Absence of HER4 Expression Predicts Recurrence of Ductal Carcinoma *In situ* of the Breast

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ABSTRACT

The type 1 tyrosine kinase receptor HER2 (c-*erb*B2/*neu*) is associated with resistance to hormone therapy and poor survival in invasive breast cancer, whereas HER4 expression is associated with endocrine responsiveness. Patterns of tyrosine kinase receptor coexpression may aid prediction of recurrence risk after surgery for ductal carcinoma *in situ* (DCIS). Women who had undergone surgery for pure DCIS were studied. Out of 129 primary tumors, 39 had recurred and 90 had not recurred after 5 years of follow-up. Primary tumors were compared for HER2, HER3, and HER4, estrogen receptor, and Ki67 by immunohistochemistry. HER2 was expressed in 58%, HER3 in 49%, and HER4 in 63% of nonrecurrent DCIS, compared with HER2 expression in 82% ($P = 0.008$), HER3 expression in 71% ($P = 0.04$), and HER4 expression in 36% ($P = 0.004$) in DCIS that subsequently recurred. Dually expressing HER2/4 DCIS was more likely to be estrogen receptor positive than HER2-only-expressing DCIS (73% versus 53%; $P = 0.05$). HER2 expression was associated with a higher percentage and HER4 expression a significantly lower percentage of proliferating DCIS cells (median, 13.8% versus 8.4%; $P = 0.001$). Coexpression of HER2 with HER4 was associated with reduced recurrence compared with HER2-only positive DCIS ($P = 0.003$). This association remained significant when analyzing only high nuclear-grade DCIS ($P = 0.015$). Low nuclear grade, low proliferation rate and presence of HER4 expression were independent predictors of nonrecurrence. Potentially, HER4 expression may identify women who could avoid radiotherapy after breast-conserving surgery for DCIS.

INTRODUCTION

The incidence of the preinvasive breast cancer ductal carcinoma *in situ* (DCIS) has increased by over five times because of the introduction of national screening programs in the 1980s (1), and now accounts for 25% of all screen-detected breast cancers (2). After breast-conserving surgery and radiotherapy, between 12% and 20% of cases recur by 10 years (3) depending on margin status. This 6-fold increase in the number of DCIS cases necessitates accurate prediction of recurrence risk. Clinicopathologic risk factors for the recurrence of DCIS have been identified, including involved or close (<1 mm) surgical excision margins (4), younger age at diagnosis, high-nuclear-grade tumors, and the presence of comedo necrosis (3, 5–7). Less is known about the molecular biological markers that could aid prediction of prognosis.

The type 1 tyrosine kinase receptors (RTK) are a group of four growth factor receptors HER1(c-*erb*B1/epidermal growth factor receptor), HER2(c-*erb*B2/*neu*), HER3(c-*erb*B3), and HER4(c-*erb*B4) characterized by their homology to the avian erythroblastosis virus transforming protein (8), which have a significant influence on the prognosis of invasive breast cancer. HER2 expression is associated with a poor prognosis, early recurrence, resistance to endocrine therapy and low estrogen receptor (ER) expression (9–13), whereas HER4 expression has been associated with increased ER expression (14) and low cell proliferation rates (15). HER2 expression has been claimed to be a univariate predictor of DCIS recurrence risk ($P = 0.012$, log-rank) being present in ~56% of all tumors (16), but the association of HER4 to DCIS recurrence has not previously been characterized. The type 1 RTKs share a common molecular structure—an extracellular ligand binding domain that contains two cystine-rich regions, a short transmembranous domain, and an intracellular tyrosine kinase domain (8)—enabling signaling across the cell membrane. The receptors can homo- or heterodimerize for activation after either ligand binding of the extracellular domain or gene overexpression (except for HER3, which has no intrinsic kinase activity and requires heterodimerization for signaling; ref. 14). With many possible receptor combinations, there is potential activation of multiple signaling pathways, the clinical effects of which are incompletely understood. In cell line experiments, when HER2-positive cancer cells were transfected to overexpress HER4, a reduction in proliferation and an increase in apoptosis was seen (17), suggesting that HER4 produces a "braking effect" on HER2 signaling activity. The effect of RTK expression on DCIS recurrence is unclear. To determine the relationship of type 1 RTKs to ER, cell proliferation, and recurrence risk of DCIS after surgical excision we looked at primary cases of pure DCIS that had either recurred or not recurred by 5 years of follow-up.

MATERIALS AND METHODS

Patient Selection. Cases were selected from a database at the University Hospital of South Manchester, composed of ~850 women diagnosed with DCIS from 1979 onward. Fifty women with subsequent recurrence were identified upon case-note review. From this, 39 formalin-fixed, paraffin-embedded

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archival blocks of the primary tumor were available for analysis; all patients had recurred within 5 years (median, 21 months; range, 10-60). Of the recurrences, 11 were invasive disease and 28 were further DCIS. These patients were matched with twice as many (100) cases of DCIS that were known not to have recurred after 5 years of follow-up. Of these, 90 blocks were available for analysis. Breast-conserving surgery had been done in 57 of the nonrecurrent and 32 of the recurrent DCIS, and mastectomy in 33 nonrecurrent and 7 of the recurrent cases. No significant differences were found in clinicopathologic variables between the initial 150 patients and the final 129 (age, tumor size, grade). Only patients with pure DCIS were studied. Ethical approval was given to study DCIS by the South Manchester University Hospital ethics board.

Immunohistochemistry. Archival blocks of formalin-fixed, paraffin-embedded tissue were selected, after confirmation that the blocks contained DCIS by an experienced breast pathologist (W.F.K). Immunohistochemical staining of HER2, ER, and Ki67 was as previously described (18). HER4 (Santa Cruz sc-283, 1:200, 1 hour, Santa Cruz, CA) staining used the same protocol as above, except for a biotinylated goat anti-rabbit secondary antibody (DAKO-432, 1:200; Ely, Cambridgeshire, United Kingdom). For HER3, the slides were pressure-cooked in Tris EDTA for antigen retrieval. The primary HER3 antibody (ab8758 AbCam, 1:20) was diluted in EnVision antibody diluent and applied to the sections, which were then incubated at 4°C overnight. After washing thoroughly in Tris buffer, the sections were incubated with the EnVision secondary, staining was visualized with EnVision 3,3′-diaminobenzidine, counterstained with hematoxylin, dehydrated through graded alcohols, cleared in histoclear, and mounted as previously described (18).

Tumor nuclear grade was determined after review of the original pathology report.

Evaluation of Immunohistochemistry. Staining was assessed by light microscopy. For ER and Ki67, the percentage of positively staining nuclei were determined; at least 1,000 cells were counted for each case using a grid graticule and cell counter at ×400 magnification. ER status was taken to be positive if ≥5% of cells were labeled. The membranous and cytoplasmic staining of HER2, and the membranous, cytoplasmic, and nuclear staining of HER4, were assessed semiquantitatively using the proportion of positively staining cells and degree of staining intensity compared with adjacent normal tissue; samples scored 0 to 3+ as previously described (18); with examples of representative staining shown in Fig. 1. HER3 staining was predominantly cytoplasmic and of uniform intensity throughout the specimen, and was scored 0 to 3+ by the degree of staining intensity. Scores of 2 to 3+ rated as positive for all type 1 RTK staining.

Statistical Analysis. The relationship between receptor coexpression tumor size, nuclear grade, ER status, and recurrence was assessed by the χ^2 test. Age, tumor size, Ki67, and recurrence were assessed by the Mann-Whitney *U* Test. The relationship between Ki67 and receptor coexpression was assessed using the Kruskal-Wallis test. Multivariate analysis was carried out using Cox proportional hazards regression analysis (forward Wald), and Kaplan-Meier survival plots were generated with log-rank significance. Significance tests were two-tailed, and 5% significance level was used throughout. Analysis was carried out by using SPSS 10.0 for windows under the guidance of the Medical Statistics department, University Hospital of South Manchester.

RESULTS

Clinicopathologic Parameters. The demographics of the nonrecurrent and recurrent DCIS groups were similar with

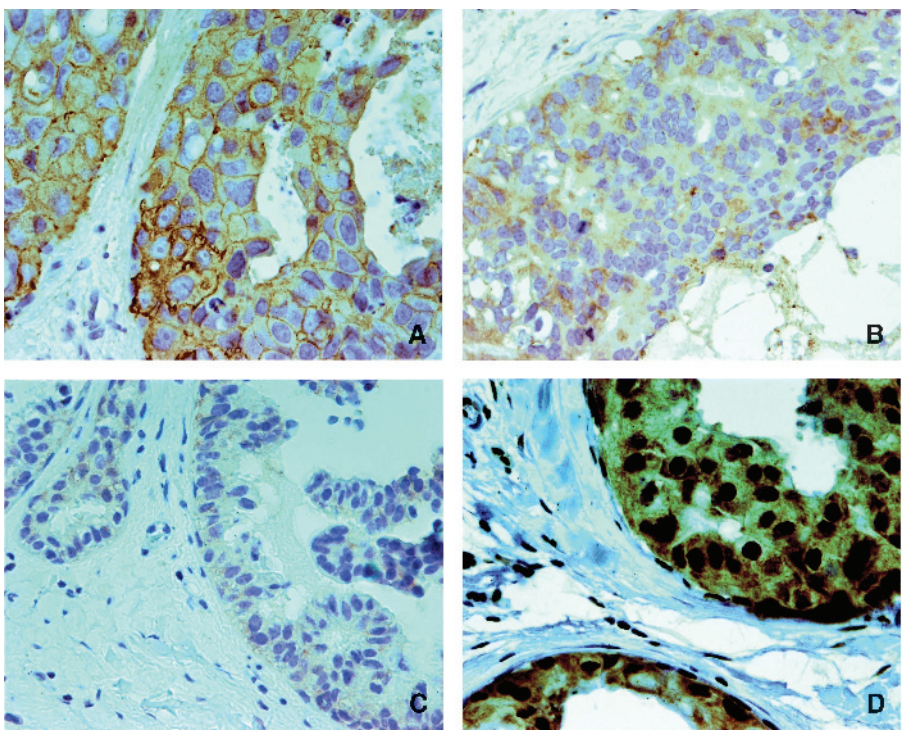

Fig. 1 Immunohistochemical staining for HER2 and HER4 in DCIS. *A,* HER2 3+. Presence of strong cytoplasmic and membrane staining in most cells. *B,* HER2 2+. Cytoplasmic but no membranous staining. *C,* HER2 1+. Weak cytoplasmic staining only. *D,* HER4 3+. Strong cytoplasmic and nuclear expression.

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respect to age, tumor size, and use of radiotherapy. In keeping with established risk factors, women that recurred tended to have close (<1 mm) or involved surgical excision margins ($P = 0.03$), with higher nuclear-grade tumors ($P = 0.001$) that showed higher rates of proliferation ($P = 0.005$; Table 1).

HER2, HER3, and HER4 Expression Frequencies. Overall, HER2 was expressed in 65% (84/129), HER3 in 56% (59/105), and HER4 in 55% (71/129) of DCIS studied. On a small proportion of blocks there was insufficient tissue available, therefore HER3 receptor status was not obtained on all DCIS studied. Of the 105 cases where expression of all three receptors was known, 4% (4/105) expressed none of the receptors, 16% (17/105) expressed HER2 alone, 5% (5/105) expressed HER3 alone, 17% (18/105) expressed HER4 alone, 18% (19/105) expressed HER2 and HER3, 8% (8/105) expressed HER2 and HER4, 8% (9/105) expressed HER3 and HER4, and 24% (25/105) expressed all three receptors.

HER2 and HER3 were expressed in a greater proportion of DCIS that subsequently recurred by 5 years than in nonrecurrent cases. HER2 was expressed in 57.8% (52/90) of the nonrecurrent tumors, compared with 82.1% (32/39) recurrent cases ($P = 0.008$), and HER3 in 49% (35/71) nonrecurrent, compared with 71% (24/34) in recurrent DCIS ($P = 0.04$). This was in contrast to the expression pattern of HER4, which showed significantly lower overall positivity in the recurrent group. Only 35.9% (14/39) of recurrent cases were HER4 positive, but this figure increased to 63.3% (57/90) expression in nonrecurrent DCIS ($P = 0.004$).

HER2/HER4 Coexpression. Tumors that were HER2 positive but HER4 negative showed reduced ER positivity (52.5%) compared with any other group. However, the percentage of ER positivity increased if the DCIS dually expressed HER2 and HER4 (73.2%; $P = 0.05$; Table 2). In addition, proliferative rate varied between the coexpression groups ($P = 0.007$; Table 2). HER2-positive/HER4-negative DCIS had a higher proliferation rate than HER2-negative/HER4-positive DCIS ($P = 0.001$; Mann-Whitney). There were no significant differences between RTK coexpression and DCIS nuclear grade (Table 2) or tumor size (data not shown).

Whereas 52.3% of the DCIS that developed recurrence within 5 years was HER2 positive/HER4 negative, only 5.1% expressed HER4 alone (Table 2). In contrast, only 24.5% of nonrecurrent DCIS solely expressed HER2, and a greater proportion of cases (30.0%) were HER2 negative/HER4 positive (Table 2). The associations of receptor coexpression and recurrence remained when analyzing only high-nuclear-grade DCIS ($P = 0.015$) or breast-conserving surgery ($P = 0.001$; Table 2). There was no influence of receptor coexpression on the ability to achieve clear margins ($P = 0.942$) and the ER status of the tumors did not influence recurrence of HER4-positive DCIS ($P = 0.8$; data not shown). When including HER3 coexpression, 63% of the cases that were HER2 positive/HER3 positive but HER4 negative recurred. In contrast, only 6% of the DCIS that was HER4 positive/HER2 and HER3 negative were in the recurrent group.

Recurrence-Free Survival. HER2-overexpressing DCIS had poorer cumulative 5-year disease-free survival than HER2-negative cases ($P = 0.0102$; Fig. 2*A*). In contrast, HER4-expressing tumors had a significant disease-free survival advantage ($P = 0.0052$; Fig. 2*B*). Expression of HER4 in the absence of HER2 (Fig. 2*C*) led to a disease-free survival advantage ($P = 0.0058$) over the other coexpression groupings. The recurrence pattern for HER3 expression was similar to that of HER2 (data not shown), and DCIS coexpressing HER2 and HER3 had the worst outcome (Fig. 2*D*).

Table 1 Summary of patient characteristics

Characteristic	Study group		*P*
	Nonrecurrent	Recurrent	
No. of patients	90	39	
Age (y), median (range)	56 (42-82)	55 (39-65)	0.198
Median recurrence time (mo), median (range)	—	21 (10-60)	—
Tumor grade (%)			
Low	12 (13.3)	0 (0.0)	0.001
Intermediate	34 (37.8)	6 (15.4)	
High	44 (48.9)	33 (84.6)	
Tumor size (mm), median (range)	20 (4 to extensive)	16 (4 to extensive)	0.208
ER status (%)			
Positive	60 (69.0)	23 (60.5)	0.358
Negative	27 (31.0)	15 (39.5)	
Ki67 (%), median (range)	10.9 (0.5-38.3)	15.5 (2.0-61.1)	0.005
Excision margin status (%)			
Involved (<1 mm)	13 (14.5)	16 (41.0)	0.03
Clear (≥1 mm)	65 (72.2)	22 (56.4)	
Unknown	12 (13.3)	1 (2.6)	
Adjuvant radiotherapy	6 (7)	2 (5)	0.9

Multivariate Analysis. Higher nuclear grade and higher DCIS proliferation rates were both independently associated with an increased risk of recurrence, as was a lack of HER4 expression, confirming that HER4 expression predicted recurrence irrespective of nuclear grade and cell proliferation (Table 3).

DISCUSSION

Common clinical factors that aid prediction of recurrence risk for DCIS include high nuclear grade, high cellular proliferation rates, involved surgical excision margins, presence of comedo necrosis, and a younger age at diagnosis (3–7). In this study, the established clinicopathologic risk factors of nuclear grade, margin status, and cell proliferation were all univariate predictors of recurrence, but on multivariate analysis only nuclear grade and proliferation rate remained independent predictors, along with lack of HER4 expression.

Little has been published on the molecular biological markers that could aid prediction of prognosis in DCIS. Recent studies have looked at cell cycle regulators, such as p21, p27 and cyclin D1 (19–22), as potential biological markers, but have published conflicting results about the role and the utility of these variables as indicators of recurrence risk. Some studies reported p21 expression to be associated with advantageous tumor factors, such as low-grade and ER positivity (19), but others with adverse factors, such as high-grade, HER2 overexpression, and recurrence (21, 22). No biological markers that have direct clinical relevance to DCIS have been reported. To our knowledge, no

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Table 2 HER2/HER4 receptor coexpression, proliferation, grade, ER status, and recurrence status

	Receptor coexpression				
HER2	–	+	–	+	
HER4	–	–	+	+	*P*
Median Ki67, % (range)	10.1 (0.5-38.8)	13.8 (2.5-61.1)	8.4 (1.6-36.0)	13.4 (2-46)	0.007*
Tumor grade (%)					
Low	0 (0.0)	2 (16.7)	6 (50.0)	4 (33.3)	
Intermediate	6 (15.0)	13 (32.5)	8 (20.0)	13 (32.5)	NS
High	10 (12.9)	27 (35.1)	15 (19.5)	25 (32.5)	
ER status (%)					
Positive	11 (73.3)	21 (52.5)†	21 (72.4)	30 (73.2)†	0.05†
Negative	4 (26.7)	19 (47.5)	8 (27.5)	11 (28.2)	
Recurrence (%)					
No. no recurrence	11 (12.2)	22 (24.5)	27 (30.0)	30 (33.3)	0.003‡
No. recurred	5 (12.8)	20 (51.3)	2 (5.1)	12 (30.8)	
Recurrence type					
DCIS	3 (10.7)	13 (46.4)	2 (7.1)	10 (35.8)	
Invasive	2 (18.2)	7 (63.6)	0 (0)	2 (18.2)	
High grade only (%)					
No. no recurrence (%)	5 (11.4)	10 (22.7)	13 (29.5)	16 (36.4)	0.015‡
No. recurred (%)	5 (15.2)	17 (51.5)	2 (6.1)	9 (27.2)	
Breast conserving surgery only (%)					
No. no recurrence	10 (17.5)	9 (15.8)	20 (35.1)	18 (31.6)	0.001‡
No. recurred	4 (12.5)	17 (53.1)	2 (6.3)	9 (28.1)	

Abbreviation: NS, not statistically significant.
*Kruskal-Wallis test across all four variables.
† χ^2 test, *P* value between HER2-positive/HER4-negative and HER2-positive/HER4-positive DCIS.
‡ χ^2 test across all four groups.

previous studies have specifically looked at type 1 RTK coexpression and recurrence in DCIS.

We have found that in DCIS, the overall expression of type 1 RTKs is higher than in invasive cancer. HER2 is expressed in ~25%, HER3 in 20%, and HER4 in 10% of invasive breast tumors (14), compared with HER2 expression in 65%, HER3 in 56%, and HER4 in 55% of DCIS, with varying receptor co-combinations linked to changes in cell proliferation and ER expression.

The initial identification of the variation in cellular proliferation rates and ER expression levels with alterations in

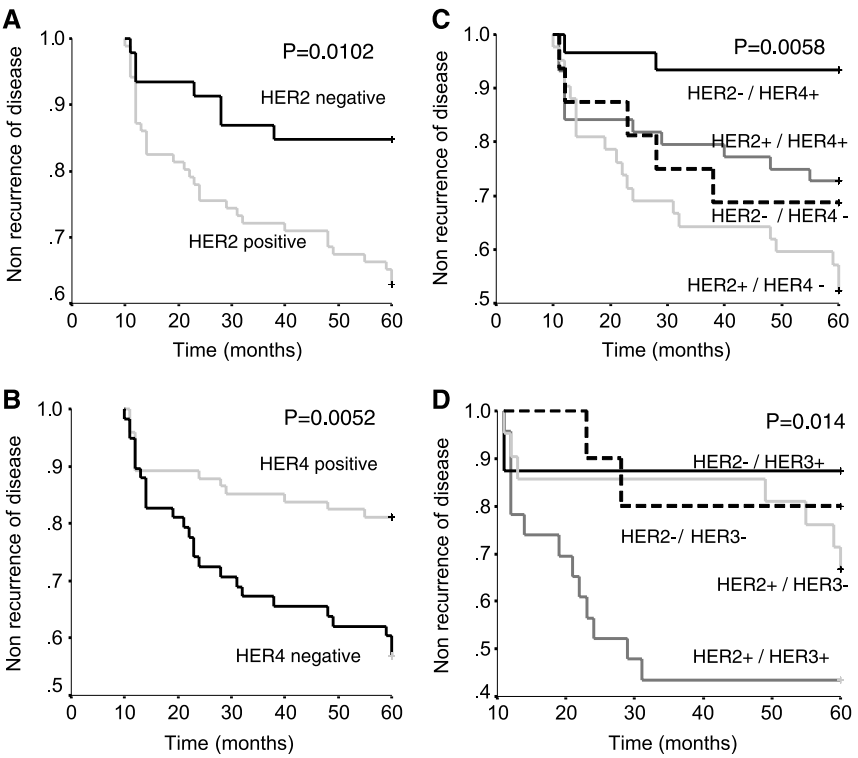

Fig. 2 Kaplan-Meier plots of cumulative local recurrence (DCIS and invasive) for the 129 patients, divided into (*A*) HER2 status, (*B*) HER4 status, (*C*) HER2/HER4 coexpression groups, and (*D*) HER2/HER3 coexpression groups. The plot generated for HER3 expression (not shown) showed the same pattern as for HER2 (*A*), which was the mirror image of HER4 expression (*B*). HER4-expressing tumors had a significant disease-free survival advantage over the other receptor combinations, with HER2-positive/HER4-negative (*C*) or HER2/HER3-coexpressing DCIS (*D*) having the poorest cumulative recurrence-free survival.

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type 1 RTK expression was shown in HER2-overexpressing cell lines that had been transfected to overexpress HER4, in which a decrease in cell proliferation and more differentiated phenotype was seen (17). HER2 expression is linked to a poor prognosis in invasive cancer and a higher risk of recurrence in DCIS (9–12, 16, 23). In this study, DCIS that was HER2 positive/HER4 negative had high proliferation, low ER expression, and increased chance of recurrence, whereas HER4-positive/HER2-negative DCIS had lower cell proliferation, higher ER expression, and better prognosis. On multivariate analysis, the expression of HER4 led to a low risk of recurrence [Exp(B), 0.69; 95% confidence interval, 0.48-0.98] regardless of HER3 and HER4 expression (HER3 expression was linked to increased risk of recurrence, HER2/HER3-expressing tumors having the greatest risk; Table 3).

Table 3 Independent predictors of DCIS recurrence

Predictor	*P*	Exp (B)	95% CI for Exp(B)
Higher grade	0.003	4.12	1.63-10.40
Ki67	0.038	1.03	1.00-1.06
HER4 positivity	0.038	0.69	0.48-0.98
Age at diagnosis	0.144		
BCS vs Mx	0.170		
Involved/close surgical margins	0.449		
HER2 positivity	0.99		
HER3 positivity	0.322		
ER status	0.77		

NOTE. BCS vs Mx, breast conserving surgery versus mastectomy; close surgical margins are those that are <1 mm from the resection edge.
Abbreviation: CI, confidence interval.

It is important to note that for this study, we selected the samples to have a 2:1 ratio of nonrecurrent cases to recurrent cases, but we did not select on the basis of type 1 RTK status. The Kaplan-Meier plots for recurrence-free survival are a useful illustration of the relative chance of recurrence between the different coexpression groups, but do not reflect overall DCIS recurrence rates in our unit, which we have previously reported (4).

One mechanism that can, in part, explain the observed differences in recurrence risk between the receptor coexpression groups is the significant difference in proliferation rates; a low percentage of proliferating DCIS cells was seen in HER4-only-expressing cases (the group that also had the lowest recurrence). However, because lack of HER4 expression is an independent predictor of recurrence risk, HER4 status is an important marker in its own right.

The variation in proliferation rates seen between the coexpression groups may reflect alterations in cell signaling dependent on receptor expression. Disparate signaling between HER2- and HER4-positive DCIS may have wider downstream consequences than alterations in cellular proliferation alone because in addition to cell proliferation, another biological factor that was influenced by RTK coexpression was ER positivity. Although it is thought that HER2-overexpressing DCIS is associated with low ER positivity (16), we have shown that the ER expression in HER2-positive cases is comparable to HER2-negative DCIS if there is coexpression of HER4. ER-positive/HER2-positive tumors are associated with tamoxifen resistance (13, 24), and ER-positive MCF7 cell lines that have been transfected to stably express HER2 have a lower response rate to tamoxifen (but remain sensitive to estrogen withdrawal; ref. 25). It has previously been shown that HER2 inhibition with trastuzumab (Herceptin) can restore tamoxifen sensitivity (26), suggesting cross talk between the HER2 and ER pathways, and aromatase inhibitors increase response rate in HER2-positive, ER-positive invasive breast cancer (13). We hypothesize that the mechanism behind this may be that these responders are HER4 positive. No studies have separated response to tamoxifen in HER2-positive disease according to HER4 status, but, potentially, this may provide a guide to response if there is also cross talk between both HER2/HER4 and ER pathways. These potential changes in signaling, conferred by the varying receptor homo-/heterodimerization patterns, need further clarification, and it will be important to determine the cross-over implications from DCIS to invasive breast cancer (i.e., if HER4 expression could aid prediction of response to therapy) with tamoxifen in ER-positive/HER2-positive invasive tumors or Herceptin in the metastatic setting.

Despite the publication of randomized controlled trials (3, 5–7) that adjuvant treatment, by way of radiotherapy or endocrine therapy (in ER-positive cases), reduces DCIS recurrence, it has no clear effect on overall mortality (3, 5–7) and only benefits the minority. It will therefore be desirable to avoid overtreatment of DCIS and identify subpopulations of patients with good prognostic features that could avoid radiotherapy. Indeed, none of our patients with recurrent disease have died. HER4 expression may identify a group of patients with good prognosis who could avoid the morbidity of radiotherapy. This retrospective identification of such patient groups is a hypothesis-generating study and the evidence will need confirmation by either longer-term studies from other groups or randomized controlled trial follow-up data.

Research is now under way to develop novel drugs that block the entire type 1 RTK family, but such "pan-HER" blockade may be undesirable. Because HER4 overexpression confers a reduced risk of recurrence, it may prove more advantageous to concentrate on developing selective type 1 RTK inhibitors for future therapy, which leave HER4 signaling uninterrupted.

In conclusion, type 1 RTK coexpression aids prediction of recurrence risk in DCIS, and HER4 expression is an independent predictor of a reduced risk of recurrence. Coexpression of HER4 with HER2 reduces the risk of early DCIS recurrence compared with HER2-expressing tumors lacking HER4, and is associated with both higher ER expression and lower rates of cellular proliferation. Consideration should be given to determining HER4 expression, if HER2 status is requested after surgical excision, to refine the prediction of recurrence risk. Furthermore, HER4 positivity may identify a DCIS patient population with good prognostic features that could avoid unnecessary radiotherapy.

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