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Crosstalk between Notch Signaling pathway and Epithelial-Mesenchymal Transition (EMT), Angiogenesis and Cancer stem cells in Triple Negative Breast Cancer (TNBC)

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Abstract

Notch signaling is an evolutionary conserved cell signaling pathway, when dysregulated can cause high cellular proliferation, high mobility capabilities, drug resistance which contribute to the development of TNBC. In this retrospective study, the role of Notch signaling pathway in TNBC progression was explored by analyzing the correlation of Notch reecptors with epithelial-mesenchymal transition (EMT), angiogenesis, and cancer stem cell (CSC) characteristics. Immunohistochemical analysis of Notch receptors (Notch1-4), EMT markers (E-cadherin and vimentin), angiogenesis marker (CD31), and CSC marker (Oct3/4) was conducted on tissue samples from 100 TNBC patients.Significant associations were found between Notch1, membrane Notch3, and nuclear Notch4 with vimentin expression, indicating their role in EMT induction. Moreover, Notch1 and nuclear Notch3 were positively correlated with CD31 expression, suggesting their involvement in angiogenesis regulation. Additionally, Notch1 and membrane Notch3 were associated with Oct3/4 expression, implicating their role in induction of cancer stem cell properties. These findings implicate the importance of Notch signaling in TNBC aggressiveness and suggest Notch receptors as potential therapeutic targets.

Introduction

Breast cancer is the most prevalent form of cancer among women in India and is the leading cause of cancer-related deaths among women in the country(https://gco.iarc.fr/). Breast cancer is a complex and heterogenous disease, which is classified into four molecular subtypes based on the gene expression patterns and hormone receptor status – Luminal A, Luminal B, Her2 positive and Triple Negative Breast Cancer (TNBC)[1]. Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer that accounts for approximately 10-15% of all breast cancer cases. TNBC cells lack estrogen receptors (ER), progesterone receptors (PR), and Her2 receptors, making it challenging to target with hormone therapies commonly used for other breast cancer subtypes [2]. TNBC tumors tend to be highly proliferative, high grade, highly metastatic and they often exhibit genetic instability, resulting in poor prognosis of the disease[3].

Epithelial-mesenchymal transition (EMT) is a biological process where cells undergo a phenotypic change from epithelial to mesenchymal, altering their characteristics and behavior[4].The EMT progression is primarilyregulated by the expression of various EMT transcriptional factors including Snail1, Slug (Snail2), Zeb1, Zeb2, Twist1 and Twist2. Further, these transcription factors inhibit the expression of epithelial markers such as E-cadherin, claudin, occludin, mucin-1, PTEN, RKIP, while simultaneously promoting the activation of mesenchymal markers including N-cadherin, Vimentin, Fibronectin, MMP1, MMP2 [5]. A number of signaling pathways play a pivotal role in activating the EMT process, such as EGF/EGFR pathway, FGF/FGFR pathway, HGF pathway, TGF- β pathway, WNT/β-catenin pathway, BMP pathway, Sonic Hedgehog pathway, PI3K/AKT pathway and the Notch signaling pathway [6].Angiogenesis is the process by which new blood vessels form from pre-existing vessels, plays an essential role in the growth and progression of cancer [7]. Angiogenesis is governed by various angiogenic factors including VEGF, FGF2, TNF-α, PDGF, IL8, EFG, HFG, CD31, cytokines, angiopoietins and chemokines [8]. Cancer stem cells (CSCs), also known as tumor-initiating

cells, represent a subpopulation of cells within a tumor that possess unique characteristics reminiscent of normal stem cells. Various signaling pathways, including Notch, Hedgehog, and Wnt/β-catenin, which are involved in the self-renewal and sustenance of stem cellscould potentially contribute to the development of drug resistance in cancer stem cells [9,10]

Notch signaling pathway is an evolutionary conserved cell signaling pathway which plays a critical role in fundamental developmental and biological processes across diverse species.The Notch signaling pathway is a vital regulator during embryonic development through adulthood by regulating somatogenesis, cellular differentiation and proliferation, organogenesis, stem cell maintenance, tissue homeostasis, vasculature, neurogenesis and apoptosis [11,12].Notch signaling pathway involves interactions between Notch receptors (Notch1, Notch2, Notch3, Notch4) and ligands (DLL1, DLL3, DLL4, JAG1, JAG2) on neighboring cells, initiating a cascade of molecular events. This process includes proteolytic cleavage by enzymes ADAM (a disintegrin and metalloprotease) and γ-secretase complex, releasing the Notch intracellular domain (NICD) into the nucleus, where the NICD interacts with various proteins such as CBF-1/RBPjk, Suppressor of Hairless, Lag-1 (CSL), Mastermind-like (MAML), and p300 to regulate the gene transcription[13]. The disruption of Notch signaling pathway can result in uncontrolled cell proliferation, migratory properties, altered cell fate determination, aberrant stem cell functions which play significant role in breast carcinogenesis and progression.Thus, understanding the intricate role of Notch signaling in promoting EMT, angiogenesis, and induction of cancer stem cell characteristicsoffers insightinto potential therapeutic targetfor TNBC.

Materials and Method

Patients

In this retrospective study, a total of 100 female patients diagnosed with Triple Negative Breast Cancer who had undergone surgery and treatment at the Gujarat Cancer & Research Institute, Ahmedabad, India during the period of 2014 to 2019 were included in the study. Detailed clinical and pathological history of the patients including age, menopausal status, tumor size, lymph node status, American Joint Committee on Cancer (AJCC) TNM stage, histopathological status, tumor grade, Bloom-Richardson (BR) score, perinodal extension, perineural invasion, necrosis, disease status and treatment offered was retrieved from the records maintained by Medical Record Department of the institute and was documented in the laboratory registers. Informed consent forms of all the patients enrolled in this study were obtained. This study was approved by Scientific Review and Ethics committees of the Institute.

Sample collection

Primary tumor of the enrolled patients was evaluated using Haematoxylin and Eosin (H&E) stain. Immunohistochemical analysis was performed on formalin fixed paraffin embedded (FFPE) tissue blocks containing the primary tissue. Formalin fixed paraffin embedded (FFPE) blocks were collected from archives of Department of Histopathology of the institute where the blocks were stored and preserved under optimal conditions.

Immunohistochemistry

Immunohistochemistry was used to assess the protein expression of Notch receptors (Notch1, Notch2, Notch3, Notch); EMT proteins (E-cadherin and vimentin); angiogenesis protein (CD31) and cancer stem cell protein (Oct3/4) in the tumor cells of TNBC patients.3-4µm sections of FFPE tissue samples were cutusing a microtome (Thermo-Fisher, USA) which were taken on APES (3-Aminopropyltriethoxysilane) coated glass slides, which were subsequently incubated overnight at 60°C.Immunohistochemitsry was performed using Ventana Benchmark XT autoimmunostainer(Ventana Medical Biosystems, USA)with the following protocol :- tissue sections were deparaffinized using EZ prep solution, followed by antigen retrieval with cell conditioning solution (CC1) at 95°C. Subsequently, sections were treated with UltraView DAB inhibitor for 4 minutes, and incubated with 100µl of respective primary antibodies (Table 1). After incubation, UltraView Horseradish peroxidase (HRP) multimer was then applied for 8 minutes for signal enhancement. Detection of the antigen-antibody complex was achieved using 3,3'-diaminobenzidine (DAB), followed by counterstaining with hematoxylin for 8 minutes and post-counterstaining with bluing reagent for 4 minutes. The UltraView DAB detection kit was utilized for staining of Notch2,

Notch3, Notch4, E-cadherin, Vimentin, CD31 and Oct3/4 while the OptiView DAB detection kit was employed for Notch1 staining. The OptiView DAB detection kit involved an additional incubation step with HQ linker for 8 minutes, followed by counterstaining for 16 minutes and post-counterstaining for 8 minutes. Finally, the slides were mounted using Dibutylphthalate Polystyrene Xylene (DPX) and xylene.

Table 1: List of antibodies used for IHC

Antibody	Antigen retrieval	Antibody company	Antibody dilution	Incubation time and Temperature
Notch1	Standard (64 minutes)	Invitrogen	1:50	32min at 37°C
Notch2	Standard (64 minutes)	Invitrogen	1:25	60min at RT
Notch3	Standard (64 minutes)	Invitrogen	1:50	60min at RT
Notch4	Mild (32 minutes)	Invitrogen	1:150	32min at RT
E-cadherin	Standard (64 minutes)	Cell Marque	1:150	32min at 37°C
Vimentin	Mild (32 minutes)	Invitrogen	1:100	32min at 37°C
CD31	Mild (32 minutes)	Cell Signaling Technology	1:100	32min at 37°C
Oct3/4	Mild (32 minutes)	Invitrogen	1:100	32min at 37°C

Scoring

The assessment of Notch1, Notch2, Notch3, Notch4, E-cadherin, vimentin, and Oct3/4 expression was conducted using the Immunoreactive Scoring system (IRS) under a 40x light microscope. This method involved determining the percentage of positive cells, categorized as 0 (negative staining), 1 (<10% positive cells), 2 (10% - 50% positive cells), 3 (51% - 80% positive cells), and 4 (>80% positive cells). Additionally, staining intensity was rated as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). The Immunoreactive Score (IRS) for each case was calculated by multiplying the percentage of positive cells by the staining intensity, yielding scores ranging from 0 to 12. For CD31 evaluation, intratumoral vessels displaying CD31 positivity were counted in high-power fields, and the counts from three fields were averaged. A median score was computed for all markers, with cases scoring below the median considered as exhibiting negative or low expression, while those scoring above the median were classified as showing overexpression.

Statistical Analysis

Statistical analysis was performed using SPSS 26.0 (SPSS, Inc., Chicago, USA). The Chi-square test was to used determine the association between two parameters and the correlation between the two parameters was determined by Pearson's correlation coefficient (r).P value ≤0.05 was considered as statistically significant.

Results

Incidence of protein markers in TNBC

As shown in our previous study, positive expression of Notch1 was detected in (18/100, 18%) patients, Notch2 in (31/100, 31%) patients, Notch3 in (84/100, 84%) patients and Notch4 in (71/100, 71%) patients of TNBC. Notch1 was localized in the nucleus, Notch2 and Notch4were present in the cytoplasm and nucleus while, Notch3 was present in membrane, cytoplasm and nucleus. Further, membranous expression E-cadherin was detected in (78/100, 78%), while cytoplasmic expression of vimentin was noted in (39/100, 39%) TNBC patients. Moreover, positive expression of CD31 was noted in (35/100, 35%) TNBC patients and nuclear expression of Oct3/4 was noted in (!9/100, 19%) TNBC patients.

Correlation of Notch receptors with EMT markers (E-cadherin and Vimentin)

With respect to Notch1, a significant positive correlation was detected between patients expressing high Notch1 (72%, 13/18) and vimentin overexpression than patients with low incidence of Notch1 (32%, 26/82, $\chi 2$=10.18, r=0.31, p=0.001).Moreover, a significant positive association was observedbetween patients with high incidence of membrane Notch3 (67%, 12/18) and patients with high incidence of vimentin as compared to patients with decreased membrane Notch3 expression (33%, 27/82, $\chi 2$=7.06, r=0.26, p=0.008).Additionally, a significant association was noted between patients with high incidence of nuclear Notch4 (69%, 09/15) and high incidence of vimentin than patients with lowincidence of nuclear Notch4 (35%, 30/87, $\chi 2$=5.74, r=0.24, p=0.01). However, no significant association was detected between Notch receptors and E-cadherin (Table 2).

Table 2: Correlation of Notch receptors wit EMT markers

Protein		**E-cadherin**		**Vimentin**	
		Low N (%)	**High N (%)**	**Low N (%)**	**High N (%)**
Notch1	**Low**	36 (44)	46 (56)	56 (68)	26 (32)
	High	10 (56)	08 (44)	05 (28)	13 (72)
		χ^2=0.80, r=-0.09 p=0.37		χ^2=10.18, r=0.31 **p=0.001**	
Notch2 Cytoplasmic	**Low**	34 (46)	40 (54)	47 (63)	27 (37)
	High	12 (46)	14 (54)	14 (54)	12 (46)
		χ^2=0.001, r=-0.002 p=0.98		χ^2=0.75, r=0.08 p=0.39	
Notch2 Nuclear	**Low**	44 (47)	50 (53)	58 (62)	36 (38)
	High	02 (33)	04 (67)	03 (50)	03 (50)
		χ^2=0.41, r=0.06 p=0.52		χ^2=0.32, r=0.05 p=0.57	
Notch3 Membrane	**Low**	35 (43)	47 (57)	55 (67)	27 (33)
	High	11 (61)	07 (39)	06 (33)	12 (67)
		χ^2=2.01, r=-0.14 p=0.15		χ^2=7.06, r=0.26 **p=0.008**	
Notch3 Cytoplasmic	**Low**	30 (49)	31 (51)	34 (56)	27 (44)
	High	16 (41)	23 (59)	27 (69)	12 (31)
		χ^2=0.63, r=0.08 p=0.43		χ^2=1.81, r=-0.13 p=0.18	
Notch3 Nuclear	**Low**	45 (46)	52 (54)	60 (62)	37 (38)
	High	01 (33)	02 (67)	01 (33)	02 (67)
		χ^2=0.20, r=0.04 p=0.65		χ^2=0.99, r=1.00 p=0.32	
Notch4 Cytoplasmic	**Low**	27 (44)	34 (56)	38 (62)	23 (28)
	High	19 (49)	20 (51)	23 (59)	16 (41)
		χ^2=0.19, r=-0.04 p=0.66		χ^2=0.11, r=0.03 p=0.74	
Notch4	**Low**	39 (45)	48 (55)	57 (65)	30 (35)
	High	07 (54)	06 (46)	04 (31)	09 (69)

Nuclear		χ^2=0.37, r=-0.06 p=0.54	χ^2=5.74, r=0.24 **p=0.01**

Correlation of Notch receptors with Angiogenesis marker (CD31)

A significant high incidence of CD31 expression was observed in patients with high incidence of Notch1 (56%, 10/18) as compared to patients with low incidence of Notch1 (17%, 14/18, χ2=11.98, r=0.34, p=0.001). Further, high incidence of CD31 was significantly associated with patients with high incidence of nuclear Notch3 (100%, 03/03) than in patients with low incidence of nuclear Notch3 (22%, 21/97, χ2=9.79, r=0.31, p=0.002) expression. Moreover, a significant association was noted between patients with high incidence of CD31 and low cytoplasmic Notch3 expression (34%, 21/61) than in patients with high cytoplasmic expression (08%, 03/39, χ2=9.32, r=-0.30, p=0.002) (Table 3).

Table 3: Correlation of Notch receptors with angiogenesis marker

Protein		**CD31**	
		Low **N (%)**	**High** **N (%)**
Notch1	**Low**	68 (83)	14 (17)
	High	08 (44)	10 (56)
		χ^2=11.98, r=0.34 **p=0.001**	
Notch2 **Cytoplasmic**	**Low**	57 (77)	17 (23)
	High	19 (73)	07 (17)
		χ^2=0.16, r=0.0 4 p=0.68	
Notch2 **Nuclear**	**Low**	71 (75)	23 (25)
	High	05 (83)	01 (17)
		χ^2=0.18, r=-0.04 p=0.66	
Notch3 **Membrane**	**Low**	65 (79)	17 (21)
	High	11 (61)	07 (39)
		χ^2=2.66, r=0.16 p=0.10	
Notch3 **Cytoplasmic**	**Low**	40 (66)	21 (34)
	High	36 (92)	03 (08)
		χ^2=9.32, r=-0.30 **p=0.002**	
Notch3 **Nuclear**	**Low**	76 (78)	21 (22)
	High	00 (00)	03 (100)
		χ^2=9.79, r=0.31 **p=0.002**	
Notch4 **Cytoplasmic**	**Low**	46 (75)	15 (25)
	High	30 (77)	09 (23)
		χ^2=0.03, r=-0.01 p=0.86	

Notch4 Nuclear	**Low**	65 (75)	22 (25)
	High	11 (85)	02 (15)
		χ^2=0.60, r=-0.07 p=0.44	

Correlation of Notch receptors with cancer stem cells (CSC) marker (Oct3/4)

A significant high incidence of Oct3/4 expression was noted in patients with high incidence of Notch1 (39%, 07/18) as compared to patients with low incidence of Notch1 (15%, 12/18, χ2=5.64, r=0.23, p=0.01). Also, patients with high incidence of membrane Notch3 expression (39%, 07/18) demonstrated significantly high incidence of Oct3/4 than patients with low membrane incidence of Notch3 expression (15%, 12/82, χ2=5.64, r=0.23, p=0.01).Further, a trend of high incidence of Oct3/4 was noted in patients with low cytoplasmic Notch3 expression (25%, 15/61) than in patients with high cytoplasmic expression (10%, 04/39, χ2=3.17, r=-0.17, p=0.07) (Table 4).

Table 4: Correlation of Notch receptors with CSC marker

Protein		**Oct3/4**	
		Low N (%)	**High N (%)**
Notch1	**Low**	70 (85)	12 (15)
	High	11 (61)	07 (39)
		χ^2=5.64, r=0.23 **p=0.01**	
Notch2 Cytoplasmic	**Low**	62 (84)	12 (16)
	High	19 (73)	07 (27)
		χ^2=1.43, r=0.12 p=0.23	
Notch2 Nuclear	**Low**	76 (81)	18 (19)
	High	05 (83)	01 (17)
		χ^2=0.02, r=-0.01 p=0.88	
Notch3 Membrane	**Low**	70 (85)	12 (15)
	High	11 (61)	07 (39)
		χ^2=5.64, r=0.23 **p=0.01**	
Notch3 Cytoplasmic	**Low**	46 (75)	15 (25)
	High	35 (90)	04 (10)
		χ^2=3.17, r=-0.17 p=0.07	
Notch3 Nuclear	**Low**	79 (81)	18 (19)
	High	02 (67)	01 (33)
		χ^2=0.41, r=0.06 p=0.52	
Notch4	**Low**	47 (77)	14 (23)
	High	34 (87)	05 (13)

Cytoplasmic		χ^2=1.58, r=-0.12 p=0.21	
Notch4 Nuclear	**Low**	70 (81)	17 (19)
	High	11 (85)	02 (15)
		χ^2=0.12, r=-0.03 p=0.72	

Discussion

The Notch signaling pathway is a highly conserved intercellular signaling pathway that plays a crucial role in controlling a number of biological developmental processes, including homeostasis, apoptosis, stem cell maintenance, cellular proliferation, and cell fate determination[14]. Aberrant Notch signaling is involved in pathogenesis and tumorigenesisby promoting tumor growth, epithelial-mesenchymal transition, angiogenesis and cancer stem cell maintenance. In our previous study, it was observed that Notch1, and membrane Notch3 were significantly associated with unfavorable clinicopathological characteristics, while Notch2 was a poor prognostic indicator for TNBC. Moreover, it was also noted that cytoplasmic Notch3 and Notch4 were associated with favorable clinicopathological characteristics[15].

Thus, this study explored the role of Notch receptors in hallmarks of cancer such as EMT, angiogenesis and induction of cancer stem cell phenotype by correlating the protein expression of Notch receptors with EMT markers (E-cadherin and Vimentin), angiogenesis marker (CD31) and stem cell marker (Oct3/4) in TNBC.The findings of the present study reveal a significant positive correlation between the expression of Notch1, membrane Notch3, and nuclear Notch4 and vimentin overexpression in TNBC patients. Similar to the results of this study, it wasrevealed that Notch1 induces EMT in breast cancer as tumor cells expressing Notch1 exhibited decreased expression of epithelial markers including E-cadherin and occludin, whereas increased expression of mesenchymal markers including N-cadherin, vimentin and fibronectin was noted [16]. Moreover, it was revealed that Notch1 overexpression was associated with decreased E-cadherin expression in MDA-MB-231 cell line and further, when Notch1 was inactivated by DAPT increased levels of E-cadherin were detected[17].Further, knockdown of Notch1 by siRNA resulted in decreasedexpression of vimentin [18,19] and increased expression of E-cadherin,causing reversal of EMT in MDA-MB-231 cell line [20]. Also, Zhang et al. (2010) revealed that decreased expression of Notch3 mRNA in metastatic breast cancer cells MDA-MET led to decreased expression of vimentin and fibronectin, whereas E-cadherin expression was not correlated with Notch3 [21].Further, Strati et al. (2017) stated that no significant correlation was noted between cytoplasmic Notch3, membrane Notch3 and E-cadherin in TNBC patients [22].Concomitantly, overexpression of Notch4 protein was significantly correlated with high vimentin expression in pancreatic ductal adenocarcinoma [23].Also, it wasrevealed that Notch4 mRNA upregulated Slug and in turn induced epithelial-mesenchymal transition in TNBC [24]. Further, knockdown of Notch4 resulted in up-regulation of E-cadherin and downregulation of Vimentin, Twist1 and VE-cadherin in melanoma cells [25].

The results of the present study further revealed that Notch1 and nuclear Notch3 are significantly associated with high incidence of CD31, while cytoplasmic Notch3 is inversely correlated with CD31. In concordance with these results, Notch1 overexpression was significantly correlated with microvessel density, evaluated by CD31 which leads to promotion of angiogenesis in melanoma [26]. Also, Notch1 was significantly associated with vascular endothelial growth factor (VEGF) and microvessel density evaluated by CD34 in tongue cancer [27]. Concomitantly, *in vivo* studies revealed that xenografts with low Notch3 expression showed reduced microvessel density, which was evaluated by CD31 and VEGFR2 in hepatocellular carcinoma [28]. Also, overexpression of Notch3 mRNA and protein promoted angiogenesis in bladder cancer via activation of PI3K/AKT pathway [29]. These findings indicate that Notch1 and nuclear Notch3 may promote angiogenesis in TNBC. Also, as cytoplasmic Notch3 is inversely correlated with CD31 expression, it can be suggested that Notch3 has not translocated to nucleus to trigger the genetic transcription.

Further, the results of the present study reveal that Notch1 and membrane Notch3 are significantly associated with cancer stem cell marker Oct3/4. Concurrently, an immunohistochemical study revealed that Notch1 was significantly correlated with stem cell marker ALDH1[30]. Further, it was demonstrated that Notch1 mRNA was significantly correlated with Oct3/4 mRNA in patients with hypoxia which, further promoted formation of cancer stem cells and tumor growth in glioma[31]. Also, tumor cells exhibiting Notch1 expression and cancer stem cell marker Oct4 were involved in cancer stem cell maintenance, invasiveness, chemoresistance and metastasis in gastric cancer [32]. Moreover, it was revealed that high incidence of Notch3 mRNA upregulated cancer stem cell markers including Oct3/4, Nanog, Rex1, Klf4, NAC1, SALL4 in ovarian cancer [33] Further, it was demonstrated that cancer stem cells overexpress PD-L1 via high Notch3 and mTOR activation, which in turn regulate cancer stem cells in breast cancer cell lines [34].

Conclusion

In conclusion, the findings of this study indicate that prominently Notch1, membrane Notch3 and nuclear Notch4 may contribute to the acquisition of mesenchymal features in TNBC cells, which can enhance their migratory and invasive capabilities. Further, Notch1 and nuclear Notch3 promote angiogenesis via the regulation of CD31 expression and Notch1 and membrane Notch3 may regulate the expression of Oct3/4 which confer drug resistance in TNBC. Therefore, it can be postulated that aberrant Notch signaling can drive cancer hallmarks including EMT, angiogenesis and induction of cancer stem properties and thereby, contributing to the aggressiveness of TNBC. Hence, Notch receptors may serve as novel therapeutic target for TNBC.

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