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

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Translational relevance

There is increasing concern about the current overtreatment of ductal carcinoma in situ (DCIS). Although numerous prognostic markers for DCIS have been reported, none have shown to be of value for clinical implementation. One of the main reasons for this is the frequently introduced bias caused by the lack of sufficiently large patient cohorts. In the context of a nation-wide cohort, we performed a nested case-control study including patients treated by breast conserving surgery alone with long-term follow-up. We found a 4-fold higher prevalence of subsequent ipsilateral invasive breast cancer (iIBC) for women diagnosed with $HER2^{+}/COX\text{-}2^{high}$ DCIS as compared to women with $HER2^{-}/COX\text{-}2^{low}$ DCIS lesions. Furthermore, patients with $COX\text{-}2^{low}$ DCIS were at lowest risk of iIBC as their risk was comparable to the general population. These prognostic markers are excellent candidates for validation and, ultimately, use in personalized patient risk stratification.

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Clin Cancer Res. 2018 Aug 1;24(15):3593-3601

CHAPTER 3

Clinicopathological risk factors for an invasive breast cancer recurrence after ductal carcinoma in situ - A nested case-control study

Abstract

Purpose: Ductal carcinoma in situ (DCIS) is treated to prevent progression to invasive breast cancer. Yet, most lesions will never progress, implying that overtreatment exists. Therefore, we aimed to identify factors distinguishing harmless from potentially hazardous DCIS using a nested case-control study.

Experimental Design: We conducted a case-control study nested in a population-based cohort of DCIS patients treated with breast conserving surgery (BCS) alone (n = 2,658) between 1989-2005. We compared clinical, pathological, and IHC DCIS characteristics of 200 women who subsequently developed ipsilateral invasive breast cancer (iIBC; cases) and 474 women who did not (controls), in a matched setting. Median follow-up time was 12.0 years (interquartile range 9.0-15.3). Conditional logistic regression models, were used to assess associations of various factors with subsequent iIBC risk after primary DCIS.

Results: High COX-2 protein expression showed the strongest association with subsequent iIBC (OR = 2.97, 95% confidence interval [95% CI] 1.72-5.10). In addition, HER2 overexpression (OR = 1.56, 95% CI 1.05-2.31) and presence of periductal fibrosis (OR = 1.44, 95% CI 1.01-2.06) were associated with subsequent iIBC risk. Patients with $HER2^{+}$/$COX\text{-}2^{high}$ DCIS had a 4-fold higher risk of subsequent iIBC (vs. $HER2^{-}$/$COX\text{-}2^{low}$ DCIS), and an estimated 22.8% cumulative risk of developing subsequent iIBC at 15 years.

Conclusions: With this unbiased study design and representative group of DCIS patients treated by BCS alone, COX-2, HER2, and periductal fibrosis were revealed as promising markers predicting progression of DCIS into iIBC. Validation will be done in independent data sets. Ultimately, this will aid individual risk stratification of women with primary DCIS.

Introduction

Ductal carcinoma *in situ* (DCIS) is a potential precursor of invasive breast cancer (IBC). It is characterized by proliferation of ductal epithelial cells confined within the ductal-lobular system. Most women (80-85%) are diagnosed with DCIS by screening mammography in which breast abnormalities are found without the women having clinical symptoms.[1] In the western world, the incidence of DCIS has increased almost 6-fold with the introduction of population-based breast cancer screening, and accounts for about 20-30% of all newly diagnosed breast neoplasms.[2–7]

Although DCIS is not life threatening, it does increase a woman's risk of developing IBC later in life, which subsequently could lead to a breast cancer-specific death.[8] However, we are currently unable to distinguish DCIS lesions that will progress to IBC from those that will not, since there is only limited information on the long-term natural history of DCIS.[9] As a consequence, almost all DCIS is treated by mastectomy or breast conserving surgery (BCS) with or without radiotherapy. This is done under the assumption that this will prevent IBCs and subsequently breast cancer-specific deaths, despite the fact that breast cancer-specific mortality after DCIS is uncommon: <2%.[10,11] On top of that, the long-term benefit of treatment of asymptomatic DCIS that may or may not progress to IBC is difficult to quantify.[12] As a result, screening programs are nowadays criticized for being associated with overdiagnosis and overtreatment.[13,14]

Distinguishing, at diagnosis, DCIS that might cause life-threatening disease from indolent DCIS is therefore of great importance. A multitude of studies have tried to find markers that could predict local recurrence or progression of DCIS.[15] In a few studies, investigators showed that various histopathologic characteristics of DCIS, such as lesion size, marginal status, histologic grade, architectural patterns, and presence of necrosis were associated with recurrence.[16,17] However, these studies did not discriminate between invasive and *in situ* recurrences. Furthermore, due to limited patient numbers and lack of validation studies, none of the markers studied to date show sufficiently strong evidence for an association with subsequent ipsilateral IBC (iIBC).

The primary objective of this study was to identify clinical and histologic characteristics of the initial DCIS lesion that are associated with the development of subsequent iIBC. Here, we report the results of our case-control study nested within a large nation-wide population-based cohort of Dutch women with DCIS treated by BCS alone between 1989 and 2005.

Patients and Methods

Study population and design

The study population has been previously described.[18] In brief, we used a nation-wide population-based patient cohort derived from the Netherlands Cancer Registry (NCR), in

which we included all women diagnosed with primary DCIS, and treated with BCS alone within the Netherlands from January 1, 1989 to December 31, 2004. Patients did not receive tamoxifen or other anti-hormonal adjuvant treatment. According to the Dutch guidelines, patients diagnosed and treated for DCIS were followed by undergoing annual mammograms for at least 5 years. If no recurrence occurred, women could participate in population-based screening again, if applicable regarding age group. Patients with adjacent invasive disease or a prior cancer diagnosis except for nonmelanoma skin cancer were not included. This resulted in 2,658 eligible female participants.

Data provided by the NCR included information on age at and date of diagnosis, histology and treatment for DCIS, and any subsequent IBCs. Follow-up for subsequent iIBC 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). 374 of the 2,658 women developed subsequent iIBC, as first invasive cancer, after a primary diagnosis of DCIS.[18] At the start of this present study, the first 316 women with a subsequent iIBC were identified and the remaining 58 cases were identified when data collection of this study was completed. These 316 women were included in this study and were considered "cases". Controls were matched to cases based on age in years at DCIS diagnosis (exact) using a variable matching ratio. Controls had to have remained free from ipsilateral and contralateral IBC for at least as long as the initial DCIS diagnosis to iIBC development of the case they were matched to. Controls were selected with replacement, so some individuals were a control for more than one case.

Cases and controls originated from 58 hospitals within the Netherlands. We could not obtain FFPE tissue blocks from some hospitals, either because tissue blocks were unavailable or because the hospital refused to provide tissue for research (61 cases and 388 controls; Supplementary Table S1). Furthermore, some patients were excluded during pathology review because: no DCIS component was found, a (micro)invasive component or LCIS was present, or because the specimen was not assessable (53 cases and 173 controls). Finally, some patients were excluded because no matched case or control was available for case-control sets they belonged to (2 cases and 268 controls).

Together with the tissue blocks, pathology reports were retrieved from the participating hospitals. Pathology reports were reviewed for measurements of lesion size and margin status. Notable, data on lesion size was often not routinely described for DCIS in these old retrospective series.

We categorized year of DCIS diagnosis into two time periods: 1989-1998 (screening implementation phase) and 1999-2004 (full nationwide coverage phase). Clinical presentation of DCIS was subdivided into screen-detected (mean age 59 years; range 50–74) and non-screening-related (mean age 54 years; range 30-89). This information was available for 91% of the included patient group.

The study was approved by the review boards of the NCR (request K12.281; 03-01-2013) and PALGA (LZV990; 16-04-2013). The secondary use of tissue and data under

an opt-out regime in this study is conform Dutch regulations and the Code of Conduct of Federa-COREON.[19]

Pathology review

New haematoxylin and eosin-stained whole slides were prepared for all tissue specimens and subsequently histopathologic re-examined by three consultant breast pathologists (J. Wesseling, E.J. Groen, and K. van de Vijver). Slides were assessed on morphological characteristics, including DCIS architecture and nuclear grade, the presence of calcifications and necrosis, and microenvironmental characteristics like stromal features and the presence of lymphocytes. This was done blinded of case or control status. In addition, for every patient a representative part of the lesion was selected for further evaluation. Clinical characteristics of patients in- and excluded in this study are presented in Supplementary Table S2. Pathology review data was available for 200 case-control sets, including at least one control per case, resulting in a case-control series of 200 cases and 474 controls, which was representative of the original case-control selection (Supplementary Table S3).

IHC assessment

IHC staining was used to identify DCIS phenotypes using slides from FFPE tissue blocks of 185 DCIS cases and 420 DCIS controls. Some tissue specimens were excluded because insufficient tissue material was available for IHC (12 cases and 26 controls; Supplementary Table 1). DCIS lesions were scored for estrogen receptor (ER) and progesterone receptor (PR) status, overexpression of human epidermal growth factor 2 (HER2), Ki67, and expression of the tumor suppressor proteins p16 and p53, and cyclooxygenase 2 (COX-2). These markers were selected because the antigens have been associated with subsequent IBC after DCIS, based on multivariable analyses, and these results had been reported previously in at least two papers.[15,20] Moreover, the ability to perform good quality IHC on FFPE material was decisive. Details about the used antibodies, IHC staining procedure, and scoring criteria can be found in Supplementary Materials and Methods. All antibodies used in this study were previously tested in our laboratory using normal tissue and tumor samples known to contain the antigens.

Statistical Analysis

Logistic regression models, conditional on matched sets, were used to assess associations of various clinical and histopathologic characteristics with subsequent iIBC risk after primary DCIS. Wald-based 95% confidence intervals (95% CI) and *P*-values are reported for overall effect for factors with more than two categories.

Variables were selected for inclusion in multivariable models based on a p-value ≤0.1 in univariate analyses. Due to the amount of missing data margin status and lesion size were excluded from the multivariable models. In addition, since histologic grade was correlated (*r*

> 0.4) with necrosis and periductal fibrosis, this variable was excluded from the multivariable models. Subsequently, the likelihood ratio (LR) chi-square was used to identify the models with the strongest association with subsequent iIBC development.

Approximate cumulative incidence of subsequent iIBC by HER2 and COX-2 status was estimated using the iIBC ORs for HER2 and COX-2 status and cumulative risk of iIBC in the entire cohort. Death due to causes other than breast cancer was considered as a competing risk in this analysis. The expected cumulative incidence of breast cancer for our study population was derived from age-specific breast cancer incidence and all-cause mortality rates in the Dutch female population using the Hakulinen method.[21]

All statistical analyses were performed using Stata/SE (version 13.1, statacorps, Texas). *P* values ≤0.05 were considered statistically significant.

Results

Patient characteristics

Clinical characteristics of cases and controls were comparable (Table 1): For both cases and controls median age was 57 years, main period of DCIS diagnosis was 1989-1998, and around 50% of DCIS was screen-detected. For cases, the median time to iIBC was 6.2 years (range 0.5-19.2). Ninety-five percent of all subsequent iIBC lesions recurred at or near the side of the DCIS excision (data not shown). Furthermore, of 141 cases (71%), we were able to assess the ER status of the matched subsequent IBC, which showed an agreement of 89% between the primary DCIS and matched IBC (data not shown).

Univariate results of characteristics associated with subsequent iIBC

The presence of periductal fibrosis was associated with increased risk of subsequent iIBC (OR = 1.44, 95% CI 1.01-2.06) compared to women who did not develop iIBC (Table 2). Furthermore, there was a trend towards a larger lesion size (*P* = 0.08) and more frequent positive resection margins (*P* = 0.06) among cases as compared to controls. However, it should be stressed that around 65% of data on lesion size and around 15% of margin data is missing within our case-control series. There was a trend for necrosis (*P* = 0.06), periductal lymphocytes (*P* = 0.13), and high histologic grade (*P* = 0.08) to be more often present in DCIS lesions of women who subsequently developed iIBC. DCIS architecture, calcifications, and periductal lymphocytes were not associated with subsequent invasive disease. The IHC markers HER2 (OR = 1.56, 95% CI 1.05-2.31) and COX-2 (OR = 2.97, 95% CI 1.72-5.10) were associated with subsequent iIBC risk, but ER, PR, p16, and p53 expression and immunohistochemical subtype were not associated with subsequent iIBC risk (Table 3). Ki67 was excluded from the analysis because of unreliable staining results.

Table 1. Clinical characteristics of female primary DCIS patients treated with BCS alone, who subsequently did (DCIS cases) or did not (DCIS controls) develop subsequent iIBC

	DCIS cases (*n* = 200)		DCIS controls (*n* = 474)	
Characteristics	***n***	**(%)**	***n***	**(%)**
Age at DCIS diagnosis (years)				
<40	14	(7.0)	30	(6.3)
40-49	27	(13.5)	60	(12.7)
50-59	79	(39.5)	204	(43.0)
60-69	55	(27.5)	125	(26.4)
70-79	19	(9.5)	41	(8.6)
≥80	6	(3.0)	14	(3.0)
Year of DCIS diagnosis, mean (range)	1996	(1989 - 2004)	1997	(1989 - 2004)
Period of DCIS diagnosis *				
1989-1998 (screening implementation phase)	147	(73.5)	335	(70.7)
1999-2004 (full nationwide coverage)	53	(26.5)	139	(29.3)
Clinical presentation of DCIS				
Screen-detected	96	(48.0)	245	(51.7)
Non-screening-related	89	(44.5)	184	(38.8)
Unknown	15	(7.5)	45	(9.5)
Time to iIBC, mean in years (range)	6.2	(0.5 – 19.2)	-	-

NOTE: Controls were matched to cases on the basis of age at diagnosis (exact), using a variable matching ratio, and followed at least as long as the case they were matched to , by conditional logistic regression analysis.
iIBC: Ipsilateral invasive breast cancer.
*: Based on the gradual implementation of the national breast cancer screening program in the Netherland for women >50 years of age, we divided year of DCIS diagnosis into two time periods: 1989-1998 , which was the period of implementation of the national mammographic screening program within the Netherlands; and within the period of 1999-2004 the screening program was fully implemented.

Multivariable results of characteristics independently associated with subsequent iIBC

COX-2 was also significantly associated with the risk of subsequent iIBC in multivariable analyses (Table 4 and Supplementary Table S4). Subsequent invasive disease was significantly associated with high COX-2 expression in combination with: (1) overexpression of HER2 (LR chi^2 = 6.47; OR = 3.98); (2) the presence of periductal fibrosis (LR chi^2 = 6.34; OR = 4.87); or (3) the presence of necrosis (LR chi^2 = 5.18; OR = 5.76). Combination of periductal fibrosis, HER2 and COX-2 was also significantly associated with subsequent iIBC, although achieving a lower LR chi-square ratio (LR chi^2 = 4.98; OR = 4.63; Table 4). When combining COX-2, HER2, periductal fibrosis, and necrosis, the addition of necrosis deteriorated the performance of the model, since necrosis is positively correlated with HER2 (data not shown). Eighty-seven percent of DCIS lesions associated with subsequent iIBC (DCIS cases) showed high expression of COX-2. Of this subset of COX-2^{high} DCIS lesions, 34% was HER2 positive, and in 37% periductal fibrosis was present (Table 2 and 3). HER2 overexpression was most frequently accompanied with high COX-2 expression. In contrast, high COX-2 expression was independent of HER2 overexpression. The cumulative risk of subsequent iIBC of HER2^+/COX-2^{high} DCIS was almost 4 times higher as compared to the

Table 2. Univariate results of histopathologic characteristics associated with subsequent iIBC

	DCIS cases (*n* = 200)		DCIS controls (*n* = 474)			
Characteristics	**n**	**(%)**	**n**	**(%)**	**OR (95% CI)[a]**	***P*[b]**
Lesion size, millimeter, mean (range)	13	(2-30)	10	(1-70)	1.03 (0.98-1.07)	0.25
Lesion size	200		474			
≤10 mm	29	(14.5)	122	(25.7)	1.00 (reference)	
11-20 mm	27	(13.5)	41	(8.6)	2.04 (0.95-4.34)	
>20 mm	6	(3.0)	8	(1.7)	3.35 (0.78-14.33)	0.08
Unknown	138	(69.0)	303	(63.9)		
Margin status						
Free	136	(68.0)	376	(79.3)	1.00 (reference)	
Not free	28	(14.0)	47	(9.9)	1.70 (0.99-2.91)	0.06
Unknown	36	(18.0)	51	(10.8)		
Growth pattern						
Solid	76	(38.0)	150	(31.6)	1.00 (reference)	
Cribriform	14	(7.0)	33	(7.0)	0.89 (0.45-1.77)	
(Micro)papillary	9	(4.5)	13	(2.7)	1.24 (0.45-3.42)	
Clinging	2	(1.0)	8	(1.7)	0.62 (0.13-3.04)	
Mixed	99	(49.5)	270	(57.0)	0.76 (0.53-1.07)	0.52
Dominant growth pattern						
Solid	119	(59.5)	275	(58.0)	1.00 (reference)	
Cribriform	45	(22.5)	110	(23.2)	0.93 (0.61-1.41)	
(Micro)papillary	24	(12.0)	54	(11.4)	1.02 (0.59-1.78)	
Clinging	12	(6.0)	35	(7.4)	0.83 (0.42-1.66)	0.95
Histologic grade						
Low (grade 1)	29	(14.5)	96	(20.3)	1.00 (reference)	
High (grade 2 and 3)	171	(85.5)	378	(79.7)	1.49 (0.94-2.37)	0.08
Necrosis						
Absent	45	(22.5)	141	(29.7)	1.00 (reference)	
Present	155	(77.5)	333	(70.3)	1.44 (0.98-2.11)	0.06
Microcalcification						
Absent	51	(25.5)	115	(24.3)	1.00 (reference)	
Present	149	(74.5)	358	(75.5)	0.95 (0.64-1.43)	0.82
N/A	0	(0.0)	1	(0.2)		
Periductal fibrosis						
Absent	124	(62.0)	336	(70.9)	1.00 (reference)	
Present	76	(38.0)	137	(28.9)	1.44 (1.01-2.06)	<0.05
N/A	0	(0.0)	1	(0.2)		
Periductal lymphocytes						
Absent	136	(68.0)	353	(74.5)	1.00 (reference)	
Present	64	(32.0)	120	(25.3)	1.33 (0.92-1.92)	0.13
N/A	0	(0.0)	1	(0.2)		

Abbreviations: HR: hormone receptors, ER and PR, where HR+ are ER+ and/or PR+ lesions, and HR- are ER- and PR- lesions. N/A: Not assessable (N/As and unknowns were not included in the analysis).
[a]: Comparisons between DCIS cases and DCIS controls were made by univariate conditional logistic regression in which matching was taken into account; [b]: For variables with >2 categories the P-value for overall effect was calculated by Wald-test, for variables with only 2 categories the prob >chi2 was used.

Table 3. Univariate results of IHC markers associated with subsequent iIBC

	DCIS cases (*n* = 185)		DCIS controls (*n* = 420)			
Characteristics	***n***	**(%)**	***n***	**(%)**	**OR (95% CI)[a]**	***P*[b]**
ER						
Negative	35	(18.9)	79	(18.8)	1.00 (reference)	
Positive	149	(80.5)	341	(81.2)	0.99 (0.63-1.55)	0.95
N/A	1	(0.5)	0	(0.0)		
PR						
Negative	74	(40.0)	149	(35.5)	1.00 (reference)	
Positive	108	(58.4)	264	(62.9)	0.83 (0.57-1.20)	0.31
N/A	3	(1.6)	7	(1.7)		
HER2						
Negative	120	(64.9)	310	(73.8)	1.00 (reference)	
Positive	62	(33.5)	104	(24.8)	1.56 (1.05-2.31)	0.03
N/A	3	(1.6)	6	(1.4)		
Subtypes						
HR+ HER2-	115	(62.2)	284	(67.6)	1.00 (reference)	
HR+ HER2+	33	(17.8)	54	(12.9)	1.50 (0.92-2.47)	
HR- HER2+	29	(15.7)	50	(11.9)	1.47 (0.87-2.49)	
HR- HER2-	5	(2.7)	26	(6.2)	0.44 (0.16-1.20)	0.06
N/A	3	(1.6)	6	(1.4)		
p16						
Low	90	(48.6)	228	(54.3)	1.00 (reference)	
High	93	(50.3)	187	(44.5)	1.29 (0.90-1.85)	0.16
N/A	2	(1.1)	5	(1.2)		
p53						
<30% positive cells	100	(54.1)	240	(57.1)	1.00 (reference)	
30-70% positive cells	40	(21.6)	61	(14.5)	1.67 (1.01-2.77)	
>70% positive cells	25	(13.5)	58	(13.8)	1.08 (0.62-1.87)	
Negative	17	(9.2)	58	(13.8)	0.78 (0.43-1.40)	0.13
N/A	3	(1.6)	3	(0.7)		
COX-2						
Low	19	(10.3)	106	(25.2)	1.00 (reference)	
High	161	(87.0)	306	(72.9)	2.97 (1.72-5.10)	<0.001
N/A	5	(2.7)	8	(1.9)		

Abbreviations: N/A: Not assessable (N/As were not included in the analysis).
[a]: Comparisons between DCIS cases and DCIS controls were made by univariate conditional logistic regression; [b]: For variables with >2 categories the P-value for overall effect was calculated by Wald-test, for variables with only 2 categories the prob >chi2 was used.

risk for HER2^{-}/COX-2^{low} DCIS lesions (Table 4). Furthermore, analysis of IHC data of 141 DCIS and matched IBC pairs showed that of the 43 patients with HER2^{+}/COX-2^{high} DCIS that subsequently developed an iIBC, 35% developed an ER negative invasive recurrence (Table 5).

In our study group, the estimated overall 10-year and 15-year cumulative incidence of iIBC were 10.9% and 13.8%, respectively. For patients with HER2^{-}/COX-2^{low} DCIS, the estimated 10-year and 15-year cumulative incidence of iIBC were 4.8% and 6.0%, respectively; for patients with HER2^{+}/COX-2^{low} DCIS, 4.5% and 5.6%; for patient with HER2^{-}/COX-2^{high} DCIS, 11.3% and 14.3%; and for patients with HER2^{+}/COX-2^{high} DCIS, 18.1% and 22.8%, respectively (Figure 1). Within our study group, 29.7% of cases and 18.3% of controls had this unfavorable DCIS subtype of HER2^{+}/COX-2^{high} DCIS.

The positive predictive value of the HER2^{+}/COX-2^{high} DCIS subtype is 42% (sensitivity: 31%; specificity: 81%), indicating that more than half of the HER2^{+}/COX-2^{high} DCIS lesions are not associated with invasive recurrence and thus are false positives. The strength of this marker combination is most likely in its negative predictive value (NPV = 73%), indicating that the risk of subsequent iIBC after DCIS is low in the non-HER2^{+}/COX-2^{high} subgroup.

Table 4. Multivariable results of histopathologic characteristics and IHC markers independently associated with subsequent invasive disease

	DCIS cases		DCIS controls			
	n	**(%)**	***n***	**(%)**	**OR (95% CI)[a]**	***P*[b]**
Periductal fibrosis/Necrosis						
Absent/Absent	34	(17.0)	127	(26.8)	1.00 (reference)	
Present/Absent	11	(5.5)	14	(3.0)	2.75 (1.12-6.75)	
Absent/Present	90	(45.0)	209	(44.1)	1.59 (1.01-2.49)	
Present/Present	65	(32.5)	123	(25.9)	1.88 (1.16-3.07)	0.04
N/A	0	(0.0)	1	(0.2)		
HER2/COX-2						
Negative/Low	14	(7.6)	77	(18.3)	1.00 (reference)	
Positive/Low	5	(2.7)	26	(6.2)	0.94 (0.30-2.95)	
Negative/High	105	(56.8)	227	(54.0)	2.44 (1.30-4.59)	
Positive/High	55	(29.7)	77	(18.3)	3.98 (2.01-7.91)	<0.001
N/A	6	(3.2)	13	(3.1)		
Periductal fibrosis/HER2/COX-2						
All other groupings	19	(10.3)	103	(24.5)	1.00 (reference)	
Negative/Negative/High	79	(42.7)	174	(41.4)	2.54 (1.42-4.54)	
Positive/Negative/High	26	(14.1)	52	(12.4)	2.45 (1.22-4.92)	
Negative/Positive/High	23	(12.4)	37	(8.8)	3.51 (1.69-7.29)	
Positive/Positive/High	32	(17.3)	40	(9.5)	4.63 (2.26-9.50)	<0.001
N/A	6	(3.2)	14	(3.3)		

Abbreviations: N/A: Not assessable (N/As were not included in the analysis).
[a]: Comparisons between DCIS cases and DCIS controls were made by multivariable conditional logistic regression; [b]: P-values for overall effect were calculated by Wald-test.
LR chi2 corrected for degrees of freedom were 2.85 for periductal fibrosis/necrosis, 6.47 for HER2/COX-2, and 4.98 for periductal fibrosis/HER2/COX-2.

Discussion

In this study we identified promising risk factors for progression of DCIS into iIBC, by conducting a nested case-control study comparing women who did and did not develop invasive disease after primary DCIS. To avoid confounding by radiation effects, we analyzed all women treated by BCS alone, within our well-characterized nation-wide population-based cohort of women diagnosed with primary DCIS between 1989 and 2005 in the Netherlands with a median follow-up time of 12.0 years. The large size of our series, the design applied, the comprehensive data, and the long term-follow-up are essential to overcome limitations due to bias and lack of power of small sample series with a relatively short follow-up.

We found that initial DCIS lesions with $HER2^{+}/COX\text{-}2^{high}$ expression were associated with increased risk of subsequent iIBC, which are often ER negative (35%). This is an important finding, as ER negative tumors have in general a worse prognosis than ER positive breast cancers. Women diagnosed with this unfavorable DCIS subtype had a 4-fold higher risk and an estimated 22.8% cumulative 15-year risk of developing subsequent iIBC. This was higher than the overall cumulative incidence of iIBC in this patient cohort, i.e. 13.8% at 15 years. The estimated cumulative risk for patients with $HER2^{-}/COX\text{-}2^{low}$ and $HER2^{+}/COX\text{-}2^{low}$ DCIS was comparable to the risk for the general population, i.e. 4-6% at 15 years. The positive and negative predictive value of the HER2/COX-2 marker combination is 42% and 73%, respectively. For clinical purposes the predictive values should definitely be improved.

Extensive granular cytoplasmic expression of COX-2 was the marker which had the strongest association with subsequent iIBC both in univariate and multivariable analysis. This is in line with one previous study that showed an association between COX-2 and subsequent invasive breast cancer in univariate analysis.[22] We also found an association of subsequent iIBC with HER2 positive primary DCIS, and presence of periductal fibrosis. HER2 overexpression was associated with subsequent iIBC in univariate analysis, which was also found in two previous studies.[23,24] Interestingly, results from our multivariable analysis showed that HER2 overexpression is not predictive for subsequent iIBC in the absence of high COX-2 expression. This indicates that the prognostic value of HER2 overexpression in the risk of subsequent iIBC is probably limited and the increase in risk is primarily driven by COX-2 overexpression. Another prognostic factor we found was periductal fibrosis. This microenvironmental factor was associated with subsequent iIBC in both univariate and multivariable analysis and is supported by a previous study.[24] So far, diagnosis of breast disease has been limited to the morphological interpretation of epithelial cells and the assessment of epithelial tissue architecture, in which the stromal compartment is largely ignored. The role of periductal fibrosis described here underline the importance of assessment of the DCIS stromal compartment.

There is biological support for a role of COX-2 in invasive breast cancer recurrence. COX-2 is a cytoplasmic enzyme involved in prostaglandin synthesis. It is induced rapidly in

response to growth factors, tumor promotors, hormones, and cytokines.[25] It has been shown that overexpression of COX-2 leads to an increased prostaglandin E2 (PGE2) level which can potentially affect most of the key processes in cancer development, including proliferation, resistance to apoptosis, angiogenesis, immune suppression and invasion.[26] Furthermore, overexpression of COX-2 has been shown to result in p16-mediated cell cycle arrest through the p16/Rb-signaling pathway. When the p16/Rb-signaling pathway is disrupted, cellular proliferation continues, resulting in additional high Ki67 expression in the presence of high p16 and high COX-2 expression.[27] It has previously been shown that $p16^{+}$/$COX\text{-}2^{+}$/$Ki67^{+}$ DCIS is associated with subsequent iIBC.[28] Unfortunately, in our study we were unable to assess p16/COX-2/Ki67 protein expression, since Ki67 suffers from loss of antigenicity over time and is very sensitive to improper formalin fixation.[29,30]

Table 5. HER2/COX-2 status DCIS related to ER status of subsequent iIBC

	Invasive breast cancer					
	ER pos n (%)		ER neg n (%)		Total	*p*
DCIS						
HER2+/COX-2high	28	(65.1)	15	(34.9)	43	<0.001
All other groupings	91	(92.9)	7	(7.1)	98	
Total	119	(84.4)	22	(15.6)	141	

NOTE: Comparison between DCIS and invasive breast cancer were made by marginal homogeneity test.

Next to the factors described above, a wide range of other prognostic factors have been reported in literature related to recurrent disease, albeit with small effect sizes.[1,15] In contrast to our study, most studies did not discriminate between invasive and *in situ* recurrences as a primary endpoint.[31–33] As invasive recurrences may lead to breast cancer mortality, it is of utmost importance to make a distinction between *in situ* and invasive recurrences in risk prediction.

Our study group comprised of DCIS patients diagnosed between 1989 and 2005 and treated by BCS alone. Regarding this time period, we have to keep in mind that treatment strategies and screening techniques for DCIS have evolved over the years, which may have impacted treatment or other care for these patients. The main period of DCIS diagnosis of our patient group was 1989-1998 and about 50% of DCIS was screen-detected. Within the time period 1989-1998, guidelines for DCIS treatment in the Netherlands recommended mastectomy or BCS alone. From 1999, the addition of radiotherapy after BCS was included. Clinical trials have shown that adjuvant radiotherapy reduces the risk of both *in situ* and invasive recurrence with about 50%.[34,35] Moreover, our group has shown that women diagnosed with DCIS between 1999 and 2004 were less likely to develop iIBC than women diagnosed between 1989 and 1998, regardless of treatment and age.[18] With the introduction of digital mammography, the coverage and sensitivity of screening improved significantly

and led to an increase in the percentage of screen-detected DCIS.[36]

In our study group, the estimated overall 10-year and 15-year cumulative incidence of iIBC were 10.9% and 13.8%, respectively. This is comparable to the 10-year cumulative incidence reported in two non-randomized prospective studies of women with DCIS treated by BCS alone.[37,38]

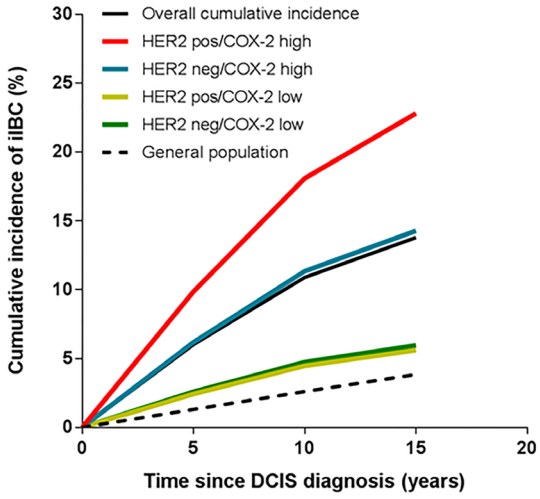

Figure 1. Cumulative incidence per category of HER2/COX-2 status. Cumulative incidence of iIBC among women with an initial diagnosis of DCIS treated by BCS alone. Approximate cumulative incidence of subsequent iIBC by HER2 and COX-2 status was estimated using the iIBC ORs for HER2 and COX-2 status from the current study and cumulative risk of iIBC and death due to other causes derived from the entire cohort.

HER2/COX-2	10-year	15-year
Overall cumulative incidence (–)	10.9%	13.8%
Positive/High (–)	18.1%	22.8%
Negative/High (–)	11.3%	14.3%
Positive/Low (–)	4.5%	5.6%
Negative/Low (–)	4.8%	6.0%
General population (- -)	2.6%	3.9%

Our study has several strengths. First, our study is nested in a large, unique population-based study of women with DCIS treated by BCS alone that provides information on clinical, histopathological and immunohistochemical characteristics, and focusses specifically on subsequent ipsilateral *invasive* breast cancer with a median follow-up time of 12.0 years. This well-annotated nature of our patient series enabled us to prevent a great amount of bias often seen in previous studies. Second, we were able to collect 85% of the requested tissue blocks, from which new whole slides were developed for re-assessment by specialized breast pathologists. Third, our large sample allowed us to assess the combinations of clinical, histopathological, and immunohistochemical characteristics, that were independently associated with subsequent invasive disease, by using multivariable models.

Our study also has some limitations. First, we were only able to assess prognostic factors for a subset of all women included in the case-control study since this depended on the

availability of FFPE tissue blocks in the participating hospitals. Fortunately, this did not cause significant bias, as these samples were missing randomly (hospital participation) and the group for which we could successfully collect the tissue blocks was an excellent representation of the complete case-control selection based on the patients' clinical characteristics. Second, the interpretation of immunohistochemical markers can be challenging because of the heterogeneous expression of certain proteins. Nevertheless, we succeeded in minimizing the inter-observer variability by our well designed scoring method. Third, margin status and size of the DCIS lesion were not known in 15% and 65% of the cases, respectively, as these data were not always routinely described in these old retrospective series. As far as margin status and DCIS lesions size were known, no statistically significant differences were present between the two groups, i.e. cases and controls. Missing data were also equally randomly distributed among these groups (Table 2), and thus was not related to the outcome of interested. Therefore, resulting over- or underrepresentation of some factors is unlikely. Fourth, we did not have data on BRCA status or family history of breast cancer of our patient group. Yet, it has to be taken into account that BRCA status could be a confounding factor in subsequent iIBC development.

Conclusions

In summary, identification of prognostic factors has the potential to improve the clinical management of women diagnosed with DCIS. We found a prognostic role for COX-2, HER2, and periductal fibrosis. In addition, women diagnosed with $HER2^{+}/COX\text{-}2^{high}$ DCIS and treated by BCS alone had a 4-fold higher prevalence of subsequent iIBC than women with $HER2^{-}/COX\text{-}2^{low}$ DCIS lesions. Furthermore, $HER2^{+}/COX\text{-}2^{high}$ DCIS was associated with ER negative invasive recurrences. Our results underline the importance of assessment of the DCIS stromal compartment and protein expression of HER2 and COX-2 to estimate the risk of subsequent invasive disease after a diagnosis of DCIS. Patients with $COX\text{-}2^{low}$ DCIS are at lowest risk of iIBC as their risk is comparable to the general population. Our study design and unique retrospective patient series provided us with excellent candidate prognostic markers for use in personalized patient risk stratification. As a next step, these prognostic markers will need to be validated in independent data sets, as a major step to distinguish harmless from potentially hazardous DCIS.

Article information

Funding: This work was supported by Pink Ribbon (grand number 2013.WO29); and A Sisters Hope (grand number 2011.WO19.C88).

Acknowledgements: We thank all patients whose stored residual material was used in this study and their treating physicians, all the collaborating hospitals and especially the pathology departments, and all persons who helped in the data collection and analysis. Specifically, we acknowledge Otto Visser, Annemarie Eeltink and the registration teams of the Netherlands Comprehensive Cancer Organization for the collection of data for the Netherlands Cancer Registry; Lucy Overbeek and PALGA, the nationwide histopathology and cytopathology data network and archive, for providing pathology data; the pathologists of the PALGA-group: Dr. P. Bult, Dr. C.H.M. van Deurzen, Dr. E. Geuken, and Dr. C.M. van Dijk for their cooperation; Frank Nieboer, Danah Kamphuis, and Chaima Loukili for their assistance; and the NKI-AVL Core Facility Molecular Pathology & Biobanking (CFMPB) for their lab support.

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Supplementary materials and methods

Pathology review and assessment of inter-observer variability

A random sample of 50 DCIS specimens were reviewed by all pathologists to assess the inter-observer agreement. The concordance between the pathologists was around 85% with an interclass correlation coefficient of >0.4 for every characteristic assessed. Subsequently, slides from each included patient were reviewed by one of these pathologists, unaware of case or control status.

Immunohistochemical staining procedure

Immunohistochemical staining of ER, PR, HER2, Ki67, p16, p53, and COX-2 was performed using a Benchmark ULTRA autostainer (Ventana Medical Systems, AZ, USA). For each lesion and for each staining, a 3 µm thick whole slide paraffin section was heated at 75 °C for 28 minutes and deparaffinized in the instrument with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1; Ventana Medical Systems) for 64 min at 95 °C. Next, the primary antibody, indicated in Table M1, was applied to the tissue section. Additionally, for the p16 staining signal amplification was performed using the Optiview Amplification kit (Ventana Medical Systems). For the PR staining the slides were additionally incubated with normal antibody diluent (ABB999, Immunologic) to reduce the background signal. Reactions were detected using the UltraView Universal DAB Detection kit (#760-500; Roche) for visualization of ER, PR, and HER2, or the OptiView DAB Detection kit (#760-700; Roche) for visualization of Ki67, p16, p53 and COX-2. Finally, the slides were counterstained with Hematoxylin II and Bluing Reagent (Ventana Medical Systems).

Table M1. Primary antibody sources and dilutions used in this study

Antigen	Clone	Dilution	Manufacturer
ER	SP1	Ready-to-use	Ventana Medical Systems
PR	1E2	Ready-to-use	Ventana Medical Systems
HER2	4B5	Ready-to-use	Ventana Medical Systems
Ki67	MIB1	1/250	DAKO
p16	JC8	1/800	Santa Cruz
p53	DO-7	1/7000	DAKO
COX-2	CX294	1/100	DAKO

ER, estrogen receptor; PR, progesteron receptor; HER2, human epidermal growth factor receptor 2; p16, cyclin-dependent kinase inhibitor 2A; COX-2, cyclooxygenase-2.

Assessment of immunohistochemistry and inter-observer variability

IHC stained slides were scanned using an Aperio AT2 Slide Scanner (Leica Biosystems) and subsequently the digital images were scored by a team of 7 observers, including 5 pathologists; all were blinded of clinical outcome. All 7 observers scored ER, PR, HER2, Ki67, p16 and p53 stains. A random sample of 20 DCIS specimens were scored by all observes to assess the inter-observer agreement. The concordance between the observers was around 97% with an interclass correlation coefficient (ICC) of >0.8 for ER, PR, HER2, and p53, and an ICC of 0.7 for p16. Ki67 was excluded for further scoring because of unreliable staining results. COX-2 stains were scored by two observers (LLV, CB). To assess the inter-observer agreement, a random sample of 20 DCIS specimens were scored by both observes. With this, COX-2 reached a concordance between the two observers of 94% with a *k* statistic of 0.7. Consequently, the remaining ER, PR, HER2, p16, and p53 stains were distributed over all observers, and the remaining COX-2 stains were distributed over two observers (LLV, CB), in which each patient was scored by one observer.

Immunohistochemical scoring criteria

Representative examples of each immunohistochemical marker and their corresponding staining categories can be found in Figure M1. For ER and PR, the percentage of luminal epithelial cells that showed staining of any intensity was assessed, and were considered positive when at least 10% of the cells nuclei showed staining. Similarly, p53 positive staining was assessed based on the percentage of nuclei that showed moderate to strong staining. p53 protein accumulation was considered when 70% or more nuclei showed moderate to strong staining.

HER2 overexpression was analyzed according to the American Society of Clinical Oncology and College of American Pathologists (ASCO-CAP) 2013 recommendations: HER2-positive staining was considered positive if circumferential membrane staining was complete, intense, and within more than 10% of tumor cells (HER2 3+), or if circumferential membrane staining was incomplete and/or weak/moderate and within more than 10% of tumor cells (HER2 2+) for which the overexpression could be confirmed by HER2 SISH. HER2 was considered negative when incomplete membrane staining was faint/barely visible and within >10% of the tumor cells (HER2 1+) or when no staining was observed (HER2 0).[13]

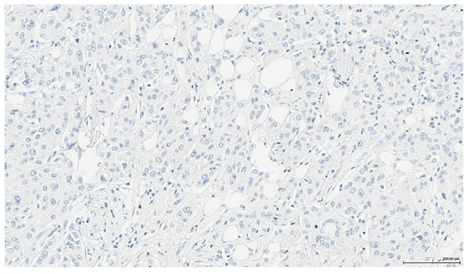
For the assessment of p16 a semi-quantitative approach was used in which H-scores were generated by multiplying the staining intensity (0 = no staining, 1 = weak, 2 = moderate, 3 = intense) by the percentage of positive cells (0-100%). H-scores below or equal to 100 were categorized as score 1, H-scores between 101-200 were categorized as score 2, H-scores between 201-300 were categorized as score 3. Score 1 was considered low p16 expression and score 2 and 3 were considered high p16 expression.

COX-2 expression was evaluated according to the following criteria (adapted from

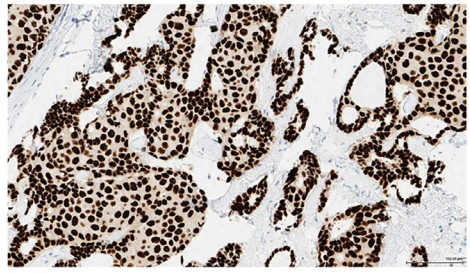
Ristimäki et al.[14]): 1 = weak diffuse cytoplasmic staining that may contain moderate to strong granular cytoplasmic staining in less than 10% of tumor cells; 2 = moderate to strong granular cytoplasmic staining in 10-90% of the tumor cells; 3 = moderate to strong granular cytoplasmic staining in over 90% of the tumor cells. Score 2 and 3 are considered high expression of COX-2 and score 1 low COX-2 expression.

Figure M1. Representative examples the immunohistochemical markers and their corresponding staining categories

ER

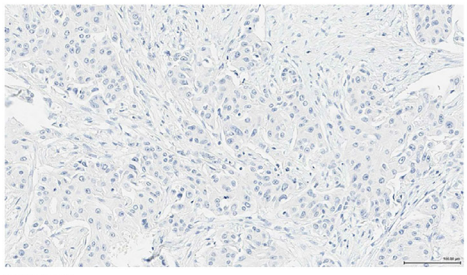


Negative

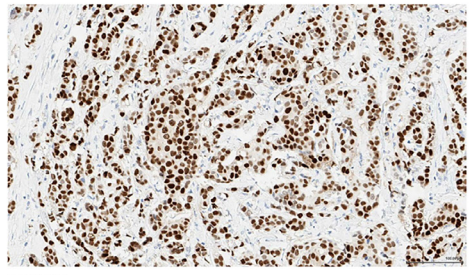


Positive

PR

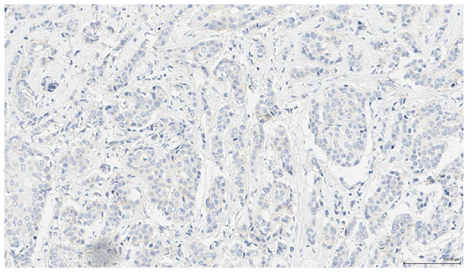


Negative

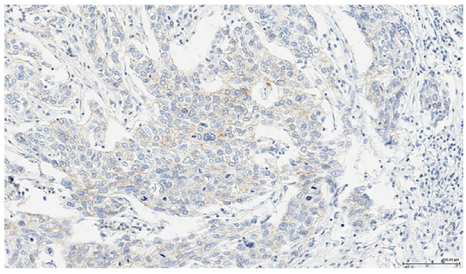


Positive

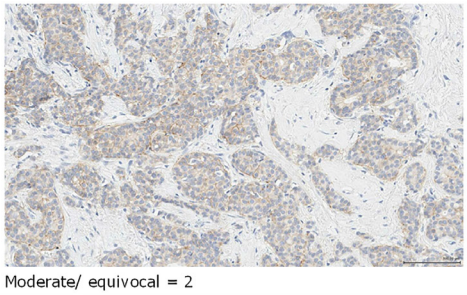
HER2



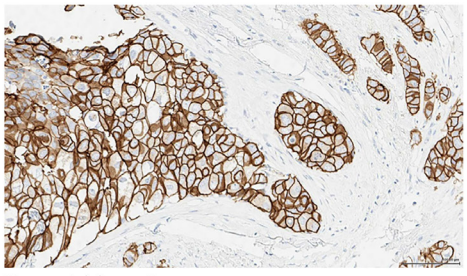
No staining = 0



Weak staining = 1

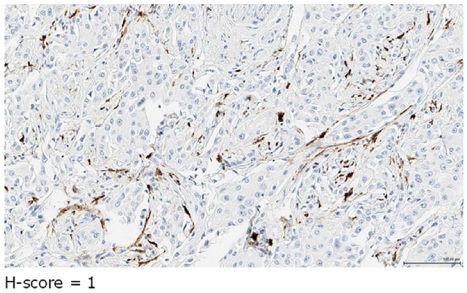


Moderate/ equivocal = 2

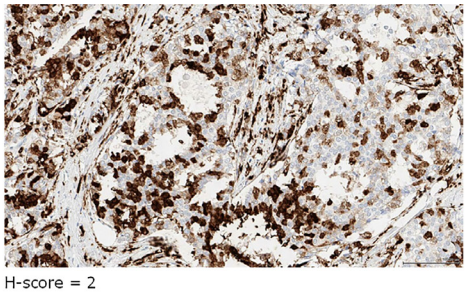


Strong staining = 3

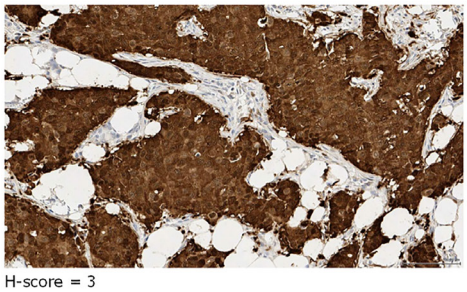
p16



H-score = 1

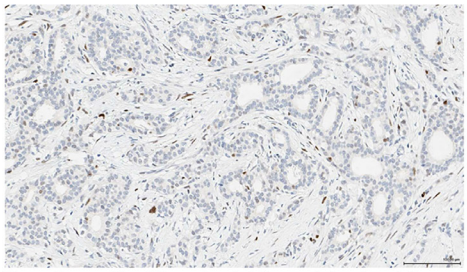


H-score = 2

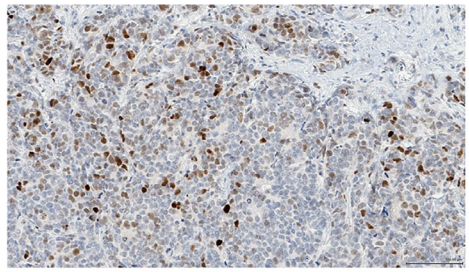


H-score = 3

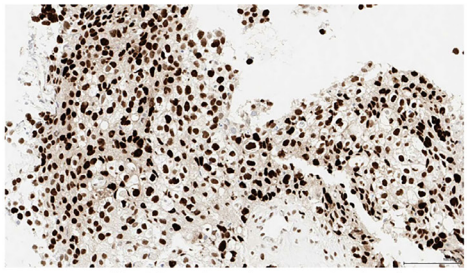
p53



Percentage cells with moderate/strong stained nuclei; 1-30%

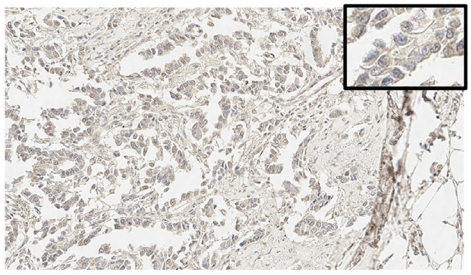


Percentage cells with moderate/strong stained nuclei; 30-70%

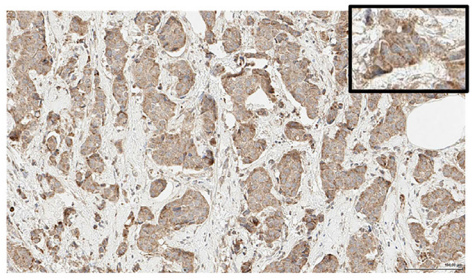


Percentage cells with moderate/strong stained nuclei; >70%

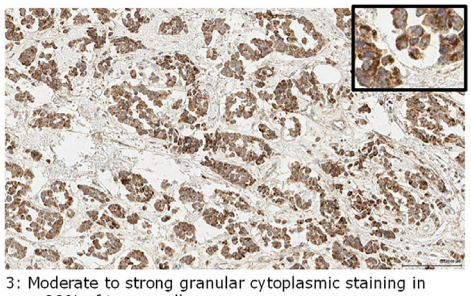
COX-2



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 tumor cells

Supplementary tables

Table S1. Distribution of cases and controls who were initially selected for the case-control study by final study status

A. Collected tissue specimens and pathology review

	Number of Cases		Number of Controls	
	No.	**%**	**No.**	**%**
Initially selected	**316**	**100.0**	**1303**	**100.0**
Excluded	**114**	**36.1**	**561**	**43.1**
- No tissue blocks available	61	19.3	388	29.8
- No DCIS component found	18	5.7	74	5.7
- (Micro)invasive component present	6	1.9	11	0.8
- LCIS present	9	2.8	31	2.4
- Papillary lesion other than DCIS	14	4.4	34	2.6
- Specimen not assessable	5	1.6	12	0.9
- Other reason	1	0.3	11	0.8
Administrative reason for exclusion	**2**	**0.6**	**268**	**20.6**
- Selected as case, controls excluded	2	0.6	-	-
- Selected as control, case excluded	-	-	268	20.6
Total excluded	**116**	**36.7**	**829**	**63.7**
Total included in study	**200**	**63.3**	**474**	**36.4**
Duplicate controls	**-**	**-**	**113**	**23.8**
Case as control	**-**	**-**	**33**	**7.0**
- Once	-	-	28	5.9
- Twice	-	-	5	1.1
Control	**-**	**-**	**72**	**15.2**
- Once	-	-	65	13.7
- Twice	-	-	6	1.3
- Three times	-	-	1	0.2
Total number of unique individuals in study	**200**	**100.0**	**361**	**76.2**

Table S1. Distribution of cases and controls who were initially selected for the case-control study by final study status

B. Immunohistochemistry (IHC) assessment

	Number of Cases		Number of Controls	
	No.	**%**	**No.**	**%**
Initially selected for IHC	**200**	**100.0**	**474**	**100.0**
Excluded	**12**	**6.0**	**26**	**5.5**
- Insufficient tissue available	12	6.0	26	5.5
Administrative reason for exclusion	**3**	**1.5**	**28**	**5.9**
- Selected as case, controls excluded	3	1.5	-	-
- Selected as control, case excluded	-	-	28	5.9
Total excluded	**15**	**7.5**	**54**	**11.4**
Total included in IHC analysis	**185**	**92.5**	**420**	**88.6**
Duplicate controls	**-**	**-**	**97**	**23.1**
Case as control	**-**	**-**	**27**	**6.4**
- Once	-	-	23	5.5
- Twice	-	-	4	1.0
Control	**-**	**-**	**61**	**14.5**
- Once	-	-	54	12.9
- Twice	-	-	6	1.4
- Three times	-	-	1	0.2
Total number of unique individuals in IHC analysis	**185**	**100**	**323**	**76.9**

Table S2. Clinical characteristics of 561 unique patients included in this study and of 669 patients not included in this case-control study.

Characteristics	Included, No. (%)		Not included, No. (%)		*P* [a]
Overall	561	(100.0)	669	(100.0)	
Patient group					
Cases	200	(35.7)	116	(17.3)	
Controls	361	(64.3)	553	(82.7)	<0.001
Age at DCIS diagnosis, mean (range)	58	(30 - 89)	59	(30 - 89)	0.008
Age at DCIS diagnosis (years)					
<40	33	(5.9)	29	(4.33)	
40-49	74	(13.2)	108	(16.1)	
50-59	225	(40.1)	210	(31.4)	
60-69	160	(28.5)	178	(26.6)	
70-79	52	(9.3)	105	(15.7)	
≥80	17	(3.0)	39	(5.8)	<0.001
Year of DCIS diagnosis, mean (range)	1996	(1989 - 2004)	1997	(1989 - 2004)	0.17
Period of DCIS diagnosis					
1989-1998 (screening implementation phase)	393	(70.1)	441	(65.9)	
1999-2004 (full nationwide coverage)	168	(29.9)	228	(34.1)	0.12
Clinical presentation of DCIS					
Screen-detected	282	(50.3)	273	(40.8)	
Non-screening-related	230	(41.0)	332	(49.6)	0.001
Unknown	49	(8.73)	64	(9.57)	

[a]: For continuous variables, the p-value was calculated by unpaired T-test, and for categorical variables the p-value was calculated by chi-square test; Unknown clinical presentation was not included in the analysis.

Table S3. Comparison of the 316 cases and 914 controls (unique) initially selected for the case-control study with the unique cases and controls included in this study

Cases					
Characteristics	**Initial selection** (*n* = 316)		**Included in study** (*n* = 200)		
	n	(%)	n	(%)	***P*** [a]
Age at DCIS diagnosis (years)					
<40	16	(5.1)	14	(7.0)	
40-49	49	(15.5)	27	(13.5)	
50-59	109	(34.5)	79	(39.5)	
60-69	85	(26.9)	55	(27.5)	
70-79	41	(13.0)	19	(9.5)	
≥80	16	(5.1)	6	(3.0)	0.48
Year of DCIS diagnosis, mean (range)	1996	(1989 - 2004)	1996	(1989 - 2004)	0.88
Period of DCIS diagnosis					
1989-1998 (implementation phase)	234	(74.1)	147	(73.5)	
1999-2004 (full nationwide coverage)	82	(25.9)	53	(26.5)	0.92
Clinical presentation of DCIS					
Screen-detected	135	(42.7)	96	(48.0)	
Non-screening-related	155	(49.1)	89	(44.5)	0.23
Unknown	26	(8.2)	15	(7.5)	

Controls					
Characteristics	**Initial selection** (*n* = 914)		**Included in study** (*n* = 361)		
	n	(%)	n	(%)	***P*** [a]
Age at DCIS diagnosis (years)					
<40	46	(5.0)	19	(5.3)	
40-49	133	(14.6)	47	(13.0)	
50-59	326	(35.7)	146	(40.4)	
60-69	253	(27.7)	105	(29.1)	
70-79	116	(12.7)	33	(9.1)	
≥80	40	(4.4)	11	(3.0)	0.30
Year of DCIS diagnosis, mean (range)	1997	(1989 - 2004)	1997	(1989 - 2004)	0.53
Period of DCIS diagnosis					
1989-1998 (implementation phase)	600	(65.6)	246	(68.1)	
1999-2004 (full nationwide coverage)	314	(34.4)	115	(31.9)	0.41
Clinical presentation of DCIS					
Screen-detected	420	(46.0)	186	(51.5)	
Non-screening-related	407	(44.5)	141	(39.1)	0.07
Unknown	87	(9.5)	34	(9.4)	

[a]: For continuous variables, the p-value was calculated by paired T-test, and for categorical variables the p-value was calculated by chi-square test; Unknown clinical presentation was not included in the analysis.

Table S4. Multivariable analysis of histopathological characteristics and immunohistochemical markers independently associated with subsequent invasive disease

	DCIS cases		DCIS controls			
	n	(%)	n	(%)	**OR (95% CI)** [a]	***P*** [b]
Periductal fibrosis/COX-2						
Absent/Low	11	(5.9)	86	(20.5)	1.00 (reference)	
Present/Low	8	(4.3)	20	(4.8)	3.26 (1.13-9.42)	
Absent/High	102	(55.1)	213	(50.7)	3.82 (1.94-7.51)	
Present/High	59	(31.9)	92	(21.9)	4.87 (2.37-9.99)	<0.001
N/A	5	(2.7)	9	(2.1)		
Necrosis/COX-2						
Absent/Low	3	(1.6)	39	(9.3)	1.00 (reference)	
Present/Low	16	(8.6)	67	(16.0)	2.34 (0.63-8.66)	
Absent/High	37	(20.0)	76	(18.1)	5.07 (1.48-17.33)	
Present/High	124	(67.0)	230	(54.8)	5.76 (1.75-18.95)	0.001
N/A	5	(2.7)	8	(1.9)		
Periductal fibrosis/HER2						
Absent/Negative	89	(48.1)	244	(58.1)	1.00 (reference)	
Present/Negative	31	(16.8)	65	(15.5)	1.25 (0.75-2.09)	
Absent/Positive	24	(13.0)	54	(12.9)	1.26 (0.74-2.17)	
Present/Positive	38	(20.5)	50	(11.9)	2.04 (1.23-3.38)	0.050
N/A	3	(1.6)	7	(1.7)		
Necrosis/HER2						
Absent/Negative	35	(18.9)	107	(25.5)	1.00 (reference)	
Present/Negative	85	(45.9)	203	(48.3)	1.26 (0.80-1.99)	
Absent/Positive	5	(2.7)	9	(2.1)	1.72 (0.52-5.63)	
Present/Positive	57	(30.8)	95	(22.6)	1.84 (1.10-3.09)	0.12
N/A	3	(1.6)	6	(1.4)		
Periductal fibrosis/Necrosis/COX-2						
All other groupings	19	(10.3)	106	(25.2)	1.00 (reference)	
Negative/Negative/High	28	(15.1)	66	(15.7)	2.39 (1.21-4.71)	
Positive/Negative/High	9	(4.9)	10	(2.4)	4.60 (1.62-13.03)	
Negative/Positive/High	74	(40.0)	147	(35.0)	2.90 (1.62-5.21)	
Positive/Positive/High	50	(27.0)	82	(19.5)	3.39 (1.81-6.34)	0.002
N/A	5	(2.7)	9	(2.1)		
Fibrosis/Necrosis/HER2						
All other groupings	40	(21.6)	116	(27.6)	1.00 (reference)	
Negative/Positive/Negative	62	(33.5)	149	(35.5)	1.19 (0.74-1.91)	
Positive/Positive/Negative	23	(12.4)	53	(12.6)	1.15 (0.62-2.12)	
Negative/Positive/Positive	21	(11.4)	45	(10.7)	1.39 (0.73-2.66)	
Positive/Positive/Positive	36	(19.5)	50	(11.9)	2.02 (1.14-3.56)	0.18
N/A	3	(1.6)	7	(1.7)		

(Continues on next page)

Table S4. Multivariable analysis of histopathological characteristics and immunohistochemical markers independently associated with subsequent invasive disease

	DCIS cases		DCIS controls			
	n	(%)	n	(%)	**OR (95% CI)** [a]	***P*** [b]
Necrosis/HER2/COX-2						
All other groupings	13	(7.0)	65	(15.5)	1.00 (reference)	
Positive/Negative/Low	11	(5.9)	43	(10.2)	1.14 (0.45-2.89)	
Negative/Negative/High	32	(17.3)	71	(16.9)	2.12 (1.00-4.50)	
Positive/Negative/High	73	(39.5)	156	(37.1)	2.23 (1.13-4.41)	
Positive/Positive/High	50	(27.0)	72	(17.1)	3.43 (1.66-7.08)	0.004
N/A	6	(3.2)	13	(3.1)		

[a]: Comparisons between DCIS cases and DCIS controls were made by multivariate conditional logistic regression; [b]: P-values for overall effect were calculated by Wald-test.
N/A: Not assessable. N/As were not included in the analysis.
LR chi2 corrected for degrees of freedom were 6.34 for periductal fibrosis/COX-2, 5.18 for necrosis/COX-2, 2.60 for periductal fibrosis/HER2, 1.95 for necrosis/HER2, 4.39 for periductal fibrosis/necrosis/COX-2, 1.57 for periductal fibrosis/necrosis/HER2, and 3.83 for necrosis/HER2/COX-2.