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High Notch1 protein expression is an early event in breast cancer development and is associated with the HER-2 molecular subtype

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Aims: Activation of Notch signalling results in hyperplasia and tumorigenesis in murine mammary epithelium. However, there is little information regarding the expression of Notch1 in premalignant lesions and early breast cancer. We investigated expression of Notch1 in breast cancer development and its association with molecular subtypes.

Methods and results: Immunohistochemical expression of Notch1 was determined in a murine model of mammary carcinogenesis and in breast tissue from two cohorts of breast cancer patients, the first (n = 222) comprising a histological progression series and the second an outcome series of 228 patients with operable invasive ductal carcinoma. Enhanced expression of Notch1 protein was an early event in both murine and human breast cancer development with progressive increases in expression with the development of hyperplasia and malignancy. High Notch1 was not prognostic in the outcome cohort. There was, however, a highly significant association of high Notch1 protein with the HER-2 molecular subtype of breast cancer (P = 0.008).

Conclusions: These data demonstrate that aberrant Notch regulation is an early event in mammary carcinogenesis and is associated with the HER-2 molecular subtype of breast cancer, and suggest the Notch signalling pathway may be a potential therapeutic target worthy of further investigation.

Keywords: breast cancer, developmental signalling pathways, Notch1

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Abbreviations: ADH, atypical ductal hyperplasia; CK, cytokeratin; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester; DCIS, ductal carcinoma *in situ*; EGFR, epidermal growth factor receptor; ER, oestrogen receptor; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence *in situ* hybridization; IDC, invasive ductal carcinoma; LOESS, Locally weighted scatterplot smoothing; MIN, mammary intraepithelial neoplasia; N1ICD, Notch1 intracellular domain; PR, progesterone receptor; TMA, tissue microarray; UDH, usual ductal hyperplasia

Introduction

There is increasing evidence that dysregulation of developmental signalling pathways such as Notch, Wnt and Hedgehog is associated with the development and progression of malignancy in a number of organs. These developmental signalling pathways regulate self-renewal and maintenance of normal stem cells in most tissues and are proposed to contribute to the maintenance of cancer stem cells.[1]

Notch signalling is critical in mammalian embryonic development, particularly in neurogenesis, angiogenesis and in development of the breast, heart and lymphoid systems.[2] More recently, Bouras *et al.*[3] have described a key role for Notch signalling in the regulation of mammary progenitor cells.

Notch signalling occurs through two separate pathways, CSL-dependent signalling (CBP/RBP-jκ in vertebrates, Suppressor of Hairless in *Drosophila* and Lag-1 in *Caenorhabditis elegans*) and Deltex protein signalling, with the majority of signalling occurring through the CSL-dependent pathway.[4] In humans, Notch signalling is mediated through the Notch transmembrane receptors (Notch 1–4), which are activated by the Delta-like (Delta-like 1, 3, 4) and Jagged (Jagged 1, 2) ligands. Binding of ligand causes cleavage of the transmembrane receptor by an ADAM metalloproteinase and γ-secretase, leading to release of the intracellular domain (NICD)[5] and translocation to the nucleus where it forms a trimeric activation complex with CSL and Mastermind to induce transcription activation of the *HES*[6] and *HERP*[5] gene families.

There is increasing evidence that aberrations in the Notch pathway, in particular the Notch1 receptor, are implicated in breast carcinogenesis. Constitutive activation of Notch1 causes mammary hyperplasia[3] and carcinogenesis in mice,[7] and Notch1 overexpression has been associated with a significantly poorer prognosis in human breast cancer.[8] Activation of Notch signalling in mouse mammary epithelial cells is associated with disorganized and invasive growth in matrigel and collagen assays,[9] and with transformation of human breast epithelial cell lines through loss of E-cadherin expression and resistance to apoptosis.[10] There is relatively little published data regarding the role of Notch signalling in early human breast carcinogenesis; Farnie *et al.*[11] have reported that primary culture of human ductal carcinoma *in situ* (DCIS) mammospheres had higher levels of Notch activation than normal duct-derived mammospheres, and increased Notch signalling in DCIS was associated with an increased risk of recurrence.

Gene expression profiling has identified intrinsic molecular phenotypes of breast cancer.[12] There is speculation that the breast cancer molecular subtypes, i.e. luminal, basal-like, HER-2 and normal breast-like cancers observed in expression profiles, may reflect distinct cells of origin,[13] although this is not universally accepted.[14] However, there is relatively little evidence to date to link breast cancer molecular subtypes with particular developmental pathways. For example, there are conflicting data regarding the association of Notch signalling pathway components with specific breast cancer subtypes. Reedijk *et al.*[8] observed that breast cancers with high levels of Jagged1 ligand, Notch1 and Notch3 receptors were almost exclusively triple negative [oestrogen receptor (ER)-negative, progesterone receptor (PR)-negative, HER-2-negative] and that high levels of Jagged1 were associated with expression of basal cytokeratins (CK). Dontu *et al.*[15] reported that Notch pathway activity drove early progenitor cells towards the development of myoepithelial cells, while Bouras *et al.*[3] have recently demonstrated that Notch signalling regulates luminal cell-fate commitment. In contrast, others have reported that Notch activity is linked with HER-2 overexpression, suggesting both positive[16,17] and negative[18] relationships between these oncogenes. Most recently, Magnifico *et al.*[19] identified that Notch1 directly regulates HER-2. Thus, there is a need to clarify further the role of aberrant Notch signalling in the development of breast cancer and any association with particular molecular subtypes of the disease. Here, we investigated the potential associations of immunohistochemical expression of Notch1 and clinicopathological parameters and disease outcome in tissue from two series of breast cancer patients.

Materials and methods

STUDY POPULATIONS

Two separate cohorts of breast cancer patient tissues were used in these studies. The first cohort comprised

a histological 'progression' series of 222 patients diagnosed with invasive ductal carcinoma (IDC) or DCIS between 1996 and 2005 at Royal Prince Alfred Hospital (Sydney, NSW, Australia) retrospectively identified for this study. This series was developed to include lesions associated with increasing risk of developing breast cancer, rather than implying a direct neoplastic progression. A range of preinvasive and invasive lesions was identified in the associated formalin-fixed paraffin-embedded (FFPE) resected breast tissue using standard diagnostic criteria by experienced breast pathologists (S.A.O'T., E.K.A.M. or D.M.). This series comprised 724 independent lesions that were suitable for interpretation of Notch1 expression: 66 morphologically normal ducts adjacent to invasive carcinoma, four atypical ductal hyperplasias (ADH), 15 usual ductal hyperplasias (UDH), 511 cases of DCIS and 101 IDCs. ADH was excluded from the analysis due to the small number of cases. The use of clinical specimens and associated data was approved by the Ethics Committee of the Royal Prince Alfred Hospital (X05-0115).

The second cohort consisted of 292 patients with operable IDC treated by a single surgeon (P.C.) between February 1992 and August 2002 and has been previously reported.[20] The clinicopathological features of the subset of 228 patients suitable for assessment of Notch1 staining from this cohort are summarized in Table 1. Prior approval for this study was obtained from the Human Research Ethics Committee of St Vincent's Hospital, Sydney (HREC SVH H94/080, HREC 06336 SVH H00036).

Table 1. Clinicopathological features of the invasive ductal carcinoma cohort (n = 228)

Clinicopathological features	*n*
Median length of follow-up (months)	68 (range 0–159)
Median age (years)	54 (range 24–87)
Tumour size (mm)	
0–10	38/228 (17%)
11–20	96/228 (42%)
21–50	85/228 (37%)
>50	9/228 (4%)
Tumour grade	
I	39/228 (17%)
II	89/228 (39%)
III	100/228 (44%)
Lymph node metastasis	
0	128/228 (56%)
1–3	68/228 (30%)
4–10	22/228 (10%)
>10	10/228 (4%)
Oestrogen receptor positive	156/227 (69%)
Progesterone receptor positive	133/228 (58%)
HER-2+ (FISH)	38/218 (17%)
Total recurrences	54/228 (24%)
Breast cancer deaths	36/228 (16%)
Five-year recurrence-free survival	79%
Five-year breast cancer-specific survival	85%
Notch1 positive	88/228 (39%)

FISH, fluorescence *in situ* hybridization.

TISSUE MICROARRAYS AND IMMUNOHISTOCHEMISTRY

Two separate sets of tissue microarrays (TMAs) of FFPE tumour tissue blocks were constructed for each cohort. For the IDC cohort, each patient was represented by two to six 1-mm cores. For the progression series four representative cores from each donor block were taken and deposited in the recipient array block for sampling of DCIS. Three representative cores were taken when sampling IDC.

TMAs were produced using the MTA-1 Manual Tissue Arrayer (Beecher Instruments, Sun Prairie, WI, USA). Haematoxylin and eosin slides for all arrays were prepared and individual cores were reviewed by an experienced breast pathologist (S.A.O'T. or E.K.A.M.). A small number of whole tissue sections were stained for Notch1 and compared visually with TMAs and the pattern of immunoreactivity was found to be concordant.

Antigen retrieval was performed using Dako solution pH 9.0, (s2367; Dako, Carpinteria, CA, USA) in a boiling water bath for 40 min, followed by cooling for 15 min in a running waterbath. Following a thorough wash in distilled water, endogenous peroxidase activity was eliminated with 3% hydrogen peroxide for 5 min. Slides were incubated for 1 h with the Notch1 H-131 antibody diluted 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following a buffer wash, detection employed Dako Envision+ rabbit secondary reagent (Dako) for 30 min at room temperature,

followed by Dako diaminobenzidine (DAB)+ chromogen for 10 min. This antibody was selected because it has previously been shown to be highly specific for Notch1 expression in a study of transgenic mice with inducible inactivation of Notch1.[21] In our study, endothelial cells[22] were used as the positive tissue control and a matched concentration isotype IgG was used as the negative control.

ER, PR, CK5/6 and epidermal growth factor receptor (EGFR) were also stained using the following antibodies: ER 1:100 (clone 6F11; Dako) and PR 1:200 (clone PgR 636; Dako), CK5/6 1:80 (clone MAB1602; Chemicon Int., Temecula, CA, USA), and EGFR 1:100 (clone H11; Dako). HER-2 fluorescence *in situ* hybridization (FISH) was assessed in the Australian National Reference Laboratory. (Department of Pathology, St Vincent's Hospital, Sydney) using the Vysis PathVysion HER-2 DNA dual colour probe kit. A HER-2:chromosome 17 ratio >2.2 was classified as HER-2 amplification.

Cytoplasmic immunoreactivity for Notch1 was assessed by two independent observers (S.J.Z. and S.A.O'T.), including an experienced breast pathologist (S.A.O'T.) and described in terms of the intensity (0 = negative, 1+ = weak, 2+ = moderate and 3+ = strong) and percentage of immunopositive cells. From these indices a simplified 'H score' (i.e. intensity × percentage of positive cells) was calculated for each core and a mean and median score for each index calculated for each tumour.[20] ER and PR were assessed as positive if they had an H score of >10. CK5/6 and EGFR were assessed as positive if there was any positive cytoplasmic or membranous immunoreactivity present at any intensity.

DEFINITION OF INTRINSIC MOLECULAR PHENOTYPE OF BREAST CANCER

This was approximated using a surrogate 'signature' similar to the immunohistochemical signature recently described by Cheang *et al.*,[23] but also using FISH to determine HER-2 status.[20] Five different subgroups were defined: luminal A = ER+ and/or PR+, HER-2−; luminal B = ER+ and/or PR+, HER-2+; HER-2 = ER− and PR−, HER-2+; basal-like = ER−, PR−, HER-2−, CK 5/6+ and/or EGFR+; unclassified = negative for all five markers.

NOTCH PATHWAY GENES mRNA EXPRESSION AND OUTCOME

Gene expression data from two publically available datasets, the Naderi[24] and Wang[25] studies, were employed to establish relationships between *NOTCH1* mRNA levels and breast cancer outcome. The Naderi cohort comprised 135 patients, with a median tumour size of 18 mm and a median follow-up of 122 months with 70% ER positivity. Data were presented from Agilent Human 1A arrays which were available as raw scanner data files and sourced from Array Express (http://www.ebi.ac.uk/microarray-as/ae/) accession E-UCON-1. Using the limma R package,[26] background-subtracted data were normalized by the global locally weighted scatterplot smoothing (LOESS) technique applied to non-control spots only. To combine information from duplicate dye swap arrays, a linear model was fitted to the normalized data using limma. Model fit coefficients for each sample were then used as final expression estimates, expressed relative to a pooled reference RNA. The *NOTCH1* probe was analysed for frequency distribution of mRNA and its associations with patient outcome using Cox proportional hazards for univariate analysis. The mean score was used to dichotomize the data to generate Kaplan–Meier curves.

The dataset of Wang *et al.*[25] comprised 286 patients with lymph node-negative primary breast cancer with a mean age of 52 years, and 73% ER positivity. The data presented were from the Affymetrix (Santa Clara, CA, USA) oligonucleotide microarray U133a GeneChip, which were scanned using standard protocols. Expression values were calculated by use of the Affymetrix GeneChip analysis software MAS 5.0. For chip normalization, probe sets were scaled to a target intensity of 600.

MOUSE MAMMARY GLAND TISSUES

The immunohistochemical expression of Notch1 was also examined in a mouse model of mammary carcinogenesis. The C3/SV40T mouse model directs transgenic expression of the SV40 large T antigen to the distal mammary duct and prostatic ductal epithelium, resulting in multistage ductal dysplasia and ultimately invasive cancer.[27] Whole mammary gland tissue sections from these mice at various stages of carcinogenesis were harvested, and routinely prepared as previously described.[28] All experiments involving mice were performed under the supervision of and in accordance with the regulations of the Garvan/St Vincent's Animal Experimentation Committee.

Essentially the same immunohistochemical protocol for Notch1 was used as described above, with minor modification due to the significantly shorter fixation period of the mouse tissues; antigen retrieval was performed in Dako pH 9 target retrieval solution for 15 min in a boiling water bath. Expression of Notch1 was assessed as described for the human tissues by an experienced breast pathologist (S.A.O'T.).

STATISTICAL ANALYSIS

Statistical evaluation was performed using Statview 5.0 Software (Abacus Systems, Berkeley, CA, USA). A *P*-value of <0.05 was accepted as statistically significant. Baseline characteristics of the cohort were defined using simple frequency distributions.

Univariate analyses using the Kaplan–Meier and Cox proportional hazards model were applied to the cohort for established clinicopathological variables using recurrence, distant relapse and breast cancer-related death as end-points.

Descriptive statistics for Notch1 protein expression in the invasive carcinoma cohort were determined and the frequency distribution of the scores assessed. No apparent break points were observed to suggest an obvious cut-point for dichotomization. The optimal cut-point determination technique using serial survival analysis (log rank *P*-value in Kaplan–Meier analysis) was then used to search for a biologically significant cut-point, but none was identified. Therefore the mean H score (H > 85) was used to dichotomize the data for univariate survival analysis. This cut-point of H ≥85 was subsequently also applied to the 'progression' cohort.

Subgroup analysis, to determine any prognostic significance of Notch1 expression, was also performed where possible. However, the smaller subgroups of HER-2 and basal-like breast cancers lacked sufficient statistical power for analysis. Clinicopathological variables were dichotomized using standard parameters.

Correlations between the determined cut-points for each variable and clinicopathological variables were tested for statistical significance using contingency tables, applying the χ^2 test. The relationship between Notch1 expression as a continuous variable and breast cancer intrinsic phenotypes was explored using an analysis of variance (ANOVA) with Fisher's *post hoc* test of significance. H score was also used as a continuous variable in ANOVA of the progression series to characterize the changing levels of Notch1 protein expression with increasing architectural and cytological atypia over the whole cohort of lesions.

Results

NOTCH1 OVEREXPRESSION IS AN EARLY EVENT IN THE DEVELOPMENT AND PROGRESSION OF BREAST CANCER

Although Notch pathway activation has been reported to induce murine mammary gland hyperplasia and neoplasia, there are relatively few data reporting the expression of Notch pathway components in proliferative and premalignant tissues of the human breast.

Notch1 expression was observed in the cytoplasm of epithelial cells ranging from weak (1+) to moderate (2+) and strong (3+) (Figure 1B–D, respectively). The specificity of immunoreactivity was confirmed by the presence of immunopositivity in endothelial cells, with no expression in smooth muscle cells (Figure 1A). There was a range of expression of Notch1 observed in normal, premalignant and invasive breast lesions; using the mean H score of 85 (derived from the invasive carcinoma cohort) to dichotomize the data in the 'progression series', high Notch1 was seen in only nine of 66 cases (13.6%) of morphologically normal ducts with a progressive increase in the proportion of lesions showing high relative Notch1 protein expression with 33% of UDH, 30% of nuclear grade 1 DCIS, 57% of nuclear grade 2 DCIS and 59% of nuclear grade 3 DCIS with high Notch1 (Figure 2Bii). Intriguingly, there was a small but statistically significant ($P = 0.005$) decline in Notch1 expression in IDC when compared with DCIS, with 39% of IDCs in the progression series showing high Notch1 (Figure 2Bi).

Photomicrographs showing the pattern of expression in the human breast progression cohort are also presented in Figure 2A with a graphical representation of the ANOVA of Notch1 H score in each of the human breast lesions shown in Figure 2Bi. There was also an increase in Notch1 protein expression between different nuclear grades of DCIS (Figure 2Bii) with a progressive increase between nuclear grades 1 and 2 DCIS ($P < 0.001$) and between DCIS grades 2 and 3 ($P = 0.03$).

We next investigated the expression of Notch1 in a murine model of mammary carcinogenesis and progression, the C3/SV40T mouse model, and found an almost identical pattern to that seen in the human breast lesion progression series (Figure 2C,D). These mice develop atypia of the mammary ductal epithelium at about 8 weeks of age, progressing to mammary intraepithelial neoplasia (MIN; analogous to human DCIS) at about 12 weeks of age with the development of invasive carcinomas at about 16 weeks of age in 100% of female mice. The carcinomas have a similar morphology to human infiltrating ductal carcinomas.[27]

Mammary glands from 15 C3/SV40T transgenic mice were evaluated and the expression of Notch was significantly elevated in hyperplastic lesions ($n = 14$), MIN (a DCIS-like lesion, $n = 12$) and invasive carcinoma ($n = 7$) compared with morphologically normal ducts ($n = 15$) ($P < 0.05$, Figure 2C,D). Furthermore, ducts from syngeneic wild-type mice showed no expression of Notch1 (Figure 2Ci). These data suggest that aberrant expression of Notch1 is an early event in both human and murine mammary neoplasia and

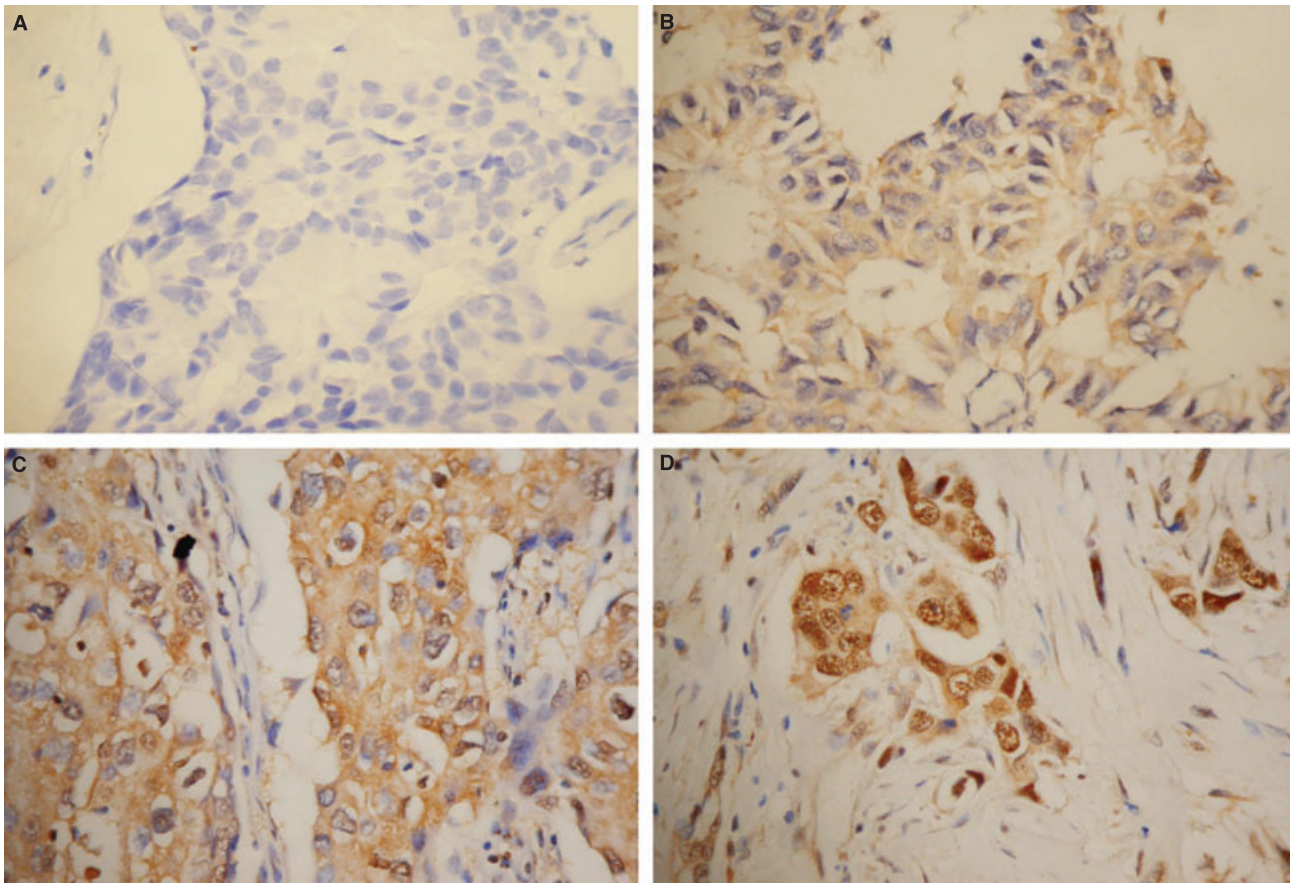

Figure 1. Range of expression of Notch1 in invasive ductal carcinoma. **A**, Notch1– IgG control. **B**, Weak Notch1 expression, intensity score 1 (matching section from IgG control in **A**). **C**, Moderate Notch1 expression, intensity score 2. **D**, Strong Notch1 expression, intensity score 3 (H&E).

progressively increases with the development of hyperplasia and malignancy.

HIGH RELATIVE NOTCH1 PROTEIN EXPRESSION IS A COMMON EVENT IN BREAST CANCER BUT IS NOT PROGNOSTIC

There have been a handful of reports describing the expression of Notch1 in human breast cancer.[8,29,30] However, to date the association with molecular subtypes of breast cancer has only been rarely explored. We investigated the expression of Notch1 by immunohistochemistry in 292 patients from a well-characterized, single-surgeon IDC cohort ($n = 292$). Once uninformative cores (folded or missing) were excluded, expression of Notch1 was assessable in 228 patients. The clinicopathological features of this group are shown in Table 1.

There was a range of Notch1 expression in this cohort; 4% of cases showed no expression of Notch1, whereas 46% showed weak (1+), 37% showed moderate (2+), and 13% showed strong expression (3+) (Figure 1). The mean H score in the IDC cohort was 85. In comparison, the mean Notch1 H score in morphologically normal ducts was 33.9, showing there is significantly higher relative expression of Notch1 in invasive carcinoma. We found a very similar mean Notch1 H score of 82.7 in cases of IDC within an independent cohort, the 'progression' series. Using the mean H score to dichotomize the Notch1 data, 39% of the IDC cohort showed high relative Notch1 protein expression.

There was no prognostic significance of Notch1 expression, either on univariate survival analysis, using Cox proportional hazards model or on Kaplan–Meier survival analysis, in the cohort as a whole or in subgroup analysis, where possible. The Kaplan–Meier curves for breast cancer recurrence and breast cancer-specific death are shown in Figure 3A,B.

χ^2 analysis was performed to determine any association between high relative Notch1 protein expression and clinicopathological characteristics of the patients. There was no association between Notch1

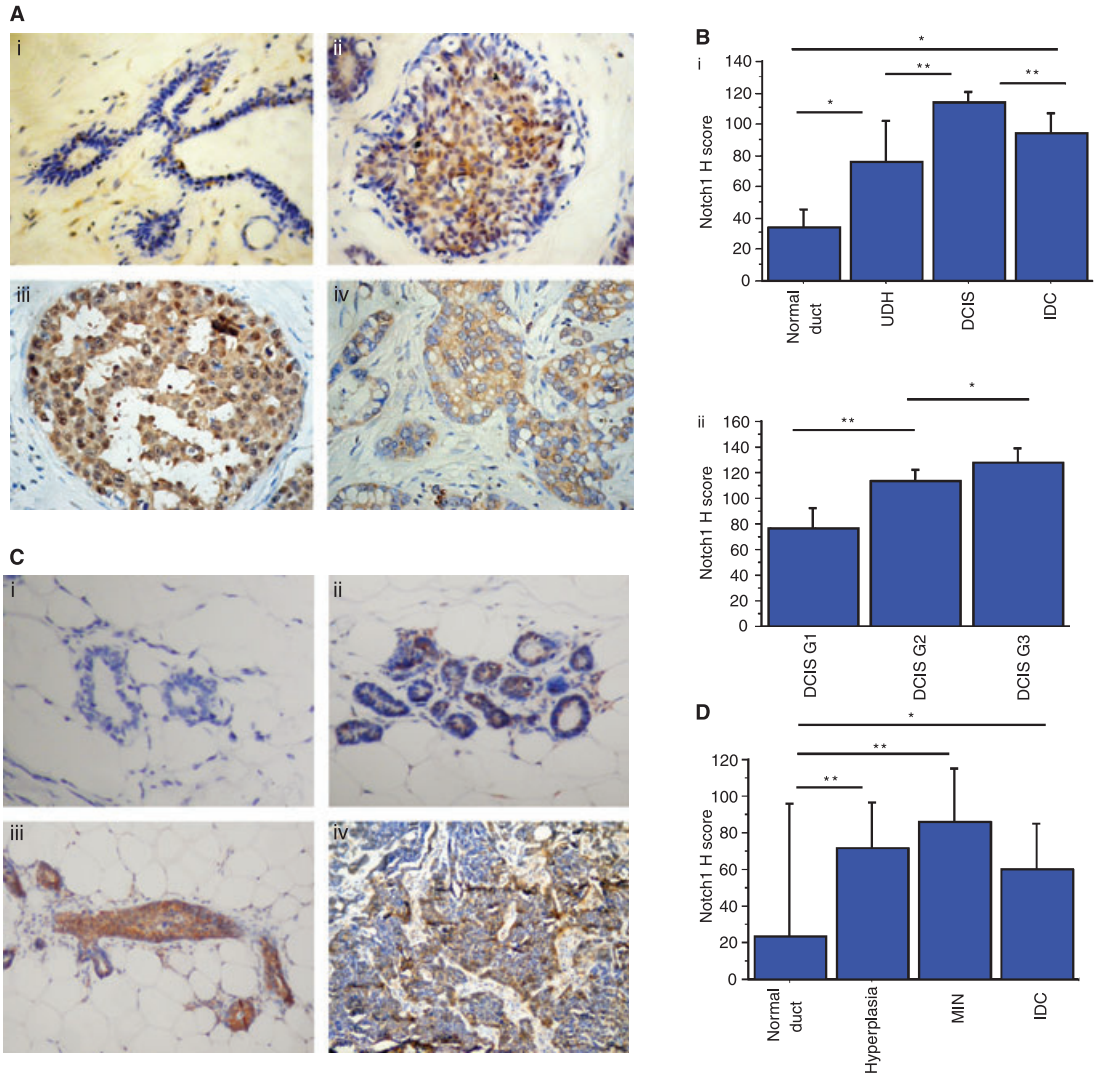

Figure 2. Immunohistochemistry for Notch1 in human and murine mammary cancer development. **A**, Photomicrographs of (i) normal duct, (ii) usual ductal hyperplasia, (iii) ductal carcinoma *in situ* (DCIS) and (iv) invasive ductal carcinoma. **B**, (i) ANOVA of Notch1 expression in human breast lesions and (ii) specifically in grades 1–3 DCIS. **C**, Photomicrographs of (i) normal ducts, (ii) hyperplasia, (iii) mammary intraepithelial neoplasia and (iv) invasive carcinoma in the C3/SV40T mouse. **D**, ANOVA of Notch1 expression in C3/SV40T mouse. $^{*}P < 0.05$; $^{**}P < 0.01$.

overexpression and tumour grade, tumour size, patient age, lymph node status, hormone receptor status or Her-2 immunohistochemistry or HER-2 FISH (P all >0.05). However, ER− patients who were HER-2+ by FISH or immunohistochemistry showed a significant association with high Notch1 protein expression ($P = 0.02$ FISH and $P = 0.049$ immunohistochemistry). In contrast, ER+, HER-2 FISH+ cases showed no association with high Notch1 ($P = 0.33$ FISH and $P = 0.99$ immunohistochemistry).

In view of previous studies reporting that Notch1 overexpression is associated with a poor prognosis,[8,29,30] we investigated the expression of NOTCH1 mRNA in two publically available breast cancer gene expression array datasets.[24,25] The probes for NOTCH1 in either dataset showed no prognostic significance ($P = 0.58$ for breast cancer recurrence in the Wang cohort and $P = 0.51$ for breast cancer-related death in the Naderi dataset, Figure 3C,D).

NOTCH1 IS ASSOCIATED WITH THE HER2 SUBTYPE

In view of our hypothesis that dysregulation of developmental signalling pathways may be associated with specific breast cancer subtypes, the association of Notch1 with each molecular subtype was then

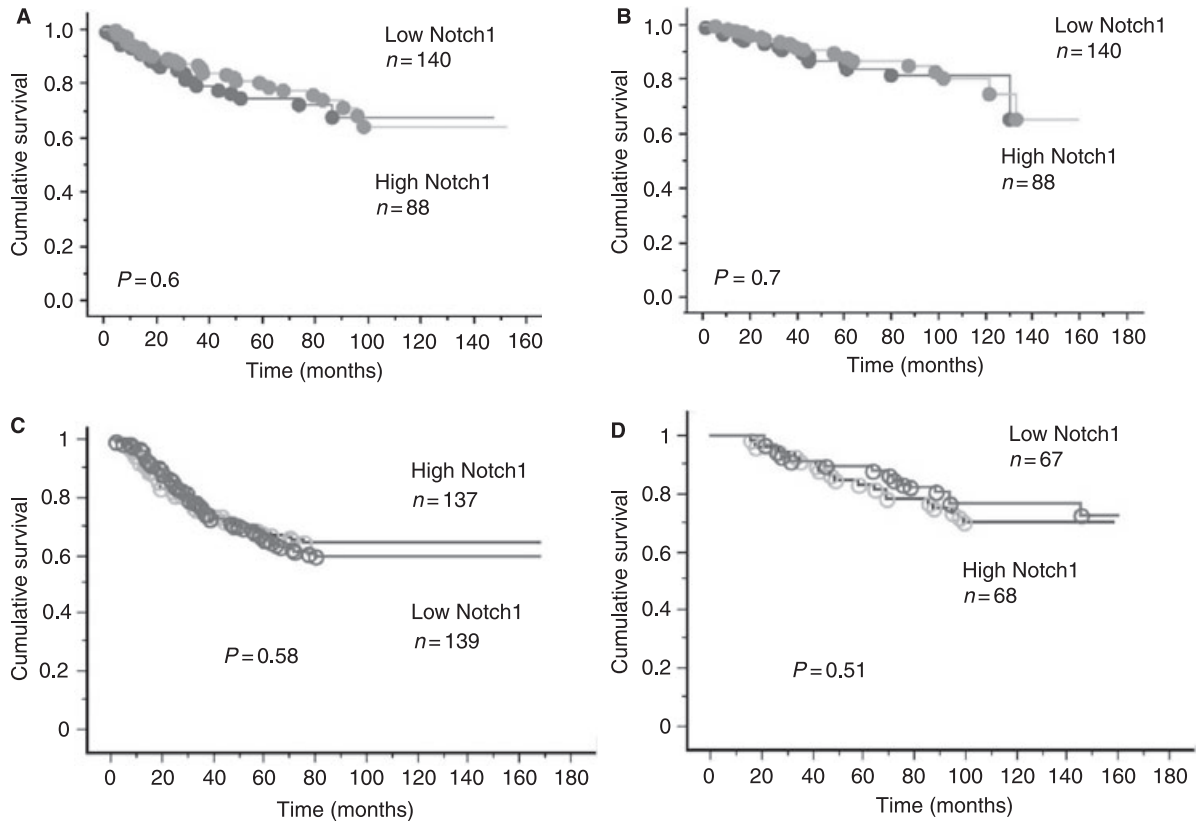

Figure 3. Relationship between expression of Notch1 and breast cancer patient outcome. Kaplan–Meier plots illustrating the relationship between Notch1 protein expression and **A**, breast cancer recurrence or **B**, breast cancer-specific death and between NOTCH1 mRNA expression and **C**, breast cancer recurrence in the Wang dataset[23] and **D**, breast cancer-related death in the Naderi cohort.[22]

determined. χ^2 analysis demonstrated that high relative Notch1 protein expression was significantly associated with the HER-2 subtype (defined as ER− and PR−, HER-2+ FISH); $P = 0.008$. There was no association between Notch1 and any other subtype (Table 2).

Analysis of variance was then used to explore the relationship between the Notch1 H score (as a continuous variable) and the breast cancer subtypes, using our modified definition of the molecular subtypes. Figure 4 shows that there is enrichment of high relative Notch1 protein expression in the HER-2 subtype, which is significantly elevated in this group compared with all other subtypes ($P < 0.05$).

Discussion

There has been considerable recent interest in the role of Notch signalling in breast cancer development and progression. Bouras *et al.*[3] have recently reported that Notch signalling regulates mammary stem cell function and that constitutive activation of Notch1 in luminal precursor cells results in the development of hyperplastic nodules and subsequently invasive cancer. Here we report that high expression of Notch1 protein is a very early event in both human and murine mammary carcinogenesis. Elevated expression of Notch1 was noted first in morphologically normal ducts in breast tissue containing invasive carcinoma and showed a progressive increase in lesions of UDH and DCIS. We also noted an increase in the expression of Notch1 from DCIS grade 1 to grade 2, with a further increased level noted in grade 3 DCIS. A similar pattern was also observed in preinvasive lesions from the C3/SV40T mouse model of mammary carcinogenesis, which shares significant homology with similar lesions in the human breast. Interestingly, ducts from syngeneic wild-type mice showed no expression of Notch1, in contrast to that detected in morphologically normal ducts from the C3/SV40T mice, suggesting that increased Notch1 expression accompanies very early events in the development of mammary carcinoma.

There are relatively little previously published data regarding a potential role for Notch early in breast cancer development. Although aberrant activation of the Notch signalling pathway results in the development of adenocarcinomas in murine models, and

Table 2. Associations of high relative Notch1 protein expression with breast cancer subtypes

Breast cancer subtype	Number (%) cases with high Notch1	χ^2 *P*-value
Luminal A	54/141 (38)	0.40
Luminal B	5/21 (23)	0.24
HER-2	12/17 (70)	0.008
Basal-like	11/31 (35)	0.84
Unclassified	2/14 (14)	0.09

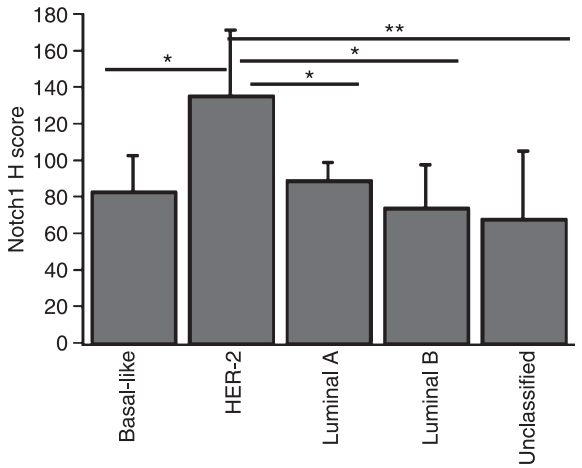

Figure 4. Notch1 protein expression in breast cancer subtypes. Analysis of variance demonstrates a statistically significant increase in Notch1 expression in the HER-2 subtype of breast cancer, compared with all other molecular subtypes (basal-like, luminal A, luminal B and unclassified). *$P < 0.05$; **$P < 0.01$.

overexpression of activated forms of the Notch1 receptor can transform normal human epithelial cells *in vitro*, only one report has previously supported a role in preinvasive human disease.[11] In this study Farnie and coworkers developed a novel method of primary culture of DCIS from human lesions, using techniques similar to the mammosphere assay. Elevated levels of the Notch1 intracellular domain (N1ICD) were noted in these mammospheres, when compared with those derived from normal breast tissue. The growth of DCIS mammospheres could be inhibited by *N*-[*N*-(3, 5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), a γ-secretase inhibitor specific to Notch pathway activity. These authors also examined expression of the N1ICD in a cohort of 50 patients with DCIS and found that elevated N1ICD was associated with a higher risk of recurrence. Our data add further support to these reports postulating a significant role for Notch signalling in early breast cancer development and progression.

In contrast to the earlier findings of Reedijk *et al.*,[8] who reported that high levels of NOTCH1 mRNA were associated with a poorer prognosis, which had even greater prognostic significance when combined with high levels of JAG1 mRNA, our data failed to demonstrate any prognostic significance for high Notch1 protein expression. There are a number of possible reasons for these differences. Although the cohorts in both these studies were of comparable size, (n = 184 and 228, respectively), there were significant differences in cohort composition as evidenced by the clinicopathological variables. For example, the patients in one of the Reedijk cohorts had larger tumours (average size 37 mm compared with 21 mm in our cohort) as well as a higher proportion of grade 3 tumours (63% versus 44%). Furthermore, it has been demonstrated for JAG1 that there is a poor correlation between mRNA levels and protein expression,[31] which may also be true for Notch1.

To test this possibility, we performed *in silico* analysis of the prognostic significance of relative mRNA levels of NOTCH1 in two publically available gene expression array data for patient cohorts with similar clinicopathological characteristics to our cohort. In keeping with our immunohistochemical findings, we found no prognostic significance of high relative NOTCH1 mRNA expression in either cohort.

Another novel finding of our study is a significant association between elevated expression of Notch1 and the HER-2 subtype of breast carcinoma. There is some experimental evidence to support a role of Notch pathway signalling in HER-2-expressing tumours. Chen *et al.*[32] identified that a palindromic binding site within the promoter region of HER-2 (ERBB-2) was identical to that for RBPJκ, which transports the intracellular domain of Notch receptors to the nucleus, where it acts as a site-specific DNA binding partner leading to transcription of Hes1. Binding of RBPJκ to the promoter region of HER-2 resulted in increased transcriptional activity, which was further augmented by addition of Notch-IC. In the light of these data, it is possible that elevated Notch1 receptor expression may be linked mechanistically to augmented HER-2 transcription.

However, there are conflicting data regarding an association between Notch1 and HER-2. Osipo *et al.*[18] in studies in human breast cancer cell lines demonstrated an inverse relationship between levels of HER-2 and Notch transcriptional activity, such that inhibition of HER-2 resulted in elevation of Notch activity, which was abrogated by a Notch inhibitor, γ-secretase. It is

interesting to note that this group also reported that inhibition of Notch activity (either by a γ-secretase inhibitor or by a Notch1 siRNA) increased sensitivity of cell lines to the growth-inhibitory effects of trastuzumab, and suggested that inhibition of Notch signalling may be a promising therapeutic approach in cases of trastuzumab-resistant HER-2+ cancers. This disparity with our findings is not entirely surprising, as there are likely to be significant differences between Notch1 transcriptional activity as measured by a luciferase reporter gene in three breast cancer cell lines[18] and Notch1 protein levels as assessed by immunohistochemistry in human breast tissue samples. This is also supported by a recent report by Rizzo *et al.*,[33] who found a poor correlation between Notch-induced transcriptional activity and Notch receptor levels. It is also interesting that a recent abstract presented by the Osipo group[18] reports that constitutively active Notch1 increased tyrosine phosphorylated ErbB-2 (HER-2) and reversed the inhibition of a γ-secretase inhibitor,[16] showing a direct positive relationship between Notch1 and HER-2, in contrast to their recent paper,[18] and these data may provide evidence of a negative feedback loop between Notch and HER-2.

Yamaguchi *et al.*[34] reported no evidence of growth inhibition of ERBB2 (HER-2)-positive cell lines following treatment with siRNA targeting NOTCH1, although they found that a NOTCH3 siRNA inhibited the growth of some ERBB2− cell lines. In contrast, a positive association between Notch2 and HER-2 protein expression was reported by Florena *et al.*[17] in a small cohort of 98 invasive breast cancers.

Our data are also supported by the recent findings of Magnifico *et al.*,[19] who have identified that Notch1 signalling directly regulates HER-2 expression levels in tumour initiating cells, so that inhibition of Notch1 resulted in a reduction of HER-2 cell surface levels.

There are also extensive data linking Notch signalling to the basal-like subtype of breast carcinoma. Notch pathway activity in a mammosphere assay, an *in vitro* model of stem cell and early mammary development, increased the differentiation of progenitor cells into a myoepithelial lineage,[15] while Rizzo *et al.*[33] demonstrated that in a series of breast cancer cell lines, Notch receptor levels are highest in ER−, HER-2 non-overexpressing cells, analogous to clinical triple-negative phenotype, a group that includes most basal-like breast cancers. The apparent discrepancies between these data and those reported here may be attributable to examination of different components of the pathway, different parameters of expression, i.e. protein compared with mRNA, and the different criteria employed to assign patients to the different molecular subtypes. Reedijk's group defined the HER-2 subgroup as any patients with positive HER-2 expression, basal-like tumours as those lacking ER and expressing CK5, whereas luminal cancers were simply defined as those expressing ER. These definitions are less stringent than the surrogate molecular signatures as described by Livasy,[35] and more recently by Cheang *et al.*,[23] and have had a significant influence on the proportion of patients assigned to each phenotypic subgroup. For example, Reedijk's definition of luminal excludes luminal B patients (ER+ and HER-2+), whereas the HER-2 group will include luminal B patients. A further confounder is the method of assessment of HER-2 status in Reedijk's cohort, in that three different methods were used to assess HER-2 status–polymerase chain reaction, Southern blot and slot blot, whereas HER-2 status in our cohort was performed using FISH, currently regarded as best practice of assessment of HER-2 status.[36] Validation of these findings in a larger cohort would add substantially to our understanding of this relationship.

In summary, these data indicate that there is an association between aberrant expression of Notch1 and HER-2, and that high relative Notch1 protein expression is an early event in both human and murine mammary development and progression. Although it remains to be determined whether high Notch1 receptor protein expression correlates with Notch pathway activity, our data combined with other recent reports[16,18] suggest that Notch signalling may present a novel therapeutic target for the future management of breast cancer, particularly in the poor prognosis HER-2 subtype.

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