High Wilms' tumor 1 mRNA expression correlates with basal-like and ERBB2 molecular subtypes and poor prognosis of breast cancer

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Abstract. The role of Wilms' tumor 1 (WT1) in breast cancer and the relationship between WT1 expression and clinico-pathological factors, molecular subtypes and prognosis of breast cancer patients have not been clarified to date. We used publicly available microarray datasets of 266 early breast cancer patients to perform bioinformatics analysis on the relationship between WT1 mRNA expression and breast cancer. Results showed that WT1 mRNA expression was correlated with higher histological grades, ER-negative and basal-like and ERBB2 molecular subtypes in breast cancer. With regard to disease-free survival analysis, the WT1 high expression group showed worse prognosis than the low expression group in univariate analysis, and WT1 was demonstrated to be an independent prognostic indicator in multivariate analysis. This study confirms an oncogenic role of WT1 and demonstrates a possible relation between WT1 and progression of breast cancer.

Introduction

Breast cancer is the most common cancer and the leading cause of cancer death among women all over the world (1). Development and progression of breast cancer depend on the balance of oncogenes and tumor suppressor genes (2). Identification and characterization of these genes will lead to discovery of new markers and potential therapeutic targets for prevention and treatment of breast cancer (3).

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Wilms' tumor 1 (WT1) gene was originally identified as a tumor suppressor gene, which is responsible for Wilms tumor (4,5). However, accumulating evidence indicated that WT1 may play an oncogenic role in leukemogenesis and tumori-genesis (6-11). It was shown that the growth of leukemic cells and a variety of solid cancer cells was inhibited by knockdown of *WT1* expression, while forced expression of WT1 promoted cell growth and motility, suppressed apoptosis and induced leukemia in *WT1*-transgenic mice (9).

WT1 protein and mRNA were firstly found to be expressed in the normal breast tissue, but absent in >90% of the breast cancer (12), and WT1 inhibited proliferation and tumorigenesis of breast cancer cells, indicating WT1 served as a tumor suppressor gene in breast cancer (13-15). On the contrary, a number of studies demonstrated that wild-type WT1 gene plays an important role in the development of breast cancer (16). Loeb *et al* reported that WT1 could be detected in 87% of primary breast carcinomas, but not in normal breast epithelium (17). Miyoshi *et al* found that WT1 mRNA significantly correlated with the poor prognosis of breast cancer (18). In addition, WT1 could promote the proliferation and restrain the apoptosis of breast cancer cells (19-21), all of which indicate that WT1 might serve as an oncogene in breast cancer. Therefore, it is necessary to clarify the oncogenic or tumor suppressive role of WT1 in breast cancer. Moreover, relationship between WT1 expression and clinicopathological parameters and prognosis of breast cancer was inconsistent so far (12,18,22,23), and the possible correlation between WT1 expression and molecular subtypes has not been reported.

In this study, we analyzed the WT1 mRNA expression in a microarray dataset of 266 early breast cancer patients and investigated its possible relationship with the clinicopatho-logical parameters, molecular subtypes and prognosis so as to explore the role of WT1 gene in breast cancer.

Materials and methods

Tumor samples. The publicly available microarray dataset, GSE21653, was collected from National Center for Biotech-nology Information (NCBI) Gene Expression Omnibus (GEO).
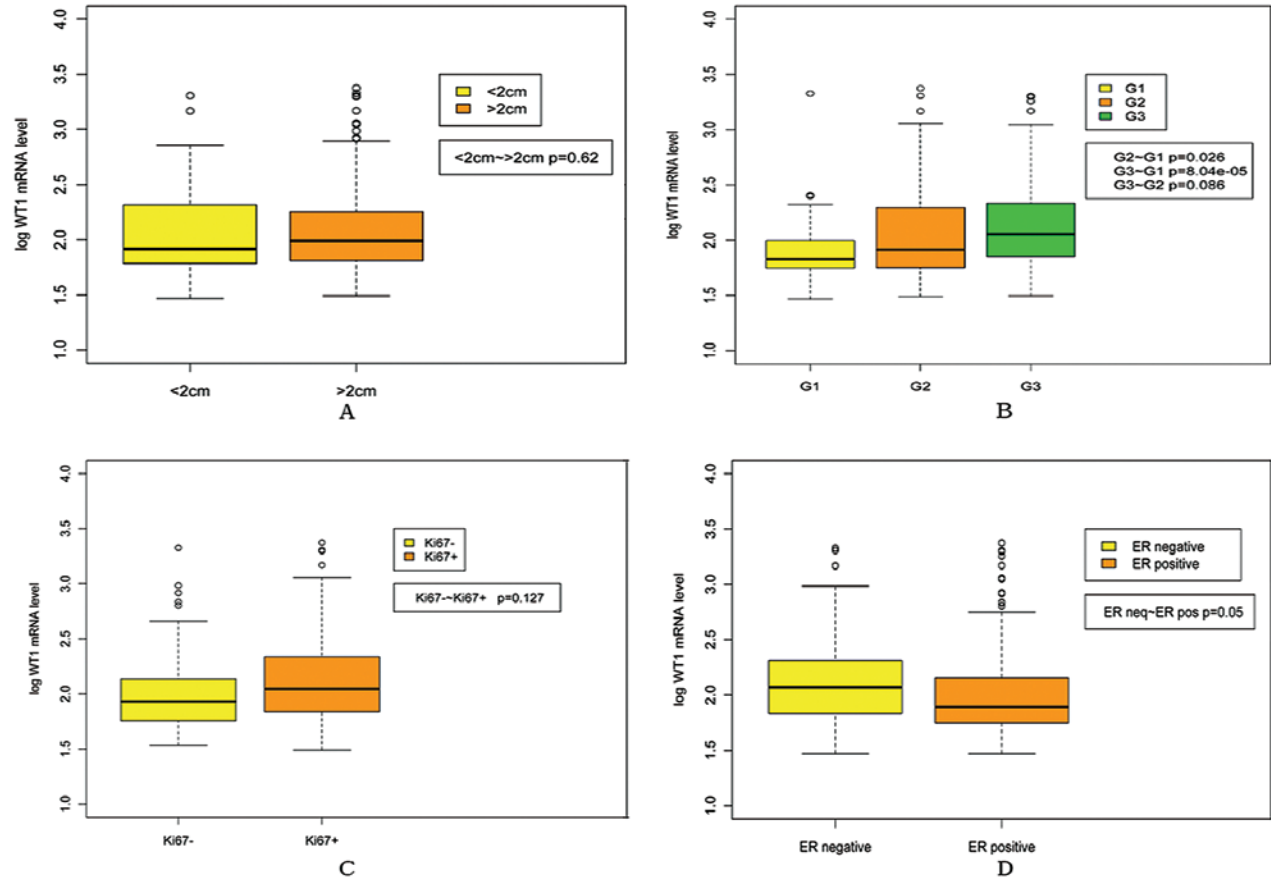
Figure 1. Relationship between WT1 mRNA expression and clinicopathological factors of breast cancer in GSE21653. Comparison of WT1 mRNA expression among (A) pT <2 cm and >2 cm groups, (B) G1, G2 and G3 groups, (C) Ki67 negative (-) and Ki67 positive (+) groups, and (D) ER negative (-) and ER positive (+) groups.

Table I. Univariate analysis of relationship between WT1 mRNA expression and DFS.

Cutoff value	Survival	HR	95% CI	Log-rank (P-value)	High level (N)	Low level (N)	N
2.1	DFS	1.226	(0.794, 1.895)	0.357	106	146	252
3.1	DFS	3.294	(1.198, 9.055)	0.014	7	245	252

DFS, disease-free survival; HR, hazard ratio; CI, confidence interval.

GSE21653 involved 266 early breast cancer patients, who underwent initial surgery in Institut Paoli-Calmettes (IPC) institution between 1992 and 2004 (24). They included 227 cases previously reported and 39 additional cases, all of which were similarly profiled using Affymetrix U133 Plus 2.0 human oligonucleotide microarrays (25). Clinicopathological characteristics of the patients have been described previously (24,25). Among them, a total of 252 patients had the DFS information.

Microarray analysis. Regarding the Affymetrix-based datasets, GSE21653, we used Robust Multichip Average (RMA) with the nonparametric quantile algorithm as normalization parameter (26). RMA was applied to the raw data from the IPC series. Quantile normalization or RMA was done in R using bioconductor (27) and associated packages. Then the

$\log^2$ transformed data for the following WT1 probes were evaluated. Patients were stratified based on the main available prognostic factors for breast cancer, such as pathological tumor size (pT), histological grades (Grade 1, G1; Grade 2, G2; Grade 3, G3) according to Searff-Bloom-Richardson (SBR) staging system, Ki67, estrogen receptor (ER), molecular subtypes (basal-like, ERBB2, luminal A, luminal B and normal-like; luminal: luminal A and luminal B) and disease-free survival (DFS).

Statistical analysis. T-test was used to investigate whether the WT1 gene expression values were significantly different between any two compared groups of different disease characteristics with P<0.05 (28,29). DFS analyses were performed during the period from the date of diagnosis to the first obser-
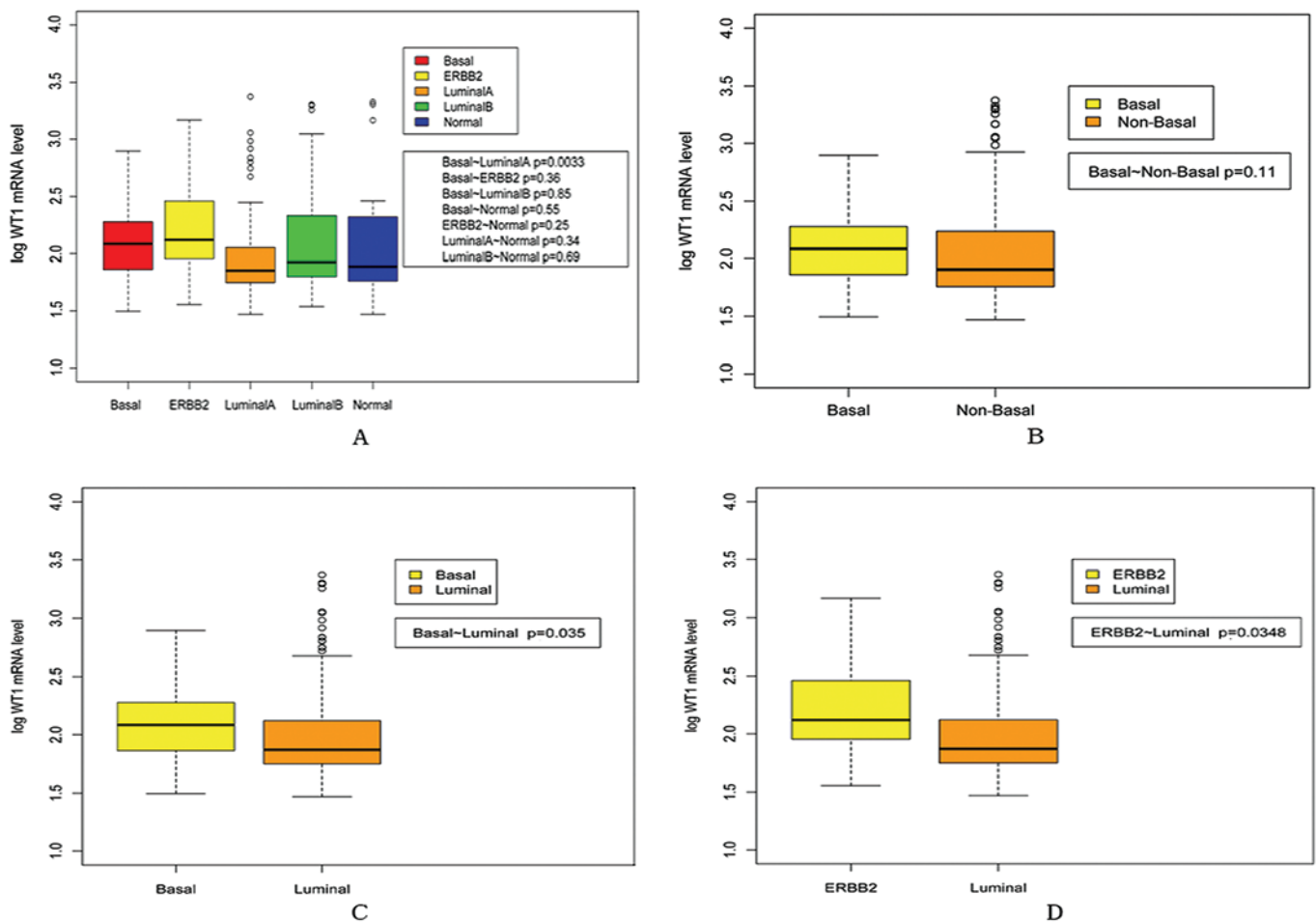
Figure 2. Relationship between WT1 mRNA expression and molecular subtypes of breast cancer in GSE21653. Comparison of WT1 mRNA expression among (A) basal-like, ERBB2, luminal A, luminal B and normal subtypes, (B) basal-like and non-basal-like subtypes, (C) basal-like and luminal subtypes, and (D) ERBB2 and luminal subtypes.

vation of any metastasis. DFS was estimated according to the Kaplan-Meier method and analyzed by log-rank (Mantel-Cox) test. Hazard ratio (HR) and 95% confidence interval (CI) were estimated by use of a stratified Cox regression analysis. Multivariate analysis was done by incorporating all variables with a P-value <0.05 in univariate analysis.

All statistical tests were two-sided at the 5% level of significance. Statistical analysis was done using the survival package (version 2.36) in the R software (version 2.12.1).

Results

Relationship between WT1 mRNA expression and clinicopathological factors and molecular subtypes of breast cancer. As shown in Fig. 1, WT1 mRNA expression increased in relation to histological grades (G2 vs. G1, P=0.026; G3 vs. G1, P=8.04e-5). In addition, WT1 mRNA expression was significantly higher in ER negative group than in ER positive group (P=0.050). However, no correlation was found between WT1 mRNA expression and pT (<2 cm vs. >2 cm, P=0.620) and Ki67 expression (Ki67 positive vs. Ki67 negative, P=0.127). As shown in Fig. 2, WT1 mRNA expression was significantly higher in basal-like and ERBB2 subtypes than in luminal subtype (P=0.003 for basal-like vs. luminal

A, P=0.035 for basal-like vs. luminal, P=0.035 for ERBB2 vs. luminal).

Univariate analysis of WT1 mRNA expression and DFS in GSE21653. Patients were divided into the WT1 high and low mRNA expression groups according to the cutoff value of 2.1, which corresponded to the mean of the WT1 mRNA levels in total breast cancer tissues and the cutoff value of 3.1, which corresponded to the mean +2 standard deviation of the WT1 mRNA levels of the normal-like breast subtype, considering its intrinsic gene expression patterns (30,31) and WT1 expression level in this study (Fig. 2A). Kaplan-Meier analyses of the probability that patients would remain free of distant metastases were carried out between WT1 high and low expression groups. As shown in Fig. 3 and Table I, there was no significant difference of DFS between the WT1 high and low expression group (HR=1.226, 95% CI=0.794-1.895, P=0.357) with the 2.1 cutoff value, however, WT1 high expression group showed a significantly lower DFS than the low expression group (HR=3.294, 95% CI=1.198-9.055, P=0.014) with the 3.1 cutoff value.

Multivariate analysis of WT1 mRNA expression and DFS in GSE21653. Using the Cox proportional hazards model, multivariate analysis demonstrated that grades are the
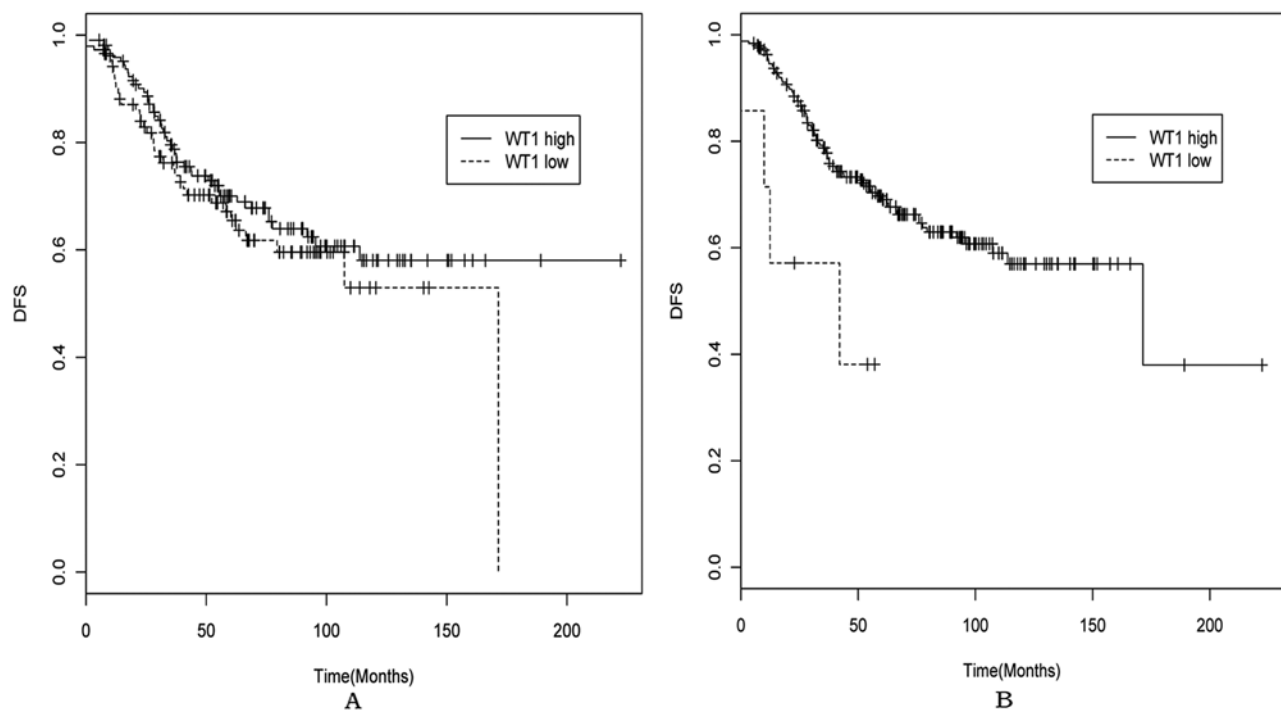
Figure 3. Univariate analysis of relationship between WT1 mRNA expression and DFS of breast cancer patients in the GSE21653. (A and B) stand for survival curve when using the 2.1 and 3.1 cutoff value to define high and low WT1 mRNA expression group, respectively.

Table II. Multivariable proportional-hazards analysis of DFS based on 2.1 cutoff value.

Variable	HR (95% CI)	P-value
WT1 values (high vs. low)	1.093 (0.687-1.740)	0.708
Grade (G2 vs. G1)	**2.611 (1.133-6.019)**	**0.024**
Grade (G3 vs. G1)	**2.993 (1.249-7.171)**	**0.014**
pT (<2 cm vs. >2 cm)	1.162 (0.662-2.040)	0.601
ER (ER positive vs. ER negative)	0.675 (0.380-1.197)	0.179
Subtype (basal vs. non-basal)	1.357 (0.703-2.619)	0.363

pT, pathological tumor size; ER, estrogen receptor; HR, hazard ratio; CI, confidence interval.

Table III. Multivariable proportional-hazards analysis of DFS based on 3.1 cutoff value.

Variable	Hazard ratio (95% CI)	P-value
WT1 values (high vs. low)	**3.454 (1.203-9.918)**	**0.021**
Grade (G2 vs. G1)	2.385 (0.961-5.924)	0.061
Grade (G3 vs. G1)	**3.121 (1.236-7.883)**	**0.016**
pT (<2 cm vs. >2 cm)	0.967 (0.523-1.789)	0.916
ER (ER positive vs. ER negative)	0.628 (0.330-1.196)	0.157
Subtype (basal vs. non-basal)	1.203 (0.572-2.534)	0.626

pT, pathological tumor size; ER, estrogen receptor; HR, hazard ratio; CI, confidence interval.

strongest predictor of the likelihood of DFS (HR=2.611, 95% CI=1.133-6.019, P=0.024 for G2 vs. G1; HR=2.993, 95% CI=1.249-7.171, P=0.014 for G3 vs. G1), when using the 2.1 cutoff value (Table II). However, with the 3.1 cutoff value, multivariate analysis demonstrated that both grades (HR=3.121, 95% CI=1.236-7.883, P=0.016 for G3 vs. G1) and WT1 mRNA

expression (HR=3.454, 95% CI=1.203-9.918, P=0.021 for high expression group vs. low expression group) are independent prognostic indicators for breast cancer (Table III).

Discussion

The WT1 gene, located at chromosome 11p13, encodes a DNA-binding protein, which contains an NH2-terminal glutamine and proline-rich domain involved in transcriptional repression and activation and a C-terminal domain composed of four Cys-Cys-His-His-type zinc finger domains (ZF) involved in DNA and RNA binding and protein-protein interactions (32). WT1 regulates a diverse array of genes through ZF domain at GC-rich sites (32), playing an important role in cell growth and development (6-11,33).

Though originally isolated as a tumor suppressor gene responsible for Wilms' tumor, WT1 is found to be overexpressed in primary human leukemia and a variety of solid tumors, including lung, colon, liver, thyroid and pancreatic ductal cancer (9,34,35). In addition, knockdown of WT1 by shRNA induced mitochondrial damage and the resultant apoptosis in several WT1-expressing solid tumor cells, indicating that WT1 might play an oncogenic role in these tumors (36).

Up to now, the role and function of WT1 in breast cancer have been not clarified. As for expression of WT1 in breast tissues, the groups of Silberstein and Loeb obtained contrary results in the clinical reports (12,17). As for function of WT1 in breast cancer cells, a promoting or repressing effect of WT1 on breast cancer cells was observed in the experimental studies, making it hard to conclude on role of WT1 in breast cancer. In addition, some studies have focused on WT1 expression in breast cancer, but results are not clear enough to demonstrate its possible relationship with tumor biology.

In this study, we found that WT1 expression was higher in ER-negative patients than in ER-positive patients, which may be explained by the fact that overexpression of WT1 directly resulted in the down-regulation of ER expression (22). In addition, our results also demonstrated that WT1 tended to be overexpressed in tumors with high histological grades. It has been well established that breast cancer patients with ERBB2-overexpression and basal-like molecular subtypes had worse prognosis than luminal subtypes (31). Of note, our present data showed that WT1 mRNA expression was much higher in patients with ERBB2 and basal tumors. To the best of our knowledge, this is the first report exploring the possible relationship between WT1 and molecular subtypes, and the preliminary results suggest a potential role of WT1 in progression of breast cancer. Though Miyoshi et al (18) found that the prognosis of patients with high WT1 expression was significantly worse than that in patients with low WT1 expression, Camcı et al failed to confirm this finding in a later study (23). In the present study, we concluded that WT1 mRNA high expression predicted poor prognosis of breast cancer patients when using 3.1 as cutoff value, which might be due to the facts that WT1 could promote proliferation, invasion, migration, response to hypoxia and drug resistance of cancer cells (19-22,35,37,38).

Conclusively, our study demonstrates association between WT1 mRNA levels and histological grade, ER status, molecular subtype and clinical outcome of breast cancer, consistent with the hypothesis that WT1 plays an oncogenic role in breast cancer. However, further research is required to confirm the current findings and clarify the functions and relevant mechanisms of WT1 in human breast cancer.

Acknowledgements

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