Numb protein expression correlates with a basal-like phenotype and cancer stem cell markers in primary breast cancer.

Rennstam, Karin; McMichael, Nicole; Berglund, Pontus; Honeth, Gabriella; Hegardt, Cecilia; Rydén, Lisa; Luts, Lena; Bendahl, Pär-Ola; Hedenfalk, Ingrid

Published in:
Breast Cancer Research and Treatment

DOI:
10.1007/s10549-009-0568-x

2010

Karin Rennstam, Nicole McMichael, Pontus Berglund, Gabriella Honeth, Cecilia Hegardt, Lisa Rydén, Lena Luts, Pär-Ola Bendahl, Ingrid Hedenfalk

"Numb protein expression correlates with a basal-like phenotype and cancer stem cell markers in primary breast cancer."

Breast cancer research and treatment, 2009, Oct 1

http://dx.doi.org/10.1007/s10549-009-0568-x

Numb protein expression correlates with a basal-like phenotype and cancer stem cell markers in primary breast cancer

Karin Rennstam[1], Nicole McMichael[1], Pontus Berglund[1], Gabriella Honeth[1], Cecilia Hegardt[1,2], Lisa Rydén[3], Lena Luts[4], Pär-Ola Bendahl[1], Ingrid Hedenfalk[1,*]

[1]Department of Oncology, Clinical Sciences, Lund, Lund University, SE-221 85 Lund, Sweden

[2]Lund Strategic Research Center for Stem Cell Biology and Cell Therapy, Lund University, SE-221 85 Lund, Sweden

[3]Department of Surgery, Clinical Sciences, Lund, Lund University, SE-221 85 Lund, Sweden

[4]Department of Clinical Pathology, Clinical Sciences, Lund, Lund University, SE-221 85 Lund, Sweden

*Corresponding author:

Dr. Ingrid Hedenfalk

Department of Oncology, Clinical Sciences, Lund

Lund University

Barngatan 2B

SE-221 85 Lund, Sweden

Phone: +46–46-178551

Fax: +46–46-147327

E-mail: Ingrid.Hedenfalk@med.lu.se

Key words: breast cancer, basal-like, Numb, cancer stem cell, CD44/CD24, *BRCA1*

Abstract

Decreased expression of Numb, resulting in activation of the proto-oncogene Notch1 and reduction of the tumor suppressor p53, has been demonstrated in mammary carcinomas. The aim of this study was to investigate the relationship between Numb protein expression and clinicopathological characteristics, tumor biological subtypes and putative cancer stem cell markers in a well-characterized cohort of primary human breast cancers. Immunohistochemistry was performed on tissue microarrays of primary invasive breast tumors using a polyclonal anti-Numb primary antibody. Of the 241 tumors evaluated, 50 (21%) displayed deficient or reduced Numb immunoreactivity. Retained Numb expression was significantly correlated to estrogen (ER) and progesterone receptor (PR) positivity (P<0.001 and P=0.004, respectively). Interestingly, we found that a higher percentage of the tumors with deficient or reduced Numb expression belonged to the triple-negative (ER-/PR-/HER2-) subgroup compared to tumors with retained Numb expression (P=0.004). Transcriptional profiling of a subset of these tumors linked *NOTCH1* and *BIRC5*, both downstream targets of Numb, to the triple-negative subgroup in an inverse manner. Typically, subgroups characterized by low expression of Numb expressed higher levels of *NOTCH1* and *BIRC5* (encoding survivin). We also found deficient expression of Numb in a significantly higher proportion of *BRCA1* dependent tumors, which are usually triple-negative, compared to sporadic tumors. The expression of Numb in 14 breast cancer cell lines correlated similarly to their respective molecular subtypes. We further established an inverse correlation between Numb expression levels and the CD44+/CD24- cancer stem cell phenotype (P=0.05) in primary tumors. Finally, decreased Numb expression was associated with poorer distant disease-free survival (P=0.01). Taken together, our results indicate that loss of Numb expression is a marker of tumor aggressiveness, potentially linked to *BRCA1* status and a cancer stem cell phenotype in primary breast cancer.

Introduction

The protein encoded by the *NUMB* gene plays an important role in the determination of cell fate during development by antagonizing the activity of the plasma membrane receptor Notch1. Numb acts as a repressor of Notch1, binding to the Notch1 intracellular domain (NICD), thereby preventing it from entering the nucleus where it normally stimulates the transcription of Notch1 target genes (*e.g. BIRC5* [encoding survivin], *CCND1*, *ERBB2*; Fig. 1). Notch signaling plays an important role, mediated through various effects on differentiation, survival and/or proliferation, in the normal development of many tissues and cell types. Since alterations in the control of differentiation, survival and/or proliferation underlie malignant transformation, altered Notch signaling contributes to cancer development in several different ways [1]. The development of poorly differentiated adenocarcinomas in murine mammary glands following increased Notch signaling has been shown, and increased Notch signaling is sufficient to transform normal breast epithelial cells, most likely through the suppression of apoptosis [2]. Pece *et al.* recently showed that Numb-mediated control of Notch signaling is lost in approximately 50% of human mammary carcinomas due to enhanced Numb ubiquitination and proteasomal degradation [3]. Inhibition of Notch signaling may be a viable therapeutic strategy for specific targeting of Notch dependent tumors as the transformed phenotype of human breast cancer cell lines can be reversed upon inhibition of Notch signaling [2]. Furthermore, a previously unknown function of human Numb as a regulator of the tumor suppressor p53 was recently reported. Numb interacts with MDM2, preventing it from blocking the transcriptional activity of p53 [4]; at the same time Numb prevents the proteasomal degradation of the p53 protein [5] (Fig. 1). Interestingly, in primary breast tumor cells, loss of Numb expression correlated with decreased p53 levels and increased chemo-resistance; this was found to result in an aggressive tumor phenotype as illustrated by poor prognostic outcome for these patients [5]. Targeting the degradation of

Numb may prove to be an attractive therapeutic approach due to the possible dual effect of Numb on the inhibition of Notch signaling and the stabilization of p53.

The aim of the present study was to further elucidate, according to the REMARK recommendations [6], the significance of Numb protein expression in relation to clinical outcome in primary human breast cancer, and to investigate the correlation between Numb protein expression and (i) the triple-negative phenotype described in primary breast cancers and (ii) the occurrence of putative cancer stem cells in the breast epithelium.

Materials and Methods

Patient and cell line material

We studied 241 primary breast tumors from a cohort of 445 patients surgically treated for stage II breast cancer (age 31 to 81 years), diagnosed in the South Swedish Health Care Region between 1985 and 1994 and originally participating in two randomized clinical trials [7, 8]. All patients in the cohort received two years of adjuvant tamoxifen treatment, without stratification according to ER status. The median follow-up time for patients alive and free from metastases at the last follow-up visit was 5.3 years. The 241 patients included in the present study did not differ significantly from the 204 excluded cases (due to lack of remaining tissue) with regard to age at diagnosis, tumor size, lymph node status, distant disease-free survival (DDFS), S-phase fraction or ER and PR status.

Also included in the study were breast tumors from 24 *BRCA1* and 15 *BRCA2* mutation carriers (three *BRCA1* tumors were excluded due to lack of remaining tissue; n=21), as well as 14 breast cancer cell lines (BT474, HCC1937, HCC1428, JIMT-1, L56Br-C1, MCF7, MCF10A, MDA-MB-231, MDA-MB-361, MDA-MB-436, SK-BR-3, T47D, PMC42 and ZR75:1). All cell lines except for L56Br-C1, JIMT-1 and PMC42 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). L56Br-C1 was established at

Lund University [9], and JIMT-1 was established at Tampere University [10, 11] and purchased from the German Collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). PMC42 was received through a generous donation from Dr Anna Git at the Breast Cancer Functional Genomics Laboratory, Cancer Research UK, Cambridge Research Institute and Department of Oncology, University of Cambridge, UK. The current study was approved by the Lund University Medical Ethics Committee.

Tumor characteristics and tissue microarrays

Fresh-frozen tumor tissue was used for routine determination of the S-phase fraction using DNA flow cytometry, as described earlier [12]. A pathologist re-evaluated the histological type on whole formalin-fixed paraffin-embedded tissue sections and routine immunohistochemical determination of ER and PR status was performed as described previously [13]. Steroid receptor (SR) negativity was defined as tumors negative for ER and PR, and consequently SR positive tumors were positive for ER and/or PR. HER2 status was determined by chromogenic *in situ* hybridization (CISH) on whole tissue sections [14, 15]. Cores of 0.6 mm diameter from formalin-fixed, paraffin-embedded tumor tissue were used to generate tissue microarrays (TMAs) of the 445 cases. Three cores from each individual tumor were arrayed. These TMAs have been used for immunohistochemical staining of CK5, CK14, EGFR and cytokeratin clone AE1/AE3, as described previously [16-18]. The *BRCA1* and *BRCA2* tumors were similarly arrayed in a separate TMA. Patient data and tumor characteristics are described in detail in Table 1.

Cell lines were grown in RPMI1640 medium (Invitrogen, Carlsbad, CA) with the addition of 10-20% fetal bovine serum (HyClone, Logan, UT) and otherwise according to recommended conditions, washed with PBS, scraped off and fixed in 4% formalin for 40 min. After a brief wash in water, a cell pellet was formed using the Shandon Cytoblock Kit (Thermo Fisher

Scientific Inc., Waltman, MA) and was dehydrated in 70%, 96% and 100% ethanol for 30, 60 and 45 min, respectively, followed by xylene for 2x45 min. The cells were thereafter embedded in paraffin and three 1.0 mm cores from each cell line were transferred to a recipient paraffin block.

Immunohistochemical staining

Sections (4 μm) of the TMA blocks were mounted on Dako REAL™ Capillary Gap Microscope Slides (DAKO Denmark A/S, Glostrup, Denmark), deparaffinized in xylene and rehydrated in ethanol. Heat-mediated antigen retrieval was achieved using a 2100 Retriever (Prestige Medical Ltd, Blackburn, England) by placing slides in Tris-ethylenediamine tetraacetic acid buffer (pH 9.0; S2367; DAKO) at 125°C. Numb was detected with the rabbit polyclonal primary antibody (anti-NUMB; HPA002874; Atlas Antibodies AB, Stockholm, Sweden), amplified using a mouse-anti-rabbit antibody (M0737, DAKO) and visualized by the EnVision™ system (K5007; DAKO) with diaminobenzidene (DAB) as a substrate. Stainings were performed on a TechMate500™ (Ventana Medical Systems, Inc., Tucson, AZ). Double-immunostaining with antibodies for detection of CD44 and CD24 has been described previously and tumors were considered positive for either marker if any stained tumor cells were detected [18]. All slides were counterstained with hematoxylin for visualization of nuclei.

Immunohistochemical evaluation

The scoring was performed twice by one person (NM) in a blinded fashion. All unclear cases were discussed with a pathologist (LL). In cases of discrepant staining between the three cores from the same patient, the overall staining was assessed. Membranous staining and cytoplasmic staining were evaluated separately. Scoring was performed as follows: 0, <10%

positive tumor cells; 1, 10-50% positive tumor cells and 2, >50% positive tumor cells. Examples of the different staining patterns are shown in Fig. 2. In cases with strong cytoplasmic staining, detection of membranous Numb was difficult; therefore the number of tumors positive for membranous Numb may be underestimated. As a consequence, we found little or no correlation between membranous Numb staining and the most common prognostic markers. In fact, membranous Numb was only significantly correlated to HER2 status (in a positive manner; Fisher's exact test; P=0.01). We will from now on only refer to the cytoplasmic expression of Numb and use the terms deficient (0), reduced (1) and retained (2) Numb expression, respectively, throughout this report.

Global gene expression analysis

For a subset of the tumors included in this study, mRNA expression analyses had previously been performed using cDNA microarrays with 27,648 reporters [14, 19]. This data is publicly available through the Gene Expression Omnibus database (accession numbers GSE6577 and GSE5325). For sixty-three of these tumors we had information on CD44+/CD24- status and Numb staining, and for these tumors we extracted the information on the gene expression levels of *NOTCH1* and *BIRC5* (encoding survivin), both downstream targets of Numb, for further analysis.

Statistical analyses

Statistical analyses used to assess correlations between Numb expression and tumor and patient characteristics included Fisher's exact test, Chi2 test for linear trend, the two-sample t-test and Kruskal-Wallis test, when applicable. DDFS was evaluated using the Kaplan-Meier method and a comparison of survival between the groups was performed using the log-rank test for trend. Uni- and multivariate Cox regression analyses were used to estimate hazard

ratios (HR) and Schoenfeld's test was used to check proportional hazards (PH) assumptions. Since significant deviations from PH were observed for Numb, ER status and HER2, the follow-up was restricted to the first five years in the Cox regression analyses. For this cut-off, which was not optimized, the PH assumption was reasonably well fulfilled for all covariates used in the multivariate model. The tests were all two-sided and $P<0.05$ was considered significant. Statistical analyses were carried out using Stata 10.1 software (Stata Corporation, College Station, TX).

Results and Discussion

We evaluated the level of cytoplasmic expression of the Numb protein in primary invasive breast tumors from a well-characterized and clinically annotated cohort of patients. Of the 241 tumors evaluated for Numb expression, 191 displayed retained expression (score 2; 79.2%), 32 displayed reduced expression (score 1; 13.3%) and 18 were Numb deficient (score 0; 7.5%). Taken together, approximately 21% of the tumors displayed reduced or deficient levels of the Numb protein. These findings are in agreement with Pece *et al*., who showed that normal breast parenchyma displayed strong and homogeneous Numb staining, while breast tumors displayed heterogeneous, and often absent, Numb immunoreactivity [3]. The associations between Numb expression and patient and tumor characteristics in the studied cohort are summarized in Table 1. There was a significant positive correlation between Numb expression (deficient and reduced *vs.* retained) and ER and PR status (Fisher's exact test; $P<0.001$ and $P=0.004$, respectively). Numb expression also correlated to menopausal status, with a higher proportion of the Numb deficient or reduced tumors occurring in pre-menopausal women (Fisher's exact test; $P<0.001$). Accordingly, the mean age was significantly higher in patients with retained Numb expression (62 years *vs.* 58 years; t-test; $P=0.006$). To further investigate the correlation between Numb levels and disease

aggressiveness, we performed a Kaplan-Meier analysis of DDFS (Fig. 3). An association was found between deficient or reduced levels of Numb and poorer DDFS (log rank test for trend; *P*=0.01) and consequently, patients with Numb deficient tumors presented a higher risk of developing distant metastases compared to patients with tumors expressing reduced or retained Numb (44%, 31% and 24% respectively). This finding is also in agreement with previously published data on the inverse correlation between Numb expression levels and indicators of aggressive disease [3].

The effect of Numb on DDFS was estimated using a Cox regression model with two dummy variables contrasting deficient and reduced expression of Numb to retained expression. Follow-up was restricted to the first five years and a significant prognostic effect was seen for deficient *vs*. retained expression (HR=4.0, 95% CI: 1.9-8.9, *P*<0.001) whereas the effect for reduced *vs*. retained expression was weaker (HR=1.7, 95% CI: 0.79-3.8, *P*=0.17). When adding lymph node status, age, tumor size, HER2 status and ER status to the model, the effect of deficient *vs*. retained Numb expression was still a factor 2, but no longer significant (HR=2.2, 95% CI: 0.90-5.3, P=0.08). While adjustment for a proliferation marker would have been interesting, meaningful statistical analysis was not possible since values for S-phase fraction were missing for 33% of the tumors in the study (including 8/18 tumors with deficient Numb expression).

It has been shown that breast cancers can be subdivided into biologically and clinically relevant subgroups based on global transcriptional profiles [20, 21], and also based on protein levels of molecular markers such as the steroid hormone receptors ER and PR, the growth factor receptors HER2 and EGFR, and the basal cytokeratins 5/14 (basal-like phenotype: positive for CK 5/14 and/or EGFR; non-basal-like negative for both CK5/14 and EGFR [18]). Interestingly, we found a significant difference in Numb expression between the different subgroups, here based on protein markers (Fisher's exact test in a 5-by-2 table; *P*=0.001). A

higher percentage of the tumors from the triple-negative (SR-/HER2-) subgroup displayed reduced or deficient Numb expression compared to tumors in the other subgroups (P=0.004). The largest difference was seen between tumors from the triple-negative subgroup and tumors in the SR+/HER2- subgroup (44% [11/25] of the basal-like and 27% [4/15] of the non-basal-like triple-negative subgroups *vs.* 14% [21/146] of the SR+/HER2- subgroup). Only one of the tumors with deficient, and none of the tumors with reduced Numb expression were SR+/HER2+, and hence all but one of the SR+/HER2+ tumors displayed retained Numb expression (Table 2). Due to missing subtype information from 21 patients, these cases were excluded from this analysis, resulting in a non-random distribution of included patients with regards to tumor size, lymph node status and ER/PR status. Importantly, however, there were no differences in age, DDFS or S-phase fraction between included and excluded cases. Nevertheless, these findings should be interpreted with caution and require confirmation in larger, independent patient groups.

Numb deficiency was also seen more frequently in tumors from *BRCA1* mutation carriers, whose tumors are most often triple-negative, compared to sporadic tumors (Chi2-test for trend; P<0.001). The *BRCA2*-dependent tumors did not differ significantly from either sporadic or *BRCA1*-dependent tumors. However, due to the small size of this group significant differences were not expected, and hence this result should be interpreted cautiously (Table 3).

Numb staining of 14 different breast cancer cell lines supported the observed association between Numb expression and molecular subtype (cell line classification according to Neve *et al.,* Jönsson *et al.* and Mackay *et al.* [22-24]). The basal-like cell lines L56Br-C1, HCC1937, HCC1428 and MDA-MB-231 were all Numb deficient, as was JIMT-1 (which is classified as borderline between HER2+ and basal-like) and MCF10A (a non-tumorigenic epithelial cell line whose gene expression profile is correlated to the normal-like phenotype, but also to the

basal-like and HER2+ profiles). The basal-like cell lines MDA-MB-436 and PMC42 both displayed reduced Numb expression, while the luminal cell lines BT474, SK-BR-3, MCF7, MDA-MB-361, T47D and ZR-75-1 displayed retained Numb levels.

The CD44+/CD24- phenotype of breast tumor cells has been associated with cancer stem cell-like characteristics [25] and in the present study we established an inverse correlation between Numb expression levels and this phenotype (P=0.04; Table 4). The 41 patients excluded from this analysis due to missing information on CD44/CD24 status were not significantly different from those included regarding age, tumor size, lymph node status, DDFS, S-phase fraction and ER/PR status (data not shown), strengthening this finding. Sixty-three of the tumors included in this study had previously been analyzed by global gene expression profiling [14, 19], examined for their content of CD44+/CD24- cells [18] and were divided into tumor biological subgroups according to their SR:HER2:CK5/14:EGFR status. Interestingly, all of these tumors displayed an intermediate level of *NUMB* mRNA expression (data not shown), supporting the notion that regulation of the Numb protein may be controlled by ubiquitination and proteasomal degradation. However, the levels of *NOTCH1* and *BIRC5* (encoding survivin) differed significantly between the five subgroups (Kruskal-Wallis; P=0.05 and P=0.01, respectively), and the subgroup with the most aberrant expression of both *NOTCH1* and *BIRC5* compared to the four other subgroups was the basal-like subgroup (Mann-Whitney; P=0.007 and P=0.01, respectively; Fig. 4). The largest differences in expression were seen between the luminal tumors and the basal-like tumors, where the median level of *NOTCH1* expression was 1.9 times higher in the basal-like than in the SR+/HER2- subgroup (corresponding to luminal A), and 1.45 times higher than in the SR+/HER2+ subgroup (largely corresponding to luminal B). The levels of *BIRC5* differed even more between the subgroups, the median expression being 2.8 and 1.9 times higher in the basal-like tumors than in the SR+/HER2- and SR+/HER2+ subgroups, respectively.

Altered Notch signaling has been linked to many human diseases, including breast cancer. Stylianou *et al.* show compelling evidence that aberrant Notch signaling is frequent in a wide variety of breast cancers, and that reduction of Notch signaling reverts the altered phenotype of human breast cancer cell lines, suggesting that inhibition of Notch signaling may be a viable therapeutic strategy for Notch dependent tumors [2]. Lee and co-workers were able to link activation of Notch signaling in ER negative but not in ER positive breast cancer cells to a direct transcriptional up-regulation of the apoptosis inhibitor and cell cycle regulator survivin, and also showed that ER negative tumors become dependent upon Notch-survivin signaling for their survival [26]. We did not observe any difference in DDFS between the three Numb classes when ER positive and ER negative tumors were analyzed separately, as the present cohort was too small to allow for meaningful statistical analyses within the ER positive and negative subgroups respectively. However, we found an inverse correlation between retained expression of Numb and the CD44+/CD24- phenotype (Fisher's exact; P=0.01) in ER positive tumors. This finding was not observed in the ER negative tumors, a fact that may be explained by the small number of cases in this group.

As Pece *et al.* recently showed, the Numb protein has a negative regulatory effect on Notch signaling. Numb operates as an onco-suppressor, decreasing Notch signaling when expression of Numb is enforced in Numb deficient/Notch reliant cells, through binding to the Notch intracellular domain (NICD). They also presented evidence that growth suppression can be induced in Numb negative but not Numb positive cells by restoration of physiological Numb levels, and that restoration of Numb function may be obtained by pharmacological inhibition of the enzyme(s) responsible for its degradation.

Modulation of the Notch pathway by increasing the expression of Numb has a double appeal as Numb is also positively associated with the tumor suppressor p53. Colaluca and co-workers showed that Numb forms a tri-complex with p53 and the E3 ubiquitin ligase MDM2,

thereby preventing the ubiquitination and degradation of p53, resulting in increased p53 protein levels and activity [5].

In this study we have shown that loss or decrease of Numb expression is correlated to negative prognostic factors in breast cancer, such as hormone receptor negativity, a basal-like phenotype and an increased proportion of cells with a CD44+/CD24- phenotype. This group of breast tumors typically has the worst prognosis, are clinically aggressive and relapse-prone and hence especially difficult to treat. Targeting Numb degradation, thereby increasing Numb levels, is especially appealing for this difficult-to-treat group of tumors as it may both increase the activity of the tumor suppressor p53 and block the Notch-survivin pathway associated with enhanced cell proliferation and heightened viability at cell division. Targeting the degradation of Numb may prove to be clinically significant and may offer a targeted therapeutic approach for a group of patients for whom we today lack an effective treatment regimen as triple-negative tumors lack targets for anti-hormonal (anti-estrogen and aromatase inhibitors) and HER2-targeted therapies.

In summary, our results show markedly heterogeneous levels of Numb expression in primary human breast cancers. Reduced levels of Numb expression correlated with decreased disease-free survival, as well as with a higher risk of developing distant metastases. Furthermore, we have demonstrated a significant variation in cytoplasmic Numb levels between mammary carcinomas of different tumor biological subgroups. Numb deficient tumors more frequently belong to the subgroups negative for hormone receptors and HER2, suggesting that loss of Numb expression is more common in less differentiated and consequently more aggressive tumors. This notion is further supported by the positive correlation between Numb negativity and pre-menopausal status, the CD44+/CD24- phenotype, mutations in the *BRCA1* gene, occurrence of distant metastases and poor survival rates. The findings of the present study are, in our view, highly interesting and add to the overall knowledge about the role of Numb in

breast cancer. The study design was, however, exploratory and the results should therefore be interpreted primarily as new hypotheses to test in independent and preferably larger patient groups.

Acknowledgements

We thank the participating departments of the South Sweden Breast Cancer Group for their contribution of breast cancer samples, Mårten Fernö and Åke Borg for providing TMA sections and Kristina Lövgren and Mats Jönsson for technical assistance. The study was supported by funds from the Swedish Cancer Society, the Swedish Research Council, the G Nilsson Cancer Foundation, the B Kamprad Cancer Foundation, the Å Wiberg Foundation, the University Hospital of Lund Research Foundation and Governmental Funding of Clinical Research within National Health Service. KR and IH were supported by the Swedish Cancer Society.

References

1. Allenspach EJ, Maillard I, Aster JC, Pear WS (2002) Notch signaling in cancer. Cancer Biol Ther 1: 466-76
2. Stylianou S, Clarke RB, Brennan K (2006) Aberrant activation of notch signaling in human breast cancer. Cancer Res 66: 1517-25
3. Pece S, Serresi M, Santolini E, Capra M, Hulleman E, Galimberti V, Zurrida S, Maisonneuve P, Viale G, Di Fiore PP (2004) Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. J Cell Biol 167: 215-21
4. Momand J, Zambetti GP, Olson DC, George D, Levine AJ (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 69: 1237-45
5. Colaluca IN, Tosoni D, Nuciforo P, Senic-Matuglia F, Galimberti V, Viale G, Pece S, Di Fiore PP (2008) NUMB controls p53 tumour suppressor activity. Nature 451: 76-80
6. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM (2006) REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast Cancer Res Treat 100: 229-35
7. Ryden L, Jonsson PE, Chebil G, Dufmats M, Ferno M, Jirstrom K, Kallstrom AC, Landberg G, Stal O, Thorstenson S, Nordenskjold B (2005) Two years of adjuvant tamoxifen in premenopausal patients with breast cancer: a randomised, controlled trial with long-term follow-up. Eur J Cancer 41: 256-64
8. (1996) Randomized trial of two versus five years of adjuvant tamoxifen for postmenopausal early stage breast cancer. Swedish Breast Cancer Cooperative Group. J Natl Cancer Inst 88: 1543-9

9. Johannsson OT, Staff S, Vallon-Christersson J, Kytola S, Gudjonsson T, Rennstam K, Hedenfalk IA, Adeyinka A, Kjellen E, Wennerberg J, Baldetorp B, Petersen OW, Olsson H, Oredsson S, Isola J, Borg A (2003) Characterization of a novel breast carcinoma xenograft and cell line derived from a BRCA1 germ-line mutation carrier. Lab Invest 83: 387-96

10. Tanner M, Kapanen AI, Junttila T, Raheem O, Grenman S, Elo J, Elenius K, Isola J (2004) Characterization of a novel cell line established from a patient with Herceptin-resistant breast cancer. Mol Cancer Ther 3: 1585-92

11. Rennstam K, Jonsson G, Tanner M, Bendahl PO, Staaf J, Kapanen AI, Karhu R, Baldetorp B, Borg A, Isola J (2007) Cytogenetic characterization and gene expression profiling of the trastuzumab-resistant breast cancer cell line JIMT-1. Cancer Genet Cytogenet 172: 95-106

12. Ferno M, Stal O, Baldetorp B, Hatschek T, Kallstrom AC, Malmstrom P, Nordenskjold B, Ryden S (2000) Results of two or five years of adjuvant tamoxifen correlated to steroid receptor and S-phase levels. South Sweden Breast Cancer Group, and South-East Sweden Breast Cancer Group. Breast Cancer Res Treat 59: 69-76

13. Chebil G, Bendahl PO, Idvall I, Ferno M (2003) Comparison of immunohistochemical and biochemical assay of steroid receptors in primary breast cancer--clinical associations and reasons for discrepancies. Acta Oncol 42: 719-25

14. Gruvberger-Saal SK, Bendahl PO, Saal LH, Laakso M, Hegardt C, Eden P, Peterson C, Malmstrom P, Isola J, Borg A, Ferno M (2007) Estrogen receptor beta expression is associated with tamoxifen response in ERalpha-negative breast carcinoma. Clin Cancer Res 13: 1987-94

15. Isola J, Tanner M, Forsyth A, Cooke TG, Watters AD, Bartlett JM (2004) Interlaboratory comparison of HER-2 oncogene amplification as detected by chromogenic and fluorescence in situ hybridization. Clin Cancer Res 10: 4793-8

16. Jumppanen M, Gruvberger-Saal S, Kauraniemi P, Tanner M, Bendahl PO, Lundin M, Krogh M, Kataja P, Borg A, Ferno M, Isola J (2007) Basal-like phenotype is not associated with patient survival in estrogen-receptor-negative breast cancers. Breast Cancer Res 9: R16

17. Dihge L, Bendahl PO, Grabau D, Isola J, Lovgren K, Ryden L, Ferno M (2008) Epidermal growth factor receptor (EGFR) and the estrogen receptor modulator amplified in breast cancer (AIB1) for predicting clinical outcome after adjuvant tamoxifen in breast cancer. Breast Cancer Res Treat 109: 255-62

18. Honeth G, Bendahl PO, Ringner M, Saal LH, Gruvberger-Saal SK, Lovgren K, Grabau D, Ferno M, Borg A, Hegardt C (2008) The CD44+/CD24- phenotype is enriched in basal-like breast tumors. Breast Cancer Res 10: R53

19. Saal LH, Johansson P, Holm K, Gruvberger-Saal SK, She QB, Maurer M, Koujak S, Ferrando AA, Malmstrom P, Memeo L, Isola J, Bendahl PO, Rosen N, Hibshoosh H, Ringner M, Borg A, Parsons R (2007) Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. Proc Natl Acad Sci U S A 104: 7564-9

20. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. Nature 406: 747-52

21. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D,

Eystein Lonning P, Borresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98: 10869-74

22. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10: 515-27
23. Jonsson G, Staaf J, Olsson E, Heidenblad M, Vallon-Christersson J, Osoegawa K, de Jong P, Oredsson S, Ringner M, Hoglund M, Borg A (2007) High-resolution genomic profiles of breast cancer cell lines assessed by tiling BAC array comparative genomic hybridization. Genes Chromosomes Cancer 46: 543-58
24. Mackay A, Tamber N, Fenwick K, Iravani M, Grigoriadis A, Dexter T, Lord CJ, Reis-Filho JS, Ashworth A (2009) A high-resolution integrated analysis of genetic and expression profiles of breast cancer cell lines. Breast Cancer Res Treat
25. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100: 3983-8
26. Lee CW, Raskett CM, Prudovsky I, Altieri DC (2008) Molecular dependence of estrogen receptor-negative breast cancer on a notch-survivin signaling axis. Cancer Res 68: 5273-81

Table 1 Associations between Numb expression and patient and tumor characteristics.

Characteristic	*All tumors (n=241)*	*Deficient or reduced Numb (n=50)*	*Retained Numb (n=191)*	*P value*[a]
Mean age	61.4	58.0	62.2	**0.006**[b]
Menopausal status				**0.001**
Premenopausal	42 (17%)	17 (34%)	25 (13%)	
Postmenopausal	199 (85%)	33 (66%)	166 (87%)	
Tumor Size				**0.03**
>20 mm	183 (76%)	44 (88%)	139 (73%)	
≤20 mm	58 (24%)	6 (12%)	52 (27%)	
Lymph node status				0.18
Positive (n>0)	159 (66%)	29 (58%)	130 (68%)	
Negative (n=0)	82 (34%)	21 (42%)	61 (32%)	
S-phase fraction				1.0
High (≥12%)	43 (27%)	9 (27%)	34 (26%)	
Low (<12%)	119 (73%)	24 (73%)	95 (74%)	
Missing	79			
Histological type				0.24
DCIS	1 (-)	0 (-)	1 (1%)	
Ductal	175 (76%)	33 (73%)	142 (77%)	
Lobular	31 (14%)	9 (20%)	22 (12%)	
Lobular + ductal	11 (5%)	0 (-)	11 (6%)	
Medullary	9 (4%)	3 (7%)	6 (3%)	
Other	2 (1%)	0 (-)	2 (1%)	
Missing	12			
Estrogen receptor				**<0.001**
Positive	166 (70%)	21 (42%)	145 (77%)	
Negative	72 (30%)	29 (58%)	43 (23%)	

Missing	3			
Progesterone receptor				**0.004**
Positive	113 (48%)	14 (29%)	99 (53%)	
Negative	123 (52%)	34 (71%)	89 (47%)	
Missing	5			
HER2				0.37
Amplified	35 (15)	9 (19%)	26 (14%)	
Non-amplified	195 (85%)	38 (81%)	157 (86%)	
Missing	11			
Cytokeratin 5/14				**<0.001**
Strong	29 (14%)	16 (34%)	13 (8%)	
Weak/negative	185 (86%)	31 (66%)	154 (92%)	
Missing	27			
EGFR				0.22
Strong	19 (9%)	6 (15%)	13 (8%)	
Weak/negative	194 (91%)	35 (85%)	159 (92%)	
Missing	28			

[a]All *P* values were calculated using Fisher's exact test, except when calculating correlation between Numb expression and age when a t-test was used ([b]). Tumors with missing values were excluded from the analyses. DCIS, ductal carcinoma *in situ*.

Table 2 Correlations between Numb protein expression and tumor biological subgroups. Tumors were divided into subgroups according to their steroid receptor (ER and PR) and HER2 status. SR+/HER2- and SR-/HER2+ corresponding to the molecular subtypes luminal A and HER2 positive, respectively, and SR+/HER2+ largely corresponding to luminal B. The triple-negative tumors were further subdivided into basal-like (positive for CK 5/14 and/or EGFR) and non-basal-like (negative for both CK5/14 and EGFR; the resulting subgroups corresponded well to gene expression subtypes [18]). The proportion of tumors with retained Numb expression differed significantly between the five subgroups (Fisher's exact test; P=0.001).

	Subgroup					
Numb expression	***SR+/HER2-***	***SR+/HER2+***	***SR-/HER2+***	***SR-/HER2- non-basal-like***	***SR-/HER2- basal-like***	***Total***
Deficient/ reduced	21 (14%)	1 (6%)	7 (39%)	4 (27%)	11 (44%)	44 (20%)
Retained	125 (86%)	15 (94%)	11 (61%)	11 (73%)	14 (56%)	176 (80%)
Total	146	16	18	15	25	220

Table 3 Numb score of sporadic, *BRCA2* and *BRCA1* deficient tumors, respectively. A significantly higher proportion of Numb deficient cells was detected in the *BRCA1* deficient tumors compared to sporadic tumors (Chi2 test for trend; $P<0.001$).

Numb expression	***Sporadic***	***BRCA2***	***BRCA1***
Deficient	18 (7%)	2 (13%)	10 (48%)
Reduced	32 (13%)	2 (13%)	0 (0%)
Retained	191 (79%)	11 (73%)	11 (52%)
Total	241	15	21

Table 4 Correlations between Numb expression and the CD44+/CD24- (cancer stem cell) phenotype. We found a significant negative correlation between the expression of Numb and the CD44+/CD24- (cancer stem cell) phenotype (Chi2 test for trend; *P*=0.04), such that a higher proportion of Numb deficient cells displayed the cancer stem cell phenotype than did tumors with retained Numb expression. This trend is, however, not linear; the cancer stem cell phenotype was equally common in tumors with deficient and reduced Numb expression but significantly less common in tumors with retained Numb expression (*P*=0.05; Fisher's exact test).

	Numb expression			
CD44+/CD24- phenotype	***Deficient***	***Reduced***	***Retained***	***Total***
Positive (>0%)	5 (45%)	12 (46%)	45 (28%)	62 (31%)
Negative (0%)	6 (55%)	14 (54%)	118 (72%)	138 (69%)
Total	11	26	163	200

Figure legends

Fig. 1 Schematic overview of the interactions between Numb, Notch and p53.

Ligand binding to the extracellular domain of Notch causes cytoplasmic release of the Notch intracellular domain (NICD). NICD then enters into to the nucleus where it binds to the nuclear transcription factor CSL. By doing so it recruits co-activators (CoA) to the complex and stimulates the transcription of Notch target genes. Numb acts as a docking protein for NICD, preventing NICD from translocating to the nucleus, thereby inhibiting intracellular Notch signaling. At the same time Numb interacts with the p53 regulating protein MDM2. MDM2 inhibits p53 activity by blocking its transcriptional activity, favors its nuclear export and stimulates its degradation through poly-ubiquitination. It has recently been shown that Numb forms a trimeric complex with p53 and MDM2, thereby regulating the stability of p53.

Fig. 2 Numb expression in primary breast cancers.

Examples of a) deficient (score 0, <10% positive tumor cells), b) reduced (score 1, 10-50% positive tumor cells), and c) retained (score 2, >50% positive tumor cells), expression of Numb.

Fig. 3 Kaplan-Meier survival estimates of DDFS by Numb protein expression.

Tumors were subdivided into three categories based on the fraction of Numb positive cells: Deficient, <10% positive tumor cells; Reduced, 10-50% positive tumor cells; Retained, >50% positive tumor cells. We found a significant difference between the three subgroups, with the shortest time to progression for the group of patients with Numb deficient primary tumors (log-rank test for trend; P=0.01). The numbers below the plot represent the number of patients at risk at each time point, and the total number of recurrences in each group.

Fig. 4 Gene expression levels of *NOTCH1* (a) and *BIRC5* (b).

Expression levels of *NOTCH1* and *BIRC5* in 63 of the tumors included in the study differed significantly between the five subgroups (Kruskal-Wallis; P=0.05 and P=0.01, respectively). The basal-like tumors, with the highest expression of *NOTCH1* and *BIRC5* differed significantly from the four other subgroups (Mann-Whitney; P=0.007 and P=0.01, respectively). The y-axes were drawn on a log2 scale.