Ductal carcinoma in situ of the breast : cancer precursor or not?

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CHAPTER 5

Discordant marker expression between invasive breast carcinoma and corresponding synchronous and preceding DCIS

Abstract

Ductal carcinoma in situ (DCIS) is considered a potential precursor of invasive breast carcinoma (IBC). Studies aiming to find markers involved in DCIS progression generally have compared characteristics of IBC lesions with those of adjacent *synchronous* DCIS lesions. The question remains whether *synchronous* DCIS and IBC comparisons are a good surrogate for *primary* DCIS and *subsequent* IBC. In this study, we compared both primary DCIS and synchronous DCIS with the associated IBC lesion, based on immunohistochemical marker expression. Immunohistochemical analysis of ER, PR, HER2, p53, and cyclo-oxygenase 2 (COX-2) was performed for 143 primary DCIS and subsequent IBC lesions, including 81 IBC lesions with synchronous DCIS. Agreement between DCIS and IBC were assessed using kappa, and symmetry tests were performed to assess the pattern in marker conversion. The primary DCIS and subsequent IBC more often showed discordant marker expression than synchronous DCIS and IBC. Strikingly, 18 out of 49 (36%) women with HER2-positive primary DCIS developed a HER2-negative IBC. Such a difference in HER2 expression was not observed when comparing synchronous DCIS and IBC. The frequency of discordant marker expression did not increase with longer time between primary DCIS and IBC. In conclusion, comparison of primary DCIS and subsequent IBC yields different results than comparison of synchronous DCIS and IBC, in particular regarding HER2 status. To gain more insight into the progression of DCIS to IBC, it is essential to focus on the relationship between *primary* DCIS and *subsequent* IBC, rather than comparing IBC with *synchronous* DCIS.

Keywords: Ductal carcinoma in situ; invasive breast carcinoma; local recurrence; synchronous lesions; immunohistochemistry

Introduction

Ductal carcinoma in situ (DCIS) is generally accepted as a non-obligate precursor of invasive breast carcinoma (IBC).[1] This because they are frequently found next to each other sharing the genetic alterations as well as risk factors (e.g. age, family history of breast carcinoma, etc).[2–6] While DCIS itself is not life-threatening, it does increase a woman's risk of developing IBC later in life, which subsequently could lead to breast cancer-specific death.[7] To prevent progression to invasive disease, almost all DCIS lesions are treated by mastectomy or breast conserving surgery with or without adjuvant radiotherapy and/or endocrine therapy.

If it holds true that DCIS directly progresses to IBC, one would expect that primary DCIS and subsequent ipsilateral IBC share multiple features, for example hormone receptor and HER2 status. It has been shown that the histological grade of the DCIS component adjacent to invasive disease (*synchronous* DCIS) and the grade of the IBC lesion are significantly correlated, i.e. well differentiated DCIS relates to grade I IBC and poorly differentiated DCIS to grade III IBC.[8,9] Therefore, it is thought that, if progression occurs, well differentiated DCIS will give rise to grade I IBC and poorly differentiated DCIS to grade III IBC. Nonetheless, comparison of marker expression shows conflicting results. Allred, et al. found that HER2 overexpression is more frequently observed in DCIS than in IBC.[10] Multiple studies have tried to address this, mostly by comparing synchronous DCIS and IBC.[3,11–13] Many of these studies showed that synchronous DCIS and IBC components were, however, very similar on the quantitative level and discordant HER2 status was rarely observed.

Several studies aimed to find markers involved in DCIS progression to IBC, mostly by comparing IBC lesions and an adjacent DCIS component, referred to as *synchronous* DCIS.[14] However, to our knowledge, it has never been investigated whether the *synchronous* DCIS and IBC comparisons are a good surrogate for primary DCIS and *subsequent* IBC. Therefore, we performed a comparative analysis between primary DCIS and subsequent ipsilateral IBC, and between this IBC and the adjacent synchronous DCIS component based on immunohistochemical marker expression (Figure 1). With this, we aimed to (1) assess the concordance in marker expression between primary DCIS and subsequent ipsilateral IBC, and IBC and synchronous DCIS and (2) to identify factors that may explain the potential discordance in marker expression.

Patients and Methods

Study population and design

The source population was derived from the Netherlands Cancer Registry (NCR), linked to the nation-wide network and registry of histology and cytopathology in the Netherlands (PALGA). It included all women diagnosed with primary DCIS within the Netherlands and

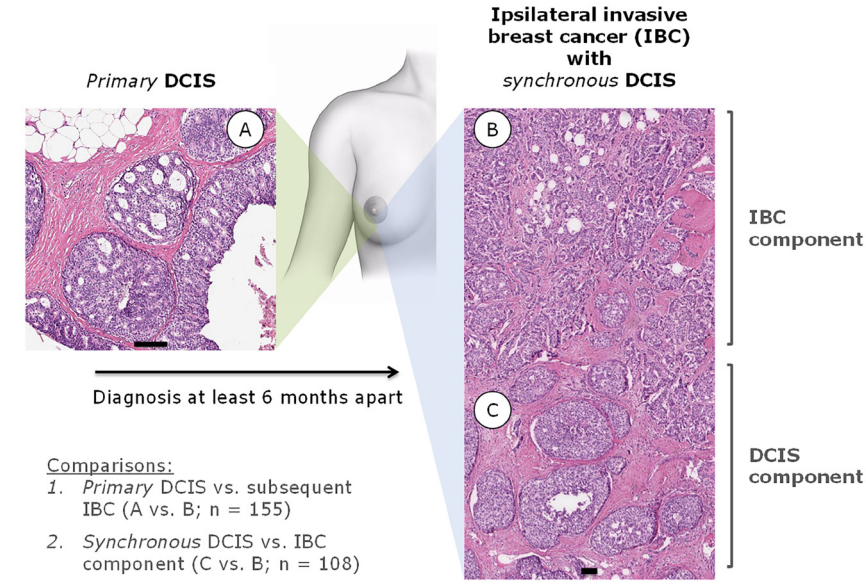

Figure 1. Set-up of the study and the number of included lesions. Out of 155, there were 108 IBC lesions that harbored a synchronous DCIS component.

treated with breast conserving surgery alone between January 1, 1989 to December 31, 2004 (n=2,658).[7] The NCR provided information on age at diagnosis, date of diagnosis, treatment of DCIS, and the development of subsequent IBC. Follow-up and vital status were complete until at least January 1, 2011. The median follow-up was 12.0 years (interquartile range, 9.0-15.3). In total, 374 of 2,658 women developed ipsilateral IBC as first invasive carcinoma after a primary diagnosis of DCIS.[7] At the tissue block collection for this current study, the first 328 women with a subsequent iIBC had been identified.

Formalin-fixed paraffin-embedded (FFPE) tissue samples from both the primary DCIS and the corresponding subsequent IBC were requested from 58 pathology laboratories within the Netherlands. All specimens were histopathologic re-examined by a team of consultant breast pathologists (JW, EJG, KvdV), using new hematoxylin and eosin-stained whole slides. Slides were assessed for histological grade: DCIS was graded according to the World Health Organization criteria (WHO; 2012).[15] IBC was graded according to Elston/Nottingham modification of the Bloom-Richardson system, based on tumor tubule formation, number of mitotic figures, and nuclear polymorphism.[16] We were unable to collect tissue blocks from some hospitals, either because the hospital refused to provide tissue for research or because tissue blocks were unavailable (n=61 pairs excluded). Women for whom DCIS and/or IBC diagnoses could not be confirmed by pathology were excluded from this study (n=59), as

were DCIS including a (micro)invasive component or LCIS (lobular carcinoma in situ; n=53). This resulted in 155 patient-matched primary DCIS and subsequent IBC pairs.

The study was approved by the review board on the NKR (request K12.281; January 3, 2013) and PALGA (LZV990; April 16, 2013). Secondary use of tissue and data for this study was done under an opt-out regime which is conform Dutch regulations and the Code of Conduct of Federa-COREON.

Immunohistochemistry

Both primary DCIS and the corresponding invasive breast carcinoma were immunohistochemically stained for estrogen receptor (ER; SP1, ready-to-use, Ventana Medical Systems) and progesterone receptor (PR; 1E2, ready-to-use, Ventana Medical Systems) status, overexpression of HER2 (4B5, ready-to-use, Ventana Medical Systems), Ki67 (MIB1, 1/250, DAKO), and expression of tumor suppressor protein p53 (DO-7, 1/7000, DAKO), and COX-2 (CX294, 1/100, DAKO), using a Benchmark ULTRA autostainer (Ventana Meducan Systems, AZ, USA), using 3-µm thick whole slides. Some DCIS tissue specimens were excluded because insufficient tissue was available for immunohistochemistry (IHC; n=13). Details on the IHC staining procedure can be found elsewhere.[17] Positive and negative controls were included in all staining runs.

IHC assessment was performed by a team of 7 observers, including 5 pathologists. Assessment of inter-observer agreement is described elsewhere.[17] For ER, PR, HER2, Ki67 and p53 a concordance was reached of 97% with an interclass correlation coefficient (ICC) of >0.8. Ki67 was excluded for further scoring because of unreliable staining results. COX-2 reached a concordance of 94% with a *k* statistic of 0.7. ER and PR status were considered positive when ≥10% of the luminal epithelial cells showed nuclear staining, based on scoring guidelines by FCCC pathologists from 2007-2011. Similarly, p53-positive staining was assessed based on the percentage of cells that showed moderate to strong nuclear staining. The presence of >70% positive cells or complete lack of p53 expression was considered as mutant p53 expression, and 1-70% positive cells was considered wild-type (WT) p53 expression.[18,19] HER2 overexpression was assessed according to the American Society of Clinical Oncology and College of American Pathologist (ASCO-CAP) 2013 recommendations.[20] A membrane score of 3+ was considered as HER2 positive, as was a membrane score of 2+ for which the overexpression could be confirmed by HER2 CISH (chromogenic in situ hybridization). A membrane score of 0 and 1+ was considered HER2 negative. COX-2 expression was evaluated according to criteria adapted from Ristimäki et al: 1= weak diffuse cytoplasmic staining that may contain moderate to strong granular cytoplasmic staining in less than 10% of tumor cells; 2= moderate to strong granular cytoplasmic staining in 10-90% of the tumor cells; 3= moderate to strong granular cytoplasmic staining in over 90% of the tumor cells. Score 1 was considered low COX-2 expression and score 2 and 3 were considered as high expression of COX-2.[21]

DCIS and IBC lesions were categorized into the following IHC subtypes: hormone receptor (HR)+ HER2-, HR+ HER2+, HR- HER2+, and HR- HER2-. Lesions were classified as HR+ when ER and/or PR was scored as positive. Lesions were classified as HR- when ER and PR both were negative.

To assess intra-lesional heterogeneity within our study cohort, we randomly selected 10 DCIS ducts within one primary DCIS lesion and independently scored the IHC markers for these ducts. Subsequently, we used the ER, PR, and HER2 status to classify the DCIS duct into the IHC subtypes. When there were different IHC subtypes present within a single DCIS lesions, we considered these DCIS lesions heterogeneous, and the DCIS ducts with different IHC subtypes were considered separate DCIS subclones.

Statistical Analysis

Concordance of marker expression was determined by calculating the agreement between DCIS and IBC samples using kappa. In addition, symmetry tests were performed to assess whether there was a pattern in the marker conversion in case of discordant expression. For variables with two categories an asymptotic symmetry test was performed. When a variable was composed of more than two categories then a marginal homogeneity test was used. Chi-square tests were performed to compare time to event and the frequency of discordant marker expression. Time to event was defined as a categorical variable i.e. the study group was divided into two groups at the median time to ipsilateral IBC diagnosis: ≤6.1 vs. >6.1 years. These two groups were compared with regard to the frequency of marker expression concordance. All statistical analyses were performed using Stata/SE (version 13.1, StataCorp). *P* values ≤0.05 were considered statistically significant.

Results

Baseline characteristics

155 DCIS patients with a subsequent ipsilateral IBC were included in this study, which is a good representation of all 328 patients that developed an invasive recurrence after primary DCIS within our cohort (Supplementary Table S1). 108 out of these 155 lesions (69.7%) had a DCIS component adjacent to the invasive disease (synchronous DCIS). The mean time to invasive recurrence was 6.3 years (range 0.5 to 17.0 years). 79.4% of the invasive recurrences occurred in the same quadrant as the initial DCIS (77 of 97; 58 pairs location not specified; Supplementary Table S2).

The primary DCIS was well differentiated in 28 lesions (18.1%), intermediately differentiated in 94 lesions (60.6%) and poorly differentiated in 33 lesions (21.3%). Of the subsequent invasive breast carcinomas, 131 (84.5%) were invasive carcinoma of no special type, 10 (6.5%) were invasive lobular carcinoma and 14 (9.0%) were mixed.

Immunohistochemical staining was performed for 142 out of 155 primary DCIS and

subsequent IBC pairs and 81 out of 108 IBC and synchronous DCIS pairs. The frequency of ER, PR, and COX-2 positivity was similar in primary DCIS, IBC, and synchronous DCIS (Table 1). HER2 was overexpressed in 34.5% of primary DCIS, 26.1% of IBC, and 24.7% of synchronous DCIS. In line with previous literature, low-grade lesions (both DCIS and IBC) were associated with ER and PR positivity and low expression of HER2, p53, and COX-2.[22,23] High-grade lesions were often ER and PR negative, frequently overexpressed HER2 and p53, and had high expression of COX-2 (Supplementary Table S3).

Table 1. Overview of immunohistochemical marker expression of primary DCIS, invasive breast cancer, and synchronous DCIS

Characteristics	**Primary DCIS** (*n* = 142)		**Invasive BC** (*n* = 142)		**Synchronous DCIS** (*n* = 81)	
	n	(%)	*n*	(%)	*n*	(%)
IHC subtype						
HR+ HER2-	90	(63.4)	96	(67.6)	56	(69.1)
HR+ HER2+	28	(19.7)	23	(16.2)	8	(9.9)
HR- HER2+	21	(14.8)	14	(9.9)	12	(14.8)
HR- HER2-	3	(2.1)	9	(6.3)	5	(6.2)
ER						
Negative	24	(16.9)	23	(16.2)	17	(21.0)
Positive	118	(83.1)	119	(83.8)	63	(77.8)
N/A	0	(0.0)	0	(0.0)	1	(1.2)
PR						
Negative	51	(35.9)	58	(40.8)	31	(38.3)
Positive	90	(63.4)	84	(59.2)	50	(61.7)
N/A	1	(0.7)	0	(0.0)	0	(0.0)
HER2						
Negative	93	(65.5)	105	(73.9)	61	(75.3)
Positive	49	(34.5)	37	(26.1)	20	(24.7)
p53						
0% positive cells (mutant)	12	(8.5)	18	(12.7)	6	(7.4)
1-70% positive cells (WT)	108	(76.1)	101	(71.2)	65	(80.2)
>70% positive cells (mutant)	21	(14.8)	23	(16.2)	10	(12.4)
N/A	1	(0.7)	0	(0.0)	0	(0.0)
COX-2						
Low	16	(11.3)	17	(12.0)	13	(16.0)
High	125	(88.0)	124	(87.3)	67	(82.7)
N/A	1	(0.7)	1	(0.7)	1	(1.2)

*: HER2 positive group includes all synchronous DCIS lesions with membrane score 3+; WT: wild-type

Discordant marker expression is more frequently observed between primary DCIS and subsequent IBC as compared to synchronous DCIS and IBC

To assess the frequency of discordant marker expression, we compared IHC marker expression between primary DCIS and subsequent IBC pairs and synchronous IBC and DCIS pairs (Table 2 and 3). IBC and synchronous DCIS were discordant for ER, PR, HER2, p53, and COX-2 marker expression in 5.0%, 19.7%, 4.9%, 12.3%, and 12.5% of the pairs, respectively (Table 2 and 4). For all five markers, conversion was observed both from positive/high to negative/low as well as from negative/low to positive/high. Four IBC and synchronous DCIS pairs consisted of ER positive IBC and an ER negative synchronous DCIS component (p = 0.046; Table 2), although this number is too small to draw any conclusions from. 9.9% of the synchronous DCIS and IBC pairs did not share the same IHC subtype (Supplementary Table S4).

Marker expression of primary DCIS and the subsequent ipsilateral IBC were discordant for ER, PR, HER2, p53, and COX-2 expression in 12.0%, 27.7%, 16.9%, 22.7%, and 19.3% of the pairs, respectively (Table 3 and 4). Notably, 18 out of 49 patients (36%) had a HER2-positive primary DCIS which was followed by HER2-negative IBC (symmetry p=0.014; Table 3). This also caused a conversion of the IHC subtype in that 33 of the 142 primary DCIS and IBC pairs (23.2%) did not share the same IHC subtype (symmetry p=0.040; Supplementary Table S5), which was irrespective of the presence of synchronous DCIS adjacent to the IBC. The discordant marker expression rates between primary DCIS and synchronous DCIS were comparable to those of primary DCIS and subsequent IBC (Table 4; Supplementary Table S6-7).

Table 2. Marker expression of synchronous DCIS related to invasive component

		Invasive component (*n*)		**Agreement**	**Symmetry**
		Negative/ Low/WT	Positive/ High/Mutant	**(%)**	***P***
Synchronous DCIS (*n*)					
ER	Negative	13	4		
	Positive	0	63	95.0	0.046
PR	Negative	25	6		
	Positive	10	40	80.3	0.32
HER2	Negative	58	3		
	Positive	1	19	95.1	0.32
P53	WT	57	8		
	Mutant	2	14	87.7	0.06
COX-2	Low	7	6		
	High	4	63	87.5	0.53

Agreement was calculated by non-weighted kappa; *p*-values were calculated by asymptotic symmetry test. Total number of pairs included: ER n=80; PR n=81; HER2 n=142; p53 n=140; COX-2 n=140. ER/PR positive: >10% positive cells; HER2 positive: membrane score 3 or membrane score 2 confirmed by CISH; p53 wild-type (WT): 1-70% positive cells; p53 mutant: >70% positive cells or complete lack of p53 expression; COX-2 high: score 2-3.

Table 3. Immunohistochemical marker expression of primary DCIS related to subsequent invasive breast cancer

		Invasive breast cancer (*n*)		Agreement	Symmetry
		Negative/ Low/WT	Positive/ High/Mutant	(%)	*P*
Primary DCIS (*n*)					
ER	Negative	15	9		
	Positive	8	110	88.0	0.81
PR	Negative	35	16		
	Positive	23	67	72.3	0.26
HER2	Negative	87	6		
	Positive	18	31	83.1	0.014
P53	WT	88	29		
	Mutant	12	21	77.3	0.16
COX-2	Low	3	13		
	High	14	110	80.7	0.85

Agreement was calculated by non-weighted kappa; *p*-values were calculated by asymptotic symmetry test
Total number of pairs included: ER n=142; PR n=141; HER2 n=142; p53 n=140; COX-2 n=140. ER/ PR positive: >10% positive cells; HER2 positive: membrane score 3 or membrane score 2 confirmed by CISH; p53 wild-type (WT): 1-70% positive cells; p53 mutant: >70% positive cells or complete lack of p53 expression; COX-2 high: score 2-3.

Table 4. Percentage discordance in grade and marker expression between pairs of DCIS and IBC

	sDCIS vs. IBC		pDCIS vs. IBC		pDCIS vs. sDCIS	
	Discordance (%)	Symmetry *P*-value	Discordance (%)	Symmetry *P*	Discordance (%)	Symmetry *P*
IHC						
ER	5.0	0.046	12.0	0.81	13.7	0.76
PR	19.7	0.32	27.7	0.26	29.6	0.68
HER2	4.9	0.32	16.9	0.014	13.6	0.007
p53	12.3	0.06	22.7	0.16	22.5	0.64
COX-2	12.5	0.53	19.3	0.85	25.0	0.37
Subtype	9.9	0.09	23.2	0.040	16.0	0.17

pDCIS: primary DCIS; sDCIS: synchronous DCIS.

Discordance of marker expression is not associated with time to event

When comparing primary DCIS with a subsequent IBC, a time factor is obviously present, which is absent when comparing synchronous DCIS and adjacent IBC. Thus, we questioned whether time to event could play a role in the higher frequency of discordant marker expression between primary DCIS and subsequent IBC. For this, the study group was divided by the median time to IBC. While 56.9% of the women that developed IBC within 6.1 years after their DCIS diagnosis showed discordant marker expression between primary DCIS and subsequent IBC involving at least one IHC marker, this was 64.3% in the

group of women that developed IBC after more than 6.1 years after their DCIS diagnosis (p=0.37).

These data suggest that the probability of discordant marker expression between the primary DCIS and subsequent IBC does not increase with longer time to IBC.

Specific subclones might be responsible for the invasive outgrowth

From previous studies we know that this intra-lesional heterogeneity already exists at the DCIS stage.[24,25] Discordant marker expression could be caused by heterogeneity within the DCIS lesion. Therefore, we assessed IHC staining in ten 10 individual ducts per DCIS lesions. In 10 out of 94 DCIS lesions (10.6%) we observed heterogeneity, defined by the presence of multiple IHC subtypes, or subclones, within one DCIS lesion. We compared the IHC subtypes of the DCIS subclones, to the IHC subtype of the subsequent IBC lesion (Figure 2). In nine of the 10 pairs, the subtype of the IBC lesion was shared with a subclone of the DCIS lesion. In two of these DCIS lesions, four different IHC subtypes were present and seven DCIS lesions consisted of two different IHC subtypes. In one pair, the subtype of the IBC lesion was not shared with any of the DCIS subclones.

These results show that intra-lesional heterogeneity exists within DCIS lesions. This may be causative for the discordant marker expression between DCIS and IBC.

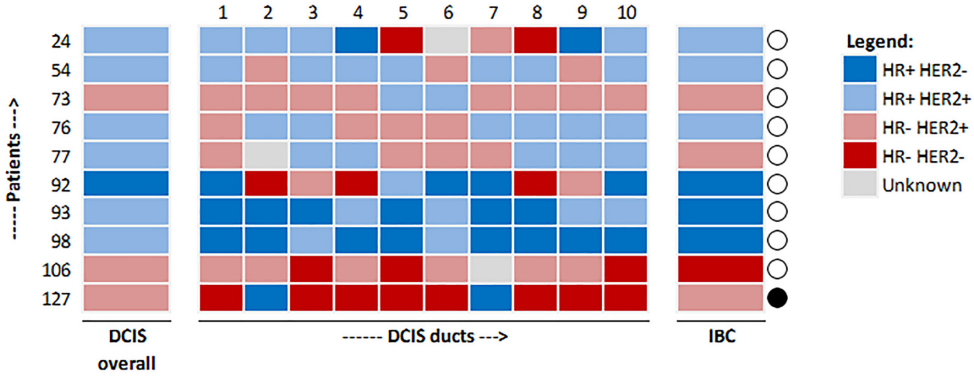

Figure 2. Matrix table of the IHC subtypes of 10 DCIS and subsequent IBC pairs in which intra-lesional heterogeneity was found in the DCIS lesion. For every DCIS lesion 10 individual DCIS ducts were assessed for IHC subtype using hormone receptor (HR) and HER2 status. On the left, columns are individual DCIS ducts (n=10); rows are individual patients. On the left, the overall IHC subtype of the DCIS lesion is shown. On the right, the IHC subtype of the corresponding IBC lesion is shown. Circle without fill indicates pairs of which the subtype of the IBC lesion was shared with a subclone of the DCIS lesion. Circle with black fill indicates a pair of which the subtype of the IBC lesion was not shared with any of the DCIS subclones.

Discussion

In this study, we demonstrated that comparative analysis between primary DCIS and subsequent ipsilateral invasive breast carcinoma (IBC) versus IBC and adjacent synchronous DCIS yields different results. This was most prominently illustrated for HER2, as we found that 36% of HER2-positive primary DCIS lesions were followed by HER2-negative IBC. Such a difference was not observed in our comparison between synchronous DCIS and IBC.

Our finding that HER2-negative IBC is preceded by HER2-positive DCIS is challenging our current understanding of the role of HER2 in the progression of DCIS to invasive breast carcinoma, as HER2 has been described as a predictor of recurrence after DCIS.[17,26,27] If the overexpression of HER2 plays a major role in DCIS progression, the overexpression of HER2 in IBC might be expected to be equal or exceeding the level of the preceding DCIS. This emphasizes that the role of HER2 in progression of DCIS to IBC remains to be elucidated. It could be hypothesized that HER2-overexpression promotes a higher proliferative rate, but does not lead a higher invasive potential of DCIS. Bijker, et al. and Karlsson, et al. performed the same comparison, but the number of matched DCIS and IBC pairs in these two studies were too small to notice the major findings presented here.[22,28]

Our group recently reported that high COX-2 expression was strongly associated with development of subsequent IBC.[17] In the current study, we showed that the level of COX-2 expression is almost similar when comparing primary DCIS and subsequent IBC. This may suggest that COX-2 could play a role in the invasive outgrowth of DCIS.

Overall, marker expression between *primary* DCIS and *subsequent* IBC was less concordant than *synchronous* DCIS and IBC. Yet, the frequency of discordant marker expression between primary DCIS and subsequent IBC did not increase with longer time to IBC. However, we found that DCIS intra-lesional heterogeneity exists in DCIS lesions, suggesting that discordant marker expression may be caused by heterogeneity. This makes it plausible that subsequent invasive disease arises from only one or just a few of these subclones. We were unable to assess multiple ducts of synchronous DCIS, as our intra-lesional heterogeneity analysis was based on lesions with at least 10 ducts. Yet, there is no reason to assume that our findings on heterogeneity do not apply to synchronous DCIS. Obviously, it is highly likely that synchronous DCIS has the potential to become invasive, as the DCIS is present in or in the close proximity of the IBC. This might imply that the DCIS subclone responsible for the invasive outgrowth is just a small minority subclone within this synchronous DCIS lesions or that it is even not existing anymore after the invasive outgrowth. As a consequence, one could argue that the comparative analyses of *synchronous* DCIS and IBC is biased and thus the preferred analysis is the comparison of *primary* DCIS and *subsequent* IBC.

In the current study, we used a 10% cut-off for ER and PR positivity, as established in the guidelines by FCCC pathologists. Using a 1% cut-off would have minimal impact on the

results of ER (concordance 10% cut-off, 88.1%; 1% cutoff, 86.1%; Supplementary Figure S1) and moderate impact on the results of PR (concordance 10% cut-off, 72.3%; 1% cutoff, 83.0%), but without changing our conclusion.

We intentionally refrained from the comparative analysis of grade between paired DCIS and IBC lesions. Previous histopathological studies of synchronous DCIS and IBC have shown a close link between the grade of the in situ and invasive component.[8,9] However, histopathological studies of primary DCIS and subsequent IBC have shown only moderate correlation between the grades of the subsequent invasive tumor and the original DCIS.[22,29] A likely explanation is that the criteria used for grading DCIS and IBC are different. For DCIS grading multiple classification methods are in use.[30–33] These grading systems are predominantly based on nuclear polymorphism, cell polarization, and also, in some, on the presence or absence of necrosis. None of the classification systems for DCIS is evidently the golden standard as they are all based on subjective criteria. In contrast, invasive breast carcinoma is graded according to a standardized classification system, i.e. the Elston and Ellis modification of Bloom-Richardson system, which is based on more objective features, i.e. tumor tubule formation, number of mitotic figures, and nuclear polymorphism.[15] Although in the current study we re-examined all DCIS lesions using a single DCIS grading system, the problem of the comparability of grading systems for DCIS and IBC still stands. Indeed, we and others, found a closer association was found between the nuclear polymorphism score for the subsequent invasive carcinoma and the original DCIS grade (Supplementary Table S8).[29]

Previous studies comparing receptor status between primary invasive breast carcinoma and the corresponding recurrence demonstrated discordances for ER, PR, and HER2 in a range of 10-37%, 24-48%, and 3-30%, respectively.[34–41] However, as almost all of these primary invasive breast carcinomas were treated by radiotherapy and/or chemotherapy, part of these discordances could be caused by treatment.[42] Furthermore, the difference in frequency of HER2 overexpression in DCIS and IBC was investigated by previous studies using synchronous DCIS and IBC.[3,11–13] In line with our study, none of these studies found a significant difference of HER2 overexpression between synchronous DCIS and IBC. Another explanation for this change in HER2 status is that the invasive component arose from a DCIS subclone that did not harbor HER2 amplification in the first place. The results from our IHC heterogeneity analysis at least suggest that this might be a valid option.

Our study has some limitations. First, our study group consisted of women that were all treated for DCIS by BCS alone. DCIS treated by BCS carries a risk of recurrent disease, but the origin of the subsequent IBC after primary DCIS could be: (1) from residual DCIS that was left behind after BCS, or (2) unrelated to the preceding DCIS, and thus be a second primary tumor. In the current study, the contribution of second primary tumors after treatment of DCIS is unknown and should be further elucidated by molecular analysis of the primary DCIS and subsequent IBC. Second, we cannot exclude the possibility of receptor

measurement error as the source of discordance in marker expression. Third, for the intra-lesional heterogeneity analysis, inclusion of more heterogeneously expressed IHC markers would be more informative when assessing heterogeneity within DCIS lesions, as now we only found 10 cases of heterogenous DCIS based on IHC subtype.

Our study has several strengths. First, we made use of a unique, large series of 155 patient-matched primary DCIS and subsequent IBC pairs, derived from a well-defined cohort of patients treated for DCIS with BCS alone. Within this patient group we were able to compare both primary DCIS versus subsequent IBC and synchronous DCIS versus the IBC component. Second, for all tissue specimens included in this study new H&E whole sides were developed, which were reassessed by specialized breast pathologists. Third, new IHC stained whole slides were used for IHC assessment, to reassure that all stains of the different samples were performed using the same protocol. Fourth, a good agreement was achieved in the interobserver analysis of IHC assessment.

In summary, this study demonstrated that marker expression between *primary* DCIS and *subsequent* IBC is less concordant than *synchronous* DCIS and IBC. This indicates that synchronous and subsequent lesions are not that similar after all. HER2 marker expression showed the largest discrepancy: 36% of HER2-positive *primary* DCIS lesions were followed by HER2-negative IBC. Surprisingly, the frequency of discordant marker expression between primary DCIS and subsequent IBC did not increase with longer time to IBC. Intra-lesional heterogeneity was identified as a possible cause of the observed discordant marker expression. We suggest that future studies investigating the progression of DCIS to IBC, should study primary DCIS and subsequent IBC, instead of synchronous DCIS and IBC lesions. Only this comparison could result in the identification of solid markers for DCIS progression.

More research is needed to assess the contribution of second primary tumors after treatment of DCIS. Molecular analysis of a large, well-annotated patient series including patient matched primary DCIS and subsequent IBC, is of high importance. Currently, there are multiple initiatives that are setting up study cohorts including primary DCIS and subsequent IBC. These include the Sloane project, the PRECISION (PREvent ductal Carcinoma In Situ Invasive Overtreatment Now) initiative, and also noninferiority trials (i.e. LORD, LORIS, and COMET; http://www.cancerresearchuk.org/ funding-for-researchers/how-we-deliver-research/ grand-challenge-award/funded-teams-wesseling).[43–46] These initiatives are highly relevant in finding markers for DCIS progression. Additionally, as copy number alterations are acquired at early stages of tumorigenesis, comparative analysis of these alterations and mutations between these matched pairs would be the preferable choice to assess the clonal-relationship between DCIS and IBC.[47,48]

Article information

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Supplementary tables and figures

Table S1. Clinical variables of all patients and of the selection used in this paper

Characteristics	Selection		All patients		*P*	
	n= 155		*n* = 328			
Age	58	(32 - 87)	59	(30 - 89)	0.32	[b]
Year of DCIS diagnosis	1996	(1989 - 2004)	1996	(1989 - 2004)	0.58	[b]
Time to event						
0-3 years	37	(23.9%)	96	(29.3%)		
4-6 years	40	(25.8%)	81	(24.7%)		
7-9 years	44	(28.4%)	78	(23.8%)		
≥10 years	34	(21.9%)	73	(22.3%)	0.56	[c]
Screen-detection [a]						
Yes	73	(47.1%)	139	(42.4%)		
No	67	(43.2%)	161	(49.1%)		
Unknown	15	(9.7%)	28	(8.5%)	0.48	[c]

[a]: Group of screen-detected lesions include women >50 years. DCIS of women <50 years were not detected via screening; [b]: *p*-value calculated by unpaired T-test; [c]: p-value calculated by Fisher's exact test

Table S2.

Available with the published article.

Table S3. Immunohistochemical marker expression by DCIS histological grade and IBC grade

	Primary DCIS						Invasive breast cancer					
	Histological grade						Grade					
	Well		Interm.		Poor		I		II		III	
	(n = 26)		(n = 86)		(n = 30)		(n = 27)		(n = 65)		(n = 48)	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
ER												
Negative	0	(0)	11	(12.8)	13	(43.3)	1	(3.7)	10	(15.4)	11	(22.9)
Positive	26	(100.0)	75	(87.2)	17	(56.7)	26	(96.3)	55	(84.6)	37	(77.1)
PR												
Negative	5	(19.2)	25	(29.1)	21	(70.0)	4	(14.8)	24	(36.9)	28	(58.3)
Positive	21	(80.8)	60	(69.8)	9	(30.0)	23	(85.2)	41	(63.1)	20	(41.7)
N/A	0	(0)	1	(1.2)	0	(0)	0	(0)	0	(0)	0	(0)
HER2												
Negative	24	(92.3)	64	(74.4)	5	(16.7)	24	(88.9)	55	(84.6)	25	(52.1)
Positive	2	(7.7)	22	(25.6)	25	(83.3)	3	(11.1)	10	(15.4)	23	(47.9)
p16												
Low	17	(65.4)	41	(47.7)	14	(46.7)	14	(51.9)	37	(56.9)	27	(56.3)
High	9	(34.6)	45	(52.3)	16	(53.3)	13	(48.1)	27	(41.5)	21	(43.8)
N/A	0	(0)	0	(0)	0	(0)	0	(0)	1	(1.5)	0	(0)
p53												
0% positive cells (mutant)	2	(7.7)	4	(4.7)	6	(20.0)	1	(3.7)	7	(10.8)	9	(18.8)
1-70% positive cells (WT)	22	(84.6)	72	(83.7)	14	(46.7)	25	(92.6)	50	(76.9)	26	(54.2)
>70% positive cells (mutant)	2	(7.7)	9	(10.5)	10	(33.3)	1	(3.7)	8	(12.3)	13	(27.1)
N/A	0	(0)	1	(1.2)	0	(0)	0	(0)	0	(0)	0	(0)
COX-2												
Low	2	(7.7)	10	(11.6)	4	(13.3)	6	(22.2)	7	(10.8)	3	(6.3)
High	24	(92.3)	76	(88.4)	25	(83.3)	21	(77.8)	57	(87.7)	45	(93.8)
N/A	0	(0)	0	(0)	1	(3.3)	0	(0)	0	(0)	0	(0)

P-values were calculated by Fisher's exact test. N/As were not included in the statistical analysis.
Note: Grade could not be assessed for two invasive lesions because of poor fixation.

Table S4. Immunohistochemical subtype synchronous DCIS related to invasive component

	Invasive component				
	HR+ HER2-	HR+ HER2+	HR- HER2+	HR- HER2-	Total
Synchronous DCIS					
HR+ HER2-	53	3	0	0	56
HR+ HER2+	0	8	0	0	8
HR- HER2+	0	3	8	1	12
HR- HER2-	1	0	0	4	5
Total	54	14	8	5	81

Agreement of 90.1%; marginal homogeneity (symmetry) test $p = 0.09$

Table S5. Immunohistochemical subtype primary DCIS related to invasive recurrence

	Invasive breast cancer				
	HR+ HER2-	HR+ HER2+	HR- HER2+	HR- HER2-	Total
Primary DCIS					
HR+ HER2-	81	4	2	3	90
HR+ HER2+	9	16	2	1	28
HR- HER2+	5	3	10	3	21
HR- HER2-	1	0	0	2	3
Total	96	23	14	9	142

Agreement of 76.8%; marginal homogeneity test $p = 0.040$

Table S6. Marker expression primary DCIS related to synchronous DCIS

		Synchronous DCIS		% Agreement	*P*
		Negative/ Low/WT	Positive/ High/Mutant		
Primary DCIS					
ER	Negative	11	5		
	Positive	6	58	86.3	0.76
PR	Negative	20	13		
	Positive	11	37	70.4	0.68
HER2	Negative	51	1		
	Positive	10	19	86.4	0.007
p53	WT	54	8		
	Mutant	10	8	77.5	0.64
COX-2	Low	1	8		
	High	12	59	75.0	0.37

Comparison between DCIS and DCIS-IBC were made by marginal homogeneity test.

Table S7. IHC subtype primary DCIS related to synchronous DCIS

	Synchronous DCIS				
	HR+ HER2-	HR+ HER2+	HR- HER2+	HR- HER2-	Total
Primary DCIS					
HR+ HER2-	48	0	1	1	50
HR+ HER2+	5	6	3	1	15
HR- HER2+	3	2	8	1	14
HR- HER2-	0	0	0	2	2
Total	56	8	12	5	81

Agreement of 79.0%; marginal homogeneity p=0.034

Table S8. Percentage discordance in grade between pairs of DCIS and IBC

	sDCIS vs. IBC		pDCIS vs. IBC		pDCIS vs. sDCIS	
	%	Symmetry *P*	%	Symmetry *P*	%	Symmetry *P*
PA						
Grade	37.7	0.06	56.2	0.015	45.4	0.17
Tumor tubule formation*	58.9	<0.001	66.9	<0.001	-	-
Number of mitotic figures *	55.7	<0.001	60.2	<0.001	-	-
Nuclear polym. *	29.9	0.001	52.6	<0.001	-	-

*: Histological grade DCIS vs. the different components of the grade of invasive disease (tumor tubule formation, number of mitotic figures, and nuclear polymorphism); discordance (%) = 100-(% Agreement calculated by kappa).
pDCIS: Primary DCIS; sDCIS: synchronous DCIS.

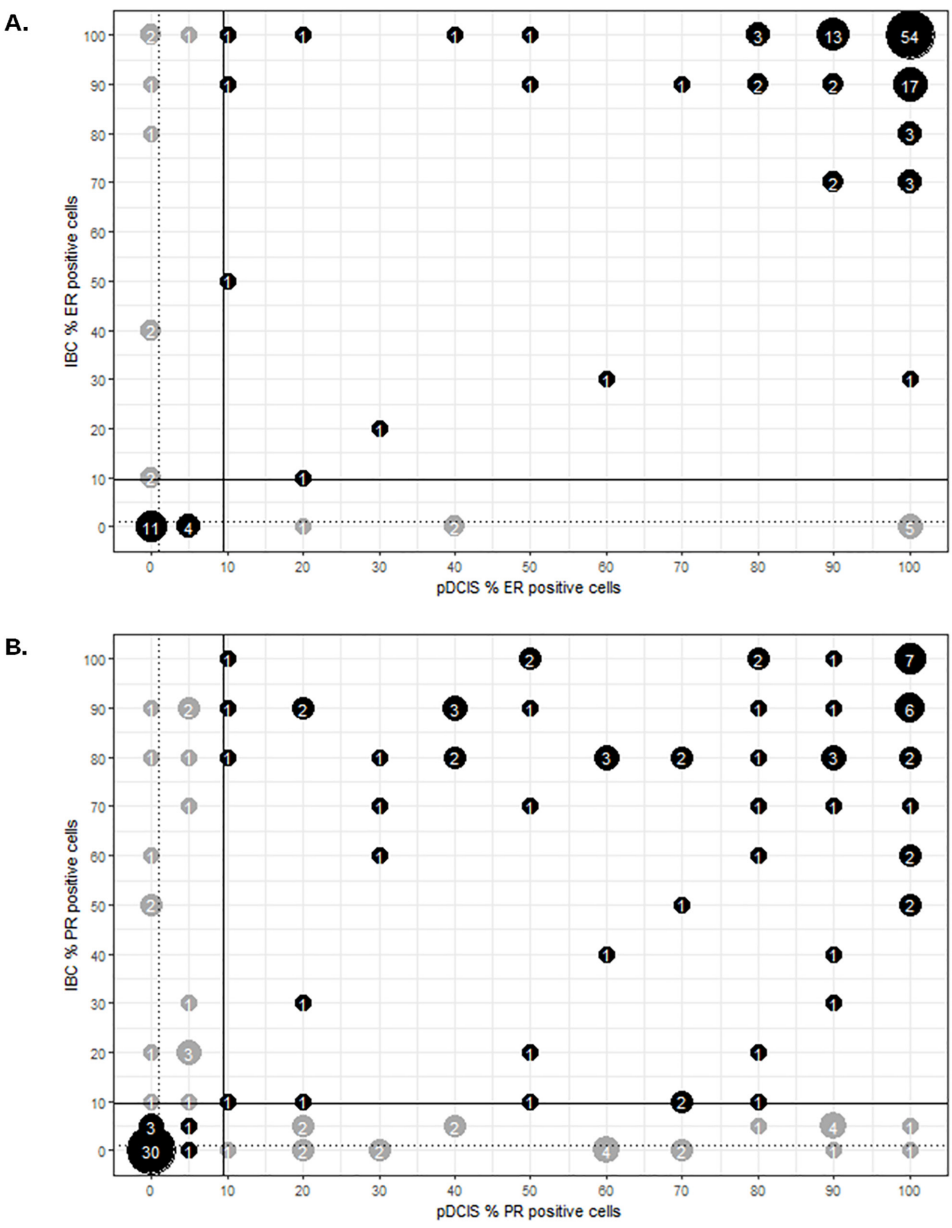

Figure S1. Continuous marker expression primary DCIS related to invasive recurrence. Solid lines represents 10% cut-off and dotted lines represent 1% cut-off for ER and PR positivity. Gray filled circles represent pairs with discordant marker expression and black filled circles represent pairs with concordant marker expression. Numbers represent number of pairs. **A.** ER expression: 10% cut-off, discordant n=17; 1% cutoff, discordant n=20. **B.** PR expression: 10% cut-off, discordant n=39; 1% cutoff, discordant n=24.