β-Catenin Stability, Cyclin D1 and Frizzled Proteins Expression in Human Breast Cancer and Their Relation with the Prognosis

İnsan Meme Kanserinde β-Katenin Stabilitesi, Siklin D1 ve Frizzled Proteinlerinin Ekspresyonları ve Prognozla İlişkisi

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ABSTRACT Objective: Aberrant Wnt signaling pathway activation and stabilization of β-catenin protein is associated with tumorigenesis and human breast tumors. We have examined β-catenin, cyclin D1, sFRP1 and sFRP2 expressions in the breast cancer and effects of these factors on prognostic parameters in patients with breast cancer. **Material and Methods:** One hundred and seventeen consecutive female malignant breast tumor patients were enrolled into the study. The mean age was 50.0 (±18.0) years. Immunohistochemical staining method was used to investigate the stability and location of β-catenin, expressions of sFRP1 and sFRP2 and cyclin D1 proteins. **Results:** Percentage expression of β-catenin, siklin D1, sFRP1 and sFRP2 were 60.0 ± 55.0, 40.0 ± 64.0, 15.0±48.0 and 25.0 ± 53.0, respectively. Menopausal status, progesterone receptor positivity, lymph node involvement and TNM staging did not show any statistically significant relation with the expression of β-catenin, cyclin D1, sFRP1 or sFRP2. However, significantly higher percentages of expression of cyclin D1 were determined in patients showing estrogen receptor positivity and cerbB-2 over-expression (p= 0.008). Additionally β–catenin expression was significantly higher only in the p53-positive group (p= 0.03). None of the parameters used in this study showed a significant effect on clinicopathological prognostic parameters. **Conclusion:** These findings suggested that expression levels and staining results of β-catenin, cyclinD1, sFRP1 and sFRP2 in tumor cells were independent from histological grade, lymph node involvement, and TNM stage. We concluded that β-catenin accumulation, cyclin D1, sFRP1 and sFRP2 expressions were not affected by the Wnt signaling pathway.

Key Words: Breast neoplasm; β-catenin; cyclin D1; secreted frizzled related proteins

ÖZET Amaç: Wnt sinyal yolağının aberan aktivasyonu ve β-katenin proteininin stabilizasyonu tümör gelişimi ve insan meme tümörleri ile ilişkilidir. Bu çalışmada, meme kanserinde β-katenin, siklin D1, sFRP1 ve sFRP2 ekspresyonlarını ve bu faktörlerin hastaların prognostik parametreleri üzerine etkilerini incelemeyi amaçladık. **Gereç ve Yöntemler:** Ortalama yaşı 50 ± 18 olan ardışık 117 meme kanserli kadın olgu çalışmaya alındı. İmmünositokimyasal yöntemle β-katenin stabilitesi, sFRP1, sFRP2 ve siklin D1 proteinlerinin ekspresyonları değerlendirildi. **Bulgular:** β-katenin, siklin D1, sFRP1 ve sFRP2'nin ekspresyonları sırasıyla %60.0 (±55.0), %40.0 (±64.0), %15.0 (±48.0) ve %25.0 (±53.0) idi. Menopozal durum, progesteron reseptörü, lenf nodu tutulumu, TNM evresi ile β-katenin, siklin D1, sFRP1 veya sFRP2 ekspresyonu arasında istatistiksel olarak anlamlı bir ilişki bulunmadı. Bununla birlikte olgularda östrojen pozitifliği ve cerbB-2 pozitifliği ile siklin D1 arasında istatistiksel olarak anlamlı ilişki varlığı izlendi (p= 0.008). β-katenin ekspresyonu ile p53 pozitifliği arasında da benzer anlamlı ilişki varlığı dikkati çekti (p= 0.03). β-katenin, siklin D1, sFRP1 ve sFRP2 ile kliniko-patolojik prognostik parametreler arasında anlamlı ilişki gözlenmedi (p> 0.05). **Sonuç:** β-katenin, siklin D1, sFRP1 ve sFRP2 ekspresyon düzeylerinin ve boyanma sonuçlarının, tümörün histolojik derecesi, lenf nodu tutulumu ve TNM evrelemesi gibi kliniko-patolojik parametrelerden bağımsız olduğu saptanmış, ve bunların Wnt sinyal yolağından etkilenmeyebileceği düşünülmüştür.

Anahtar Kelimeler: Meme neoplazmları; β-katenin; siklin D1; secreted frizzled related protein

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Breast cancer is one of the leading causes of cancer mortality in women. In the recent 20 years, achievements are obtained in understanding the mechanisms, diagnosis and therapy modalities of breast cancer.[1] A number of genetic alterations are predisposing factors for tumorigenesis.[2] Most of these genetic events still need further investigation to be completely clarified. Wnt signaling pathway components, adenomatous polyposis coli (APC) and β-catenin protein defects have been previously studied and demonstrated in malignant melanoma, colon carcinoma, hepatoblastoma, and medulloblastoma. First data on the oncogenic potential of β-catenin is its abnormal activation in colon cancers over-expressing Myc oncogene.[2,5,7,18-21] Potential role of β-catenin in breast carcinoma development was also assessed in several studies.[2,3,8,22,23]

β-catenin is an element in Wnt pathway mechanism and its accumulation in the cytoplasm is a major step in its oncogenic activation.[2-5] Oncogenic activation of β-catenin may result from three events; a) inactivation of APC tumor suppressor gene, b) mutations localized to N-terminal region of β-catenin, and c) activation of Wnt signaling pathway.[7,22] All of these three mechanisms trigger the cytoplasmic stabilization and nuclear translocation of β-catenin.[2,4,6-16]

Behrens et al.[17] demonstrated the translocation of cytoplasmic β-catenin into the nucleus and subsequent activation of genes having the T-cell factor/lymphocyte enhancing factor (TCF/LEF) transcription activator binding sites. Lin et al.[22] reported that cyclin D1 was one of the targets of β-catenin in breast cancers. Their data can explain the demonstration of cyclin D1 overexpression in 50% of breast cancers while its amplification was demonstrated in only 15-20%.[22] Previous knowledge about the cyclin D1 overexpression in breast cancers and data obtained on the regulatory effect of β-catenin on cyclin D1 gene urged us to study the β-catenin activities in this type of tumors. Since mutations in APC and in the N-terminal region of β-catenin in breast cancer have been previously studied and no clear relation was identified, we have decided to investigate the other components of the Wnt signaling pathway.[24]

Frizzled related protein (FRP) group proteins compose another group of secreted proteins which are responsible for the negative control of Wnt pathway. Secreted frizzled related proteins (sFRP) are secreted proteins that have a region resembling the cysteine-rich extra cellular domain (CRD) of frizzled receptors.[25-28] Zhou et al.[29] demonstrated that increased expression of human sFRP had an inhibitory effect on cellular growth, and FRP inhibition caused aggressive progression in breast cancers. The role of sFRP downregulation in breast cancers has been further confirmed by a number of authors.[30-33]

In this study, we investigated; 1- Expression levels of β-catenin, cyclin D1, sFRP1 and sFRP2 in the breast cancer tissue, 2- The influence of menopausal status, estrogen receptor, progesterone receptor, c-erbB2, p53 positivity, lymph node involvement and clinical TNM stage on the expression of β-catenin, cyclin D1, sFRP1 and sFRP2, 3- The effect of the evaluated parameters on β-catenin, clinicopathological prognostic parameters including histological grade, lymph node involvement and TNM stage.

MATERIALS AND METHODS

PATIENTS

Tumor specimens obtained from 117 consecutive female breast cancer patients in Izmir Bozyaka Training and Research Hospital, I. Surgery Clinic through 1997 to 2001 were studied. Age range of the study group was 24-77 years with a mean of 50.0±18.0 years. Median follow-up duration of the patients was 7.26±3.12 years. Numbers of pre- and post-menopausal women were similar (58 patients vs. 59 patients, respectively). Stage II (58 patients-50.0%) and stage III (47 patients-40.5%) patients represented the majority of the patients. Lymph node involvement was noted in 70 (60.9%) patients. Invasive ductal carcinomas constituted the largest group with 73 (62.4%) patients (Table 1).

TABLE 1: Characteristics of patients

Characteristics	Patient N (%) 117 (100)
Age (mean±SD) (years)	50.0±18.0
(min-max)	(24-77)
Menopausal status	
Premenopausal	58 (49.6)
Postmenopausal	59 (50.4)
Estrogen receptor (+) / (-)	58 (49.6) / 59 (50.4)
Progesteron receptor (+) / (-)	57 (48.7) / 60 (51.3)
c-erbB2 (+) / (-)	32 (28.3) / 81 (71.7)
p53 (+) / (-)	30 (27.0) / 81 (73.0)
Ki67 (+) / (-)	49 (47.1) / 55 (52.9)
Stage (AJCC)	
Stage I	11 (9.5)
Stage II	58 (50.0)
Stage III	47 (40.5)
Lymph node involvement	
Positive	70 (60.9)
Negative	45 (39.1)
Histopathological diagnosis	
Invasive ductal carcinoma	73 (62.4)
Mixed breast carcinoma (ductal+lobular)	19 (16.2)
Signet ring carcinoma	7 (6.0)
Atypical medullary carcinoma	5 (4.3)
Mucinous carcinoma	4 (3.4)
Other (invasive lobular carcinoma, Medullary carcinoma etc)	9 (7.7)

N: Number of patients, AJCC: American Joint Committee on Cancer.

IMMUNOHISTOCHEMICAL ANALYSIS

Hematoxylin-eosin stained pathological material has been reviewed and 4 to 5-μ thick serial-sections were taken. Immunohistochemical stains were performed in serial sections of all patients with anti-estrogen (M-7047-1D5), anti-progesteron (M-3569- PGR636), anti-c-erbB2 (A-0485-Polyclonal), anti-p53 (M-7001- DO-7) and anti-Ki67 (M-7240- MIB-1) supplied from Dako Inc, Denmark, and anti-β-catenin [(E-5): sc7963 Lot:A212 mouse], anti-cyclin D1 [(A-12): sc8396 Lot:A282 mouse monoclonal], anti-FRP1 [(H90): sc13939 Lot: D172 rabbit], and anti-FRP2 [(H140): sc13940 Lot: B262 rabbit polyclonal] from Santa Cruz Biotechnology Inc (USA, CA). Primary antibody binding was followed by addition of the biotin bound secondary antibody and subsequent addition of peroxydase conjugated avidin and 3-amino 9-ethyl carbazole. Counter-staining was performed by Mayer's Hematoxylin. Immunohistochemical stained slides were reviewed and assessed for ratio of ER+, PR+, c-erbB2+, p53+, β-catenin+, cyclin D1+, sFRP1+, and sFRP2+ cells. The cells were counted at high power magnification (x400), at random every 100 tumor cells in three separate cell rich areas by two pathologists without any knowledge of the clinical data. Nuclear staining in more than %1 of the cells was regarded as positive. In order to maximize the consistency of the results, only nuclei showing moderate or strong staining in ER, PR, p53, and cyclin D1 stained sections were regarded as positive. Only cytoplasmic and nuclear staining was evaluated as positive in β-catenin stained sections. sFRP1 and sFRP2 posivity were evaluated as cytoplasmic staining of invasive tumor cells. Immunohistochemical stainings for ER+, PR+, c-erbB2+, Ki67+, β-catenin, cyclinD1, sFRP1 and sFRP2 were recorded as percentages. This study is approved by local ethic committee.

STATISTICAL ANALYSIS

Frequency tests were used to delineate the patients' characteristics. Normal distribution of numeric data (β Catenin, cyclin-D1, sFRP1 and sFRP2 percentage values) was tested with Shapiro-Wilk test. To compare the two groups, Mann-Whitney-U test without normal distribution was performed in numeric variables. Kruskal-Wallis test was used in stage based (stage 1, 2 and 3) group comparisons. 'SPSS 15.0 for Windows' package program was used with 95% reliability .

RESULTS

Median percentages of β-catenin, cyclin D1, sFRP1 and sFRP2 expression of 117 patients were 60.0%±55.0, 40.0%±64.0, 15.0%±48.0 and 25.0±%53.0,respectively. When we evaluated the effect of these factors on the parameters such as menopausal status, progesterone receptor, c-erbB2 positivity, lymph node involvement, and

TNM stage; we found that only β-catenin expression was significantly higher in the p53 positive patients when compared to negative p53 patients (p= 0.03). Similarly, a statistically significant relation was found between c-erbB2 and cyclin-D1 expressions (p= 0.008). Additionally, significant higher percentages of expressions of cyclin D1 were determined in estrogen-positive cases (p= 0.008) (Table 2). No other significant relations were found between the evaluated variables and clinicopathological parameters including histological grade, lymph node involvement, and TNM stage (Table 3, 4, 5).

In the study group; 2, 5 and 10 years overall survival values were calculated as 90%, 81% and 74%, respectively. Disease free survival probabilities were defined as 93%, 86% and 61% in 2, 5 and 10 years follow-up periods, respectively. Metastases developed in 34 of 117 patients (29.0%), and 28

TABLE 2: Comparison of patients according to β catenin, cyclin D1, sFRP1 and sFRP2 expression percentages and disease characteristics

	N	β Catenin (%) Median±IR	Cyclin D1 (%) Median±IR	sFRP 1 (%) Median±IR	sFRP 2 (%) Median±IR
All patients	117	60.0±55.0	40.0±64.0	15.0±48.0	25.0±53.0
Menopausal status					
Premenopausal	58	60.0±60.0	35.0±69.0	15.0±40.0	25.0±45.0
Postmenopausal	59	60.0±60.0	50.0±65.0	15.0±60.0	30.0±59.0
		p= 0.353	p = 0.058	p = 0.782	p = 0.537
Estrogen receptor					
Negative	59	55.0±55.0	25.0±65.0	10.0±35.0	20.0±48.0
Positive	58	62.5±60.0	50.0±65.0	25.0±55.0	30.0±55.0
		p = 0.802	p = 0.008	p = 0.074	p = 0.337
Progesteron receptor					
Negative	60	55.0±54.0	42.5±69.0	15.0±44.0	30.0±59.0
Positive	57	65.0±55.0	40.0±60.0	20.0±55.0	20.0±43.0
		p =0.366	p =0.742	p= 0.679	p = 0.532
c-erbB2					
Negative	81	60.0±58.0	25.0±69.0	15.0±43.0	20.0±50.0
Positive	32	65.0±48.0	60.0±48.0	20.0±48.0	30.±53.0
		p= 0.521	p = 0.008	p = 0.274	p = 0.073
p53					
Negative	81	50.0±55.0	35.0±65.0	15.0±55.0	20.0±53.0
Positive	30	65.0±50.0	52.5±68.0	12.5±46.0	32.5±51.0
		p= 0.039	p = 0.164	p = 0.679	p = 0.191
Lymph node involement					
Negative	45	65.0±65.0	35.0±60.0	15.0±50.0	25.0±60.0
Positive	70	50.0±50.0	50.0±66.0	20.0±50.0	27.5±40.0
		p= 0.116	p = 0.161	p = 0.402	p = 0.413
Disease Stage					
Stage I	11	60.0±75.0	30.0±55.0	30.0±60.0	5.0±60.0
Stage II	58	60.0±60.0	32.5±67.0	15.0±41.0	20.0±56.0
Stage III	47	50.0±50.0	50.0±65.0	20.0±50.0	35.0±40.0
		*p = 0.690	*p = 0.201	*p = 0.557	*p = 0.498

N: Number of patients, IR: Interquartile range, p: Mann-Whitney-U Test, *p: Kruskall-Wallis test.

TABLE 3: Relation of histological grade with β-catenin, Cyclin D1, sFRP1 and sFRP2 expressions

Histological Grade	β Catenin Mean %/SD	Cyclin-D1 Mean %/SD	FRP 1 Mean %/SD	FRP 2 Mean %/SD
HG 1	48.33/32.53	41.67/33.29	28.33/30.13	30.00/26.45
HG 2	56.10/31.75	37.88/29.20	25.15/26.80	32.30/28.92
HG 3	51.52/33.91	54.36/39.11	32.18/29.19	26.52/25.04
	p=0.80	p=0.12	p=0.56	p=0.66

SD: Standard deviation.

TABLE 4: Analysis of lymph node involvement of β-catenin, Cyclin D1, sFRP1 and sFRP2 expressions

Lymph Node Classification	β Catenin Mean %/SD	Cyclin-D1 Mean %/SD	FRP 1 Mean %/SD	FRP 2 Mean %/SD
N0	59.89/34.08	36.04/30.41	22.96/26.37	29.78/29.92
N1	47.91/27.74	39.41/34.24	22.94/24.83	28.63/28.23
N2	56.75/33.49	51.60/35.70	33.85/28.98	32.85/27.95
N3	46.26/33.13	53.89/36.28	27.21/30.05	40.53/22.47
	p=0.27	p=0.13	p=0.44	p=0.47

SD: Standard deviation.

TABLE 5: β-catenin, Cyclin D1, sFRP1 and sFRP2 expressions in various clinical TNM stages

TNM Stage	β Catenin Mean %/SD	Cyclin-D1 Mean %/SD	FRP 1 Mean %/SD	FRP 2 Mean %/SD
Stage 1	51.36/35.50	35.00/26.45	30.45/30.28	29.55/32.66
Stage 2A	64.06/33.34	35.53/31.87	19.63/23.90	26.25/27.20
Stage 2B	45.31/27.13	41.77/37.98	26.31/28.15	33.31/32.26
Stage 3A	54.17/30.24	46.96/31.33	28.75/24.46	27.58/24.57
Stage 3B	66.25/47.14	45.00/42.03	26.25/43.08	52.50/31.22
Stage 3C	44.45/33.25	51.20/37.31	25.85/29.88	38.50/23.68
	p=0.19	p=0.60	p=0.82	p=0.39

SD: Standard deviation.

(23.9%) of them have died. The expression status of β-catenin, cyclin D1, sFRP1 and sFRP2 antibodies was not found to have a significant effect on clinicopathological parameters.

Additionally, localization of β-catenin in the cell (cytoplasmic or nuclear) was evaluated in 64 patients. Intracellular (cytoplasmic and/or nuclear) localization of β-catenin was not found to have a significant effect on clinicopathological parameters.

DISCUSSION

In this study, we focused on the β-catenin amount and localization in breast cancer cells, and aimed to investigate the effect of sFRP1 and sFRP2 expressions on β-catenin as inhibitors of Wnt signaling and relation between sFRP1, sFRP2 expressions and cytoplasmic-nuclear β-catenin positivity. In addition, we aimed to investigate the relation between cyclin D1 expression in the cells

and transcription activating capacity of β-catenin. In order to investigate these parameters and mechanisms, we used immunohistochemical staining with different specific antibodies and evaluated the results in stained slides.

In our study, no correlations were identified between β-catenin expression and estrogen receptor, progesterone receptor, c-erbB2 over-expression, histological grade, lymph node involvement or TNM staging. On the other hand, Zhang et al.[34] reported a significant correlation between β-catenin expression and c-erbB2 overexpression and lymph node involvement. In the study of Fanelli et al.,[35] although no relation was determined between β-catenin expression and tumor grade, lymph node involvement and β-catenin expression were found to be correlated, and this finding was in accordance with Zhang's study. In our study, a significantly higher level of expression of β-catenin was determined only in the p53-positive group. However Fanelli et al.[35] did not find a significant relation between p53 and β-catenin, in contrast to the findings of our study.[35] A possible explanation of this discrepancy may be the details of the immunohistochemical technique and/or the selected cut-off value of immunohistochemical results used in these studies.

In the presented study, the β-catenin expression was also analyzed for its relation with the parameters effecting survival. There were no significant differences in terms of parameters –histological grade, lymph node involvement, and TNM stage-between β-catenin positive and negative patients group. Nakapolaou et al.[40] and Lin et al.[22] have reported that aberrant β-catenin expression was correlated with poor prognosis. Lin et al.[22] and Lee et al.[36] reported that the patients with normal membranous β-catenin expressions had better survival.

Higher cyclin D1 expressions were found in patients with positive estrogen receptors, and similarly, higher cyclin D1 was found in patients with c-erbB2 over-expression in the presented study. Similarly, statistically significant relations were reported between the estrogen receptor, c-erbB2 expression and cyclin D1 expression in Lee et al.'s study.[36] Rudas et al.[37] reported a lower ratio for overall and disease-free survival for cyclin D1-positive tumors in patients with hormone receptor positivity. Additionally, Rudas et al.[37] have emphasized that cyclin D1 expression affected patient's prognostic factors. Similarly, in the study performed by Aaltonen et al.[38] it is reported that cyclin D1 expression had adverse effects on prognosis. However, on the contrary, Penault-Llorca et al.[39] did not find a statistically significant relation in patients between with cyclin D1 expression and disease-free and overall survival. In our study, cyclin D1 is not found to be related with the prognostic factors such as histological grade, lymph node involvement and TNM staging. The effect of cyclin D1 expression on the prognosis of breast carcinoma cases remains vague due to the presence of divergent findings in different studies. Our data suggested that cyclin D1 over-expression might not be derived from the active Wnt signaling, and might show no significant correlation among these parameters. Translocation of β-catenin into the nucleus-cytoplasm was not in concordance with cyclin D1 overexpression, although in most cases cytoplasmic β-catenin (not bound to E-cadherin) accumulation was evident.

We did not find any statistically significant relation between sFRP1, sFRP2 and estrogen receptor, progesterone receptor, cerbB-2, p53, Ki67 and prognostic parameters such as histological grade, lymph node involvement and TNM staging. Some authors suggested that sFRP1 and sFRP2 were mostly negative in patients with breast carcinoma, and they suggested that the lack of its production may be related to malignant transformation. The Frizzled-related gene FRP1/FRZB, was turned off in 78% of breast carcinomas, suggesting that the lack of its product may be associated with malignant transformation.[30] According to Suzuki et al.[41] Wnt antagonist genes may be a useful marker in breast cancer.

As a result of the current study, overexpression of β-catenin and p53 positivity were found to be correlated. Additionally, cyclin D1 expression was found to be related to estrogen receptor

positivity and c-erbB2. However, no statistically significant relations were found between β-catenin, cyclin D1, sFRP1, sFRP2 expression and certain clinicopathological parameters such as histological grade of tumor, clinical stage and lymph node involvement stage. Limited number of patients and lack of previously determinated cut-off values of the studied markers are the limitations of our study. In the future, in the light of these findings, in order to detect and investigate the relation between Wnt signaling pathway, sFRP1 and sFRP2 expressions, cyclin D1 and cytoplasmic-nuclear β-catenin further, larger, more detailed studies should be performed. In addition, an objective cut-off value that could point out the probability of survival and recurrence needs to be determined for β-catenin, cyclin D1, sFRP1, sFRP2 expression levels in patients with breast carcinoma.

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