Clinical Significance of Notch Receptors In Triple Negative Breast Cancer

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Research Article

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Abstract

Background: The Notch signaling pathway is an evolutionary conserved cell signaling pathway that plays an indispensable role in essential developmental processes. Aberrant activation of Notch pathway is known to initiate wide array of diseases and cancers. The role of Notch receptors and the effect of their expression in Triple Negative Breast Cancer is unknown.

Methods and Results: In this study, we evaluated the association between Notch receptors and clinicopathological parameters including disease-free survival and overall survival of one hundred TNBC patients by immunohistochemistry. We observed membrane, cytoplasmic and nuclear localization of Notch receptors in tumor cells of TNBC patients. It was observed that positive expression of nuclear Notch1 receptor (18%) was found to be significantly correlated with high BR score ($p=0.009$) and necrosis ($p=0.003$) while cytoplasmic expression of Notch2 receptor (26%) was significantly correlated with local or distant metastasis ($p=0.04$), worse DFS ($p=0.05$) and poor OS ($p=0.02$) in TNBC patients. Further, it was determined that cytoplasmic (65%) and cytonuclear (3%) expression of Notch3 was significantly associated with poorly differentiated tumors ($p=0.008$) and necrosis ($p=0.04$) respectively. However, cytoplasmic Notch3 and Notch4 expression were negatively correlated with poor prognostic factors.

Conclusion: Our data indicated that Notch receptors play a key role in promoting TNBC and mainly, Notch2 receptor may contribute to poor prognosis of the disease. Hence, it is implicated that Notch2 receptor may serve as a potential biomarker and therapeutic target for TNBC.

1. Introduction

Breast cancer is the most commonly diagnosed type of cancer, accounting for 24.5% newly registered cases in women and 11.7% newly registered cases in both sexes in a year (<https://gco.iarc.fr/today/data/factsheets/populations/900-world-fact-sheets.pdf>). Globally, it is second leading cause of death in women after lung cancer (<https://gco.iarc.fr/today/data/factsheets/populations/900-world-fact-sheets.pdf>). Breast cancer is a phenotypically and biologically heterogeneous disease with diverse response to the treatments offered [1]. Immunohistochemical profiling categorizes breast cancer into four molecular subtypes – luminal A, luminal B, Her2 enriched and Triple negative breast cancer [2]. Luminal A, luminal B and Her2 enriched subtypes are characterized by hormone receptor positivity and better prognosis than TNBC. Triple negative breast cancer is defined by the lack of expression of hormone receptors (ER-, PR-, HER2-), which accounts for 15-20% of breast cancer cases [3, 4]. TNBC subtype has unfavorable prognosis and patients with TNBC are at greater risk of disease relapse or distant metastasis [5]. TNBC is characterized as an aggressive form of cancer which includes features such as higher tumor size, high histological grade, advanced stage and lymph node involvement [6]. Currently treatment available for TNBC comprises of surgery, traditional chemotherapy with anthracyclines, taxanes, platinum agents and radiotherapy [7]. Recently, Poly (ADP-Ribose) Polymerase (PARP) inhibitors are used as targeted therapy for TNBC patients with BRCA1/2 gene mutations and programmed death-ligand 1 (PD-L1) positive TNBC patients with

locally advanced or metastatic disease are benefited from PD-L1 inhibitors [8, 9]. Nonetheless, these therapies are beneficial to a small number of TNBC population.

The Notch signaling pathway is a highly conserved pathway which regulates fundamental developmental processes such as cellular differentiation, proliferation, organ formation, morphogenesis and apoptosis [10]. The Notch signaling pathway is constitutively activated by the interaction of transmembrane Notch receptors and cell-surface ligands on adjacent cells [11]. Upon ligand activation, cascade of events is triggered which includes sequential cleavage by tumor necrosis factor alpha (TNF) converting enzyme (TACE) and gamma-secretase complex, releasing the Notch intracellular domain (NICD). The cleaved NICD translocates to the nucleus and binds with transcriptional regulators such as CBF-1/RBPjk, Suppressor of Hairless, and Lag-1 (CSL), Mastermind-like (MAML), p300 in order to initiate the expression Notch target genes [12, 13]. Abnormal expression of Notch receptors is known to play a crucial role in malignant transformation in haemopoietic malignancies as well as solid tumors [14]. Notch signaling pathway is responsible for normal development of mammary glands and the deregulation of Notch signaling pathway is involved in breast carcinogenesis [15]. Studies have frequently shown that Notch1, Notch3 and Notch4 are responsible for aggressiveness of TNBC by contributing to increased cell proliferation, anti-apoptotic activity and stem cell maintenance while few studies have reported tumor suppressive role of Notch2 receptors [16]. However, the role of Notch receptors in TNBC is quite debatable. The present study evaluated Notch1, Notch2, Notch3 and Notch4 expression by immunohistochemistry and their clinical significance in TNBC.

2. Materials And Methods

2.1 Patients

One hundred female patients diagnosed with TNBC who had undergone surgery and treatment at the Gujarat Cancer & Research Institute, Ahmedabad during 2014 to 2019 were enrolled in this retrospective study. Detailed clinical history of the patients including age, menopausal status, tumor size, nodal involvement, AJCC TNM stage, histological type, histological grade and treatment offered was noted from the institutional records. Patients who had received chemotherapy or radiotherapy prior to the surgery were excluded from the study. Formalin fixed paraffin embedded (FFPE) blocks were retrieved from archives of Histopathology Department of the institute. This study was approved by Scientific Review and Ethics committees (EC/BHR/01/2020) of the institute.

2.2 Immunohistochemistry

Immunohistochemical analysis was used to detect the presence of Notch1, Notch2, Notch3 and Notch4 receptors in formalin fixed paraffin embedded (FFPE) blocks of TNBC patients. 4µm sections from FFPE tumor tissue blocks were taken using a microtome (Leica Biosystems, Germany) and the sections were mounted on 3-Aminopropyl triethoxysilane (APES) coated slides. Immunohistochemistry was performed using autoimmunostainer instrument, Ventana Benchmark XT (Ventana Medical Biosystems, USA) and Ventana reagents according to the manufacturer's protocol. Briefly, the protocol includes

deparaffinization with EZ prep solution, antigen retrieval using cell conditioning solution (CC1) at 95°C, followed by incubation with Ultra view DAB inhibitor for 4 minutes, 100µl of respective primary antibody for Notch1 (Monoclonal, 1:50, Invitrogen) for 32 minutes, Notch2 (Polyclonal, 1:25, Invitrogen) for 60 minutes, Notch3 (Polyclonal, 1:50, Invitrogen) for 60 minutes and Notch4 (Polyclonal, 1:150, Invitrogen) for 32 minutes, incubation with Ultra view Horseradish peroxidase (HRP) multimer for 8 minutes. The detection of antigen-antibody complex was carried out by 3,3'-diaminobenzidine (DAB), counterstaining using haematoxylin for 8 minutes and post counterstaining with bluing reagent for 4 minutes. Ultra view DAB detection kit was used for staining of Notch2, Notch3, Notch4 receptors and Opti view DAB detection kit was used for staining of Notch1. The protocol of Opti view DAB detection kit includes additional incubation with HQ linker for 8 minutes, counterstaining and post counterstaining for 16 minutes and 8 minutes respectively. The slides were later mounted using DPX and xylene.

2.3 Evaluation of Immunohistochemistry

Expression of Notch receptors (Notch1, Notch2, Notch3 and Notch4) was assessed by semiquantitative scoring method by employing Immunoreactive scoring (IRS) system. The percentage positive cells were graded as 0(negative staining), 1(<10% positive cells), 2(10% - 50% positive cells), 3(51% - 80% positive cells) and 4(>80% positive cells). Further, staining intensity of the cells was ranked as 0(negative), 1+(weak), 2+(moderate) and 3+(strong). The immunoreactive score (IRS) for each case was obtained by multiplying the percentage of positive cells and the staining intensity which gives the range of 0-12. Median score was calculated and cases below the median score were considered as negative or low expression and cases above the median score were considered as over expression.

2.4 Statistical Analysis

Statistical analysis was performed using SPSS 26.0 (SPSS, Inc., Chicago, USA). The Chi-square test was used to determine the association between the expression of Notch receptors and clinicopathological parameters and the association between the Notch receptors. The correlation between the two parameters was determined by Pearson's correlation coefficient (r). Disease-free survival (DFS) time was calculated from date of diagnosis of the patient to date of disease relapse or date of last follow-up. Overall survival time was calculated from date of diagnosis of the patient to last follow-up or date of death due to cancer. Univariate survival analysis was performed by Kaplan-Meier method and significance of survival differences was calculated using log-rank test. Multivariate survival analysis was performed by Cox regression model with forward stepwise selection, Wald's statistics and hazard ratio (HR) with 95% confidence interval (CI) were calculated to estimate the prognostic significance. P value <0.05 was interpreted as statistically significant.

3. Results

3.1 Correlation of Notch receptors with clinical and pathological parameters

Notch receptors were found to be localized in membrane, cytoplasm and nucleus of the tumor cells. As shown in figure1, positive expression of Notch1 was observed in 18 patients (18%), Notch2 in 31 patients (31%), Notch3 in 84 patients (84%) and Notch4 in 71 patients of TNBC (71%).

3.1.1 Notch1 receptor expression

Notch1 receptor was found to be predominantly localized only in the nucleus of the tumor cells of 18% patients as represented in Figure2. As shown in table 3, Notch1 nuclear positivity was significantly associated with high BR score (27%, 17/62, $p=0.009$) and necrosis (31%, 13/42, $p=0.003$) and a trend of association was observed between Notch1 positivity and high histological grade (23%, 15/66, $p = 0.08$).

3.1.2 Notch2 receptor expression

Notch2 was localized in cytoplasm of tumor tissue of 26% patients and in nucleus of 4% patients. In 2% patients, coexistent cytoplasmic and nuclear expression of Notch2 was detected. The incidence of cytoplasmic Notch2 expression was significantly higher in patients with local or distant metastasis (47%, 7/15, $p = 0.04$), while there was no significant association observed between nuclear and cytonuclear expression of Notch2 and clinicopathological parameters.

3.1.3 Notch3 receptor expression

It was observed that Notch3 receptor was found to be most frequently localized in the cytoplasm (65%), while 4% patients exhibited membrane expression, 13% patients and 3% patients exhibited cytomembrane and cytonuclear expression respectively. Although, membrane Notch3 expression was found in few patients, it was observed that tumors which were poorly differentiated exhibited high membrane Notch3 levels (23%, 15/62, $p=0.008$). Also, a trend of association was observed between overexpression of membrane Notch3 and metastasis (33%, 05/15, $p=0.09$). Contrary to this, trend of loss of Notch3 expression was observed in patients with high BR score (76%, 47/62, $p=0.06$). Moreover, cytoplasmic Notch3 expression was more frequently detected in patients with well differentiated to moderately differentiated tumors (43%, 16/37, $p=0.01$), low BR score tumors (52%, 14/27, $p=0.03$), early TNM stage (100%, 02/02, $p = 0.05$), lymph node negative status (51%, 29/57, $p=0.02$) and perinodal extension negative status (44%, 35/79, $p=0.03$). It was observed that cytonuclear Notch3 positivity was strongly correlated with necrosis (07%, 03/43, $p=0.04$).

3.1.4 Notch4 receptor expression

Localization of Notch4 receptor was observed in cytoplasm of 54% cases, cytonuclear expression was observed in 13% cases and cytomembrane expression was observed in 4% cases. Low incidence of cytoplasmic Notch4 expression was detected in younger patients (72%, 36/50, $p= 0.02$) and necrotic tumors (72%, 31/43, $p=0.04$). However, cytomembrane and cytonuclear expression of Notch4 did not show any significant correlation with clinicopathological parameters. The association between Notch4 and clinicopathological parameters is listed in table8.

3.2 Intercorrelation of Notch receptors

It was observed that expression of Notch2 and Notch3 receptors was significantly correlated with expression of Notch4 receptor. Patients with increased levels of Notch2 expression exhibited increased levels of Notch4 receptor (84%, $r=0.19$, $p=0.05$) and similarly, higher levels of Notch4 receptor were detected in patients with positive Notch3 expression (76%, $r=0.26$, $p=0.008$). In addition, Notch1 expression was strongly correlated with nuclear expression of Notch3 (99%, $r = 0.22$, $p=0.02$). Further, it was seen that patients with increased levels of Notch1 receptor had decreased levels of cytoplasmic Notch3 receptor (95%, $r=-0.26$, $p=0.007$).

3.3 Univariate Survival analysis for expression of Notch receptors in TNBC

The association between expression of Notch receptors and disease-free survival time and overall survival time of TNBC patients was investigated using Kaplan-Meier survival curves. The mean follow-up time was 37.05 months and maximum follow-up time was 75 months. During this period, 15 (15%) patients had local or distant metastasis and 15 (15%) patients had died due to the disease. With regard to disease-free survival, patients expressing higher cytoplasmic Notch2 (27%, 7/26, 50.71 ± 4.50 months) had worse disease-free survival than patients expressing lower cytoplasmic Notch2 (11%, 8/74, 66.83 ± 2.71 months, $\chi^2 = 3.77$, $df=1$, $p=0.05$). With regard to overall survival, it was observed that patients with higher incidence of cytoplasmic expression of Notch2 (31%, 8/26, 50.60 ± 3.87 months) had poor overall survival than patients with low expression of cytoplasmic Notch2 (10%, 7/74, 67.93 ± 2.48 months, $\chi^2 = 5.44$, $df=1$, $p=0.02$). Expression of other Notch receptors did not show any statistically significant relationship with disease-free survival and overall survival.

3.4 Multivariate Survival analysis

Multivariate survival analysis using Cox regression model with forward selection method (likelihood ratio) revealed that, with regard to disease-free survival advanced TNM stage (Wald = 9.36, HR = 0.19, 95% CI = 0.066-0.551, $p = 0.002$) entered at step 1 and high BR score (Wald = 7.94, HR = 4.93, 95% CI = 1.626-14.977, $p = 0.005$) entered at step 2, indicating their role as poor prognostic factors. With respect to overall survival, greater tumor size (Wald = 10.69, HR = 0.57, 95% CI = 0.10-0.321, $p = 0.001$) entered at step 1, positive lymph node status (Wald = 5.482, HR = 7.209, 95% CI = 1.380-37.676, $p = 0.019$) entered at step 2, high BR score (Wald = 8.355, HR = 7.787, 95% CI = 1.936-31.317, $p = 0.004$) entered at step 3 and higher grade (Wald = 8.762, HR = 0.012, 95% CI = 0.001-0.223, $p = 0.003$) entered at step 4. Multivariate analysis carried out by Cox regression model to evaluate the prognostic role of Notch receptors expression, independent of clinicopathological parameters revealed that with respect to overall survival, cytoplasmic expression of Notch2 (Wald = 4.87, HR = 3.152, 95% CI = 1.138-8.734, $p = 0.027$) entered at step 1.

4. Discussion

Notch signaling pathway is known to play a vital role in normal development and growth. Notch signaling pathway is involved in embryonic development, cellular differentiation, proliferation, homeostasis and

apoptosis. Numerous studies have reported that aberrant Notch expression is involved in neoplastic transformation and tumorigenesis. There are four known Notch paralogs which display different patterns in their expression and function across different malignancies. Oncogenic role of Notch signaling was first reported in T-ALL [17] and later reported in lung cancer, ovarian cancer, breast cancer, colorectal cancer [18, 19, 20, 21, 22] while tumor suppressive role of Notch signaling was reported in pancreatic cancer and hepatocellular carcinoma [23, 24]. However, research about relationship between Notch receptors and TNBC, and the association among the Notch receptors is limited and unclear.

In this study, we have examined the role of Notch receptors in TNBC cohort by analyzing the cellular localization of all four known Notch receptors by immunohistochemistry. We observed that Notch1 was expressed in the nucleus of tumor cells suggesting the translocation and accumulation of Notch intracellular domain (NICD1) in the nucleus of tumor cells. The accumulation of NICD1 in the nucleus was strongly correlated with high BR score and necrosis, indicating the tumor aggravating role of Notch1. Our data was in concordance with studies reporting the association of high grade tumors and poor prognosis of TNBC patients with up-regulated Notch1 [25, 26, 27].

Furthermore, upon examination of Notch2 protein it was revealed that Notch2 was localized in the cytoplasm and nucleus of tumor cells. Despite, various studies showing evidence of tumor suppressive role of Notch2 [28, 29, 30], our data suggests that Notch2 receptor plays a tumor promoting role in TNBC. It was observed that high cytoplasmic Notch2 expression was significantly correlated with local or distant metastasis, however nuclear and cytonuclear expression of Notch2 did not show any statistical significance with clinicopathological parameters. Nonetheless, our study was in line with evidence presented by Lee et al. and Sehwat et al., stating that inhibition of Notch2 was responsible for anti-apoptotic activity and inhibition of migration in TNBC cells [31, 32]. Also, our study demonstrated that high levels of cytoplasmic Notch2 was positively correlated with worse disease-free survival and poor overall survival proving its tumor promoting role. Earlier studies regarding Notch pathway emphasized that nuclear translocation of NICD was required in order to trigger the downstream target genes. Although, recently it has been demonstrated that in non-canonical Notch pathway, nuclear translocation is not necessary for the pathway to be activated and it may interact with other molecules in the cytoplasm to activate the pathway [33]. Therefore, it may be suggested that the association between cytoplasmic Notch2 expression and poor prognosis of the patients might be due to non-canonical Notch pathway.

Moreover, our data represents that majority of TNBC samples exhibited high Notch3 protein levels in the cytoplasm. Along with cytoplasmic expression, membrane, cytomembrane and cytonuclear protein expression were also detected. Notably, it was revealed that up-regulated cytoplasmic Notch3 expression in tumors was associated with low BR score tumors, early TNM stage, lymph node negative status and perinodal extension negative status indicating that patients with up-regulated cytoplasmic Notch3 might have favorable prognosis. Our data was in line with studies demonstrating tumor suppressive role of Notch3. Zhang and colleagues observed that high expression of Notch3 inhibited cellular proliferation and tumorigenesis by activating PTEN resulting in better prognosis of breast cancer patients [34]. Another study by Chen and colleagues provided evidence that overexpression of Notch3 inhibited cell cycle

progression by upregulating levels of Cdh1 in MDA-MB-231 cell lines. However, contrary to above findings, it was observed that membrane and cytonuclear expression of Notch3 were strongly correlated with poorly differentiated tumors and necrosis respectively. Also, a trend of association was observed between frequent membranous Notch3 expression and metastasis. Although, cytonuclear expression of Notch3 was not detected frequently it was strongly associated with necrotic tumors suggesting the role of nuclear translocation in tumor promoting activity of Notch3. These results were parallel to the findings of Choy and colleagues that revealed the involvement of nuclear Notch3 expression in tumor promotion [35]. The association of increased levels of Notch3 with poorly differentiated tumors might be due to the non-canonical pathway, where membrane-bound Notch3 may have interacted with cell regulators present in the cytoplasm. Contrary to that, membrane Notch3 expression was tended to associate with low BR score tumors. These results of Notch3 suggests that nuclear translocation of Notch3 is potentially involved in promoting tumor aggravating activity, whereas membrane-bound Notch3 may act as an oncogene via activation of non-canonical pathway which does not essentially require nuclear translocation. However, the strong association of high cytoplasmic Notch3 expression with low BR score tumors, early TNM stage, lymph node negative status and perinodal extension negative status might be due the immature state of the receptor or interaction of cytoplasmic Notch3 with tumor suppressing molecules which might be responsible for favorable prognosis of TNBC patients.

In addition, in the majority of the patients expressing Notch4 receptor, it was frequently detected in the cytoplasm. This study revealed that high levels of cytoplasmic Notch4 were negatively associated with younger age and necrotic tumors. Contrary, to a study by Wang et al. which stated that Notch4 was associated with poor prognosis for TNBC patients [36], our data represents that Notch4 is inversely correlated to poor prognostic factors of the disease and may function as a tumor suppressor. Concurrently, few studies have presented over expression of Notch4 receptor as tumor suppressive in melanoma and endometrial cancer [37, 38].

Further, we evaluated the co-expression patterns of Notch receptors in TNBC patients. It was observed that Notch2 and Notch3 receptors showed positive correlation with Notch4 receptor. This may be interpreted as Notch receptors are significantly associated with each other and even though Notch4 might function as a good prognostic factor, together with Notch2 and Notch3 receptors they contribute to tumor promotion and poor survival of TNBC patients. Moreover, it was observed that nuclear expression of Notch3 was positively associated with nuclear expression of Notch1 suggesting the oncogenic role of nuclear localization of both Notch1 and Notch3 receptors. Additionally, it was noted that nuclear Notch1 expression was negatively correlated with the cytoplasmic Notch3 expression indicating opposing role of both the receptors based on their localization, as cytoplasmic Notch3 is associated with better prognosis and nuclear Notch1 is linked to poor outcome of the disease. Therefore, our results imply that interplay of Notch receptors is rather complex mechanism.

In conclusion, our study demonstrated that Notch receptors exhibit a pleiotropic role in TNBC. Notch1 and Notch2 receptors play a tumor promoting role and contribute to poor outcome in TNBC patients. It was also revealed that although membrane and cytonuclear Notch3 had tumor aggravating effects,

cytoplasmic Notch3 and Notch4 receptor had tumor suppressive effect. It is implicated that the tumor promoting activity of Notch receptors might be due to the canonical Notch signaling pathway which essentially requires the nuclear translocation of NICD to trigger the transcriptional regulation of target genes or it might be due to the non-canonical pathway which is activated through the interaction of Notch receptors with transcriptional regulators present in the cytoplasm to exert its effects. The tumor suppressive effect of cytoplasmic Notch3 and Notch4 expression may be due to the inactive state of the receptor or its interaction with tumor suppressive molecules in the cytoplasm. Therefore, it is important to assess the subcellular localization of the Notch receptors as they are ubiquitously distributed in the cells in order to evaluate their individual effects in TNBC. Hence, our results strongly indicate that Notch receptors are involved in promoting TNBC and chiefly, Notch2 receptor may serve as a potential prognostic marker and therapeutic target for patients with TNBC.

Statements And Declarations

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Conflict of Interest

The authors have no conflict of interest.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ms. Heer Shah with the help of Ms. Mittal Mistry. The first draft of the manuscript was written by Ms. Heer Shah under observation of Dr. Hemangini Vora and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

This study was approved by Scientific Review and Ethics committees of The Gujarat Cancer & Research Institute and The Gujarat Cancer Society (EC/BHR/01/2020), Ahmedabad, Gujarat, India on 23/03/2020.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

The authors affirm that all the participants in the study provided informed consent for publication of their individual data including medical history and treatment.

Availability of Data and Material

Data and material of this study may not be available for another purposes.

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Tables

Table I: Antigens and antibodies details used in IHC

Antigen	Antigen retrieval	Antibody dilution	Incubation time	Staining pattern
Notch1	Standard	1:50	32min at 37°C	Nucleus
Notch2	Standard	1:25	60min at RT	Cytoplasm and/or Nucleus
Notch3	Standard	1:50	60min at RT	Membrane and /or Cytoplasm and/or Nucleus
Notch4	Mild	1:150	32min at RT	Membrane and/or Cytoplasm and/or Nucleus

Table II: Clinical and pathological data of TNBC patients (N=100)

Characteristics		N	(%)
Age (Median age: 47 years)	≤47	50	50
	>47	50	50
Menopausal status	Premenopausal	46	46
	Postmenopausal	47	47
	Perimenopausal	07	07
Tumor Size	T1	08	08
	T2	77	77
	T3	11	11
	T4	04	04
Lymph node status	N0	57	57
	N1	24	24
	N2	14	14
	N3	05	05
Stage	I	02	02
	II	75	75
	III	23	23
	IV	00	00
Histological Type	IDC	81	81
	IDC + DCIS	17	17
	IDC + Medullary	02	02
Histological Grade	I	01	01
	II	26	26
	III	66	66
	Unknown	07	07
BR Score	Low (3-5)	02	02
	Intermediate (6-7)	27	27
	High (8-9)	62	62
	Unknown	09	09

Perinodal extension	Positive	21	21
	Negative	79	79
Perineural invasion	Positive	06	06
	Negative	94	94
Necrosis	Positive	42	42
	Negative	58	58
Metastasis	Metastasis	15	15
	Remission	85	85
Survival	Dead	15	15
	Alive	85	85

Table III: Correlation of Notch1 and clinicopathological characteristics

Characteristics	Notch1 (IRS Median = 0)						
	Low expression		High expression		χ^2	r	p
	N	%	N	%			
Age (Median = 47)							
< 47 Years	40	80	10	20	0.27	-0.05	0.60
> 47 Years	42	84	08	16			
Menopausal status							
Premenopausal	36	78	10	22			
Perimenopausal	05	71	02	29	1.83	-0.04	0.68
Postmenopausal	41	87	06	13			
Tumor Size							
T1	08	100	00	00	3.03	0.001	0.99
T2	61	79	16	21			
T3	09	82	02	18			
T4	04	100	00	00			
Lymph node status							
N0	49	86	08	14	11.13	0.12	0.23
N1	18	72	07	28			
N2	13	100	00	00			
N3	02	40	03	60			
Stage							
I	02	100	00	00	0.67	0.07	0.48
II	62	83	13	17			
III	18	78	05	22			
Histopathology							
IDC	68	84	13	16	2.14	0.07	0.48
IDC + DCIS	12	71	05	29			
IDC + Medullary	02	100	00	00			
Grade							

I	01	100	00	00			
II	24	92	02	08	3.04	0.18	0.08
III	51	77	15	23			
BR Score							
Low (3-5)	02	100	00	00			
Intermediate (6-7)	26	96	01	04	7.17	0.27	0.009
High (8-9)	45	73	17	27			
Perinodal Extension							
Negative	66	84	13	16	0.60	0.07	0.44
Positive	16	76	05	24			
Perineural Invasion							
Negative	77	82	17	18	0.008	-0.009	0.93
Positive	05	83	01	17			
Necrosis							
Negative	53	91	05	09	8.23	0.28	0.003
Positive	29	69	13	31			
Metastasis							
No	69	81	16	19	0.26	-0.05	0.61
Yes	13	87	02	13			

Table IV: Correlation of cytoplasmic Notch2 and clinicopathological characteristics

Characteristics	Notch2 (IRS Median = 0)						
	Negative		Positive		χ^2	R	p
	N	%	N	%			
Age (Median = 47)							
< 47 Years	37	74	13	26	0.001	0.001	1.00
> 47 Years	37	74	13	26			
Menopausal status							
Premenopausal	36	78	10	22			
Perimenopausal	06	86	01	14	1.78	0.04	0.67
Postmenopausal	32	68	15	32			
Tumor Size							
T1	05	62	03	38			
T2	56	73	21	27	2.25	-0.11	0.26
T3	10	91	01	09			
T4	03	75	01	25			
Lymph node status							
N0	45	79	12	21			
N1	16	67	08	23	2.17	0.09	0.36
N2	09	64	05	36			
N3	04	80	01	20			
Stage							
I	02	100	00	00			
II	56	75	19	25	0.95	0.07	0.44
III	16	70	07	30			
Histopathology							
IDC	61	75	20	25			
IDC + DCIS	11	65	06	35	1.53	0.02	0.78
IDC + Medullary	02	100	00	00			
Grade							

I	01	100	00	00			
II	22	85	04	15	1.77	0.13	1.18
III	48	73	18	28			
BR Score							
Low (3-5)	02	100	00	00			
Intermediate (6-7)	21	78	06	22	1.18	0.10	0.32
High (8-9)	44	71	18	29			
Perinodal Extension							
Negative	59	75	20	25	0.09	0.03	0.76
Positive	15	71	06	29			
Perineural Invasion							
Negative	69	73	25	26	0.28	-0.05	0.59
Positive	05	83	01	17			
Necrosis							
Negative	42	72	17	28	0.59	-0.07	0.44
Positive	32	78	09	22			
Metastasis							
No	66	78	19	22	3.91	0.19	0.04
Yes	08	53	07	47			

Table V: Correlation of membrane Notch3 and clinicopathological characteristics

Characteristics	Notch3 Mem (IRS Median = 0)						
	Negative		Positive		χ^2	r	p
	N	%	N	%			
Age (Median = 47)							
< 47 Years	39	78	11	22	1.08	-0.10	0.29
> 47 Years	43	86	07	14			
Menopausal status							
Premenopausal	36	78	10	22			
Perimenopausal	06	86	01	14	0.80	-0.08	0.66
Postmenopausal	40	85	07	15			
Tumor Size							
T1	08	100	00	00	5.23	0.13	0.15
T2	62	80	15	20			
T3	10	91	01	09			
T4	02	50	02	50			
Lymph node status							
N0	47	83	10	17	1.80	0.05	0.61
N1	20	83	04	17			
N2	12	86	02	14			
N3	03	60	02	40			
Stage							
I	02	100	00	00	0.67	0.07	0.71
II	62	83	13	17			
III	18	78	05	22			
Histopathology							
IDC	67	83	14	17	0.81	0.13	0.66
IDC + DCIS	13	77	04	23			
IDC + Medullary	02	100	00	00			
Grade							

I	01	100	00	00			
II	26	100	00	00	7.31	0.27	0.008
III	51	77	15	23			
BR Score							
Low (3-5)	01	50	01	50			
Intermediate (6-7)	27	100	00	00	9.08	0.19	0.06
High (8-9)	47	76	15	24			
Perinodal Extension							
Negative	67	85	12	15	2.10	0.14	0.15
Positive	15	71	06	29			
Perineural Invasion							
Negative	76	81	18	19	1.40	-0.11	0.23
Positive	06	100	00	00			
Necrosis							
Negative	49	83	10	17	0.10	0.03	0.74
Positive	33	80	08	20			
Metastasis							
No	72	85	13	15	2.81	0.16	0.09
Yes	10	67	05	33			

Table VI: Correlation of cytoplasmic Notch3 and clinicopathological characteristics

Characteristics	Notch3 (IRS Median = 4)						
	Low expression		High expression		χ^2	R	p
	N	%	N	%			
Age (Median = 47)							
< 47 Years	29	58	21	42	0.37	-0.06	0.54
> 47 Years	32	64	18	16			
Menopausal status							
Premenopausal	26	57	20	43			
Perimenopausal	04	57	03	43	0.91	-0.06	0.55
Postmenopausal	31	66	16	34			
Tumor Size							
T1	04	50	04	50			
T2	47	61	30	29	0.76	-0.08	0.42
T3	07	64	04	26			
T4	03	75	01	25			
Lymph node status							
N0	28	49	29	51			
N1	20	83	04	17	11.69	-0.23	0.02
N2	08	57	06	43			
N3	05	100	00	00			
Stage							
I	00	00	02	100			
II	44	58	31	42	4.91	-0.18	0.05
III	17	74	06	26			
Histopathology							
IDC	49	60	32	40			
IDC + DCIS	11	65	06	35	0.20	-0.009	0.93
IDC + Medullary	01	50	01	50			
Grade							

I	00	00	01	100			
II	11	42	15	58	6.74	-0.26	0.01
III	45	68	21	32			
BR Score							
Low (3-5)	01	50	01	50			
Intermediate (6-7)	13	48	14	52	5.12	-0.22	0.03
High (8-9)	45	73	17	27			
Perinodal Extension							
Negative	44	56	35	44	4.44	-0.21	0.03
Positive	17	81	04	19			
Perineural Invasion							
Negative	57	61	37	39	0.08	-0.02	0.76
Positive	04	67	02	23			
Necrosis							
Negative	37	63	22	37	0.17	0.04	0.67
Positive	24	58	17	42			
Metastasis							
No	50	59	35	41	1.12	-0.10	0.29
Yes	11	73	04	27			

Table VII: Correlation of nuclear Notch3 and clinicopathological characteristics

Characteristics	Notch3 (IRS Median = 0)						
	Negative		Positive		χ^2	r	p
	N	%	N	%			
Age (Median = 47)							
< 47 Years	48	96	02	04	0.34	-0.05	0.56
> 47 Years	49	98	01	02			
Menopausal status							
Premenopausal	45	98	01	02			
Perimenopausal	06	86	01	14	3.29	0.11	0.26
Postmenopausal	46	98	01	02			
Tumor Size							
T1	08	100	00	00	1.81	0.68	0.50
T2	75	97	02	03			
T3	10	91	01	09			
T4	04	100	00	00			
Lymph node status							
N0	55	97	02	03	0.75	-0.06	0.51
N1	23	96	01	04			
N2	14	100	00	00			
N3	05	100	00	00			
Stage							
I	02	100	00	00	0.23	0.04	0.63
II	73	97	02	03			
III	22	96	01	04			
Histopathology							
IDC	78	96	03	04	0.72	-0.08	0.42
IDC + DCIS	17	100	00	00			
IDC + Medullary	02	100	00	00			
Grade							

I	01	100	00	00			
II	26	100	00	00	1.26	0.11	0.27
III	63	96	03	04			
BR Score							
Low (3-5)	02	100	00	00			
Intermediate (6-7)	27	100	00	00	1.45	0.12	0.25
High (8-9)	59	95	03	05			
Perinodal Extension							
Negative	76	96	03	04	0.82	-0.09	0.36
Positive	21	100	00	00			
Perineural Invasion							
Negative	91	97	03	03	0.19	-0.04	0.66
Positive	06	100	00	00			
Necrosis							
Negative	57	100	00	00	4.10	0.20	0.04
Positive	40	93	03	07			
Metastasis							
No	82	96	03	04	0.54	-0.07	0.46
Yes	15	100	00	00			

Table VIII: Correlation of cytoplasmic Notch4 and clinicopathological characteristics

Characteristics	Notch4 (IRS Median = 4)						
	Low expression		High expression		χ^2	r	p
	N	%	N	%			
Age (Median = 47)							
< 47 Years	36	72	14	28	5.08	0.22	0.02
> 47 Years	25	50	25	50			
Menopausal status							
Premenopausal	32	70	14	30			
Perimenopausal	05	71	02	29	3.68	0.10	0.28
Postmenopausal	24	51	23	49			
Tumor Size							
T1	05	62	03	08			
T2	47	61	30	39	0.53	-0.01	0.92
T3	06	54	05	46			
T4	03	75	01	25			
Lymph node status							
N0	35	61	22	39			
N1	14	58	10	42	0.14	-0.003	0.97
N2	09	64	05	36			
N3	03	60	02	40			
Stage							
I	01	50	01	50			
II	45	60	30	40	0.30	-0.05	0.59
III	15	65	08	35			
Histopathology							
IDC	52	64	29	36			
IDC + DCIS	07	41	10	59	4.43	0.08	0.41
IDC + Medullary	02	100	00	00			
Grade							

I	01	100	00	00			
II	14	54	12	46	1.39	-0.05	0.61
III	42	64	24	36			
BR Score							
Low (3-5)	01	50	01	50			
Intermediate (6-7)	14	52	13	48	1.35	-0.11	0.26
High (8-9)	40	64	22	36			
Perinodal Extension							
Negative	48	61	31	39	0.009	-0.01	0.92
Positive	13	62	08	38			
Perineural Invasion							
Negative	57	61	37	39	0.08	-0.02	0.77
Positive	04	67	02	33			
Necrosis							
Negative	31	52	28	48	3.90	-0.19	0.04
Positive	30	72	11	28			
Metastasis							
No	51	60	34	40	0.23	-0.04	0.63
Yes	10	67	05	33			

Table IX: Intercorrelation between Notch receptors

Markers	Notch1	Notch2	Notch3	Notch4
Notch1	--	r = 0.02 p = 0.81	r = 0.06 p = 0.53	r = -0.04 p = 0.65
Notch2	--	--	r = -0.002 p = 0.98	r = 0.19 p = 0.05
Notch3	--	--	--	r = 0.26 p = 0.008
Notch4	--	--	--	--

Figures

Figure 1

Total cases expressing Notch receptors

Figure 2

Localization of Notch receptors

Figure 3

Relationship between disease-free survival and Notch2

Figure 4

Relationship between overall survival and Notch2