The Wnt Pathway Components are Upregulated in Different Molecular Subtypes of Invasive Ductal Carcinoma Patients of Breast

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Abstract

Wnt/β-catenin pathway is a key pathway associated with the development of breast cancer. Hence, the aim of this study was to investigate the expression of Wnt pathway components in primary invasive ductal carcinoma (IDC) patients of breast and explore their relation with clinico-pathological parameters as well as the prognostic significance. The study comprised of 160 IDC patients classified into different molecular subtypes and who have undergone surgery as the primary treatment. The expression of important pathway components- Fzd7, β-catenin and cyclin D1 was detected by immunehistochemical staining and analyzed using H-score method. Statistical analysis was carried out using SPSS and $p \leq 0.05$ were considered significant. Fzd7 was significantly over-expressed in breast tumours with smaller size and metastatic lymph nodes. The expression of Fzd7, β-catenin and cyclin D1 were associated with grade 2 tumours than grade 3 tumours. Upon correlating with the hormone receptors, the molecules were found to be positively related to the receptor positivity. Also, the patients comprising Luminal subtypes exhibited higher expression of the molecules than the triple negative breast cancer (TNBC) subtype. Finally, Fzd7 and cyclin D1 emerged as the significant prognosticator of overall survival (OS) in patients with grade 3 tumours and TNBC, respectively. Wnt pathway components are up regulated during the early stages of the breast cancer development with a loss during the disease advancement.

Although the pathway failed to predict the prognosis in total breast cancer patients, in patient subgroups having grade 3 tumours and TNBC, Fzd7 and cyclin D1 were found to be significant predictors of OS.

Keywords: Breast; Invasive Ductal Carcinoma; Wnt/B-Catenin Pathway

Abbreviations: FZD: Frizzled; PR: Progesterone Receptor; IDC: Invasive Ductal Carcinoma; APES: 3-Amino Propyl Triethoxy Silane.

Introduction

Breast cancer has thrived to become the most common cancer in women with the second leading cause of cancer mortality worldwide [1]. The clinical parameters and pathological markers (estrogen receptor [ER], progesterone receptor [PR] and human epidermal growth factor 2 [Her2]), are routinely used by the clinicians to stratify patients for prognostic predictions and to select treatments [2]. However, inspite of various treatment modalities, breast cancer rate keeps on increasing due to the complexity of the disease. This is due to the association of a number of pathways in the development and progression of breast cancer [3]. Wnt/β-catenin signalling pathway is one of such pathways which is actively engaged in various stages of development including adult tissue organization [4].

The binding of the Wnt ligand to its receptor Frizzled (Fzd) and co-receptors at the cell membrane initiates several downstream pathways. Activation of β-catenin-dependent canonical pathway results into cytoplasmic accumulation of β-catenin and thereby its nuclear translocation. This subsequently transcribes the target genes of the pathway resulting in carcinogenesis [5]. The role of aberrant Wnt signaling is most prominent in colorectal cancer, but it is also observed in many cancer types such as non-small cell lung cancer, prostate cancer, breast cancer and so on [6]. However, the role of Wnt signaling in breast cancer is controversial with few reports showing Wnt pathway mutations in only rare cases [7].

Hence in the present study, we aimed to determine the incidence of the Wnt pathway in primary breast cancer patients by analyzing the expression of important pathway components. We studied the expression and sub-cellular localization of the Wnt ligand receptor- Frizzled7 (Fzd7), the principle protein of the pathway- β-catenin and the activated target gene- cyclin D1 in invasive ductal carcinoma (IDC) patients of breast. Further, the protein expression of these molecules was correlated with different clinicopathological parameters, hormone receptors and molecular subtypes. Also, the prognostic significance of these molecules was studied in the breast cancer patients.

Materials & Methods

Patients

A total of 160 untreated histologically confirmed breast cancer patients with Invasive Ductal Carcinoma (IDC) type registered at Gujarat Cancer & Research Institute from March 2014 to December 2015 were enrolled in the present study. The written consent forms were obtained from all the patients prior to treatment administration and the study was approved by the Institute's Ethics Committee Board. Detailed clinical and demographic history of the patients was obtained from the case files maintained at the Medical Record Department of the institute. Histopathological details such as tumour size, lymph node status, disease stage, BR score, histological grade and status of ER, PR, Her2 were evaluated and reported on routine basis by the pathologists of our institute. The patients were classified into different molecular subtypes based on the expression of hormone receptors and Her 2 in breast tumours, where Luminal A subtype comprised patients with ER+, PR+/-, and Her2-, Luminal B subtype comprised of patients with ER+, PR+/-, and Her2+, Her2 positive subtype were patients having ER-, PR-, and Her2+ while those who were negative for all the three receptors were classified as TNBC patients. Detailed clinico-pathological characteristics of the enrolled patients are enlisted in Table 1. Complete follow-up details of 69% (111/160) patients were obtained, who were included in overall survival (OS) analysis. Amongst these, 3% (2/111) patients had persistent disease and hence only 68% (109/160) patients were included for the analysis of relapse free survival (RFS).

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Table 1: Clinicopathological characteristics of IDC patients

Variables	N (%)
Total patients	160 (100)
Age (years)	
≤50	81 (51)
>50	79 (49)
Menopausal Status	
Pre-menopausal	56 (35)
Post-menopausal	104 (65)
Tumour size	
T1 (≤20 mm)	21 (13)
T2 (20-50 mm)	113 (71)
T3 (>50 mm)	17 (11)
T4 (Extension to chest wall and/or skin)	09 (05)
Node involvement	
Negative	65 (41)
Positive	95 (59)
TNM Stage	
I	12 (08)
II	86 (54)
III	61 (38)
IV	01 (06)
Tumor Grade	
Grade 1	13 (08)
Grade 2	103 (64)
Grade 3	44 (28)
ER	
Negative	72 (45)
Positive	88 (55)
PR	
Negative	100 (62)
Positive	60 (38)
Her2-neu	
Negative	91 (57)
Positive	69 (43)
Molecular subtype	
Luminal A	52 (33)
Luminal B	36 (22)
Her2-positive	35 (22)
TNBC	37 (23)
Treatment administered	
S	11 (07)
S+CT	45 (28)
S+CT+RT	27 (17)
S+CT+HT	35 (22)
S+CT+RT+HT	42 (26)

S=Surgery; CT=Chemotherapy; RT=Radiotherapy; HT=Hormonal therapy.

Immunohistochemistry

For immunohistochemical (IHC) study, formalin-fixed paraffin embedded tissue blocks of the enrolled patients were retrieved from the tissue repository of our institute's Pathology Department. The blocks were cut into 4 μm sections and placed on 3-amino propyl triethoxy silane (APES)-coated slides. IHC staining was performed for the detection of Fzd7, β-catenin and cyclin D1 using HRP/DAB (ABC) Detection IHC kit (Abcam) according to manufacturer's protocol. Briefly, antigen retrieval treatment was given by heating the sections in 10 mM sodium citrate buffer (pH-6.0) in a pressure cooker. Then after, sections were incubated overnight at 4^0 C with respective primary antibodies procured commercially, after appropriate dilutions: Fzd7 (Aviva Systems Biology, USA; 1:100), β-catenin (Genetex Inc, USA; 1:300) and cyclin D1 (Biogenex, USA; 1:50). The stained sections were mounted with DPX and observed under the light microscope. A section with intense staining for a given marker was used as a positive control, whereas negative control was obtained by omission of primary antibody.

Assessment of Fzd7, β-Catenin and Cyclin D1 Expression

The stained sections were evaluated immunohistochemically by semi-quantitative method. The scoring was done independently in a blinded fashion on the basis of staining intensity and percentage of positive cells. The staining intensity was scored on a scale of 0-3 where 0 indicated no staining obtained, 1+ for weakly stained cells, 2+ for moderately stained cells and 3+ for strong intense staining of the cells. The extent of staining was expressed by percentage of positive cells (0-100%) by 10% intervals. The final histoscore (H-score) was calculated by multiplying the staining positivity score with the staining intensity score of each section, ranging from 0 to 300. The mean value of the H-score for each of the studied protein (i.e. Fzd7, β-catenin, cyclin D1) was set as the cut-off value. The calculated mean for Fzd7 score was 130, β-catenin was 169 and cyclin D1 was 162. The H-score values above this cut-off were considered as the over-expression/upregulation of the protein and the values below the cut-off were considered as low expression.

Statistical Analysis

The data was analyzed statistically using SPSS Inc. version 23 software. The correlation between the expression of studied parameters and various clinicopathological characteristics of breast cancer patients was determined by two-tailed chi square test (χ^2) and spearman's correlation. Survival analysis was performed using Kaplan-Meier survival function and the differences in survival were tested for statistical significance using log-rank statistic. $p \leq 0.05$ was considered to be statistically significant.

Results

Incidence of Fzd7, β-Catenin and Cyclin D1

The immunostaining pattern of Fzd7 protein was intensely membranous, that of the β-catenin protein was found to be cytoplasmic whereas for cyclin D1 protein, it was in the nucleus of the breast tumour cells (Figure 1). The immunoreactivity of Fzd7, β-catenin and cyclin D1 was detected in 92% (148/160), 96% (154/160) and 96% (154/160) patients, respectively. According to the cut-off value as described previously, 48% (77/160), 56% (89/160) and 50% (80/160) patients exhibited overexpression of Fzd7, β-catenin and cyclin D1, respectively.
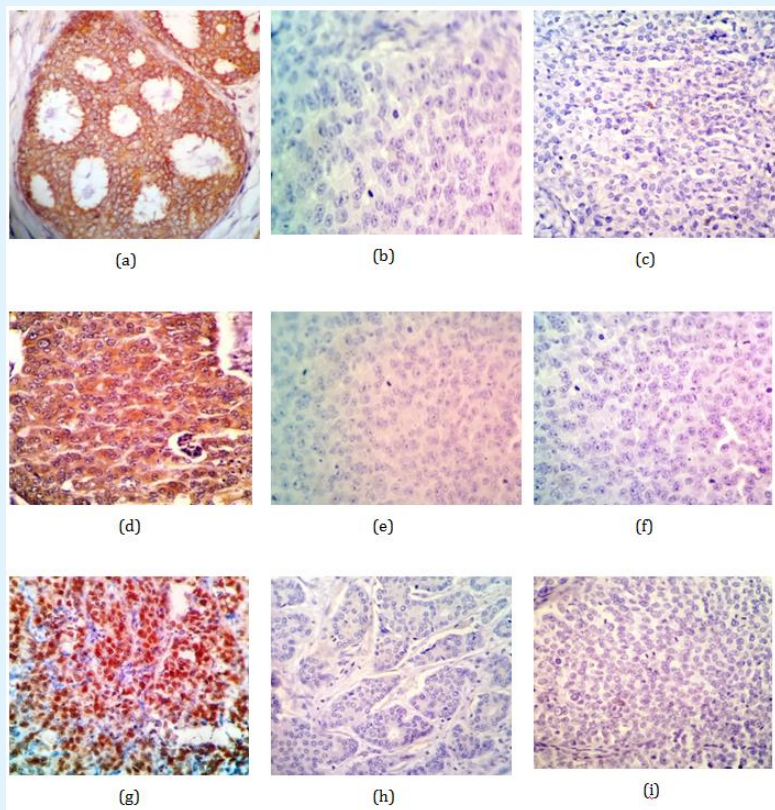

Figure 1: Representative immunohistochemical staining patterns in breast cancer patients.
a) Membranous staining of Fzd7 protein.
b) Negative control for Fzd7 staining.
c) Negative staining of Fzd7 protein in breast tumors.
d) Cytoplasmic staining of β-catenin protein.
e) Negative control for β-catenin staining.
f) Negative staining of β-catenin protein in breast tumors.
g) Nuclear staining of cyclin D1 protein.
h) Negative control for cyclin D1 staining.
i) Negative staining of cyclin D1 protein in breast tumors.

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Correlation of Fzd7, β-catenin and cyclin D1 with clinico-pathological features and molecular subtypes

Correlation of Fzd7, β-catenin and cyclin D1 with clinico-pathological features: The expression of Fzd7, β-catenin and cyclin D1 was not significantly associated with any of the clinical characteristics of breast cancer patients. However, patients with grade 1/2 tumours (low/intermediate tumours) showed significant higher expression of Fzd7 (p=0.029), β-catenin (p=0.021) and cyclin D1 (p=0.013) as compared to those with grade 3 tumours (high BR score). On the other hand, only Fzd7 showed significant upregulation in patients having lymph node metastasis (p=0.043). Moreover, a trend was observed for higher expression of Fzd7 with various tumour sizes (p=0.061) (Table 2). Furthermore, ER⁺ patients exhibited significant over-expression of Fzd7 (p=0.002), β-catenin (p=0.010) and cyclin D1 (p<0.001) as compared to ER⁻ patients. Similarly, PR⁺ patients showed significant higher expression of Fzd7 (p=0.003) and cyclin D1 (p<0.001) than PR⁻ patients. However, in case of β-catenin over-expression, only a trend was observed in PR⁺ patients (p=0.065). On the other hand, no correlations were observed for any of the protein expression with Her2 status of the tumours.

Table 2: Correlation of Fzd7, β-catenin and cyclin D1 expression with pathological features

Variables	N	Fzd7 expression		β-catenin expression		Cyclin D1 expression	
		Low	High	Low	High	Low	High
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Tumor size							
T1	21	08 (38)	13 (62)	11 (52)	10 (48)	06 (29)	15 (71)
T2	113	58 (51)	55 (49)	46 (41)	67 (59)	60 (53)	53 (47)
T3	17	11 (65)	06 (35)	11 (65)	06 (35)	11 (65)	06 (35)
T4	09	06 (67)	03 (33)	03 (33)	06 (67)	03 (33)	06 (67)
		χ^2=3.521 r=-0.144 p=0.061		χ^2=4.452 r=-0.012 p=0.883		χ^2=6.761 r=-0.074 p=0.141	
Nodal status							
Negative	65	40 (62)	25 (38)	28 (43)	37 (57)	36 (55)	29 (45)
Positive	95	43 (45)	52 (55)	43 (45)	52 (55)	44 (46)	51 (54)
		χ^2=4.095 r=0.16 **p=0.043**		χ^2=0.075 r=-0.022 **p=0.786**		χ^2=1.27 r=0.089 **p=0.263**	
Tumor grade							
Grade 2	103	48 (47)	55 (53)	39 (38)	64 (62)	48 (47)	55 (53)
Grade 3	44	29 (66)	15 (34)	26 (59)	18 (41)	29 (66)	15 (34)
		χ^2=4.607 r=-0.17 **p=0.030**		χ^2=5.632 r=-0.196 **p=0.018**		χ^2=4.607 r=-0.177 **p=0.032**	
Tumor grade							
Grade 1	13	06 (46)	07 (54)	06 (46)	00 (54)	03 (23)	10 (77)
Grade 3	44	29 (66)	15 (34)	26 (59)	18 (41)	29 (66)	15 (34)
		χ^2=1.653 r=-0.17 **p=0.205**		χ^2=0.682 r=-0.109 **p=0.418**		χ^2=7.477 r=-0.362 **p=0.006**	
ER status							
Negative	72	47 (65)	25 (35)	40 (56)	32 (44)	53 (74)	19 (26)
Positive	88	36 (41)	52 (59)	31 (35)	57 (65)	27 (31)	61 (69)
		χ^2=4.607 r=-0.17 **p=0.030**		χ^2=6.63 r=0.204 **p=0.010**		χ^2=29.192 r=0.427 **p<0.001**	
PR status							
Negative	100	61 (61)	39 (39)	50 (50)	50 (50)	64 (64)	36 (36)
Positive	60	22 (37)	38 (63)	21 (35)	39 (65)	16 (27)	44 (73)
		χ^2=8.894 r=0.236 **p=0.003**		χ^2=3.418 r=0.146 **p=0.065**		χ^2=20.907 r=0.361 **p<0.001**	

Correlation of Fzd7, β-catenin and cyclin D1 with molecular subtypes: The expression of Fzd7 (p=0.007), β-catenin (p=0.008) and cyclin D1 (p<0.001) were significantly associated with the molecular subtypes of the patients. Hence, upon correlating with the individual subtype it was observed that, the upregulation of Fzd7 (p=0.015, p=0.041), β-catenin (p=0.011, p=0.024) and cyclin D1 (p<0.001, p=0.006) were significantly higher in

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patients with Luminal A subtype as well as Luminal B subtype when compared to TNBC patients, respectively (Table 3). Further, Fzd7 (p=0.003) and cyclin D1 (p=0.005) were over-expressed in Luminal B subtype when compared to Her2-positive subtype. However, only cyclin D1 expression was significantly upregulated in Luminal A subtype when compared to Her2-positive subtype (p<0.001) (Table 3).

Table 3: Correlation of Fzd7, β-catenin and cyclin D1 expression with molecular subtypes.

Variables	N	Fzd7 expression		β-catenin expression		Cyclin D1 expression	
		Low	High	Low	High	Low	High
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Luminal A	54	24 (44)	30 (56)	19 (35)	35 (65)	13 (24)	41 (76)
Her2- positive	35	21 (60)	14 (40)	17 (49)	18 (51)	26 (74)	9 (26)
		χ^2=2.081 r=-0.155 p=0.153		χ^2=1.695 r=-0.14 p=0.197		χ^2=20.54 r=-0.486 **p<0.001**	
Luminal A	54	24 (44)	30 (56)	19 (35)	35 (65)	13 (24)	41 (76)
TNBC	37	26 (70)	11 (30)	23 (62)	14 (38)	27 (73)	10 (27)
		χ^2=5.915 r=-0.255 **p=0.015**		χ^2=6.43 r=-0.266 **p=0.011**		χ^2=21.31 r=-0.484 **p<0.001**	
Luminal B	34	13 (36)	23 (64)	13 (36)	23 (64)	14 (39)	22 (61)
TNBC	37	26 (70)	11 (30)	23 (62)	14 (38)	27 (73)	10 (27)
		χ^2=8.557 r=-0.342 **p=0.003**		χ^2=4.954 r=-0.261 **p=0.026**		χ^2=8.61 r=-0.343 **p=0.003**	
Luminal B	36	13 (36)	23 (64)	13 (36)	23 (64)	14 (39)	22 (61)
Her2-positive	35	21 (60)	14 (40)	17 (49)	18 (51)	26 (74)	9 (26)
		χ^2=4.058 r=-0.239 **p=0.045**		χ^2=1.129 r=-0.126 p=0.295		χ^2=9.039 r=-0.357 **p=0.002**	

Inter-correlation between Fzd7, β-catenin and Cyclin D1

The inter-correlation among the studied proteins reveal that significant positive correlation was observed between β-catenin and Fzd7 (p=0.022, r=+0.18), however no significant correlation was obtained between β-catenin and cyclin D1 or between Fzd7 and cyclin D1.

Survival Outcome

Survival analysis in total breast cancer patients showed that patients expressing either high or low Fzd7, β-catenin and cyclin D1 failed to discriminate better or worse disease outcome. Further, patients were stratified into various subgroups according to clinico-pathological parameters and it was observed that in patients with grade 3 tumours, low expression of Fzd7 showed a trend of increased incidence of death as compared to the high expression of Fzd7 (Log rank= 3.606, df=1, p=0.058) (Figure 2a, Table 4). Additionally, although not statistically significant, in TNBC patients, low expression of cyclin D1 showed an increased incidence of death as compared to the high expression of cyclin D1 (Log rank= 2.724, df=1, p=0.099) (Figure 2b, Table 4).

Table 4: Overall survival analysis of Fzd7 and Cyclin D1 in patient subgroups.

	N	Alive N (%)	Dead N (%)	Log rank	df	p
Fzd7 protein expression						
Patients with grade 3 tumors (N=29)						
Low	19	13 (68)	06 (32)	3.606	1	0.058
High	10	10 (100)	00 (00)			
Cyclin D1 protein expression						
TNBC patients (N=25)						
Low	17	15 (88)	02 (12)	2.724	1	0.099
High	08	05 (62)	03 (38)			

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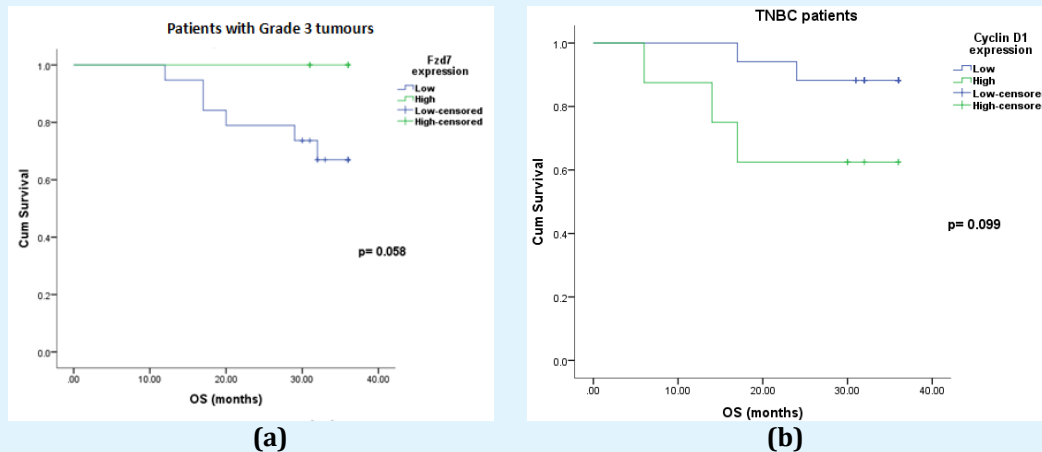
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Figure 2: (a) Overall survival curve of breast cancer patients with grade 3 tumours in relation to Fzd7 protein expression. (b) Overall survival curve of TNBC patients in relation to cyclin D1 protein expression.

Discussion

Wnt/β-catenin signalling pathway has various functions in embryonic development as well as normal homeostasis [8-10]. However, dysregulation of the pathway leads to its aberrant activation and these results in tumorigenesis [11]. Hence, the present study aimed to analyse the expression of Wnt pathway components in breast cancer patients.

The immunoreactivity of Fzd7, β-catenin and cyclin D1 were found in cell membrane, cell cytoplasm and nucleus of 48%, 56% and 50% of breast cancer patients, respectively. Similarly, Corda *et al.* also reported membranous localization of Fzd7 [12]. Wang, et al. showed cytoplasmic as well as nuclear expression of β-catenin while Lee *et al.* found the normal membranous β-catenin expression with the loss of membranous staining for abnormal β-catenin expression [13,14]. Thus the observed only cytoplasmic staining pattern in current study indicated the probability of loss of membranous pattern implying that the Wnt/ β-catenin pathway is most likely activated in breast cancers [15]. Consequently, as observed in our study, cyclin D1 has been reported to be localized in the nucleus with an overexpression in about 50% of human breast cancers [16]. In addition, Peurala *et al.* has also reported nuclear expression of cyclin D1 in 60% of breast cancer patients [17].

Further, the proteins were correlated with clinicopathological parameters, and we observed that high expression of Fzd7, β-catenin and cyclin D1 proteins were associated with smaller tumor size and node negative tumour cells. Although, there was a difference between high and low β-catenin and cyclin D1 proteins the data failed to reach statistical significance, but overall it indicates that there was a loss of protein expression with advanced disease. Additionally, higher expression of Fzd7, β-catenin and cyclin D1 were also significantly associated with tumor differentiation that is higher expression in grade 2 breast tumors than that of grade 3 tumors. Likewise, the expression was also high in grade 1 tumors than in grade 3 tumors, but difference was statistically significant for only cyclin D1. Hence the current data strengthen the above finding that there was an association of loss of the three proteins with the disease progression. In agreement with our results, Peurala, et al has observerd high cyclin D1 to be significantly expressed in lower grade of breast tumors [17]. Likewise Shapiro, et al. in non small cell lung carcinoma observed that β-catenin expression is increased in stage 1 patients [18]. On the other hand, contradictory to our results, He et al reported significant correlation of β-catenin and cyclin D1 over expression with the differentiation grade, clinical stage and lymph node status of breast cancer patients [19]. Apart from this, Zengel, et al. failed to find any correlation of clinic pathological parameters with β-catenin or cyclin D1 in breast cancer patients [20].

It is well established that in patients with breast cancer, hormone receptors and Her2 status is used for treatment decision. Corda, et al. observed over expression of Fzd6 in ER+ breast cancers [12]. Zwijsen, et al. showed an

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association of cyclin D1 over expression with the estrogen response element in breast cancer tissues. The authors hypothesized that the effects of estrogen on breast tumors may be exerted via the cyclin D1 pathway, and that ER-α may promote breast cancer occurrence through the induction of cyclin D1 [21]. On the other hand, Luo, et al. suggested that ER pathway activation may activate β-catenin, which may further activate the Wnt signaling pathway and result in the increased expression of cyclin D1 [22]. The present study too observed significant association of Fzd7, β-catenin and cyclin D1 with ER+ and PR+ status indicating that ER pathway activates Fzd7 and β-catenin resulting in activation of cyclin D1 and thereby Wnt signaling pathway. Contradictorily, some studies reported higher cyclin D1 expressions to be associated with ER-, PR- and Her2- breast tumours [20,23]. Moreover, β-catenin gene has been identified to correlate with the ER expression in breast cancer cell lines and patient derived metastatic breast cancer samples [23]. On the other hand, Zengel, et al. did not observe any correlation between β-catenin and ER/PR or Her2 expression [20].

In addition, we analyzed the expression of Fzd7, β-catenin and cyclin D1 in different molecular subtypes of breast cancer patients, where it was observed that all the three proteins were significantly higher in Luminal subtypes as compared to Her2-positive and TNBC subtypes. The probable reason for this could be that Wnt pathway is related to estrogen and Her2 signaling in breast tumors, leading to enhanced malignant properties. In literature, there are a number of contradictory reports for the activation of Wnt pathway in the molecular subtypes of breast cancer. Fzd7 over-expression is inversely associated with the high levels of Her2 in breast cancer cell lines, while there was no difference in β-catenin expression amongst the four molecular subtypes of breast cancer [24,25]. On the other hand, still another study shows β-catenin to be preferentially expressed in triple-negative/basal-like breast carcinomas [26]. Also, in accordance to our study, cyclin D1 is shown to have a positive correlation with hormone receptors as well as non-basal breast carcinomas and non-TNBC phenotype [27].

In univariate survival analysis, low Fzd7 expression was found to be significantly associated with reduced overall survival in patients with grade 3 tumours. Additionally, in the present study, low expression of cyclin D1 showed a trend of association with decreased overall survival of TNBC patients. Similarly, Guo, et al. has also shown patients with positive cyclin D1 expression to exhibit a longer survival time compared with those with

negative cyclin D1 expression [28]. However, the significance of cyclin D1 as a prognostic factor in breast cancer is controversial with studies reporting both positive and negative findings [29]. Cyclin D1 expression was not of prognostic value in early stage breast cancer patients [30]. Contradictorily, higher expression of cyclin D1 was associated with better prognosis in breast cancer patients with Her2 over expression [20, 31]. In conclusion, the over-expression of the Wnt pathway components is likely to have a role in early stages of breast cancer development leading to the cancer progression. The pathway molecules are also associated with the surface receptors with predominance in non-TNBC subtype. Also, Fzd7 and cyclin D1 emerged as a significant prognosticator in patient subgroups of poorly differentiated tumours and TNBC subtype. However, further studies with a large sample size in each of the molecular subtype of patients are required for an exact correlation of the pathway components.

Conflicts of interest

There are no conflicts of interest in the present study.

Acknowledgement

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