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European Journal of Cancer 39 (2003) 622–630

European Journal of Cancer

www.ejconline.com

Biological markers that predict clinical recurrence in ductal carcinoma *in situ* of the breast

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Received 9 May 2002; received in revised form 12 September 2002; accepted 10 October 2002

Abstract

The optimal management of ductal carcinoma *in situ* (DCIS) is controversial, due in part to our poor understanding of its natural history. We undertook to identify subgroups of DCIS based on the expression of biomarkers, which were related to the likelihood of clinical recurrence. Biomarker expression of a total of 95 DCIS lesions in a nested case–control study within a population-based cohort with up to 135 months follow-up data (median 101 months) was analysed using immunohistochemistry. ERBB2-positivity and bcl-2-, oestrogen receptor (ER)- and progesterone receptor (PR)-negativity were individually associated with the risk of clinical recurrence. The predictive value of these biomarkers was independent of cytonuclear grade. ERBB2, bcl-2, ER and PR expression were conserved in the recurrent lesions, including subsequent invasive cancers. p21-positive DCIS was also associated with clinical recurrence, independently of the associations with ERBB2/bcl-2/ER/PR expression. These data identify clinically and biologically relevant subcategories of DCIS lesions, an essential basis for improving management.

Keywords: Ductal carcinoma *in situ*; Breast cancer; ERBB2; Bcl-2; Oestrogen receptor

1. Introduction

Ductal carcinoma *in situ* (DCIS) of the breast is the proliferation of epithelial cells with all the morphological features of malignancy, but without stromal invasion [1]. Previously a rare entity accounting for $<5\%$ of breast cancers, DCIS has dramatically increased in incidence since the advent of screening mammography, now representing 15–20% of all breast cancers and up to 40% of screen-detected cancers [2].

Historically, DCIS was treated with mastectomy, leading to prevention of recurrence of DCIS and invasive cancer in almost all cases [3]. However, with the increased utilisation of local therapy for invasive cancer, it has become difficult to justify mastectomy for a pre-invasive condition that should be curable with adequate local excision. Notwithstanding, long-term series show that up to 40% of women treated with local therapy (excision ± radiotherapy) will develop recurrent disease in the ipsilateral breast [3,4]. Approximately half of these recurrences will be as invasive cancer, with the concomitant risk of metastasis and death [3,5]. Several morphological factors have been shown to predict recurrence [6–9]. However, many of these factors, including high morphological grade, are not sensitive markers of recurrence and inter-observer variability

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0959-8049/03/$ - see front matter
PII: S0959-8049(02)00666-4

affects the reproducibility of a purely morphological approach to disease stratification [10–12]. Furthermore, morphological analysis alone does not take into account the key molecular drivers of the neoplastic process. Currently therefore, we have no way of reliably identifying those patients at increased risk of developing recurrent disease and there is a need to identify biological markers which predict disease progression.

In contrast to invasive cancer, there are few publications on the role of biological markers in predicting prognosis in DCIS [13–16]. Two of the four published studies showed no relationship between expression of biological markers detected by immunohistochemistry and disease recurrence [13,14], but these studies had very limited power since there were only small numbers of recurrences (8 out of 36 patients [13], and 3 out of 81 patients [14]). The third study showed bcl-2, p53 and Ki-67 immunostaining were predictive of a shorter time interval to ipsilateral recurrence, with no effect attributable to ERBB2 expression status [15]. A fourth study showed strong p53 staining was common in DCIS cases which recurred, but again only small numbers of DCIS lesions with known recurrence were examined (five DCIS lesions of 8 cases with recurrence were p53-positive) and the ERBB2 status was not assessed [16].

We have evaluated the potential role of biological markers in predicting disease outcome in DCIS, using a nested case–control study taken from a population-based cohort with long-term follow-up.

2. Patients and methods

2.1. Subject selection

The *In Situ* and Small Invasive Breast Cancer Register (ISSIBCR) was established to examine the natural history of DCIS and invasive breast cancers less than 1 cm in size [17]. Reporting of all *in situ* and invasive cancers to the Victorian Cancer Registry is mandatory. The registry contains 457 women with DCIS reported from 1988 to 1992, 62% of which were known to have had breast conserving surgery. These subjects were followed-up for a maximum of 135 months (range 35–135 months; median 101 months), with regard to initial treatment, and the event and nature of recurrence, as described by Giles and colleagues [17]. The study was performed under appropriate institutional ethical guidelines and approval.

Recurrent disease was defined as a lesion occurring more than 3 months after the initial surgery, and regarded by the treating surgeon as being a separate disease episode. We have identified 53 women with DCIS from the ISSIBCR study managed by breast conserving therapy (wide local excision or subtotal mastectomy) with or without adjuvant radiotherapy and/or hormonal therapy, who have subsequently suffered an ipsilateral recurrence as *in situ* or invasive cancer (i.e. cases). A nested case–control study was designed in which controls were selected from the remainder of the cohort, who had not experienced a recurrence throughout follow-up, which was at least an equivalent period of follow-up to the cases. Controls were matched for age (control age at diagnosis within 3 years of the case) and date of diagnosis (control date of diagnosis within 6 months of the case). Using a risk set sampling model, some controls were matched to more than one case [18] and a total of 42 controls were used. A subset analysis was performed on the nested cohort, which attempted to distinguish the immunohistochemical features of the primary DCIS lesions dependent on the type of recurrent disease (i.e. DCIS versus invasive recurrence). Three cases had recurrence as metachronous ipsilateral DCIS and invasive disease. For the purposes of the subset analysis, these cases were classified as invasive recurrence cases. 2 cases presented with axillary metastases alone with a presumed occult breast primary and were analysed with the other invasive recurrences. In 2 cases, the recurrence was diagnosed on clinical grounds and so these cases were not included in the subset analysis.

A detailed histological review of each primary DCIS lesion was performed (unpublished data). The recurrent lesions were also recalled and scored using the same criteria. Nuclear grade was assessed using the criteria of Holland and colleagues [19].

2.2. Immunohistochemical studies

A panel of nine antibodies was used on a DAKO Autostainer (DAKO, Carpinteria, CA, USA), with or without antigen retrieval. The detailed protocol has been described elsewhere [20]. The wash steps used 50 mM Tris–HCl (pH 7.6) and 0.05% Tween 20. Sections were incubated in primary antibody for 30 min at room temperature and then incubated with biotinylated secondary antibody, followed by peroxidase-conjugate Streptavidin using DAKO LSAB2 or LSAB+ kits (DAKO, Carpinteria, CA, USA). Staining was visualised using 3-amino-9-ethyl-carbimazole, washed in water, and counterstained with haematoxylin.

Immunohistochemical staining was scored as appropriate for each antibody and either nuclear, cytoplasmic or membranous staining was assessed, depending on the known location of the specific antigen of interest. The scoring pathologists were blinded to the case/control status. The hormone receptors (androgen receptor (AR); oestrogen receptor (ER) and progesterone receptor (PR)) were assigned as positive, based on the commonly used clinical cut-off points of $\geqslant 10\%$ of nuclei staining, regardless of the intensity. For all other

antibodies, a previously reported combination score of the intensity of staining and proportion of positive cells was determined [20]. The proportion score represented the estimated fraction of positive staining cells (0: less than or equal to 10%; 1: 11–25%; 2: 26–50%; 3: 51–75%; 4: 76–90%; 5: equal to or greater than 91%). The intensity score represented the estimated average staining intensity of positive cells (0: none; 1: weak; 2: moderate; 3: strong). Samples with intensity scores of 0 or 1 were designated as negative to weak expression. For intensity scores of 2 and 3, a combined score was derived, by adding the intensity and proportion scores. Combined scores of 2 and 3 were designated as negative to weak expression; 4–6 as moderate expression and 7 or 8 as strong expression. Moderate and strong staining was regarded as positive for all antibodies except ERBB2. For ERBB2, only strong staining was regarded as positive, as this equates better to a score of 3+ using the scoring recommended for use with the DAKO HercepTest (DAKO, Carpinteria, CA, USA). ERBB2 positivity thus defined correlated well with *ERBB2* gene amplification detected by fluorescence *in situ* hybridisation (FISH) on a subset of slides from the same cohort and in a separate series of invasive breast cancers evaluated previously in our laboratory (data not shown). Appropriate positive and negative control slides were used with each antibody run.

2.3. *Fluorescence* in situ *hybridisation*

FISH was performed on paraffin-embedded, formalin-fixed slides, using an *ERBB2* commercial probe, the PathVysion HER-2 DNA probe kit (Vysis Inc., Downer's Grove, IL, USA), essentially as per the manufacturer's instructions. The slides were examined using a Zeiss Axioplan 2 epiflourescent microscope with the correct filters. All areas of DCIS on the slide were examined. The *ERBB2* signal was considered amplified when the ratio of orange to green dots was greater than 2 to 1.

2.4. *Statistical methods*

Statistical analysis was performed using STATA software. The degree of association between binary factors listed in Table 1 were assessed by odds ratios. For analyses of individual factors on recurrence, Mantel–Haenszel odds ratios (ORs) were calculated for dichotomous explanatory variables taking into account the matching. Nominal P values were calculated using the exact McNemar test. Conditional multiple linear logistic regression was used to model the concurrent effects of categorical and continuous variables. Nominal P values are presented, and the issue of multiple testing is addressed in the Discussion.

3. Results

A total of 95 subjects with DCIS were used for this study; 53 cases and 42 controls. The median length of follow-up was 101 months (range 35–135 months). 28 of the cases (53%) had an ipsilateral recurrence as DCIS and 23 (43%) had ipsilateral recurrence as invasive cancer, while a further two had recurrent disease diagnosed clinically, with no pathology to confirm type of recurrence. A further case had ipsilateral recurrent DCIS followed by ipsilateral invasive cancer. The status of lymph node metastases was known for 10 of the ipsilateral invasive cancers, and six of these were positive for metastasis. Four of the cases with invasive recurrence went on to die of their disease. The median time to recurrence was 21.8 months for DCIS (range 6.7–61.6 months), and 35.6 months for invasive cancer (range 7.4–101.4 months) (P for difference = 0.09).

Information on the method of lesion detection was available for all, but one, subject. 64 of the 94 subjects (68%) had their lesions detected by mammographical screening (60% of cases and 77% of controls; OR = 2.2, $P = 0.09$). The subjects were treated with partial mastectomy or wide local excision. Partial mastectomy was slightly more common in cases than controls (49% versus 36%, respectively; $P = 0.25$). Due to the retrospective nature of the cohort, margin status was difficult to assess on histological review by the authors. Margin status was recorded in the pathology report for 64 of the 95 subjects (62% of cases and 74% of controls), and was given as a numerical value in only 6 subjects. When margin status was divided into two categories, 42% (14/33) of cases and 49% (19/39) of controls were reported to have 'clear' margins or $\geqslant 2$ mm excision (compared with involved/close/< 2 mm; $P = 0.9$ [21–23]). In the 92 subjects where information on adjuvant therapy was available, 23 subjects received adjuvant therapy at the time of diagnosis of their primary DCIS. 10 subjects (8% of cases and 11% of controls) received radiotherapy, and 14 subjects (16% of cases and 11% of controls) received hormonal therapy. One control received hormonal therapy and radiotherapy. There was no difference in the adjuvant treatments received by the cases and controls ($P = 0.9$). These results are from the matched analysis as detailed below. The age at time of diagnosis for the study cohort ranged from 34 to 88 years.

3.1. *Correlation of staining of biological markers and nuclear grade*

An attempt was made to assess all 95 available cases and controls with the nine antibodies. However, due to several specimen archive and technical reasons, not all were available for immunohistochemical analysis, resulting in a total of 44 matched pairs.

Table 1
Proportion of positive subjects and association of expression of antibodies in all the primary DCIS lesions (cases and controls)

	Number positive	%	High grade	AR	bcl2	Cathepsin D	ERBB2	ER	p21	p53	PR
High grade	71/95	75									
AR	64/79	81	0.39 (0.08–1.90)								
bcl-2	39/78	50	0.55 (0.18–1.68)	1.79 (0.60–5.29)							
Cathepsin D	44/81	54	2.46 (0.88–6.85)	0.50 (0.16–1.58)	1.47 (0.61–3.58)						
ERBB2	27/84	32	**14.18** (1.80–111.8)**	0.59 (0.20–1.78)	**0.08*** (0.02–0.25)**	**3.75* (1.32–10.65)**					
ER	39/80	49	0.36 (0.12–1.07)	**6.17** (1.61–23.56)**	**17.29*** (5.76–51.91)**	1.02 (0.43–2.42)	**0.06*** (0.02–0.23)**				
P21	23/67	34	0.86 (0.27–2.71)	4.67 (0.96–22.74)	1.57 (0.58–4.23)	**3.06* (1.07–8.74)**	1.23 (0.43–3.54)	1.39 (0.52–3.70)			
P53	45/75	60	1.11 (0.37–3.31)	1.57 (0.53–4.67)	1.06 (0.42–2.68)	**3.64** (1.40–9.51)**	1.17 (0.44–3.12)	1.10 (0.44–2.74)	1.92 (0.66–5.59)		
PR	39/81	48	0.59 (0.21–1.65)	**5.95** (1.56–22.71)**	**4.58** (1.79–11.74)**	0.54 (0.22–1.29)	**0.07*** (0.02–0.25)**	**11.37*** (4.08–31.70)**	0.56 (0.21–1.51)	0.67 (0.27–1.70)	
PS2	29/76	38	0.72 (0.25–2.08)	0.88 (0.30–2.62)	1.80 (0.72–4.53)	1.06 (0.42–2.64)	0.60 (0.22–1.62)	**2.87* (1.13–7.30)**	0.74 (0.27–2.04)	0.96 (0.37–2.51)	2.01 (0.81–5.00)

95% CI, 95% Confidence Interval; ER, oestrogen receptor; PR, progesterone receptor; AR, androgen receptor. The odds ratio (OR) is given with the 95% CI included in parentheses. ORs at the $P < 0.05$ level are given in bold print. *indicates $0.01 < P \leqslant 0.05$, **$0.001 < P \leqslant 0.01$, ***$P \leqslant 0.001$.

The proportion of all primary DCIS lesions (cases and controls) positive for each antibody is shown in Table 1, together with the degree of association between the different antibody scores for individual primary lesions and nuclear grade. There was no difference in cytonuclear grade between cases and controls (OR = 1.11, 95% Confidence Interval (CI) 0.42–2.89; $P = 0.9$).

ERBB2 positivity was seen in 32% of the primary lesions. Importantly, our determination of ERBB2 immunohistochemical positivity was based on gene amplification status by FISH in a subset of lesions in the current cohort and as previously determined by our laboratory (data not shown). Thus, in the current cohort, 9 of 10 cases which we scored as positive by immunohistochemistry showed gene amplification by FISH. Only 1 of 10 cases with moderate immunohistochemical staining showed gene amplification and neither of the 2 cases scored weak/negative by immunohistochemistry had gene amplification. ERBB2 showed a positive association with high nuclear grade and cathepsin D positivity and a negative association with bcl2. p21 and p53 expression were also positively associated with cathepsin D.

ER, PR and AR positivity were seen in 49, 48 and 81% of primary DCIS lesions, respectively. All three steroid hormone receptors were positively associated with each other. Positive staining for bcl2 was seen in 50% of the primary DCIS lesions. ER and PR were both positively associated with bcl2 expression. ER was also positively associated with pS2.

3.2. Biological markers as a predictor of recurrence

Table 2 shows the cytonuclear and immunohistochemistry results for all cases and controls. Five of the

Table 2
Immunohistochemistry results for each antibody when cases were compared with controls, where controls did not develop a recurrence

	All recurrences				Unadjusted			Adjusted for high grade		
	Case	%	Control	%	OR		*P* value	OR		*P* value
Grade										
High	34/44	77	32/44	73	1.3	0.5–3.5	0.6	N/A		
Non-high	10/44	23	12/44	27						
AR										
Positive	30/36	83	28/36	78	1.5	0.4–7.2	0.8	1.5	0.4–5.5	0.5
Negative	6/36	17	8/36	22						
bcl-2										
Positive	12/35	34	26/35	74	0.2	0.1–0.6	**0.003**	0.2	0.1–0.6	**0.005**
Negative	23/35	66	9/35	26						
Cathepsin D										
Positive	21/38	55	19/38	50	1.2	0.5–2.9	0.8	1.2	0.8	
Negative	17/38	45	19/38	50						
ERBB2										
Positive	17/41	41	5/41	12	5	1.4–26.9	**0.008**	5	1.4–17.5	**0.01**
Negative	24/41	59	36/41	88						
ER										
Positive	14/37	38	24/37	65	0.2	0.1–0.8	**0.01**	0.2	0.1–0.8	**0.02**
Negative	23/37	62	13/37	35						
p21										
Positive	14/26	54	4/26	15	6.0	1.3–55.2	**0.01**	9.3	1.3–68.5	**0.03**
Negative	12/26	46	22/26	85						
p53										
Positive	19/34	56	19/34	56	1	0.4–2.8	1	1	0.4–2.5	1
Negative	15/34	44	15/34	44						
PR										
Positive	14/38	37	25/38	66	0.4	0.1–1.0	**0.04**	0.4	0.2–0.9	**0.03**
Negative	24/38	63	13/38	34						
pS2										
Positive	13/33	39	12/33	36	1.2	0.3–4.2	1	1.2	0.4–3.5	0.8
Negative	20/33	61	21/33	64						

Mantel–Haenszel odds ratios are given, unadjusted and adjusted for high nuclear grade, with 95% CI given in the following column. *P* values were calculated using the exact McNemar test with results of $P \leqslant 0.05$ given in bold print.

nine antibodies analysed showed differential staining between the cases and controls. Bcl2 positivity was less common in cases than in controls (OR = 0.18, $P = 0.003$), as was ER (OR = 0.2, $P = 0.01$) and PR (OR = 0.2, $P = 0.04$). p21 nuclear positivity was more common in cases than controls (OR = 6.0, $P = 0.01$). ERBB2 antibody staining was successful in 41 matched cases and controls, of which 17 cases were ERBB2-positive, compared with 5 control subjects (OR = 5.0, $P = 0.008$). All other antibodies showed no differences between cases and controls. When adjusted for cyto-nuclear grade, there was no reduction in the odds ratio estimates (Table 2).

Multiple conditional logistic regression analysis was performed to determine whether one or more of these proteins had a dominant effect in determining the risk of recurrence in DCIS. When combined with each of the other proteins (ER, ERBB2, bcl2, PR), the odds ratio for recurrence with p21 positivity showed minimal change (OR range 4.31–6.54) and p21 remained an independent predictor for recurrence ($P = 0.01$–0.02). That is, p21 positivity was associated with recurrence independently of the other proteins. However, when any of ER, ERBB2, bcl2 or PR were combined with one another, the odds ratio for recurrence of both proteins moved towards 1 and were no longer nominally significant. For example, when ERBB2 and ER were combined, the odds ratio for recurrence with ERBB2 positivity changed from 5 to 3.1, while the odds ratio with ER changed from 0.2 to 0.3 ($P = 0.10$ for both). That is, it was not possible to differentiate between this group of proteins in terms of identifying a single best predictor of recurrence. Given the correlations between these proteins shown in Table 1, it would appear that they may be indicators of factors operating on the same disease pathway.

A subset analysis was performed in order to determine whether the immunohistochemical profile of the primary DCIS lesion could be associated with recurrence as either DCIS or invasive disease. p21 positivity was shown to be more common in cases which recurred as DCIS lesions compared with invasive lesions (9/14 p21-positive cases had recurrence as further DCIS; OR for recurrence as DCIS >10, $P = 0.03$, versus OR for recurrence as invasive cancer 2.5, $P = 0.45$). ERBB2, ER and bcl-2 were associated with any recurrence, but not with recurrence as either DCIS or invasive disease. Immunohistochemical staining with all antibodies was also evaluated in the normal breast tissue adjacent to the primary DCIS lesion and showed no association with the incidence of recurrence.

3.3. Conservation of antigen expression between primary and recurrent lesions

A comparison of the staining results for primary lesions and their recurrences was performed. Not all 48 recurrent DCIS and invasive lesions could be examined immunohistochemically for all antibodies due to technical difficulties, including cutting archived lesions out of the available block. ERBB2 positivity was seen in 36% (9/25) of DCIS recurrences and 50% (10/20) of invasive recurrences. 63% (10/16) of the DCIS lesions associated with invasive cancer were also positive for ERBB2. The nodal metastases were positive in 63% (5/8) of cases. ERBB2 status was conserved between the primary DCIS lesion and 80% (20/25) of DCIS recurrences and 79% (15/19) of the invasive recurrences (Table 3). In the 5 discordant cases for invasive cancers, including 1 case with microinvasive cancer, ERBB2 staining changed from negative to positive in 4 cases. ERBB2 expression was identical to the primary DCIS in 73% (11/15) of DCIS lesions adjacent to an invasive recurrence. ERBB2 staining was preserved in 75% (6/8) of metastatic cancers compared with the original DCIS lesion.

Our data indicate that bcl-2 staining is more commonly negative in cases compared with controls. Bcl-2 negativity was seen in 38% (10/26) of recurrent DCIS lesions, and 63% (12/19) of invasive recurrences. Bcl-2 negativity in metastases was 88% (7/8). The DCIS component of invasive recurrences was negative in 9/17 (53%) cases. Bcl-2 expression in the primary and recurrence was conserved in 85% (22/26) of DCIS recurrences, 71% (12/17) of invasive recurrences and 88% (7/8) of axillary node metastases (Table 3). When discordant cases were examined, there was no obvious trend for loss of bcl-2 positivity with progression of the disease.

As detailed above, ER negativity was more common in cases compared with controls. 54% (14/26) of recurrent DCIS lesions and 42% (8/19) of invasive recurrences were ER-negative. The DCIS component adjacent to the recurrent invasive lesions was ER-negative in 41% (7/17) of cases. The metastases were ER-negative in 63% (5/8) of cases. The ER status was conserved between the primary DCIS in 85% (22/26) of

Table 3
Comparison of antibody expression in primary lesions and their recurrences

	DCIS recurrence		Invasive recurrence					
			DCIS component		Invasive component		Metastases	
bcl-2	22/26	85%	10/15	67%	12/17	71%	7/8	88%
ERBB2	20/25	80%	11/15	73%	15/19	79%	6/8	75%
ER	22/26	85%	13/15	87%	12/17	71%	6/8	75%
PR	20/25	80%	11/13	85%	8/15	53%	5/7	71%

ER, oestrogen receptor; PR, progesterone receptor; DCIS, ductal carcinoma *in situ*.
The fractions represent the proportion of recurrences with the same antibody expression as the primary DCIS lesion. The DCIS and invasive components of invasive recurrences were evaluated separately.

DCIS recurrences and 71% (12/17) invasive recurrences (Table 3). The DCIS adjacent to the invasive recurrence was identical in 93% of cases (14/15 cases available for assessment). ER expression was conserved between primary DCIS and metastases in 75% (6/8) of the metastatic lesions.

As with ER, PR negativity was more common in cases than controls. 60% (15/25) of DCIS recurrences were negative for PR, with 80% (20/25) having the same PR expression as the primary DCIS (Table 3). 56% (10/18) of invasive recurrences were PR-negative, with 53% (8/15) having the same PR expression pattern as the primary DCIS. This was the lowest level of agreement for all of the antibodies tested. In contrast, PR was negative in the DCIS component associated with recurrent invasive cancer in 6/15 (40%) cases, with 85% (11/13) concordance with the primary DCIS lesions. The DCIS adjacent to the invasive recurrence was identical to the invasive component in 92% of cases (11/12 cases available for assessment).

4. Discussion

In an effort to improve our understanding of the fundamental molecular drivers of DCIS, with a view to thereby improving stratification as a prelude to advancing management of this disease, we have evaluated biomarkers in primary DCIS lesions and recurrent disease. Only four previous studies have directly examined expression of biomarkers in DCIS and recurrence [13–16] and in all but one study [15] the numbers of recurrent cases analysed have been very few. In contrast to our own study, neither of the two studies which examined ERBB2 status found ERBB2 positivity to significantly increase the relative risk of clinical recurrence [13,15]. However, unlike these other studies, we used a stringent cut-off level for ERBB2 positivity, which we correlated to *ERBB2* gene amplification status by FISH. Hence, it is likely that only high-level ERBB2 overexpression due to gene amplification, as assessed in the current study, is able to predict recurrence. This suggests that, as with invasive cancer, *ERBB2* gene amplification as the mechanism of protein overexpression may confer biologically aggressive disease. 32% of the primary DCIS lesions in our study were positive for ERBB2. This is less than the 46–80% positivity reported in the literature [24–26], which describes studies that did not attempt to standardise ERBB2 protein expression with gene amplification.

Morphological high-grade DCIS is related to poor disease outcome [6,7,9,15]. Although ERBB2 positivity has not been previously implicated directly with increased risk of disease progression, previous studies have shown that ERBB2 and high morphological grade are associated [24,26]. We found little change in the odds ratio of ERBB2 when combined with nuclear grade in a bivariate analysis, indicating that ERBB2 positivity is not dependent on high nuclear grade. Thus, it seems that ERBB2 positivity, when related to gene amplification, is a fundamental characteristic of DCIS associated with an increased risk of clinical recurrence.

50% of the recurrent invasive cancers with preceding DCIS in our series showed ERBB2 positivity. Although our numbers are small (10/20 recurrent invasive cancers), this proportion is higher than the 10–30% rate previously reported for unselected invasive breast cancers [27–29] and in our own series of invasive breast cancers unselected for DCIS status taken from the general Australian population (21% ERBB2-positive, unpublished data). In keeping with this observation, others have described a higher incidence of ERBB2 positivity in invasive cancers with an adjacent DCIS component compared with invasive cancers without DCIS (22% versus 11%, respectively [27]). It has been proposed that ERBB2 overexpression is a fundamental abnormality of a subset of invasive cancers, whose mode of development includes a prolonged *in situ* phase [28] and our data supports this proposal. These *in situ* cancers retain ERBB2 overexpression through to invasive cancer.

We have shown that bcl-2-, ER- and PR-negativity in primary DCIS lesions are associated with clinical recurrence. A similar finding for bcl-2 was detected by Ringberg and colleagues [15]. Additionally, in our bivariate analysis with cytonuclear grade, the odds ratios of bcl-2, ER and PR in predicting recurrent disease were little changed suggesting, as with ERBB2, that these biomarkers are not dependent on cytonuclear grade and are likely to be fundamental factors in DCIS progression. In the present study, we were able to determine the association of biomarker expression in individual lesions. Thus, we found that ERBB2 positivity and ER-, PR- and bcl-2-negativity were associated with each other and that these proteins were interdependent as predictors of recurrence in DCIS. This may reflect the different biological pathways in which ERBB2, ER, PR and bcl-2 are believed to function [30]. p21 overexpression was also shown to be strongly associated with recurrent disease, particularly when recurring as DCIS. There are few studies in the literature analysing the role of p21 expression in DCIS, and these show conflicting results as to the association of p21 with nuclear grade and ER status [31,32]. p21 expression has not been previously directly related to DCIS outcome, and the role of p21 in invasive cancer is equally controversial. Some studies show an association between p21 overexpression and high-grade invasive disease with a shorter disease-free survival [33], whilst others show an improved survival time [34]. There was no association between ERBB2, bcl-2, ER or PR expression with p21 expression and p21 positivity was an independent

predictor of recurrence in DCIS, indicating that p21-positive DCIS lesions may delineate a different subset of poor-prognosis DCIS.

We have used a population-based series of DCIS cases to identify different immunophenotypes of DCIS associated with clinical recurrence. At this point in time, it is essential to use such studies in order to achieve long-term outcome data. However, such studies are disadvantaged due to the incompleteness of reporting resection margin status in the original pathology reports and the inability to ascertain the completeness of disease excision on histology review. In this current study, a numerical value of excision margin was rarely given. However, there was no difference between the number of cases and controls described as having 'clear' resection margins. Due to the incompleteness of the excision margin data, it was not possible to perform the primary matching of cases and controls by this parameter. We were, however, able to determine the type of breast conserving therapy between cases and controls (i.e. wide local excision versus partial mastectomy). Slightly more cases had a partial mastectomy compared with controls, although this did not reach statistical significance. Therefore, although it is not possible in the current study, as with the majority of other studies with long-term outcome data, to determine whether the recurrences were true second foci of disease or re-growth of residual disease, our results suggest that ERBB2-positive DCIS (32% of all DCIS cases within the study) should be offered primary treatment which takes this increased risk into account. Interestingly, a recent report has suggested that margin status is a significant predictor for recurrence as DCIS, but not as invasive cancer [21], suggesting that residual disease *per se* is insufficient for the progression to invasive cancer in the absence of other molecular changes. Despite the complex issue of resection margins, these data suggest that ERBB2 status may be a more sensitive predictor of recurrent disease than the commonly used morphological grading of DCIS (with approximately 70% of all DCIS lesions high grade, as in the current study).

One advantage of this study is that the incident cases were accrued from 1988 until 1992, before adjuvant therapy was commonly given to patients with DCIS in Australia. Hence, only 11% of all patients received adjuvant radiotherapy, and there was no difference between cases and controls. Equally, hormone therapy was rarely given in this cohort (16%) and again there was no difference between treatment of cases and controls. The latter point is important, given that we have found that a phenotype of ERBB2-positivity, bcl-2-, ER- and PR-negativity is associated with recurrence. Clearly, association of this phenotype with recurrence cannot be explained by excess hormone therapy in the control group.

The current study is essentially a hypothesis-generating study in which multiple analyses have been conducted. One problem of this type of study is that some factors may appear nominally significant due to chance alone. If we apply a Bonferroni correction factor based on there being 10 major factors under consideration, the effect of bcl-2 would remain significant at the 0.05 level (corrected to $P = 0.03$), and the evidence for associations with ERBB2-positivity become marginal (corrected to $P = 0.08$). Clearly, this study should be judged as a preliminary study highlighting biomarkers warranting further investigation.

In summary, we have shown an immunophenotype of DCIS characterised by ERBB2 overexpression and bcl-2-, ER- and PR-negativity which has an increased risk of clinical recurrence. Furthermore, there may be an additional separate DCIS phenotype also exhibiting a higher risk of clinical recurrence, which is characterised by p21-positivity. We have also shown conservation of this phenotype between the original DCIS lesion and the recurrent lesion in the majority of cases, including the recurrent invasive cancers. These findings form an important basis for further, prospective studies concerning DCIS subtype and outcome. Such studies are necessary to understand the potential implications of the immunophenotype for adjuvant therapy of DCIS. Our data suggest that the high-risk, ERBB2-positive, bcl-2-, ER- and PR-negative DCIS cases would be less responsive to hormone-related therapies, such as tamoxifen. Rather, our data implies that therapies more directly related to ERBB2 dysfunction, such as antibody-based therapies against the ERBB2 receptor, may have an important role in the management of this group of pre-invasive breast cancers.

Acknowledgements

We would like to thank Dr Gino Somers for his careful reading of the manuscript. The study would not have been possible without the collaboration and long-term support of members of the Breast Committee of the Victorian Cooperative Oncology Group. This work was supported by The Cancer Council Victoria, The Victorian Breast Cancer Research Consortium and the National Health and Medical Research Council, Australia.

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