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Expression of Notch Receptors in Primary Breast Cancer and Correlation with Pathological Features

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Abstract

Introduction: Notch signaling evolves as an important mediator of stem cell biology, tumor formation, angiogenesis and cell fate decisions. Four Notch receptors and five ligands have been identified in human, and they regulate a complex signal transduction pathway that interacts with other stem cell-related pathways as Sonic Hedgehog and Wnt. Recently, dysregulation of Notch pathway has been found to play a crucial role in breast cancer oncogenesis and it could serve as a target for treatment.

Methods: We estimated the expression of Notch receptors' at the mRNA level, in 200 human breast cancer samples from a single institution in Northern Greece. Notch 1-4 mRNA was estimated by using Real-time PCR and we correlated the level of Notch expression with breast cancer pathology (TNM, grade oestrogen and progesterone receptors and HER2).

Results: We found that Notch receptors (at the mRNA level) are differentially expressed in breast cancer subgroups. Hormone receptor positive cancers express Notch-4 whilst Notch-1 and Notch-3 receptors are involved in the development of triple-negative breast cancer. HER2-positive cancers express lower levels of Notch-1, a finding that is in concert with other reports as well.

Conclusion: Notch receptors are expressed differentially and could serve as potential targets for treatment in different molecular subtypes of breast cancer.

Keywords: Breast cancer; Notch; mRNA; Triple-negative

Abbreviations: CSC: Cancer Stem Cells; ER: Estrogen Receptor; PR: Progesterone Receptor; HER2: Human Epidermal Growth Factor Receptor-2; HR: Hormone Receptors; DP: Double-Positive (Hormone receptors and HER2); TN: Triple-Negative

Introduction

Despite recent advances regarding its management, breast cancer remains one of the most common malignancies in women and the second most common cause of cancer-related mortality. Current research in breast cancer focuses on understanding the genetic basis of malignant transformation of the breast epithelium. Breast cancers are routinely subcategorized on the basis of clinical stage, cellular morphology and immunohistochemical analysis of a small number of markers. At present breast cancer is viewed as a spectrum of different disease subtype consistent with molecular profiling of the disease. Recent experimental data suggest that cancer is a disease of the stem cell [1]. Stem cells play a crucial role in the biology, development and evolution of the mammary gland. Consistent with the cancer stem cell hypothesis, breast cancer originates from breast-specific stem cells that undergo oncogenic transformation [2]. Cancer stem cells (CSC) display particular features that may account for the development of therapeutic resistance. In order to improve the treatment of breast cancer a better understanding of the multifactorial nature of the disease is required. IN this aspect, pathways that regulate self-renewal and CSC fate (Notch, Sonic Hedgehog and Wnt/β- catenin) begin to be elucidated [3].

Notch signalling is an evolutionary conserved pathway that plays a central role in stem cell biology, tumor formation, angiogenesis and cell fate decisions [4]. Mammals have four Notch transmembrane proteins (Notch-1, 2, 3 and 4) that function as receptors for five ligands (DLL-1, 3 and 4 and JAG-1 and 2). Cell-cell contact is necessary for Notch activation by ligand binding. Binding of ligands to Notch leads to proteolytic cleavage of the receptor at a site just outside the plasma membrane by ADAM-family protease (S2 cleavage). This is immediately followed by cleavage at a site just inside the plasma membrane by the presenilin-γ-secretase complex (S3 cleavage) [5]. Thereafter, the cytoplasmic fragment of notch protein (Notch IC) is released, which can enter the nucleus and interact with the RBP-Jk/CBF-1 transcription factor complex. This interaction converts CBF-1 from a transcription repressor to an activator, resulting in increased expression of other transcriptional regulatory proteins (e.g. Hes and Hey) [6,7].

Many studies indicate that Notch plays a predominantly oncogenic role and interacts with other pathways involved in tumorigenesis [8-10], particularly in breast [11-15]. Notch pathway activation induces adenocarcinomas in the murine mammary gland [16,17] and loss of Numb expression, a negative regulator of the Notch pathway, is reported in a large proportion of breast carcinomas [18]. Overexpression of active forms of the Notch-1, Notch-3 and Notch-4 receptors cause

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mammary tumor in mice [17,19,20]. In human breast cancer, high levels of Notch-1 and its ligand, Jagged1 (mRNA) is associated with poor overall survival, including cancers that overexpress HER-2 [21]. It seems that Notch is involved in breast cancer development from the *in situ* stage [22]. On the other hand elevated levels of Notch-2 were found in well-differentiated breast tumours and correlate with a higher probability for survival [23].

Investigations of cross-talks between Notch signalling and ER or HER2 pathways have shown that Notch-1 and -4 genes are expressed differently in triple negative (that do not express estrogen and progesterone receptor or the HER2 protein) MDA-MB231 cells as compared to estrogen receptor-positive T47D cells [24,25]. In addition, increased Notch-1 expression has been linked to the development of trastuzumab-resistance in HER2 overexpressing cell lines [26] and Notch-3-mediated signalling is associated with the proliferation of ErbB2-negative breast tumour cells [27].

Although Notch receptors are known to be involved in mammary tumorigenesis, more evidence is still required in order to fully clarify their role in all breast cancer subtypes. To this end, in the present study we assessed the expression of all four Notch receptors, at the mRNA level, in 200 primary breast cancer specimens and evaluated their correlation to pathologic and molecular features.

Material and Methods

Tissue sampling

Breast cancer tissue specimens were obtained from 200 women who had undergone surgical resection at the Theagenion Cancer Hospital, Thessaloniki, Greece and were approved by the ethics committee of the Institute. All patients gave their informed consent for use of tissue in the research protocol. Five normal breast tissue samples were obtained from breast reductive surgery. Immediately after surgery, tumor samples were immersed in RNA later solution (Ambion) overnight at room temperature to allow thorough penetration of the tissue and then stored at -80°C until homogenization (Fisher Scientific PowerGen Homogenizer).

RNA extraction and reverse transcription

Total RNA was extracted using Trizol reagent (Invitrogen) as per manufacturer's instructions. After DNase treatment (Ampion), the quantity of RNA extracted was assessed with a spectrophotometer (Eppendorf Biophotometer) and RNA quality by electrophoresis. Intact rRNA subunits of 28 S and 18 S RNA was observed on the gel electrophoresis staining with ethidium bromide, indicating that the degradation of the RNA was minimal. First strand cDNA was synthesized from 2 μg of total RNA using Thermoscript Reverse Transcriptase (Invitrogen) with Random Hexamer Primers.

Real Time PCR

Notch receptor expression was evaluated with Real-time PCR assays using Hydrolysis (TaqMan probes for Notch 1,2,4 and β-actin) or SybrGreen (for Notch 3) technology (Invitrogen). Multiplex PCR-applications were performed for pairs of Notch-1 and 4 as well as Notch-2 and β-actin. Each TaqMan probes reaction mix was performed in an individual tube containing, in a final volume of 25 μl, 1 x Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 5 mM $MgCl_2$, 250 ng of cDNA and the optimal concentration of primers (500 nM for Notch-1, 400 nM for Notch-2 and 4, and 100 nM for β-actin) and probes (300 nM) for each set-pair of genes. The thermal cycling conditions were at 50°C for 2 minutes and at 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds and at 58°C for 30 seconds. The Real time PCR mix for Notch 3 contained in a final volume of 25 μl, 1x SYBRGreenER qPCR Super Mix Universal and 200 nM of the forward and reverse primers. The thermal cycling conditions were at 50°C for 2 minutes and at 95°C for 10 minutes, followed by 35 cycles at 95°C for 15 seconds and at 59.5°C for 60 seconds. Melting curve analysis was performed to control for specificity of the expected amplification product (Tm of the notch 3 amplicon 86 ± 0.4°C). All PCR reactions were performed in duplicate in a Rotor Gene 6000 Real Time analyzer, Corbett. PCR primers sets and probes were designed using the Beacon Designer programme and the ologonucleotide sequences are listed in supplementary table 1.

The reaction efficiency for each gene was calculated after obtaining standard curves for each PCR reaction by making 10-fold serial dilutions covering the range equivalent to 250-0.25 ng cDNA. Threshold cycle numbers (Cq or Ct) were obtained using Rotor Gene 6000 interface software. Beta-actin was used as reference gene to normalize for differences in the amount of total RNA in each sample. A pool of five normal breast specimens from reduction mammoplasty was used for evaluation of controls mRNA expression. Data analysis was done using the $\mathbf{2}^{-\Delta\Delta Ct}$ method according to literature (28). Results were expressed as $\mathbf{2}^{-\Delta\Delta Ct}$, with $\Delta\Delta Ct = (Ct_{notch1,\,2,\,3,\,4} - Ct_{\beta\text{-actin}})_{cancer\ samples} - (Ct_{notch1,\,2,\,3,\,4} - Ct_{\beta\text{-actin}})_{pull\ of\ normal\ breast\ tissue}$ returning the expression to the normal tissue and to beta actin. The maximum Ct value was assigned for specimens that Notch expression was below the threshold for detection in repeated qPCR reactions.

Statistical analysis

Data were tested for normality with the Kolmogorov-Smirnov test. Analysis was based on the log-transformed values in order to smooth the effect of extreme observations on the notch distributions. Differences in mRNA levels of notch 1, 2, 3, 4 between groups were analyzed using the non-parametric Mann-Whitney U test and Kruskal-Wallis test. Pearson's correlation coefficient was used to estimate the correlation between two continuous variables. Additionally, binomial logistic regression analysis was performed in order to evaluate the effects of notch 1, 2, 3, 4 on different subtypes of breast cancer (TN, ER, PR and HER2) adjusted for all the statistically significant baseline tumour and patient characteristics. In order to select the final model a careful examination of all variables, in terms of predictive power, and relationship with other covariates was performed. Due to multicollinearity effects Notch-4 was excluded from the models. Significance was set at $p<0.05$.

Results

Notch mRNA expression in breast cancer

Samples from 200 patients were used in this study. Average age was 62 years old, while average tumor size was 25.88 mm and the majority of tumors were of Grade 3 (68%). The clinicopathological characteristics of the tumors are shown in Table 1.

Expression profiling of Notch-1, 2, 3, 4 was studied in normal (a pool of five normal breast specimens from reduction mammoplasty) and breast cancer specimens. The results (Table 2) were expressed as ratio $2^{-\Delta\Delta Ct}$ returning the expression to the normal tissue and to beta actin. The variables Notch-1, 2, 3, 4 did not follow normal distribution

even though they were log-transformed before analysis. Notch-1, 2, 3 and 4 were found to be expressed in both control (normal) and breast cancer specimens. Only the Notch-2 gene was found to be overexpressed in all cancer samples compared to normal–control (mean 3.70 fold) while the Notch-3 mRNA expression was a little bit higher in all cancer samples (mean 1.41 fold). Moreover, the Notch 3 gene was found to be overexpressed in triple-negative tumors compared to normal control (mean 7.87 fold) while tumors expressing hormone receptors and overexpressing HER2 were found to have the higher levels of Notch-4 mRNA (mean 1.34 fold) compared to control. The results indicated the Notch-2 gene as the predominant receptor (at the mRNA level) in all samples whereas the Notch-3 showed the lowest mRNA expression with the widest range (Figure 1 and Table 2).

Notch 1, 2, 3 and 4 mRNA expression in comparison with ER, PR and HER2 status

We then assessed the expression of Notch receptors in correlation to ER, PR and HER2 status. We found that the ER negative samples express statistically significant higher levels of Notch-3 mRNA (p=0.007) and higher levels of Notch-1 that was not statistically significant (p=0.085). On the other hand, the ER positive tumors overexpress Notch-4 (p=0.036) (Figure 2). Furthermore, Notch-1 mRNA levels were lower in samples which express HER2 receptors but this expression wasn't statistically significant (p=0.1) (Figure 3). There was no statistically significant correlation between the expression of Notch receptors and PR status (Figure 4).

Further exploratory analysis was performed by categorizing the 200 breast cancer samples into four main subgroups based upon the presence or absence of ER, PR and HER2 receptors, namely: 1) Hormone Receptor (HR) positive (one or both of them are positive) and HER2 negative (HR+ve/HER2-ve), 2) HR negative and HER2 positive (HR-ve/HER2+ve), 3) Double Positive (HR positive and HER2 positive, DP) and 4) Triple negative (TN).

N	200
Age (mean)	61.91
Tumor size mm (mean)	25.88
Grade%	
1	1
2	31
3	68
Lymph node positive %	55.5
ER positive %	77.5
PR-positive %	59
HER2-positive %	30.5
HR-positive %	78.5
TN %	12
ILC %	9.5

ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor-2; HR: hormone receptor, TN: triple negative, ILC: in situ lobular carcinoma.

Table 1: Patient characteristics.

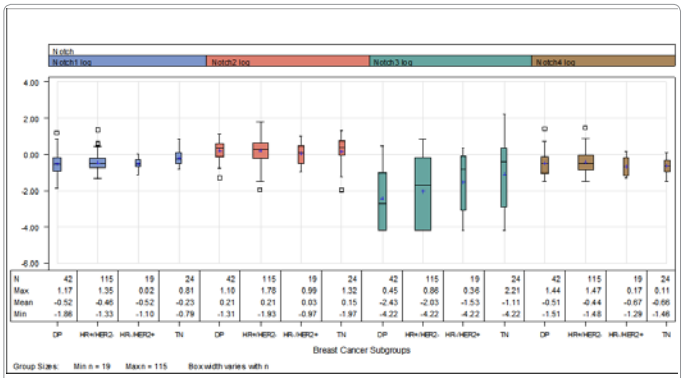

Figure 1: Box plots of notch 1-4 receptor expression in four basic breast cancer subgroups according to ER, PR and HER2 status.(HR+ve/HER2-ve, HR-ve/HER2+ve, TN and DP). Shaded boxes represent interquartile range making the 25th to 75th percentile, whiskers represent the 10th to 90th percent range, bars represent the median.

All samples	notch1	notch2	notch3	notch4
N=200				
Mean	0.75	3.70	1.41	0.95
Median	0.32	1.87	0.03	0.28
Std. Deviation	2.03	6.44	11,48	2.98
Range	22.46	60.12	162.02	29.62
HR+ve/HER2-ve	**notch1**	**notch2**	**notch3**	**notch4**
N=115				
Mean	0.71	4.22	0.63	1.03
Median	0.31	1.87	0.02	0.34
Std. Deviation	2.15	7.94	1.29	2.97
Range	22.42	60.12	7.31	29.62
HR-ve/HER2+ve	**notch1**	**notch2**	**notch3**	**notch4**
N=19				
Mean	0.37	2.21	0.49	0.37
Median	0.28	1.30	0.16	0.20
Std. Deviation	0.25	2.60	0.70	0.42
Range	0.96	9.67	2.27	1.44
DP	**notch1**	**notch2**	**notch3**	**notch4**
N=42				
Mean	0.91	2.81	0.25	1.34
Median	0.28	2.26	0.00	0.32
Std. Deviation	2.50	2.91	0.61	4.25
Range	14.92	12.50	2.81	27.25
TN	**notch1**	**notch2**	**notch3**	**notch4**
N=24				
Mean	0.93	3.96	7.87	0.33
Median	0.55	2.45	0.41	0.23
Std. Deviation	1.26	4.62	32.88	0.30
Range	6.25	20.67	162.02	1.26
ILC	**notch1**	**notch2**	**notch3**	**notch4**
N=19				
Mean	0.59	5.10	0.64	0.89
Median	0.35	2.58	0.01	0.35
Std. Deviation	0.83	10.47	1.27	1.39
Range	3.67	46.99	4.09	6.05

Table 2: Descriptive statistics of Notch 1, 2, 3 and 4 mRNA expressions between subgroups.

The descriptive characteristics of the examination of Notch receptors' mRNA expression between subgroups are reported in table 2. Statistically significant higher expression from mean was established for Notch-1 and Notch-3 only in TN tumors (p=0.005 for Notch-1 and p=0.023 for Notch-3). DP tumors expressed the least levels of Notch-3 receptor (p=0.063). Notch-4 expression was higher in HR+ve/HER2-ve tumors (p=0.055) (Figure 1).

Notch receptor expression in correlation with other clinicopathological characteristics

We checked if the type of cancer, Invasive Ductal Carcinoma (IDC) or Invasive Lobular Carcinoma (ILC), could affect the expression profile of Notch receptors. Even though there was a tendency in the ILC samples to express lower level of Notch-3 and higher level of Notch-2 and -4, the differences were not statistical significant (Figure 5). In addition the well-differentiated tumors (grade 1 and 2) exhibited increased level of Notch-4 but this was not statistically significant (p=0.09) (Figure 6). As Notch-4 was overexpressed in ER-positive tumors, this may just reflect the fact that low-grade tumors express hormonal receptors more often. We found that there was correlation between tumor size and mRNA expression of Notch-1, -3 and -4. Tumors smaller than 20 mm in diameter overexpressed Notch-1 and Notch-4 (p=0.047 for Notch-1 and p=0.004 for Notch-4) compared with those having size larger than 20 mm, which over expressed Notch-3 (p=0.031) (Figure 7). Finally, it was found that women younger than 50 years old express statistically significant higher level of Notch-2 gene (p=0.03), while there was no correlation between the expression of notch receptors and lymph node involvement.

Correlation analysis of notch receptors in breast cancer subgroups

Pearson correlation analysis was applied in different subgroups, as well as in the total sample in order to calculate the strength of the relationship between the continuous variables Notch-1, -2, -3, -4 (Table 3). We found a strong positive correlation between the variables Notch-1 and Notch-4 in all subgroups except for triple negative tumors (all cancers r=0.78, HR+ve/HER2-ve r=0.86, HR-ve/HER2+ve r=0.81,

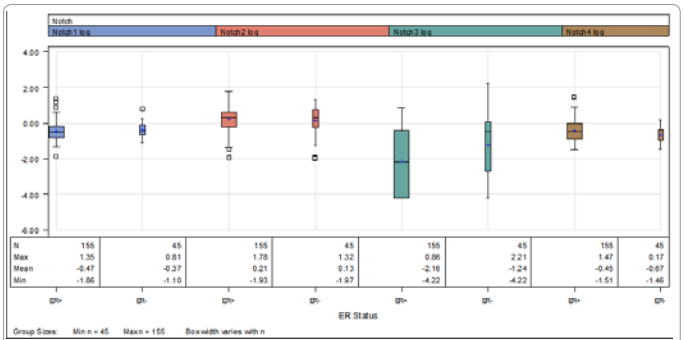

Figure 2: Box plots of notch 1-4 expression in ER+ve and ER-ve breast cancer samples.

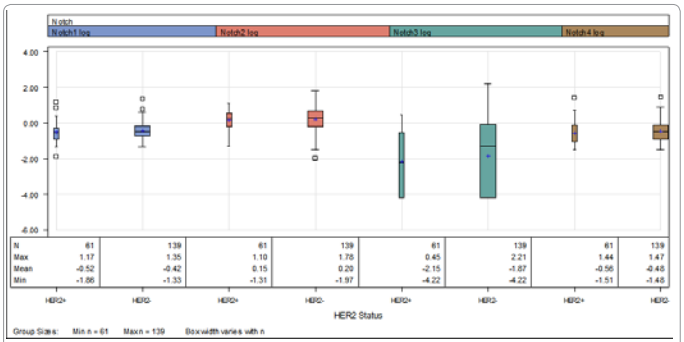

Figure 3: Box plots of notch 1-4 receptor expression in HER2+ve and HER2-ve breast cancer samples.

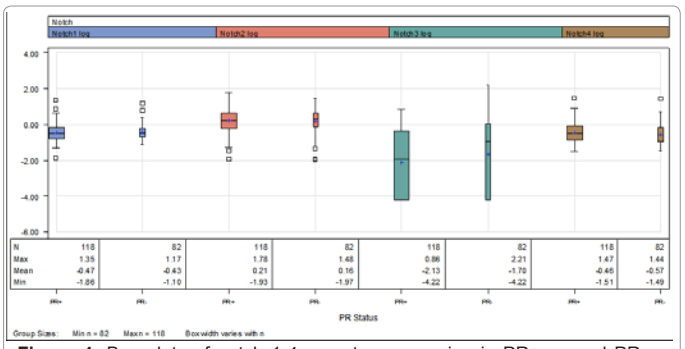

Figure 4: Box plots of notch 1-4 receptor expression in PR+ve and PR-ve breast cancer samples.

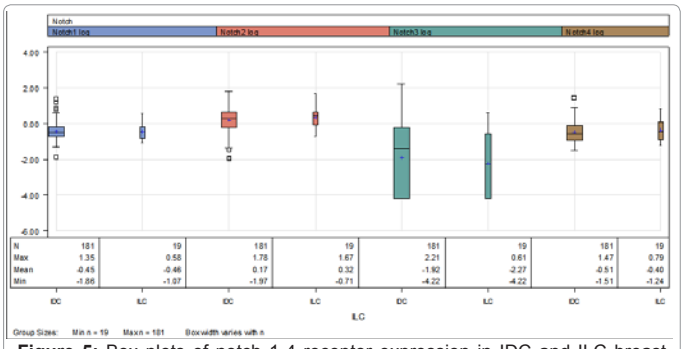

Figure 5: Box plots of notch 1-4 receptor expression in IDC and ILC breast cancer subtypes.

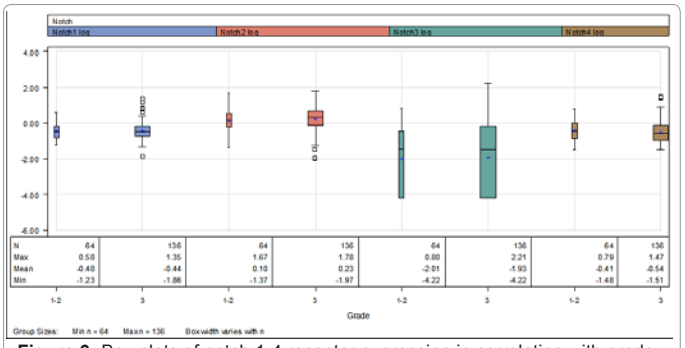

Figure 6: Box plots of notch 1-4 receptor expression in correlation with grade.

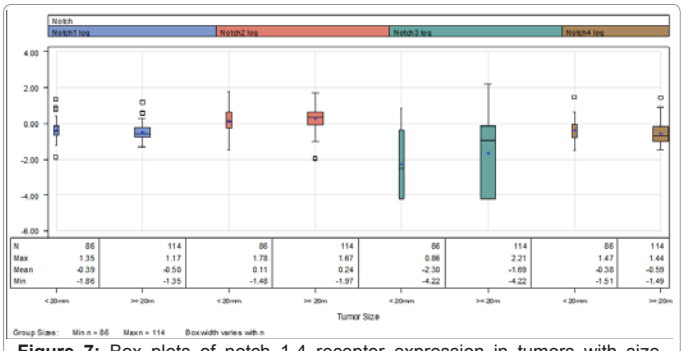

Figure 7: Box plots of notch 1-4 receptor expression in tumors with size <20mm and size >=20mm.

Group	Subgroup	Variable	With Variable	N	Sample Correlation	Lower 95% Confidence Limit	Upper 95% Confidence Limit	p Value
All patients	All patients	lognotch1	lognotch2	200	-0.16	-0.29	-0.02	0.0215
			lognotch4	200	0.78	0.72	0.83	<.0001
		lognotch2	lognotch3	200	0.21	0.07	0.34	0.0026
			lognotch4	200	-0.18	-0.31	-0.04	0.0103
		lognotch3	lognotch4	200	-0.39	-0.50	-0.26	<.0001
HR+ve/ HER2-ve	non HR+ve/HER2-ve	lognotch1	lognotch4	85	0.71	0.59	0.80	<.0001
		lognotch3	lognotch4	85	-0.38	-0.55	-0.18	0.0003
	HR+ve/ HER2-ve	lognotch1	lognotch3	115	-0.25	-0.42	-0.07	0.0062
			lognotch4	115	0.86	0.80	0.90	<.0001
		lognotch2	lognotch3	115	0.34	0.16	0.49	0.0002
			lognotch4	115	-0.21	-0.38	-0.02	0.0256
		lognotch3	lognotch4	115	-0.39	-0.53	-0.22	<.0001
HR-ve/ HER2+ve	non HR-ve/ HER2+ve	lognotch1	lognotch2	181	-0.18	-0.32	-0.04	0.0141
			lognotch4	181	0.78	0.72	0.83	<.0001
		lognotch2	lognotch3	181	0.23	0.08	0.36	0.0019
			lognotch4	181	-0.19	-0.32	-0.04	0.0110
		lognotch3	lognotch4	181	-0.37	-0.49	-0.23	<.0001
	HR-ve/ HER2+ve	lognotch1	lognotch4	19	0.81	0.56	0.92	<.0001
		lognotch3	lognotch4	19	-0.58	-0.81	-0.15	0.0087
TN	Triple negative	lognotch3	lognotch4	24	-0.48	-0.74	-0.09	0.0153
	ER or& PR or& HER2=positive	lognotch1	lognotch3	176	-0.21	-0.35	-0.07	0.0042
			lognotch4	176	0.87	0.82	0.90	<.0001
		lognotch2	lognotch3	176	0.23	0.08	0.36	0.0025
			lognotch4	176	-0.19	-0.33	-0.04	0.0105
		lognotch3	lognotch4	176	-0.37	-0.49	-0.23	<.0001
ILC	IDC	lognotch1	lognotch2	181	-0.17	-0.31	-0.03	0.0214
			lognotch4	181	0.78	0.71	0.83	<.0001
		lognotch2	lognotch3	181	0.22	0.07	0.35	0.0035
			lognotch4	181	-0.19	-0.33	-0.05	0.0089
		lognotch3	lognotch4	181	-0.35	-0.47	-0.21	<.0001
	ILC	lognotch1	lognotch3	19	-0.54	-0.80	-0.11	0.0146
			lognotch4	19	0.88	0.69	0.95	<.0001
		lognotch3	lognotch4	19	-0.75	-0.90	-0.44	<.0001
DP	non DP	lognotch1	lognotch3	158	-0.16	-0.31	-0.01	0.0410
			lognotch4	158	0.74	0.66	0.80	<.0001
		lognotch2	lognotch3	158	0.27	0.11	0.40	0.0007
			lognotch4	158	-0.18	-0.32	-0.02	0.0251
		lognotch3	lognotch4	158	-0.43	-0.55	-0.29	<.0001
	DP	lognotch1	lognotch4	42	0.89	0.81	0.94	<.0001

Table 3: Correlation of notch receptors in breast cancer subgroups (statistically significant correlations only (at 5% sig. level)).

DP r=0.89, ILC r=0.88). These correlations are significant at the 0.01 level. The scatter plots of correlations between log Notch-1 and log Notch-4 are represented in Figure 8 to Figure 13. In TN tumors, there is a weak positive correlation between Notch-3 and Notch-2 (r=0.19) and a medium negative correlation between Notch-3 and Notch-4 (r=-0.48, p=0.0153), which is observed in other subgroups as well (supplementary figures 1-5).

Prediction model in triple negative tumors

Logistic regression analysis was carried out in order to evaluate the role of Notch-1, 2, 3 and 4 as predictors for a sample to belong in TN tumors. Notch-1 (p-value=0.0015) and Notch-3 (p-value=0.0075) were the only statistically significant variables. As mentioned earlier Notch 4 was excluded from the final model since its high correlation to Notch-1 created multicollinearity effects, which reduced model validity. Furthermore, the model estimated that the effect of one unit increase in the log of Notch-1 and log of Notch-3 on the odds of belonging in the TN tumors (versus not being TN), is an increase by a factor of 6.4 (95% CI: 2.04-20.11) [unadjusted 4.26 (95% CI: 1.63-11.13)] and 1.48 (95% CI: 1.11-1.98) [unadjusted 1.43 (95% CI: 1.10-1.86)] respectively (Table 4). The sensitivity of the model when the cut-off probability of a sample to be included in TN tumors is 10% was 70.8% and the specificity 67.0%, while the respective values become 54.2% and 84.7% when the cut-off probability of inclusion is 20%. The AUC of the ROC curve was 82.6% (unadjusted 71.7%, Figure 14).

As expected, since by construction TN is the intersection of ER, PR and HER2 negative samples and ER has the lowest negativity rate, (i.e. 22.5%), ER correlates highly (logistic regression estimates from a model performed as above) with Notch-1 (p-value=0.049) and Notch-3 (p-value=0.0040). Correlation of PR and HER2 with Notch-1 and Notch-3 was not statistically significant, p-value=0.4434 and

p-value=0.1250 for PR and p-value=0.0762 and p-value=0.1764 for HER2 respectively.

Discussion

In the present study we assessed the Notch 1-4 mRNA expression in 200 primary breast cancer specimens and evaluated their correlation to pathologic features. This is to our knowledge one of the larger studies accessing the mRNA expression of all Notch receptors in breast cancer. Firstly, our findings demonstrate that all these genes are expressed in both control-normal and breast cancer specimens. Notch-2 seems to be the predominant Notch receptor in breast cancer irrespective of histological subtype (Notch-2 has been related to less aggressive tumors with a better prognosis [24]).

We found that Notch-1 and Notch-3 are expressed in hormone receptor-negative tumors. Notch-1 has been related to more aggressive tumours with a higher probability for relapse [24] and Notch-3 is thought to be involved in vessel formation [29], a known characteristic of high-grade, ER-negative breast cancers [30]. Larger tumors, which need larger blood supply and therefore rely more on angiogenesis, seem to express higher levels of Notch-3 arguing for the previous hypothesis. Our study further supports the role of these receptors in the ER-negative carcinogenesis and therefore suggests Notch 1 and

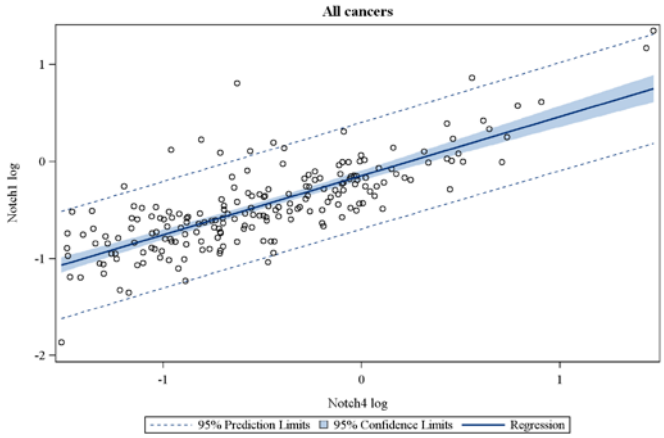

Figure 8: Correlation of Notch1 and Notch4 expression in breast cancer. Expression levels are log transformed.

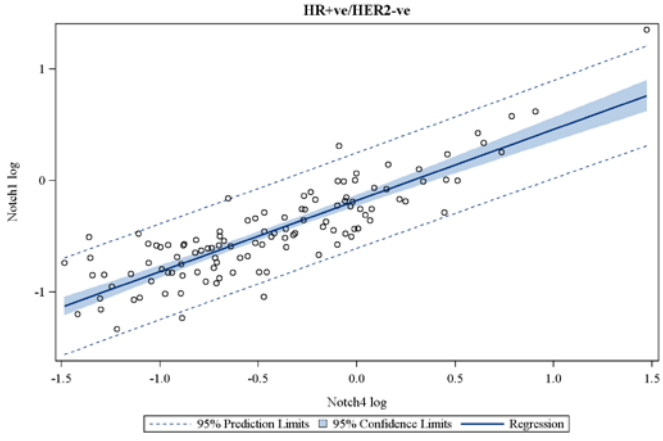

Figure 9: Correlation of Notch1 and Notch4 expression in hormone receptor-positive / HER2-negative breast cancer. Expression levels are log transformed.

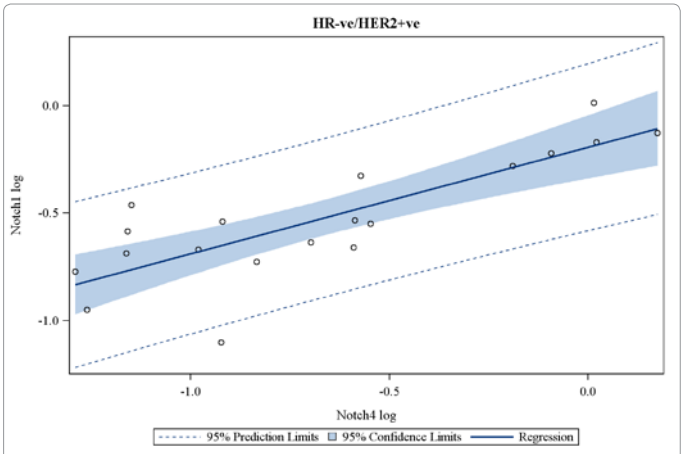

Figure 10: Correlation of Notch1 and Notch4 expression in hormone receptor-negative / HER2-positive breast cancer. Expression levels are log transformed.

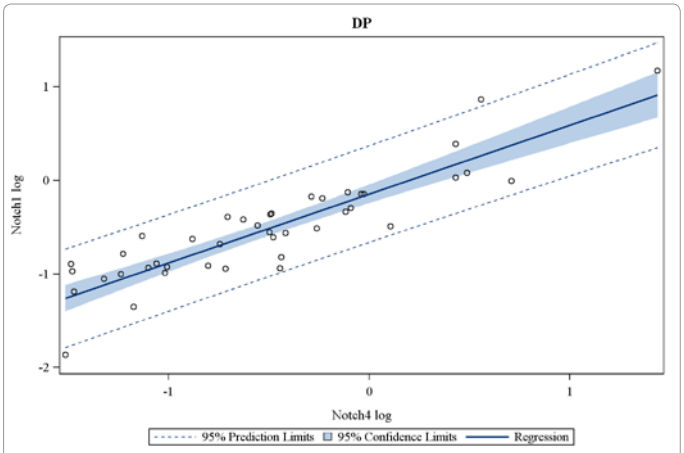

Figure 11: Correlation of Notch1 and Notch4 expression in hormone receptor-positive / HER2-positive breast cancer. Expression levels are log transformed.

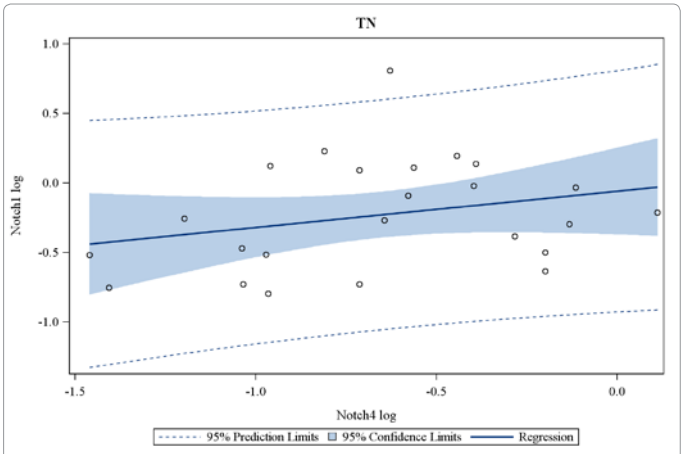

Figure 12: Correlation of Notch1 and Notch4 expression in triple-negative breast cancer. Expression levels are log transformed.

Notch-3 as possible targets for treatment in these tumors. On the other hand, Notch-4 is predominately expressed in ER-positive tumours. In the 61 HER2-positive cancers (amongst the 200 specimens - 30.5%), there was a lower expression of Notch-1. It has been previously shown

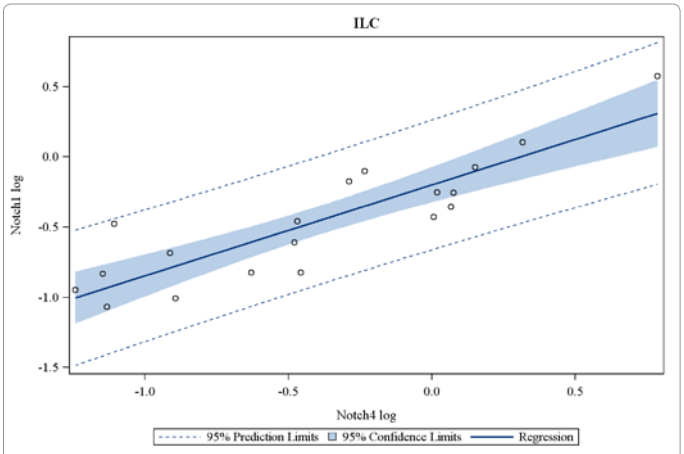

Figure 13: Correlation of Notch1 and Notch4 expression in lobular type breast cancer. Expression levels are log transformed.

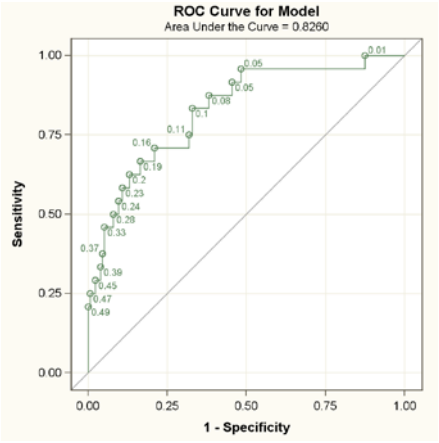

Figure 14: ROC curve of the TN logistic regression model.

	Effect	Estimate	Standard Error	Odds Ratio Estimate	Lower 95% Confidence Limit for Odds Ratio	Upper 95% Confidence Limit for Odds Ratio	Pr > Chi-Square
Adjusted	lognotch1	1.856	0.584	6.40	2.04	20.11	0.0015
	lognotch3	0.394	0.147	1.48	1.11	1.98	0.0075
Un adjusted	lognotch1	1.448	0.491	4.26	1.63	11.13	0.0032
	lognotch3	0.358	0.133	1.43	1.10	1.86	0.0071

Table 4: Logistic Regression for TN.

that HER2 promoter contains Notch-binding sequences [31], however HER-2 expression is probably not regulated via these pathways in the HER2-amplified tumours. Indeed there are reports that HER2-positive tumors express lower levels of Notch 1 and Notch 3 [32] and it has been shown that inhibiting the HER2 pathway with trastuzumab leads to activation of the Notch-1 pathway in HER2-overexpressing cells [33].

When we analyzed the results grouping the patients in four subgroups Notch-1 and Notch-3 were found to be predominately expressed in TN tumors and Notch-4 in the less aggressive, ER-positive / HER2-negative subgroup. This observation implies that Notch 1 and 3 may be a target for the treatment of triple-negative breast cancers [33], a subgroup of tumours with limited treatment options (except chemotherapy) for the moment. On the other hand, as Notch-4 seems to play an important role in breast cancer stem cell activity [34], Notch-4 could be an attractive target in hormone receptor-positive breast cancer.

When seen from the inverse point of view, tumors that express Notch-1 and Notch-3 have a higher probability of belonging to the TN subgroup, suggesting that Notch-1 and Notch-3 play a crucial role in the TN oncogenesis.

Expression of Notch-4 seems to follow Notch-1 in the whole cohort and in all breast cancer subtypes except the TN tumors. Our data are in concert with the observation that Notch-4 is a transcriptional target of Notch-1 [35]. In the case of TN tumours however, which is a small subgroup of the breast cancer population, there may be a differential activation of the Notch pathway that is not inducing Notch-4. As Notch-4 is related to less aggressive phenotypes, failing to express Notch-4 might be contributing to the worse prognosis of the TN breast cancer. Further research is needed for elucidation of the Notch pathway activation in this subtype.

Conclusion

In conclusion, Notch receptors (at the mRNA level) are differentially expressed in breast cancer subgroups. Notch-4 seems to be involved with the hormone receptor positive cancers whilst Notch-1 and Notch-3 are involved in TN breast cancer biology. Notch pathway seems to be an attractive target for treatment in TN cancers. We currently continue the study by further analyzing the activation of Notch downstream signalling in TN breast cancer, and analyze the protein expression of Notch receptors and targets as well.

Acknowledgements

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