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ORIGINAL PAPER

HIF-1α and NOTCH signaling in ductal and lobular carcinomas of the breast

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Abstract
Background NOTCH signaling is involved in every step of metazoan development and maintenance of adult tissue homeostasis. It is frequently deregulated by mutations and overexpression in different cancer types including solid tumors such as breast cancer. Another common feature of solid tumors is hypoxia, which occurs due to defective or insufficient vascularization. Hypoxia-inducible factors (HIFs) are key regulators of the homeostatic response to low oxygen levels. HIF-1α is overexpressed in many solid tumors, including breast cancer. Hypoxia-induced stabilization of HIF transcription factors has been shown to lead to NOTCH activation in vitro in different contexts and tissues, causing differentiation arrest and induction of proliferation and migration.
Methods Since the link between HIF-1α and NOTCH signalling has hardly been studied, we set out to closely investigate associations between the expression of HIF-1α and NOTCH pathway members in primary and metastatic human breast cancer specimens and their prognostic value.
Results Co-expression of NOTCH1 intracellular domain (N1ICD) and HIF-1α was associated with a high grade and a high proliferation rate in invasive breast cancer. HIF-1α expression was low in classic, but high in pleomorphic lobular cancers, which also frequently showed stromal HIF-1α expression. NOTCH1 pathway activation was prognostically unfavorable.
Conclusion In breast cancer, NOTCH pathway activation appears to be associated with a poor prognosis, but NOTCH and HIF signaling do not seem to be functionally associated.

Keywords Hypoxia inducible factor-1 · NOTCH signaling · Breast cancer · Prognosis · Metastasis

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1 Introduction

NOTCH signaling serves as a short-range cell-cell communication pathway, which is highly conserved from flies to mammals. It is involved in every step of metazoan development and maintenance of adult tissue homeostasis. NOTCH proteins are single pass type transmembrane receptors consisting of extracellular ligand binding and cytoplasmic signal transduction domains [1]. Canonical NOTCH signaling is regulated by proteolysis and initiated by the interaction of transmembrane bound ligands and receptors on neighboring cells. Upon the final intramembranous cleavage of the receptor, the NOTCH intracellular domain (NICD) is released and translocated to the nucleus. In the nucleus, NICD interacts with the transcription factor CSL (CBF1 in humans; RBP-J_k in mice) to activate the HES/HEY family of genes, which are involved in growth, proliferation, differentiation, and survival [2]. NOTCH signaling is frequently deregulated by mutation and overexpression in different cancer types [3]. Therefore, targeting NOTCH in human malignancies could be a powerful approach to counteract tumor progression.

The NOTCH pathway has also been shown to play an important role in breast cancer. NOTCH1 is overexpressed in breast cancer and the NOTCH1 locus was also identified

as a Mouse Mammary Tumor Virus (MMTV) insertion site in MMTV-Neu tumors [4]. Based on previous research, we have proposed that dose and context dependency of NOTCH pathway activation may lead to different phenotypes in different tissues [5]. Immunohistochemistry performed on human breast cancer specimens revealed frequent overexpression of NOTCH1 [6], NOTCH3 [7–9] and the NOTCH ligand JAGGED1 in most cases, together with loss of expression of NUMB, a negative regulator of NOTCH [10]. NOTCH family members seem to have different roles in cancer. For example, high NOTCH2 expression in breast cancer has been shown to correlate to a higher chance of survival [11]. In vivo studies performed in mice supported the tumor promoting role of NOTCH1, NOTCH3 and NOTCH4 [7, 12, 13].

Low oxygen tension (hypoxia) is a common feature of solid tumors and it can occur due to defective or insufficient vascularization. Hypoxia-inducible factors (HIFs) are key regulators of the homeostatic response to low oxygen levels. Hypoxia and HIF-1α, the hypoxia response-regulating unit of the HIF complex, have been proposed to play a role in breast carcinogenesis [14] and to affect breast cancer prognosis [15, 16]. Hypoxia-dependent NOTCH activation has been observed in different contexts and tissues [17–19]. Recently, Chen et al. demonstrated that hypoxia-induced stabilization of HIF transcription factors leads to expression of the NOTCH target genes HES1 and HEY1 in human breast cancer cell lines [20]. In another study, Xing et al. reported increased JAGGED2 (a NOTCH ligand) and NOTCH1 intracellular domain (N1ICD) expression in the hypoxic invasive front of breast tumors. [21]. Given these interesting results, which indicate a potential link between these two oncogenic pathways in breast cancer, we investigated the associations between HIIF-1α and N1ICD expression in primary and metastatic human breast cancer specimens, in conjunction with downstream targets of both pathways.

2 Materials and methods

2.1 Tissue samples

Formaldehyde-fixed paraffin embedded breast cancer tissue blocks of 449 cases were collected from the archives of Departments of Pathology of the University Medical Center Utrecht (Utrecht, The Netherlands) and the Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands) to prepare tissue microarrays (TMAs). This series was enriched in lobular carcinomas because of a special interest in this type of cancer by our research group. Typing was done according to the WHO, and grading was done according to the Nottingham scheme. The mitotic activity index (MAI) was assessed as previously described [22]. Representative areas containing morphologically well-defined tumor tissues were identified in haematoxylin-eosin stained reference slides by a pathologist (PJvD). Tissue cylinders of 0.6 mm were transferred from these tumor areas in each donor block to the recipient block using a tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Four μm thick serial sections were cut from the recipient blocks and transferred to Superfrost + slides to produce TMAs (Menzel and Glaeser, Germany) for immunohistochemistry. The use of anonymous or coded leftover material for scientific purposes is part of the standard treatment contract with patients in The Netherlands [23]. Ethical approval was therefore not required. Overall survival data were obtained from the Comprehensive Cancer Centre of The Netherlands (Integraal Kankercentrum Nederland, IKNL).

Material of distant metastases (including brain, lung, skin, liver and GI tract) was available for 44 out of 449 primary breast cancer cases (19 lobular, 6 ducto-lobular and 19 ductal). These were used to investigate the difference in HIF-1α and N1ICD expression in primary tumors and their corresponding metastases.

2.2 Immunohistochemistry

Immunohistochemistry was carried out for the following proteins: N1ICD, HES1, HIF-1α, and the HIF downstream proteins CAIX and GLUT1. After deparaffination and rehydration, the TMA slides were immersed in a buffer solution containing 0.3 % hydrogen peroxidase for 15 min to block endogenous peroxidase activity. Antigen retrieval was obtained by boiling for 20 min in 10 mM citrate buffer pH 6.0 for GLUT1, CAIX and N1ICD staining, or Tris/EDTA buffer pH 9.0 for HES1 and HIF-1α staining. A cooling off period of 30 min was applied before pre-incubation with 1:50 normal goat serum in PBS (pH: 7.4), containing 0.1 % sodium azide and 1 % BSA to block unspecific binding sites. This was followed by primary antibody incubation: polyclonal rabbit activated NOTCH1 antibody (Abcam, Cambridge, UK) in 1:500, HES1 antibody (Millipore, Billerica, MA, USA) 1:600, GLUT1 (DAKO, Glostrup, Denmark) in 1:200; CAIX (Abcam) 1:1,000 in PBS/1%BSA, HIF-1α (BD Transduction Labs, Breda, The Netherlands). The slides were incubated with antibody solutions either for 1 h at room temperature (GLUT1, CAIX) or overnight at 4 °C (HIF-1α, HES and NOTCH1). After that, the sections were incubated with Brightvision poly-HRP anti-mouse, rabbit, rat (DPVO-HRP, Immunologic, Duiven, The Netherlands) or the Novolink kit (Leica, Rijswijk, The Netherlands) (in the case of HIF-1α) and developed with diaminobenzidin, counterstained with hematoxylin, dehydrated in alcohol, and sealed with a coverslip. Throughout the immunohistochemical analyses, negative controls were obtained by omitting the primary

Table 1 Clinopathological characteristics of 449 invasive breast cancer patients included in this study

Feature	Grouping	N or value
Age (years)	mean	60
	range	28-88
Histological Type	IDC	290
	ILC	119
	other	40
Tumour size (cm)	≤2	179
	>2 and ≤5	203
	>5	46
	missing	21
Mitotic Index (per 2 mm^2)	≤ 12	204
	≥ 13	230
	missing	15
Lymph node status	negative	139
	positive	193
	missing	117

antibodies. For NOTCH1 and HES1 staining, normal breast tissue was used as a positive control. For GLUT1 staining, positive erythrocyte staining was used as an internal control. For CAIX and HIF-1α, a breast cancer case was included that was previously proven to be positive for these markers.

2.3 Scoring

Scoring of immunohistochemistry was done in a blinded fashion with respect to patient characteristics and other staining results. N1ICD and HES1 staining in more than 10 % of nuclei was considered as positive. The percentage of nuclei positive for HIF-1α was estimated as well. The staining was considered positive when ≥1 % nuclear staining was observed, as described before [24]. GLUT1 and CAIX expression were scored positive when membrane staining was observed. HIF-1α expression in the stroma was also scored positive when frequent nuclear staining in fibroblasts was observed.

2.4 Statistics

The SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Pearson's Chi-square test was used to examine associations between categorical variables. Percentages of nuclei expressing HIF-1α and N1ICD in primary tumors and their corresponding metastasis were compared by a paired Student's *t*-test. The graphs were made using GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Survival analysis was performed by plotting Kaplan-Meier curves and Log rank test. Two-sided p-values below 0.05 were considered as statistically significant. Multivariate survival analysis was performed using Cox regression, using entry and removal limits of 0.05 and 0.10, respectively.

Table 3 Summary of associations between expression of HIF-1α and N1ICD and their downstream targets CAIX, GLUT1 and HES1 in invasive breast cancer (chi-square test)

	CAIX	GLUTI	HES1
HIF1-α	$p<0.001$	$p<0.001$	$p=0.313$
N1ICD	$p=0.283$	$p=0.279$	$p=0.004$

3 Results

3.1 Associations between HIF-1α and NOTCH expression and clinicopathological features

Table 1 summarizes the clinicopathological characteristics of the patient material used in this study. Table 2 shows the association between HIF-1α and N1ICD and Table 3 summarizes the associations between the expression of the proteins studied. The expression of HIF-1α and that of its downstream targets CAIX and GLUTI ($p<0.001$ for both, Table 3) were significantly associated, as expected, as were those of N1ICD and its downstream target HES1 ($p=0.004$). HIF-1α expression correlated with neither N1ICD ($p=0.435$, Table 2) nor HES1 expression, and N1ICD expression did not correlate with the expression of HIF-1α targets. The only significant correlation observed was between GLUT1 and HES1 expression ($p=0.003$) (Tables 3 & 4). Also, no significant associations were noted between the expression of proteins in these pathways, when investigated in the N1ICD negative subgroup (data not shown).

Table 2 Association between expression of HIF-1α and N1ICD (chi-square test)

	HIF-1α expression		
N1ICD expression	-	+	p-value
-	50	31	0.435
+	222	128	

Table 4 Association between expression of GLUT1 and HES1 (chi-square test)

	GLUT1 expression		
HES1 expression	-	+	p-value
-	184	67	0.003
+	167	31	

Table 5 Associations between HIF-1α and NOTCH1 intracellular domain (N1ICD) co-expression and clinicopathologic features (* means $p<0.001$)

Feature	Coexpression of HIF-1α and N1ICD			
	N1ICD↓ HIF-1α↓	N1ICD↓ HIF-1α↑	N1ICD↑ HIF-1α↓	N1ICD↑ HIF-1α↑
Histological type				
Ductal	40	31	135	84
Lobular	26	6	70	30
Other	1	3	16	11
Histologic grade				
1	13	5	46	9
2	21	7	88	41
3	30	27	78	72*
Tumour size (cm)				
≤2	30	15	94	50
>2 and ≤5	32	22	93	59
>5	4	2	26	14
Mitotic Index (per 2 mm^2)				
≤ 12	29	11	128	46
≥ 13	36	28	87	76*
Lymph node status				
Negative	34	21	106	55
Positive	27	19	97	64

Co-expression of N1ICD and HIF-1α was significantly associated with a high tumor grade ($p<0.001$) and a high mitotic activity index (MAI; $p<0.001$) (Table 5). No other significant correlations with clinicopathological features were observed.

3.2 Associations between HIF-1α and NOTCH pathway proteins in primary breast cancers and distant metastases

There were no significant associations between the expression of N1ICD and HIF-1α pathway proteins in distant

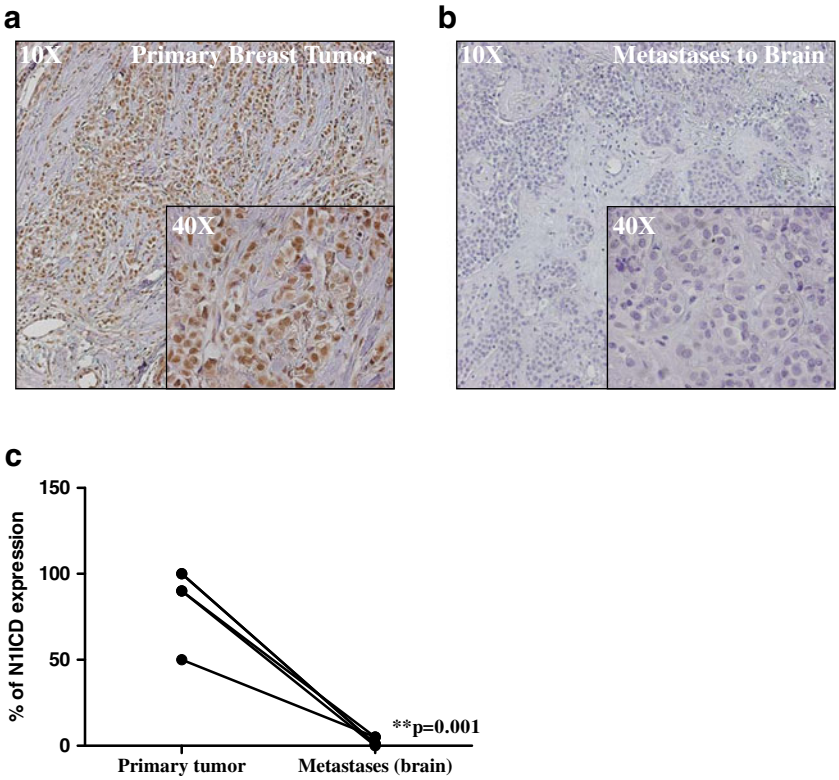

Fig. 1 High N1ICD expression in primary tumors which metastasized to brain. A representative image of N1ICD staining in a primary breast tumor case **a**) and its corresponding metastases **b**) to the brain. **c**) N1ICD expression in paired primary breast tumors and corresponding brain metastases ($p<0.001$)

Table 6 HIF-1α expression in tumor nuclei and stromal fibroblasts of pleomorphic versus classic invasive lobular cancers

	HIF-1α expression in tumor cells			HIF-1α expression in fibroblasts		
	-	+	p-value	-	+	p-value
Pleomorphic ILC	33	21	0.001	31	23	0.001
Classic ILC	33	1		34	0	

metastases (data not shown). However, primary breast tumors expressed on average higher levels of N1ICD than their corresponding metastases ($p<0.05$), especially in the cases that metastasized to brain ($p=0.001$) and skin ($p<0.05$) (Fig. 1).

3.3 HIF-1α expression in lobular breast carcinomas and surrounding stroma cells

High HIF-1α expression in tumor cells was observed more often in ductal carcinomas (109/277, 39 %) and the pleomorphic variant of lobular breast cancer (21/54, 38.8 %) as compared to classic lobular cancer cases (1/34, 2.9 %) ($p<0.001$). Strikingly, pleomorphic lobular cases more frequently exhibited stromal HIF-1α expression as compared to classic lobular breast carcinomas (42.5 % vs 0 %, $p=0.001$) (Table 6). Figure 2 shows representative images of HIF-1α expression in tumor cell nuclei (A) and in stromal fibroblast nuclei (B). Stromal HIF-1α correlated to HIF-1α expression in the tumor ($p<0.001$).

3.4 Associations between HIF-1α and NOTCH pathway proteins and survival

In all patients, there was no difference in survival between patients with no N1ICD and HIF-1α expression and those with either N1ICD expression alone or N1ICD/HIF-1α co-expression ($p=0.708$). The same applied to the subgroups of classic and pleomorphic ILC (Fig. 3). Stromal HIF-1α expression in both classic and pleomorphic lobular breast cancer patients also had no prognostic value. In the entire group, patients with high HES1 expression exhibited a significantly worse survival as compared to the low HES1 expressing group ($p=0.03$) (Fig. 4).

Upon Cox regression analysis, lymph node status and mitotic index were found to act as independent prognosticators, and none of the other variables had additional prognostic value. HES1 was, however, close to having additional prognostic value ($p=0.089$).

4 Discussion

The NOTCH and HIF signaling pathways are highly conserved through evolution, and play a role in various cellular processes [25, 26]. Several studies have demonstrated the overlapping effects of hypoxia-induced expression of HIFs and NOTCH signaling in normal development and various cancers [20, 27]. It has been suggested that NOTCH signaling is aberrantly activated during tumor progression, and that hypoxia might further stimulate its activation [20]. For example, NOTCH1 mRNA levels were found to increase upon stabilization of HIF-1α in melanoma cell lines [19], and NOTCH1 signaling was upregulated in lung cancer cell lines cultured in hypoxic conditions [17]. Very recently, Xing et al. (2011) investigated a large group of breast cancer patients and observed a strong upregulation of JAGGED2, a NOTCH ligand, and NOTCH signaling at the hypoxic invasive tumor front, although they did not show a firm correlation between HIF and NOTCH signaling by immunohistochemistry analysis in patient material [21]. Previously, Chen et al. (2010) demonstrated by chromatin immunoprecipitation (ChIP) that hypoxia-induced HIF-1α binds to the HES1 promoter, thereby inducing its activity

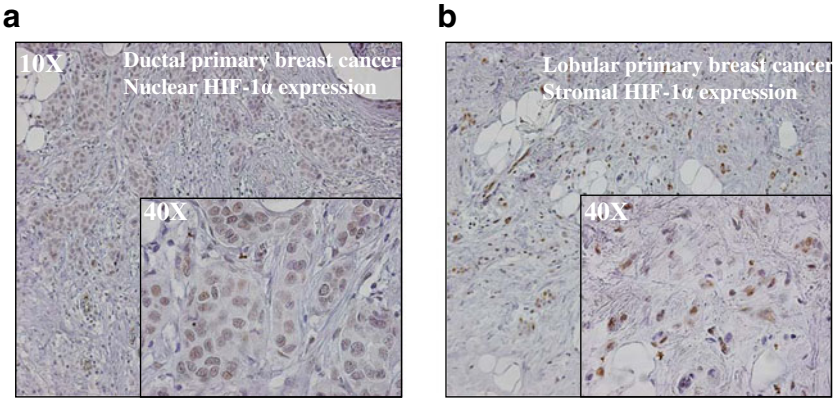

Fig. 2 HIF-1α expression in ductal and lobular breast carcinomas and surrounding stroma. Representative images of HIF1-α staining in **a**) ductal breast carcinoma and **b**) lobular breast carcinoma

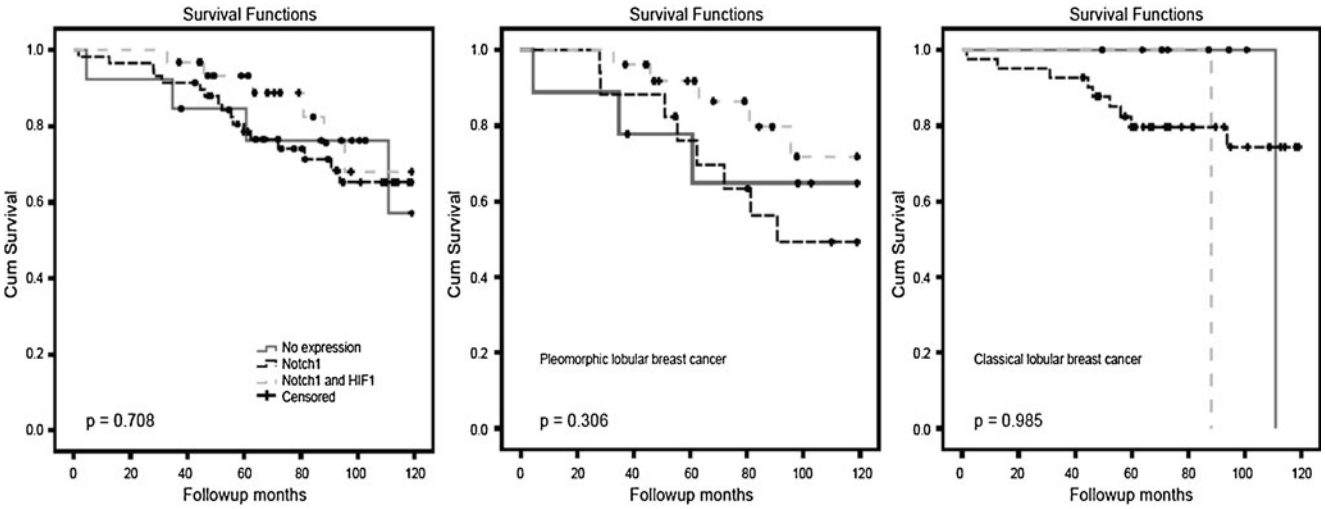

Fig. 3 Survival curves of breast cancer patients according to HIF-1α/N1ICD status (left all patients, middle and right subgroups of pleomorphic and classic ILC, respectively). There were no significance differences in survival

[20]. These researchers claimed that the mechanism behind this observation likely involves an interaction of HIFs with the NOTCH co-activator MAML1, which potentiates NOTCH activation. Our immunohistochemistry results did not show significant correlations between HIF-1α and N1ICD expression, indicating no direct functional effect of HIF-1α on the activation of NOTCH in invasive breast cancer. There was also no association observed between HIF-1α and HES1, suggesting that HIF-1α might not directly induce HES1 expression, as was suggested by the ChIP experiments of Chen et al. [20]. Our observation of the association between GLUT1 and HES1 expression is interesting in the light of the work of Efferson et al. where the group observed a correlation between GLUT1 and HES1 protein expression in HER2 positive human breast cancer specimens [28]. One of the mechanisms by which HES1 is known to regulate cell growth is through AKT/mTOR signaling pathway [29]. Moreover, glucose metabolism during cell growth is known to be influenced by AKT/mTOR signaling pathway through different anabolic processes one of which is the glucose uptake through HIF-1α transcriptional control of the GLUT1 [30]. Efferson et al. hypothesizes that the correlation between HES1 and GLUT1 protein expression is due to this influence of HES1 on AKT/mTOR signaling pathway which in turn controls GLUT1 levels [28].

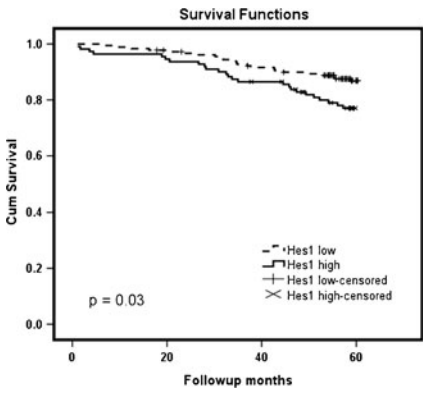

Fig. 4 Survival curves of breast cancer patients according to HES1 status. High HES1 expression, indicating activation of the NOTCH pathway, is associated with worse survival

Interestingly, co-expression of HIF-1α and N1ICD was significantly associated with high tumor grade and high mitotic activity index, suggesting that activation of both signaling pathways might be leading to a more aggressive phenotype. However, this hypothesis should be investigated and supported further by functional evidence, especially since HIF-1α and N1ICD co-expression had no prognostic value, and metastases expressed lower levels of N1ICD as compared to the primary tumors. Our group has previously shown that the immunophenotype of distant breast cancer metastases can be different from that of the primary tumor [31] with prognostic impact [31, 32]. NOTCH targeting may therefore be less effective in brain and skin metastases in breast cancer patients, and may be especially effective in preventing local recurrences and metastases to sites other than brain and skin. Previously, a similar observation was reported for NOTCH expression in human colorectal cancer [33].

It was interesting to note the prognostic value of expression of HES1 expression, a downstream target of all NOTCH members [34]. N1ICD itself had no prognostic value, which may be explained by the fact that it is highly expressed in the normal breast, where it might not be functional. The association of high HES1 expression with a poor prognosis, which had an almost additional prognostic value to lymph node status and mitotic index, may indicate that

NOTCH1 downstream activation does lead to more aggressive behavior of breast cancer. However, we cannot exclude that NOTCH family members other than NOTCH1 may have caused the activation.

It is well established now that the tumor stroma is important for regulating tumor growth [35]. The role of fibroblasts, the principal cellular components of connective tissues, in cancer progression is increasingly recognized. Recently, Chiavarina et al. (2010) speculated, based on in vitro and in vivo experiments, that HIF-1α might have a tumor promoting role in breast cancer associated fibroblasts [36]. Our observation of strong stromal HIF-1α expression, particularly in pleomorphic lobular cases, is interesting in this respect. Novel therapies targeting HIF-1α may add to the current treatment strategies of pleomorphic cases [37].

In conclusion, co-expression of N1ICD and HIF-1α is associated with a high grade and a high proliferation rate in invasive breast cancer, and activation of the NOTCH pathway is associated with a poor prognosis. HIF-1α expression is low in classic and high in pleomorphic lobular cancers, the latter of which also frequently show stromal HIF-1α expression. However, N1ICD and HIF-1α do not seem to be functionally related in breast cancer. Primary breast cancers express higher levels of N1ICD than their corresponding metastases, especially those in the brain and skin, implying that NOTCH targeting may especially be effective in preventing local recurrences and metastases at sites other than brain and skin.

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Conflict of interest The authors declare no conflict of interests.

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