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Prognostic significance of Wilms Tumor 1 (WT1) protein expression in breast cancer

Meme kanserinde Wilms Tümör 1 (WT1) protein ekspresyonunun prognostik önemi

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Abstract
Breast cancer is the most common cancer among women all over the world. Since the clinical outcome of breast cancer may differ among some women who have the same clinicopathological stage, researchers focused on additional prognostic parameters to predict the tumor behavior. The aim of this study was to investigate the prognostic value of Wilms Tumor 1 (WT1) expression in tumor tissues and to compare it with known prognostic variables in patients with breast cancer. In patients with breast cancer, we investigated the relationship between (WT1) protein expression in tumor and surrounding tissues and prognostic variables including age, pathologic type, axillary node involvement, estrogen receptor (ER) status, menopausal status, stage (TNM), tumor grade and treatment. Borderline significance was detected between WT1 monoclonal antibody (mAb) staining and premenopausal state (p=0.051). Additionally, surrounding tissue staining showed significant correlations with grade (p=0.045), stage (p=0.026), lymph node status (p=0.026), and axillary involvement (p=0.02), respectively. No correlation was demonstrated between relapse free survival, relapse sites and WT1 mAb staining of tumor and surrounding tissues (p=0.36). WT1 mAb staining was demonstrated in human breast cancer tissues, and in this study we ~~have~~ used monoclonal antibody against WT1 on paraffin-embedded tissue samples. The results indicate that WT1 expression by tumor is more evident in premenopausal state rather than postmenopausal period. To confirm the results, we need large scale studies on WT1 expression in breast cancer.
Keywords: Breast cancer; immunohistochemistry; prognosis; premenopausal; WT1

Özet
Meme kanseri tüm dünyada kadınlarda görülen en sık kanser tipidir. Aynı klinikopatolojik evredeki kadınlarda meme kanserinin klinik seyri farklı olabilir, bu yüzden araştırmacılar tümör davranışını tahmin edebilmek için ek prognostik parametrelere odaklanmışlardır. Bu çalışmanın amacı, meme kanseri hastalarında tümör dokusunda Wilms Tumor 1 (WT1) ekspresyonunun prognostik önemi ve bunun bilinen prognostik değişkenlerle karşılaştırılmasıdır. Meme kanserli hastalarda, tümor ve tümör dokusu çevresindeki dokularda WT1 protein ekspresyonu ile prognostik faktörlerden yaş, patolojik tip, aksiller lenf nodu tutulumu, östrojen reseptör (ER) durumu, menopoz durumu, evre (TNM), tümör gradı ve tedavi arasındaki ilişki araştırıldı. WT1 monoklonal antikor (mAb) ile premenopozal durum arasında orta derecede bir ilişki saptandı (p=0.051). Ek olarak, tümör çevresindeki doku ile tümör gradı (p=0.045), evre (p=0.026), lenf nodu (p=0.026) ve aksiller tutulum (p=0.02) arasında anlamlı ilişki saptandı. Nükssüz sağkalım, nüks bölgeleri ile tümör dokusu ve tümör çevresi dokudaki WT1 mAb arasında anlamlı ilişki gözlenmedi (p=0.36). İnsan meme kanseri dokularında WT1 mAb gösterilmiştir ve bu çalışmada parafindeki doku örneklerine karşı monoklonal antikorlar kullanıldı. Bu sonuçlar premenopozal dönemde tümör dokusunda WT1 ekspresyonunun postmenopozal döneme göre daha fazla olduğunu göstermektedir. Bu sonuçların teyid edilmesi için meme kanserinde WT1 ekspresyonu için daha büyük çalışmalara ihtiyaç vardır.
Anahtar kelimeler: Meme kanseri; immunohistokimya; prognoz; premenopoz; WT1

Introduction

Breast cancer is the most common cancer among women all over the world. Despite the improvements in diagnosis and therapy, breast cancer has still high morbidity and mortality rates. Since the clinical outcome of breast cancer may differ among some women who have the same clinicopathological stage, researchers focused on additional prognostic parameters to predict the tumor behavior. As cure is not possible in patients with advanced stage or relapsed disease after primary therapy, and only palliation can be achieved with chemoradiotherapy and/or other treatment modalities, the most important point is to predict the patients who will benefit from adjuvant chemo and/or radiotherapy, especially in the early stage.

Wilms tumor 1 (WT1) gene was originally isolated as a tumor suppressor gene. The expression of WT1 is limited to kidney, testis, ovary, hematopoietic and myoepithelial progenitor cells in postnatal life and WT1 is overexpressed in approximately 90% of breast cancers (1). The aim of this study was to investigate the prognostic value of WT1 gene expression in tumor tissues and to compare it with known prognostic variables in patients with breast cancer.

Material and Methods

Sixty-six biopsy samples of breast cancer were included in this study. Parameters including age, histopathologic type, axillary node involvement, estrogen receptor (ER) status, menopausal status, stage (TNM) and therapy were recorded.

Tumor tissue samples were stained with hematoxyline and eosin for routine histopathological evaluation and grading. Microscopic grading of the tumor was made with Notthgan modification of Bloom-Richardson, and ABC method was used for immunohistochemical

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evaluation. Monoclonal mouse anti-human estrogen receptor kit (DAKO code # 7047; 1:50 dilution) was used for estrogen receptor analysis. Higher than 10% staining was accepted as positive for ER status. WT (F-6) mouse monoclonal antibody kit (Santa Cruz Biotechnology cat # sc-7385; 1:200 dilution) for WT1 was used to determine the WT1 protein expression. Staining was classified as 1+ to 3+ by the experienced pathologist (SZ). Fetal kidney tissue was used as a positive control for WT1 monoclonal antibody (mAb). Statistical analysis was done by SPSS for Windows version 11.0. Correlations between clinical parameters and WT1 staining were tested with Chi-square and Fisher's exact tests, Mann-Whitney U and Kruskal-Wallis tests.

Results

Demographical, clinical and pathological parameters of patients were illustrated in Tables 1 and 2. WT1 mAb staining was shown in Figure 1.

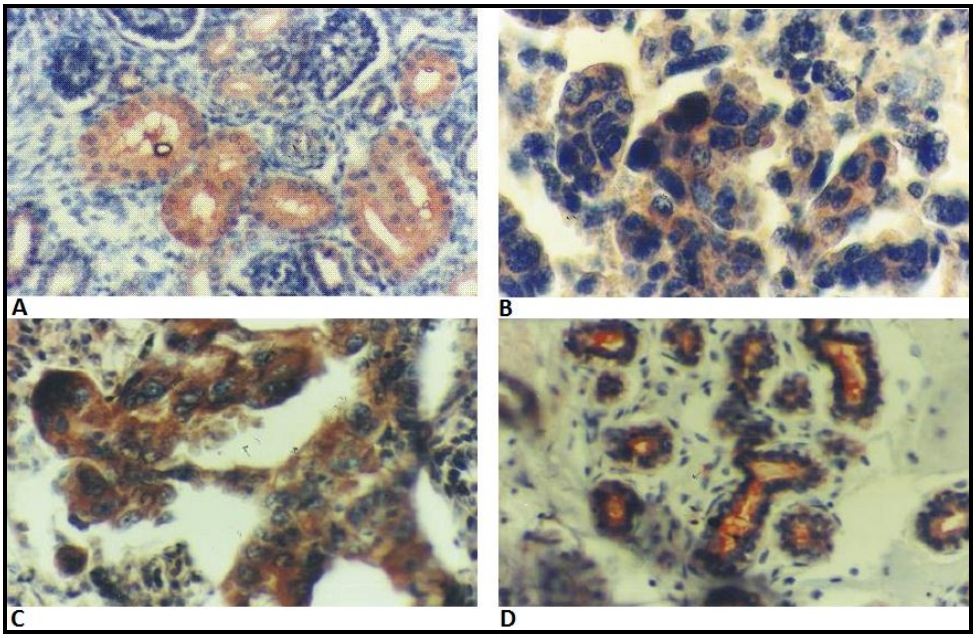

Figure 1. WT1 monoclonal antibody staining in fetal kidney (x100) (A), WT1 monoclonal antibody staining sparse cytoplasmic staining (x400) (B), WT1 monoclonal antibody staining dense cytoplasmic staining (x400) (C), WT1 monoclonal antibody staining in surrounding normal appearing tissue (x400) (D).

Table 1. Demographic and clinical features of the patients

Features	Number of patients	Mean±SD	(min-max)
Age	66	47.03±11.23	(25-85)
Premenopausal	41	40.12±5.42	(25-50)
Postmenopausal	25	58.36±8.79	(45-85)
Adjuvant therapy			
CMF±T	38		
CAF/CEF	16		
ADR/Epi+CMFx6	7		
Docetaxel+ADR	1		
AC	1		
Tamoxifen	3		
Relapse	11		
Bone	10		
Liver	2		
Pleura	2		
Soft tissue	2		
Contra lateral breast	2		

Abbreviations: CMF±T:Cyclophophamide+Methotrexate+Fluorouracil±Tamoxifen; CAF/CEF:Cyclophophamide+Adriamycin/Epirubicin+Fluorouracil; ADR/Epi+CMFx6:Adriamycin/Epirubicin+6 courses of CMF; AC:Adriamycin+ Cyclophophamide for four courses.

Table 2 Pathologic features of the patients

Features		No of patients	Premenapousal
Histopathology			
	Infiltrative ductal	55	35
	Infiltrative lobular	11	6
ER			
	Positive	33	21
	Negative	33	20
TNM staging			
	Stage I	11	4
	Stage II	34	23
	Stage III	17	13
	Stage IV	4	1
Tumor			
	Tx	1	-
	Tis	1	-
	T_1	16	9
	T_2	31	21
	T_3	10	6
	T_4	7	5
Node			
	Nx	1	-
	N_0	27	16
	N_1	28	18
	N_2	10	7
Axillary node involvement			
	<4	18	11
	≥4	18	14
Grade			
	I	7	3
	II	42	24
	III	17	14

Table 3. WT1 mAb staining of tumor and surrounding normal appearing tissue

	WT1 at tumor	WT1 at surrounding tissue*
Nostaining	34 (51.5%)	24 (36.4%)
(+)Staining	21 (31.8%)	22 (33.6%)
(++)Staining	10 (15.2%)	-
(+++)Staining	1 (1.5%)	-

*In 20 samples there was no surrounding normal appearing tissue.

WT1 monoclonal antibody staining in fetal kidney has been shown in Figure 1A. Both sparse and intense cytoplasmic (without nuclear) staining were detected in tumor samples (Figures 1B and 1C). Intense cytoplasmic and nuclear staining were detected at the surrounding normal appearing tissue (Figure 1D). All samples were re-evaluated and graded and results were illustrated in Table 3.

Correlation between clinicopathological features and WT1 mAb staining were calculated with Chi-square and Fisher's exact tests and results were shown in Table 4. Borderline significance was detected between WT1 mAb staining at tumor tissue and premenopausal status (p=0.051) (56% for premenopausal, 40% for postmenopausal). In addition, surrounding tissue staining showed significant correlation between WT1 mAb staining and grade (p=0.045), stage (p=0.026), lymph node status (p=0.026) and axillary involvement (p=0.02), respectively. Subgroup analyses were illustrated in Table 5.

Table 4. Correlation between clinicopathologic features and WT1 mAb staining at tumor and surrounding tissue

	Grade	E.R.	Relapse	Hist. Diag.	Menopause	Number of LN	T	N	Stage
tWT1	p=0.15	P=0.23	p=0.12	p=0.91	**p=0.051**	p=0.28	p=0.90	p=0.96	P=0.46
sWT1	**p=0.045**	p=0.77	p=0.49	p=0.45	p=0.39	p=0.14	p=0.92	**p=0.02**	**p=0.026**

Abbreviations: ER: Estrogen receptor, Hist.diag: Histopathologic diagnosis, Number of LN: Number of lymph node, T: Tumor diameter, N: Lymph node involvement, tWT1:WT1 staining at tumor tissue, sWT1:WT1 staining at surrounding normal appearing tissue.

Table 5. WT1 mAb staining at surrounding normal appearing tissue

	Stained/total no of pt. (%)		p
Grade			
I	4/7(57)	Grade I vs. Grade II+III	**0.045**
II	12/42(29)		
III	6/17(35)		
Stage			
I	7/8 (87.5)	Stage I vs. Stage III+IV	0.75
II	9/24(37.5)	Stage II vs. Stage III+IV	0.074
III	6/12(50)	Stage I vs. Stage II	**0.037**
IV	0/2 (0)		
Lymph node			
N_0	14/20(70)	N_0 vs. N_{1+2}	**0.026**
N_{1+2}	8/25(32)		

No correlations between relapse free survival, relapse sites and WT1 mAb staining of tumor and surrounding tissues were found (p=0.36).

Discussion

Breast carcinoma is the most common tumor among women that causes morbidity and mortality. Some clinical and pathological prognostic parameters including histopathologic type, grade, steroid receptor status, TNM staging, menopausal status and cell cycle parameters (mitotic index, S-phase fraction, Ki67, PCNA, etc) was used for this purpose (2). These parameters are valuable in clinical practice but not ideal to predict the clinical outcome in all patients. For this reason it is important to detect the additional predictive factors determining the biology of the breast cancer.

Tumor suppressor gene activities have been widely studied in breast cancer. nm23 and p53 are the most commonly studied genes, and a correlation has been shown between good prognosis and normal expression (3-6). Wilms tumor 1 gene was originally isolated as a tumor suppressor gene. The expression of WT1 is limited to kidney, testis, ovary, hematopoietic and myoepithelial progenitor cells in postnatal life (1). WT1 is a member of tumor suppressor gene family and is responsible for pediatric Wilms tumor. It is located on 11p13 chromosome. Functional loss on this gene after deletion and/or mutation has been demonstrated in some but not all cases with Wilms tumor.

Wilms tumor 1 gene expression was evaluated with PCR on some solid tumor cell lines. Two of 4 breast cancer cell lines' WT1 expressions were detected and researchers suggested that the WT1 gene plays an essential role in the growth of solid tumors and performs as an oncogenic rather than a tumor suppressor gene function (7). Wilms tumor 1 expression has been studied in solid tumors but the results were not clear enough to demonstrate a possible relation between WT1 and tumor biology. Higher WT1 expression has been demonstrated in malignant mesothelioma cells than normal mesothelial cells (8,9) and different degrees of WT1 expression have been found in leukemia (10), osteosarcoma (11), lung cancer (12), thyroid cancer (13), head and neck squamous cell carcinoma (14), colorectal adenocarcinoma (15) and desmoplastic small round cell tumors (16). Netinatsunthorn et al. (17) showed that the expression of WT1 gene may be an indicator of poor prognosis in patients with advanced serous epithelial ovarian carcinoma. Ninety nine patients were included in this study and 50.5% of patients showed WT1 staining. Five-year survival of non-staining patients and staining patients were 39.4% and 10.7% ($p < 0.00005$); five-year recurrence-free survival of these patients were 29.8% and ≤ 7.5% ($p < 0.00005$), respectively (17).

Wilms tumor 1 is overexpressed in approximately 90% of breast cancers (1). WT1 protein can suppress the transcription of some of the genes induced by growth factors. For example, the genes encoding insulin-like growth factor 1 (IGF-1) and epidermal growth factor receptor are thought to be a target for WT1. The IGF-1 receptor is important for the development and progression of breast cancer and IGF-1 receptor is downregulated by some transcription factors like WT1 protein (18). Expression of growth factors increases after WT1 inactivation and results in cell proliferation (19). Regulation of WT1 transcription is dependent on wild type p53. In the absence of this, WT1 will act as a transcription activator rather than suppressor (20). Reizner et al. showed that WT1 suppresses IGF-IR gene transcription in breast cancer cells and there was an association with estrogen receptor alpha. This finding suggested the potential role of the tumor suppressor activity of WT1 in breast cancer (21). However, another study showed that WT1 protein plays a role in progression and apoptosis on HER2/neu-overexpressing breast cancer cells. The decreased expression of WT1 led to cell cycle arrest at G1 phase and increased

apoptosis in HER2/neu-overexpressing breast cancer cells and this act was correlated with decreased cyclin D1 and Bcl-2 levels (22).

Overexpression of WT1 is associated with poor prognosis, especially worse 5-year disease-free survival in breast cancer (23). A study showed overexpression of WT1 at drug resistance cells after cytotoxic treatment (24). Levels of WT1 are correlated with proliferation of breast cancer cells and WT1 expression increase during proliferation of breast cancer cells stimulated by 17 beta-estradiol. If proliferation of cancer cells are inhibited by tamoxifen or all-trans-retinoic acid, the expression of WT1 decreased (25). Oji et al. (26) evaluated whether the overexpressed WT1 genes in breast cancer were mutant genes. They obtained breast cancer tissues from 36 patients and there were no mutations at the whole 10 exons of the WT1 gene in these cases. These results suggested that the wild-type of WT1 gene plays important role in primary breast cancer (26).

Wilms tumor 1 protein could be a target antigen for immunotherapy in some malignancies. Oka et al. (27) reported the results of a phase I clinical study of WT1 peptide-based immunotherapy for patients with lung cancer or breast cancer, acute myeloid leukemia, or myelodysplastic syndrome. They included 26 patients in this study and 18 patients completed WT1 vaccination. Twelve of the 20 patients could be assessed for the efficacy of WT1 vaccination and 12 patients showed clinical responses. These results suggested that WT1 vaccination could induce WT1-specific cytotoxic T lymphocytes (27). Moreover, Morita et al. (28) used WT1 peptide vaccine in 10 patients with refractory solid tumors at a phase I/II study. There was a partial response at 1 patient and stable disease at 5 patients with weekly WT1 peptide vaccine treatment (28). CD8+ CTL is one of the most important immune effector cells for tumor protection. WT1 is a potential target antigen for treatment of breast cancer. Gillmore et al. showed WT1 specific CTL killed only HLA-A2 (+) breast cancer cells treated with IFN-γ (1).

Loeb et al. (29) detected overexpression of WT1 protein in breast cancer cells by Western blotting method as 87 percent (27 of 31 patients). No WT1 overexpression in normal breast cells was observed and WT1 promoter has been found methylated in 32% of breast cancer cells (29). Silberstein et al. studied WT1 expression in human breast cancer by using immunohistochemistry and RT-PCR methods (30). They studied WT1 expression in 21 breast cancer and 15 normal breast tissues. In this study monoclonal antibody against amino-terminus of WT1 protein has been used, and positivity has been found in 6 samples (28.6%) of tumor tissues, however, positive staining has been found in 14 samples (93.3%) of normal breast tissues. Also Miyoshi et al. showed overexpression of WT1 in breast cancer compared with normal breast tissue ($P<0.0005$) by quantitative RT-PCR analysis (23). In this study, WT1 protein expression was detected in 32 of 66 samples (48.5%).

We have not find any correlation between WT1 expression in tumor tissue and known prognostic indicators except menopausal status ($p=0.051$). Although statistical significance was not determined, WT1 staining was more evident in ER positive premenopausal tumor samples compared with ER negative tumors. However, correlation was observed in postmenopausal women in the opposite manner.

Additionally, correlation between some prognostic variables (grade, stage and lymph node involvement) and surrounding normal appearing tissue staining was demonstrated. Although the number of available tissue sample was low, staining with WT1 mAb was higher in surrounding tissue in grade I tumors when compared with grade II and III (57%, 29% and 35%, respectively). In terms of clinicopathological stage, stage I tumors showed significant staining (87.5%, 37.5%, 50%, 0% for stage I, II, III and IV, respectively). Moreover, WT1 mAb staining was 70% in N0 tumors and 32% in N1 and N2 tumors ($p=0.026$). There was no relation between relapse free survival and WT1 mAb staining both for tumor and surrounding tissue samples.

In conclusion, WT1 mAb staining was demonstrated in human breast cancer tissues, and in this study we have used monoclonal antibody against WT1 on paraffin-embedded tissue samples. The results indicate that WT1 expression by tumor is more evident in early stage and premenoposaul state. We need the confirmation with large scale studies on WT1 expression in breast cancer.

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