen ook eerder werd gerapporteerd door voorgaande studies.[10,11] Echter, een deel van de geïdentificeerde mutaties werd alleen in de invasieve component gedetecteerd, hetgeen impliceert dat DCIS progressie wordt gedreven door een selectie van subklonen.[10,12] In andere woorden, de evolutie van DCIS zou gebaseerd kunnen zijn op het Darwiniaanse principe; bepaalde subklonen met specifieke genetische veranderingen worden geselecteerd gedurende invasieve groei. Dit resulteert in verschillen in prevalentie van specifieke mutaties tussen de neoplastische DCIS cellen en de aangrenzende

invasieve component. Deze kennis zou toekomstige studies kunnen faciliteren in het identificeren van potentiele markers die een belangrijke rol spelen in de transitie van DCIS naar invasief borstkanker.

In **hoofdstuk 7** evalueerden we de aanwezigheid, karakteristieken en prognostische relevantie van voorloper laesies aangrenzend aan invasief borstkanker bij mannen in een groot internationaal cohort, aangevuld met moleculair onderzoek. In dit cohort van mannen met invasief borstkanker werd bij een aanzienlijk deel van de patiënten een aangrenzende DCIS component aangetroffen (46% van de casus), terwijl de frequentie van andere voorloper laesies (lobulair carcinoom in situ, atypische ductale hyperplasie en cilinder cel laesies) erg laag was (< 1%). Er was een sterke positieve correlatie tussen DCIS en de naastgelegen invasieve component voor kerngradering ($p = <0.001$) en een hoge concordantie van ER, PR en HER2 receptorstatus. Deze sterk overeenkomende kenmerken tussen synchroon DCIS en invasief borstkanker bij mannen, hetgeen ook uitvoerig is beschreven bij vrouwen, impliceert dat DCIS inderdaad een voorloper laesie is van borstkanker bij mannen. Dit werd verder ondersteund door onze moleculaire analyse in een deel van de casus (n=4), waarbij in beide componenten identieke mutaties werden gedetecteerd (PIK3CA, GATA3, TP53 en MAP2K4). In de literatuur is het bestaan van cilinder cel laesies in de mannelijke borst, zoals beschreven bij vrouwen, controversieel. Bij mannen ontbreken specifieke morfologische criteria om cilinder cel laesies te definiëren, waardoor het herkennen ervan lastig is. In deze grote serie werden echter ducti herkend met overlappende kenmerken met cilinder cel laesies zoals beschreven bij vrouwen. NGS toonde identieke genetische afwijkingen (inclusief PIK3CA en GATA3 mutaties) tussen de cilinder cel laesie en de aangrenzende invasieve component. Deze overeenkomsten op zowel morfologisch als genomisch niveau suggereren een mogelijk causaal verband tussen deze laesies en ondersteunen de rol van cilinder cel laesies als een voorloper laesie in borstkanker bij mannen. Onze analyse betreffende de associatie tussen de aanwezigheid van DCIS en prognose toonde dat patiënten met luminaal A en luminaal B HER2+ type borstkanker met een aangrenzende DCIS component een betere overleving hadden dan patiënten zonder DCIS component. Dit suggereert dat invasief borstkanker met een aangrenzende DCIS component bij deze subtypes mogelijk een biologisch minder agressieve vorm van borstkanker betreft. In **hoofdstuk 8** werd een selectie van mannen met borstkanker geëvalueerd waarvan het cohort deels overlapt met het cohort beschreven in het vorige hoofdstuk. Voor deze analyse werden invasief borstkanker, DCIS aangrenzend aan invasief borstkanker en puur DCIS onderzocht

op DNA copy number veranderingen van 22 borstkanker-gerelateerde genen met behulp van multiplex ligatie-afhankelijke probe amplificatie (MLPA). Deze resultaten werden vergeleken met een voorgaande analyse van copy nummer veranderingen bij vrouwen met borstkanker. Verder werden de copy number afwijkingen gecorreleerd met clinicopathologische kenmerken en 10-jaars overleving data. Deze analyse toonde een concordante copy nummer status van 21 van de 22 genen tussen invasief borstkanker, synchroon DCIS aangrenzend aan invasief borstkanker en puur DCIS. Deze bevinding ondersteunt de klonale relatie tussen mannelijke borstkanker en aangrenzende DCIS. Verder benadrukt het de genetisch gevorderde status van DCIS; copy number veranderingen zijn een vroeg event in de carcinogenese van de borst bij mannen. Echter, 1 gen, MTHD, betrokken bij de activatie van verschillende signaal cascades inclusief PI3K/Akt, NFκβ, Wnt/βcatenin en de MAPK cascade,[13] demonstreerde een hogere copy number ratio in de invasieve component vergeleken met de synchrone DCIS component. In de literatuur wordt dit gen ook beschreven bij vrouwelijke borstkanker waar het is geassocieerd met een agressief gedrag,[14] hetgeen suggereert dat dit gen een belangrijke rol zou kunnen spelen bij de carcinogenese van borstkanker bij mannen. Verder toonden enkele genen een hogere frequentie van copy nummer afwijkingen in hooggradige laesies vergeleken met laaggradige laesies (BIRC5, ESR1 en C11ORF30). In totaal waren 2 van de 22 onderzochte genen, CCNE1 en CPD gain, geassocieerd met een verlaagde 10-jaars overleving. Echter, het aantal casus met deze genetische afwijkingen was klein dus voorzichtigheid is geboden bij de interpretatie van deze data. De resultaten van hoofdstuk 7 en 8 dragen bij aan een betere kennis van vroegtijdige events bij borstkanker bij mannen.

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PhD PORTFOLIO

Name PhD student: S. C. Doebar
Department: Pathology
Promotor: prof.dr. F.J. van Kemenade
Copromotor: dr. C.H.M. van Deurzen

1. PhD training

General courses	Year
Biomedical English Writing Course for PhD-students , Rotterdam	2014
The workshop on Photoshop and Illustrator CS6, Rotterdam	2014
Advanced Excel Workshop, Rotterdam	2016
Endnote course, Rotterdam	2016
Course on Scientific Integrity, Rotterdam	2019

Specific courses	
Course on Genome sequencing in Medical Diagnostics, Postgraduate School, Rotterdam	2014

Seminars and workshops	
Mini Symposium Sequencing Services: A Beauty Contest, Erasmus Medical centre, Rotterdam	2014
Weekly research meetings, Erasmus Medical Centre, Rotterdam	2015-2016
Weekly Josephine Nefkens Institute Oncology meetings, Erasmus Medical Centre, Rotterdam	2014-2017
Medical Oncology research meetings, EMC, Rotterdam, Netherlands	2016
Medical Business Master Class, Medical business Education School Amsterdam	2017
Course on Communication, Desiderius School, Erasmus University, Rotterdam	2017
Teach the Teacher, Desiderius School, Erasmus University, Rotterdam	2017
Interactive mamma course, Utrecht Medical University, Utrecht	2018
Course on Patient Safety, Netherlands Cancer Institute, Amsterdam	2018
Seminar Radiology MRI, Netherlands Cancer Institute, Amsterdam	2018
General Education Course Brainsnack, Netherlands Cancer Institute, Amsterdam	2018
Weekly research meetings Wesseling research group, Netherlands Cancer Institute Amsterdam	2018
Teaching on the run, Pathology Expert Centre Symbiant, Alkmaar	2019

(Inter)national conferences	Year
18th Molecular Medicine day, Postgraduate School, Rotterdam, Netherlands	2014
Breast cancer research meeting, Erasmus Medical Centre, Rotterdam, Netherlands (oral presentation)	2014
Symposium Borstkanker Behandeling Beter, Erasmus Medical Centre, Rotterdam, Netherlands (oral presentation)	2014
National pathology conference, Zeist, Netherlands	2015
Scientific day, Erasmus Medical Centre, Rotterdam, Netherlands	2015
Kick Off Symposium Daniel den Hoed Core Facilities, Erasmus Medical Centre, Rotterdam, Netherlands	2015
European Breast Cancer Conference (EBCC), Amsterdam RAI, Amsterdam, Netherlands (Poster presentation)	2016
National Pathology Conference, Zeist, Netherlands (Oral poster pitch presentation)	2016
San Antonio Breast Cancer Conference, San Antonio, United States (Poster presentation)	2016
Genitourinary and Gynaecologic conference, Florence, Italy	2019
European Pathology conference, Nice, France	2019

2. Teaching

Lecturing	Year
Research meeting, Erasmus Medical Centre, Rotterdam, Netherlands (oral presentation)	2014
Daniel den Hoed Oncology research Meeting, Erasmus Medical Centre, Rotterdam, Netherlands (oral presentation)	2016
Research meeting, Erasmus Medical Centre, Rotterdam, Netherlands (oral presentation)	2016
General Pathology meetings, Reinier de Graaf Gasthuis, Delft, Netherlands (oral presentations)	2018
General Pathology meeting, Netherlands Cancer Institute, Amsterdam, Netherlands (oral presentation)	2018

Supervising practicals and excursions, Tutoring	
Practical Skills Educational sessions, Erasmus Medical Centre, Rotterdam, Netherlands	2014-2018
Practical Gynaecology macroscopy, Pathology Expert Centre Symbiant, Alkmaar, Netherlands	2019
Supervising pathology residents and medical interns	2018- 2019

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CURRICULUM VITAE

Charlane Doebar werd op 1 februari 1986 geboren in ’s-Gravenhage. Ze deed haar middelbare school aan het Erasmus College te Zoetermeer, waar ze in 2004 afstudeerde voor haar VWO. Aansluitend begon zij met de studie geneeskunde aan de Erasmus Universiteit van Rotterdam. Zij behaalde haar artsenexamen in 2010. Het jaar hierop begon zij in 2011 met haar opleiding pathologie in het Leids Universitair Medisch Centrum in Leiden. Na 1 jaar zette zij de opleiding voort in het Erasmus Medisch Centrum te Rotterdam. Tijdens de opleiding pathologie werkte zij aan het onderzoek beschreven in dit proefschrift onder begeleiding van dr. C.H.M. van Deurzen en prof.dr. F. van Kemenade. In 2014 heeft zij haar opleiding gedurende 2,5 jaar onderbroken om fulltime aan dit onderzoek te werken. In augustus 2018 heeft ze de opleiding Pathologie afgerond en sindsdien is zij werkzaam als patholoog.

LIST OF PUBLICATIONS

Doebar SC, de Monyé C, Stoop H, Rothbarth J, Willemsen SP, van Deurzen CH. Ductal carcinoma in situ diagnosed by breast needle biopsy: Predictors of Invasion in the Excision Specimen. Breast 2016;27:15-21.

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Doebar SC, Slaets L, Cardoso F, Giordano SH, Bartlett JM, Tryfonidis K, Dijkstra NH, Schröder CP, van Asperen CJ, Linderholm B, Benstead K, Dinjens WN, van Marion R, van Diest PJ, Martens JW, van Deurzen CH. Male breast cancer precursor lesions: analysis of the EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program. Mod Pathol 2017; 30:509-518.

Vermeulen MA, **Doebar SC**, van Deurzen CHM, Martens JWM, van Diest PJ, Moelans CB. Copy number profiling of oncogenes in ductal carcinoma in situ of the male breast. Endocr Relat Cancer 2018; 25:173-184.

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Vermeulen MA, van Deurzen C, **Doebar SC**, de Leng W, Martens J, van Diest PJ, Moelans CB. Promoter hypermethylation in ductal carcinoma in situ of the male breast. Endocr Relat Cancer 2019; 26: 575-584.

Agahozo MC, Sieuwerts AM, **Doebar SC**, Verhoef E, Beaufort CM, Ruigrok-Ritstier K, de Weerd V, Sleddens HFBM, Dinjens WN, Martens J, van Deurzen C. PIK3CA mutations in ductal carcinoma in situ and adjacent invasive breast cancer. Endocr Relat Cancer 2019; 2: 471-482.

STELLINGEN

behorende bij proefschrift

DUCTAL CARCINOMA IN SITU: THE JOURNEY TO INVASIVE BREAST CANCER

1. DCIS is een heterogene ziekte

2. Synchroon DCIS en invasief borstkanker hebben veel moleculaire overeen-komsten, maar zijn niet identiek

3. Individuele risicoschatting zou een geïntegreerd onderdeel moeten zijn van het behandeltraject van patiënten met DCIS

4. DCIS bij mannen is anders dan DCIS bij vrouwen

5. Onzekerheid leidt tot overbehandeling

6. An ounce of prevention is worth a pound of cure - *Benjamin Franklin*

7. The more you know, the less you need - *Yvon Chouinard*

8. You are unique, and if that is not fulfilled, then something has been lost - *Martha Graham*

9. He will manage the cure best who has foreseen what is to happen from the present state of matters - *Hippocrates*

10. You can’t make decisions based on fear and the possibility of what might happen - *Michelle Obama*

11. Een ziekte spreekt voor ieder zijn eigen taal