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Original Article

# Characterization of *CCND1* and *TWIST1* as Prognostic Markers with the Mortality Rate of Breast Cancer

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Breast cancer is one of the most common cancers affecting women worldwide. Although the survival rate of breast cancer has increased, breast cancer still results in a high mortality rate. Breast cancer deaths are caused by metastasis that occurs in organ dysfunction. Recently, there have been many studies on circulating tumor cells (CTCs), which are related to breast cancer metastasis in the blood. Recent studies have demonstrated that some CTCs do not express epithelial markers. Therefore, in this study, total RNA was extracted from blood without separating out the CTCs, and the characteristics of the CTCs were analyzed by RT-qPCR. Cyclin D1 and twist-related protein 1 (*TWIST1*) are well-known markers for predicting the prognosis of patients with breast cancer. However, few studies have demonstrated the use of *CCND1* and *TWIST1* in blood as diagnostic and prognostic markers of breast cancer. In this study, patients with late-stage breast cancer had overexpressed *CCND1* and *TWIST1* than patients with different stages of breast cancer ($P < 0.001$ and $P < 0.01$, respectively). The relative expression level of *CCND1* in survivors was higher than in patients who died ($P = 0.06$). The relative expression level of *TWIST1* in survivors was lower than in patients who died ($P = 0.08$). Overall *CCND1* and *TWIST1* were not useful as markers for the diagnosis of breast cancer through blood. However, we showed the possibility of using *CCND1* and *TWIST1* as prognostic markers, and a large-scale study is needed to confirm the usefulness of these prognostic markers.

**Key Words:** Breast cancer, *CCND1*, *TWIST1*, Circulating tumor cells, Blood, RT-qPCR, Prognosis

## INTRODUCTION

Breast cancer is the most common cancer affecting women worldwide and is responsible for high mortality rate among all cancer (Ferlay et al., 2015). According to a World Health Organization (WHO) report of 2015, there have been 1.6 million new cases per year and 520,000 deaths due to breast cancer (Torre et al., 2015).

Improvements in diagnostic and treatment methods have reduced the mortality rate of breast cancer. However, it remains the leading cause of mortality in female patients suf-

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fering from cancer (Miller et al., 2016). Mortality in patients with breast cancer is caused by metastases that cause dysfunction of organs. Breast cancer is a systemic disease that causes micro-metastases, and also occurs in the very early stages of cancer (Hansen et al., 2009; Redig and McAllister, 2013). Tumor cells isolated from primary tumors have been known to metastasize to another organ through the bloodstream or lymph (Bonnomet et al., 2010).

Tumor cells entering the blood vessels are called circulating tumor cells (CTCs). CTCs can metastasize in two ways. The first is when the premalignant cells enter the blood vessels for metastasis to other tissues. The second is when there is the formation of primary cancer and the tumor cells simultaneously enter the blood vessels and transfer cancer to other tissues (Pantel and Speicher, 2016). Therefore, CTCs have been found not only in patients with metastatic breast cancer but also in those with early breast cancer (Barrière et al., 2012; Politaki et al., 2017).

To detect CTCs, the most widely used method for measuring the number of cells has been by capturing cells using epithelial cell adhesion molecule (EpCAM), which is a surface antigen. In particular, the CellSearch system (Veridex LLC, Raritan, NJ, USA) is the only CTC detection method approved by the US Food and Drug Administration (FDA). It has been used to predict the outcome of breast, prostate, and colon cancers. In this system, epithelial cells are immunemagnetically separated and labeled with fluorescence, and then the number of nucleated (DAPI-positive) cells that are positive for EpCAM, as well as cytokeratin (CK) 8, 18, and 19, are estimated for the number of CTCs (Raimondi et al., 2014). Another method of studying CTCs involve immunemagnetic separation followed by multiplex RT-PCR of tumor markers such as human epithelial growth factor receptor 2 (HER2), mucin-1 (MUC-1), and GA773-2 (Müller et al., 2012).

However, CTCs in the blood vessels lose their characteristic of an epithelial cell and undergo epithelial-to-mesenchymal transition (EMT) during the process of entering the blood vessels, thereby adopting the characteristics of mesenchymal cells (Krebs et al., 2014). Because of this, there are many CTCs without surface antigen or EpCAM and various other characteristics. The *CCND1* and *TWIST1* are biomarkers that have been researched to characterize CTC and breast cancer (Kim et al., 2014; Markiewicz et al., 2014).

The *CCND1* is a gene that synthesizes cyclin D1, which regulates the G1 to S phase transition of the cell cycle (Motokura et al., 1991). Many studies have reported the oncogenic function of cyclin D1 (Ma et al., 2003; Oesterreich et al., 2003). In particular, over-expression of *CCND1* is known to increase the synthesis of cyclin D1 and regulate the G1 to S phase, thereby contributing to cancer growth and affecting endocrine therapy resistance (Tian et al., 2007). The twist-related protein 1 (*TWIST1*) is a basic helix-loop-helix transcription factor that acts as an important regulator of cell migration and tissue reorganization during early embryogenesis. However, *TWIST1* is known to be reactivated in cancer (Ansieau et al., 2010). In cancer, *TWIST1* is known to be an EMT-inducing factor that inhibits E-cadherin (Bonnomet et al., 2010). Therefore, *TWIST1* causes cancer metastasis by inhibiting E-cadherin (Yang et al., 2004).

RT-qPCR is widely used in many diagnostic and prognostic marker research. RT-qPCR is a sensitive method, which can be used to detect small quantities of transcripts in the blood. Furthermore, each clinical sample can be easily normalized based on the expression levels of the reference genes.

In this study, an RT-qPCR assay targeting *CCND1* and *TWIST1* was developed and investigated to assess the relative expression levels of *TWIST1* and *CCND1* in the blood of breast cancer patients. The relationship between each marker and clinical information of breast cancer patients was analyzed. Furthermore, the association between the prognosis of patients and the two markers by comparing the relative expression levels of these genes in patients who survived versus in those who died.

## MATERIALS AND METHODS

### Breast cancer cell lines and cell culture

SKBR-3, MCF-7, BT-474, and MDA-MB-231 were used for assay development and the generation of gene-specific quantification calibrators. Human breast cancer cell lines, SK-BR3 (KCLB No. 30030), MCF-7 (KCLB No. 30022), BT-474 (KCLB No. 60062), and MDA-MB-231 (KCLB No. 30026) were obtained from the Korean Cell Line Bank

(Seoul, Republic of Korea). The SKBR-3, MCF-7, BT-474, and MDA-MB-231 cell lines were grown at 37℃ in a humidified atmosphere with 5% $CO_2$ in the air.

SKBR-3, MCF-7, BT-474, and MDA-MB-231 cells were cultured in RPMI 1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco-BRL, Carlsbad, CA, USA).

**Study samples**

A total of 152 patients with breast cancer and 56 healthy subjects were included in the study for evaluating *CCND1*, and a total of 51 patients with breast cancer and 38 healthy subjects were included in the study for evaluating *TWIST1*. Patients with breast cancer were recruited from the Yonsei Severance Hospital (Seoul, Republic of Korea) from 2013 to 2014. Healthy subjects were recruited from Yonsei Severance Hospital and Yonsei University at Wonju. This study was approved by the Institutional Ethics Committee at Yonsei Severance Hospital (approval number 4-2011-0011 for patients with breast cancer and healthy donors) and Yonsei University at Wonju (approval number 1041849-201311-BM-020-02), and all subjects provided written informed consent.

The information on estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) was collected from the pathology reports of the Yonsei University Severance Hospital. ER and PR were positive when over 10% of the cells were stained. HER2 status was determined based on the criteria from the American Society of Clinical Oncology/College of American Pathologists, as follows IHC 0 or 1+ considered as negative HER2 IHC, and 3+ was considered as positive HER2 IHC. When the HER2 IHC score was 2+, positivity and negativity were distinguished by gene amplification with fluorescence *in situ* hybridization (FISH).

**Blood collection and total RNA extraction**

All blood samples were obtained from a middle vein puncture. The blood cell samples were lysed with 1× ACK solution (0.15 M $NH_4Cl$, 1 mM $KHCO_3$, and 0.1 mM $Na_2EDTA$). Total RNA was isolated using the Isol-RNA Lysis Reagent (5 Prime, Austin, TX, USA) according to the manufacturer's instructions. The total RNA purity and concentration were determined by measuring the absorbance at 260 and 280 nm using an Infinite 200® (Tecan, Salzburg, Austria) spectrophotometer. The preparation and handling of total RNA were conducted in a laminar flow hood under RNase-free conditions. The isolated total RNA was stored at -70℃.

**Synthesis of cDNA and RT-qPCR assay**

The cDNA was synthesized using an M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) with random hexamers (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized at 25℃ for 10 min, then at 37℃ for 50 min, followed by at 70℃ for 15 min.

The relative expression of *CCND1* and *TWIST1* to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels was measured with TaqMan probes in the CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA). RT-qPCR amplification was performed with 10 μL of 2× Thunderbird probe qPCR mix (Toyobo, Osaka, Japan), 3 μL of primer and TaqMan probe mixture, 2 μL of template cDNA, and distilled water to bring the final volume to 20 μL. Each sample was tested in duplicate, and all PCR runs were performed twice. The reaction conditions were as followed: 95℃ for 3 min, followed by 40 cycles at 95℃ for 15 sec, and then at 55℃ for 30 sec. The relative gene expression was assessed using the comparative Ct method (ΔΔCt method). The amount of target gene expression, normalized to an internal housekeeping gene and relative to a calibrator, was calculated by the $2^{-\Delta\Delta Ct}$ method, which was normalized according to the following equation: $\Delta\Delta Ct = [\Delta Ct_{(test)} = Ct_{(target\ test)} - Ct_{(reference\ test)}] - [\Delta Ct_{(calibrator)} = Ct_{(target\ calibrator)} - Ct_{(reference\ calibrator)}]$

The relative expression levels of *CCND1* and *TWIST1* were referred to a calibrator, and the healthy donor blood samples were used to represent the 1× relative expression. The expression of *CCND1* and *TWIST1* were expressed as n-fold changes.

**Statistical analysis**

GraphPad PRISM software version 6.01 (GraphPad, La Jolla, CA, USA) and the Statistical Package for the Social

**Table 1.** Clinicopathologic characteristics of patients with breast cancer in the *CCND1* and *TWIST1* sets

| Characteristics | | No. of patients *CCND1* set (N=152) (percentage) | No. of patients *TWIST1* set (N=51) (percentage) |
|---|---|---|---|
| Age | < 50's | 61 (40.1) | 30 (58.8) |
| | ≥ 50's | 69 (45.4) | 19 (37.3) |
| | Unknown | 22 (14.5) | 2 (3.9) |
| Cancer stage | 0 | 46 (30.3) | 0 (0) |
| | I & II (early) | 60 (39.5) | 29 (56.9) |
| | III & IV (late) | 20 (13.1) | 14 (27.5) |
| | Neoadjuvant | 23 (15.1) | 8 (15.6) |
| | Unknown | 3 (2.0) | 0 (0) |
| HER2 IHC | Positive | 57 (37.5) | 18 (35.3) |
| | Negative | 67 (44.1) | 31 (60.8) |
| | Unknown | 28 (18.4) | 2 (3.9) |
| ER IHC | Positive (≥ 10%) | 87 (57.2) | 29 (56.9) |
| | Negative (< 10%) | 39 (25.7) | 20 (39.2) |
| | Unknown | 26 (17.1) | 2 (3.9) |
| PR IHC | Positive (≥ 10%) | 59 (38.8) | 17 (33.3) |
| | Negative (< 10%) | 66 (43.4) | 32 (62.8) |
| | Unknown | 27 (17.8) | 2 (3.9) |

Abbreviations; ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor type 2

Sciences software version 24 (SPSS Inc., Chicago, IL, USA) were used for statistical analysis. A receiver operating characteristic (ROC) curve analysis was performed to determine the optimal cut-off values. To determine whether the relative expression levels of *CCND1* and *TWIST1* were different according to the stage of breast cancer the Kruskal-Wallis test was performed because the number of patients per stage was too small for an analysis of variance (ANOVA) test. The relative expression levels of *CCND1* was analyzed by Student's *t*-test, and *TWIST1* was analyzed by the Mann-Whitney test according to the results of IHC.

## RESULTS

### Clinical characteristics of breast cancer

The characteristics of the patients involved in this study are shown in Table 1. A total of 152 and 51 patients were involved in the marker development for *CCND1* and *TWIST1*, respectively.

In the *CCND1* set, the age of patients ranged from 25 to 79 years, and the median age ± standard deviation (SD) was 51 ± 10.16 years. Among the 152 patients, 46 had stage 0 cancer, 60 had early-stage cancer (stage I & II), 20 had late-stage cancer (stage III & IV), 23 were neoadjuvant (no stage), and 3 were unknown. Furthermore, 57 patients (37.5%) were HER2-positive, 87 (57.2%) were ER-positive, and 59 (38.8%) were PR-positive by IHC examination (Table 1). In the *TWIST1* set, the age of patients ranged from 26 to 80 years, and the median age ± SD was 48 ± 12.64 years. There were no stage 0 cancer in the *TWIST1* set, and 29 (56.9%) had early stage cancer, 14 (27.5%) had late-stage cancer, and 8 (15.6%) were neoadjuvant. Among the 51 patients, 18 (35.3%) were HER2-positive, 29 (56.9%) were ER-positive, and 17 (33.3%) were PR-positive by IHC examination (Table 1).

### Comparative analyses of the relative expression levels of the *CCND1* and *TWIST1* markers between healthy subjects and patients with breast cancer

To compare the relative expression levels of *CCND1* and

*TWIST1* markers in blood samples from the patients with breast cancer and those from healthy subjects, a ROC curve analysis was used. Two groups were analyzed with a standard cut-off for the reference gene GAPDH. The GAPDH cut-off standard was set at 25, and the samples with over 25 threshold cycles (Ct) of GAPDH were excluded because the RNA was considered to be of poor quality. Of the 152 patients in the *CCND1* set, 2 samples had a Ct value of more than 25 for GAPDH, and this sample was excluded from further analysis. One out of the 51 patients in the *TWIST1* set was excluded for the same reason.

As a result, *CCND1* expression was not significantly different between patients with breast cancer (mean ± SD, 7.55 ± 35.55) and the healthy subjects (mean ± SD, 39.71

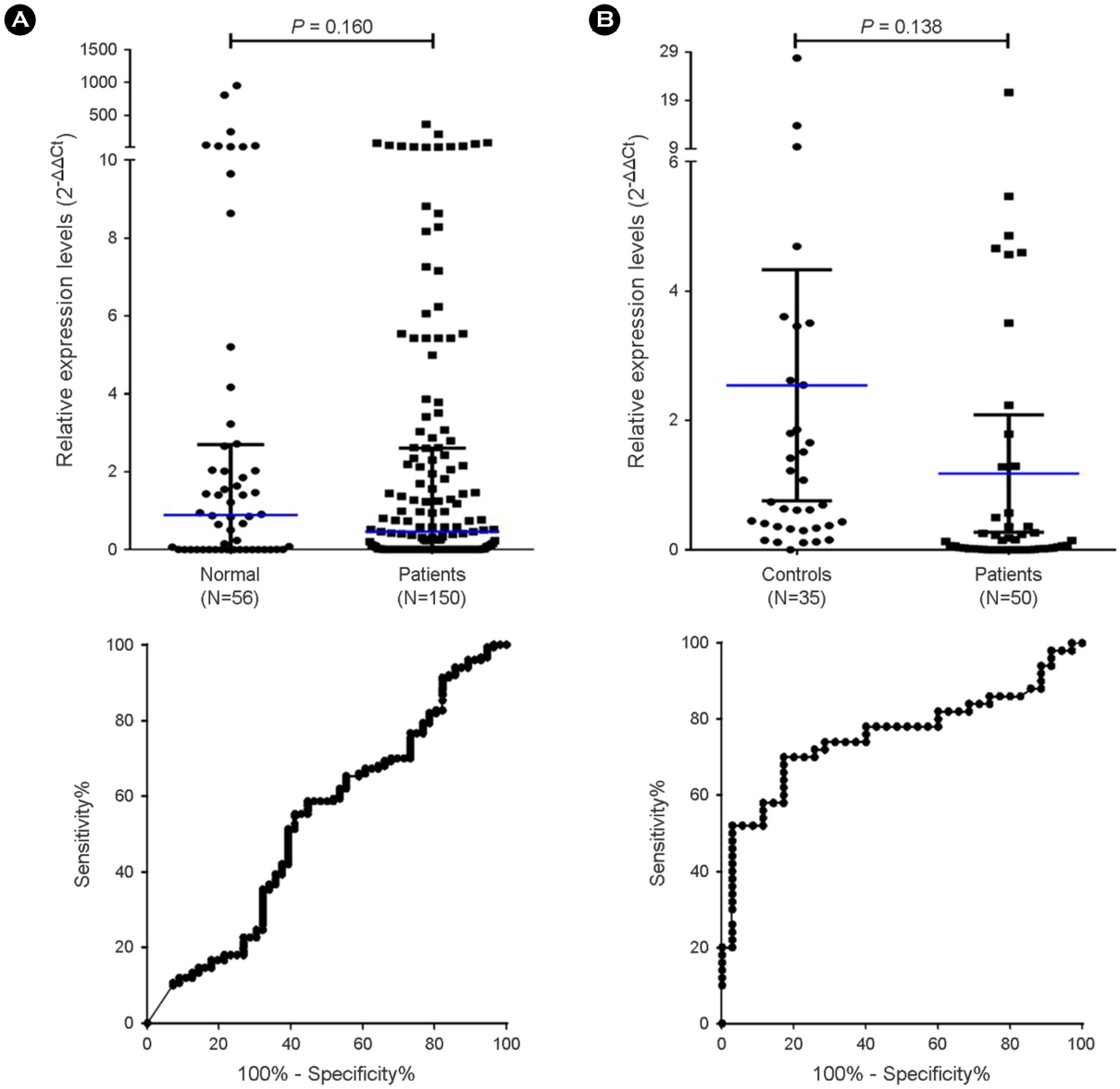


**Fig. 1.** Comparison of the relative expression of the two markers in the blood samples from patients with breast cancer with those in the blood samples from healthy subjects. (A) The relative expression levels of *CCND1* showed no significant differences between patients with breast cancer and the healthy subjects (AUC = 0.529, $P = 0.160$). (B) The relative expression levels of *TWIST1* in healthy subjects was higher than that in patients with breast cancer however, there was no statistically significant difference between healthy subjects and patients with breast cancer using the Student's *t*-test (AUC = 0.758, $P = 0.138$).

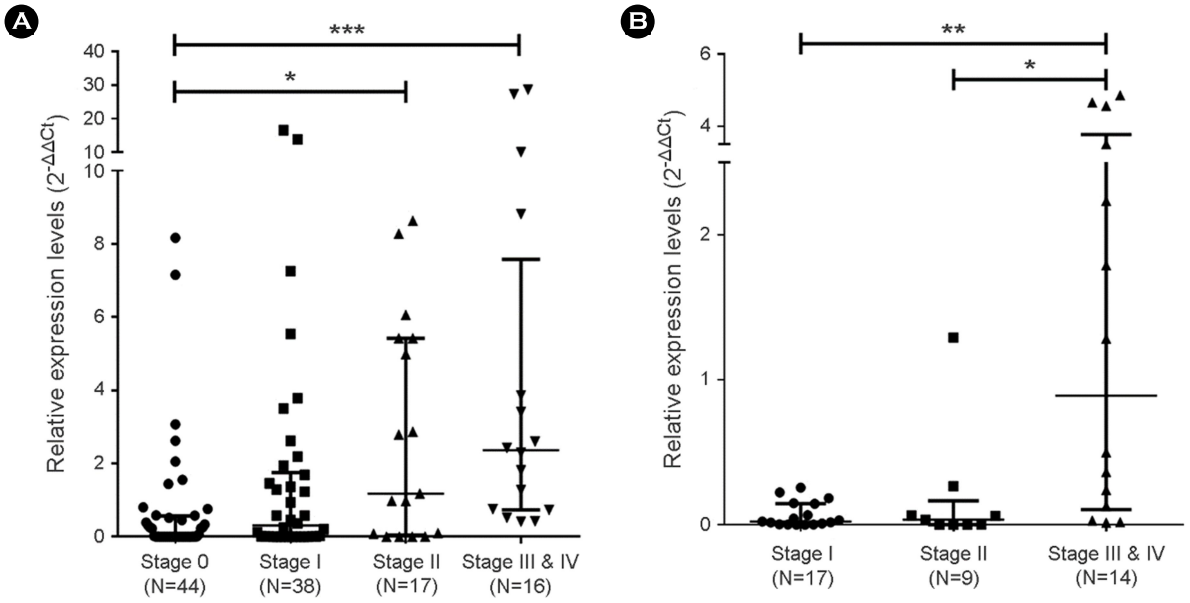


**Fig. 2.** Relative expression levels of (A) *CCND1* and (B) *TWIST1* according to breast cancer stages. In the *CCND1* set, patients with late-stage breast cancer showed higher expression of *CCND1* than in those with stage 0 breast cancer ($P = 0.002$). Patients with stage II breast cancer showed statistically higher expression of *CCND1* compared with those with stage 0 breast cancer ($P = 0.016$). In the *TWIST1* set statistically significant higher expression of *TWIST1* was seen in patients with late-stage breast cancer than in patients with stage II ($P = 0.022$) and stage I breast cancer ($P = 0.005$).

± 167.7), where the AUC was 0.529 and *P* value was 0.160 (Fig. 1A). The relative expression levels of *TWIST1* also showed no significant difference between patients with breast cancer (mean ± SD, 1.18 ± 3.20) and the healthy subjects (mean ± SD, 2.54 ± 5.20), where the AUC was 0.758 and *P* value was 0.138 (Fig. 1B).

**Relative expression levels of *CCND1* and *TWIST1* markers according to the stages of breast cancer**

In the *CCND1* set, patients with late-stage breast cancer had a higher *CCND1* expression (mean ± SD, 5.96 ± 9.04) than patients with stage 0 breast cancer (mean ± SD, 0.73 ± 1.69). Moreover, patients with stage II breast cancer had over-expressed *CCND1* (mean ± SD, 2.81 ± 3.05) than patients with stage 0 breast cancer. *TWIST1* was over-expressed in patients with late-stage breast cancer (mean ± SD, 1.73 ± 1.90) than those with stage I (mean ± SD, 0.07 ± 0.09) and stage II (mean ± SD, 0.19 ± 0.42) breast cancer. There were no significant differences in the expression of *TWIST1* between patients with stage I and stage II breast cancer, and in the expression of *CCND1* in the patients with stage 0 and stage, I breast cancer (Fig. 2).

**The relationship between the relative expression levels of *CCND1* and *TWIST1* and the results of IHC**

The association between *CCND1* and *TWIST1* markers and the results of IHC that used for hormone therapy in the patients with breast cancer were investigated.

Among the 150 patients in the *CCND1* set, 123 patients had IHC data. There were no significant differences of between the patients who were ER-negative (mean ± SD, 14.7 ± 59.9) and those who were ER-positive (mean ± SD, 6.1 ± 25.5, $P = 0.26$). The patients who were PR-negative had higher expression of *CCND1* (mean ± SD, 9.5 ± 45.8) than those who were PR-positive (mean ± SD, 7.7 ± 30.5). However, this was not statistically significant ($P = 0.80$). Furthermore, there were no differences in the relative expression levels of *CCND1* between patients who were HER2-negative (mean ± SD, 4.2 ± 12.0) and those who were HER2-positive (mean ± SD, 14.2 ± 56.7, $P = 0.16$) (Fig. 3).

Of the 50 patients in the *TWIST1* set, 48 patients had

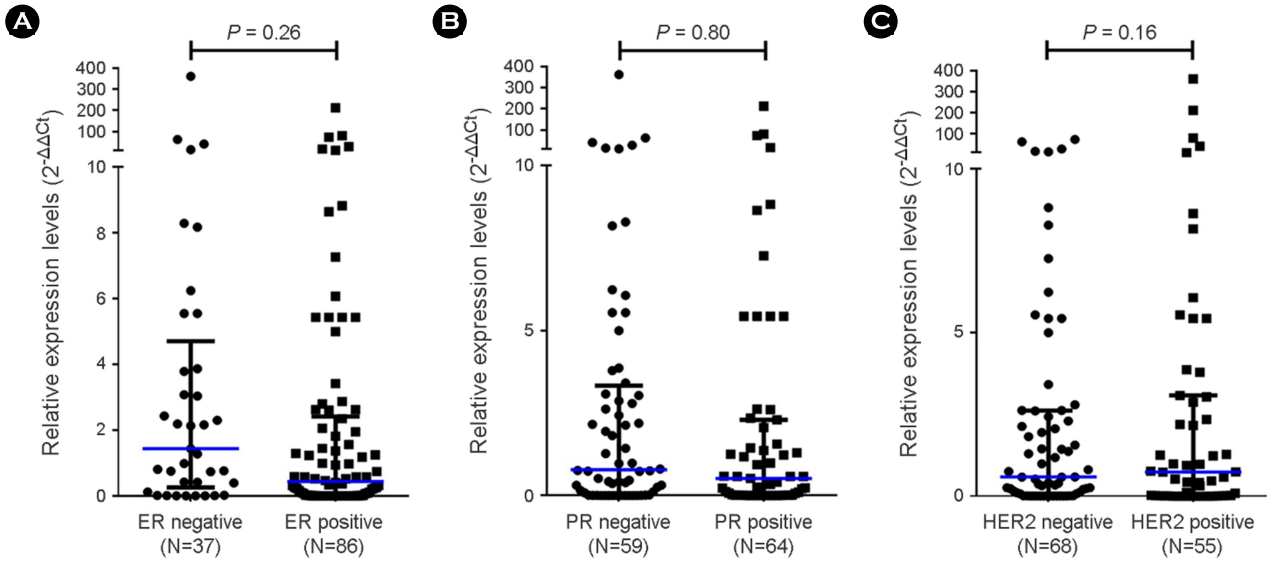


**Fig. 3.** Relative expression levels of *CCND1* markers in the blood of patients with breast cancer according to IHC results. The relative expression levels of *CCND1* showed no statistically significant differences according to (A) ER IHC data, (B) PR IHC data, and (C) HER2 IHC data (Student's *t*-test, $P = 0.26$, $P = 0.80$, and $P = 0.16$, respectively).

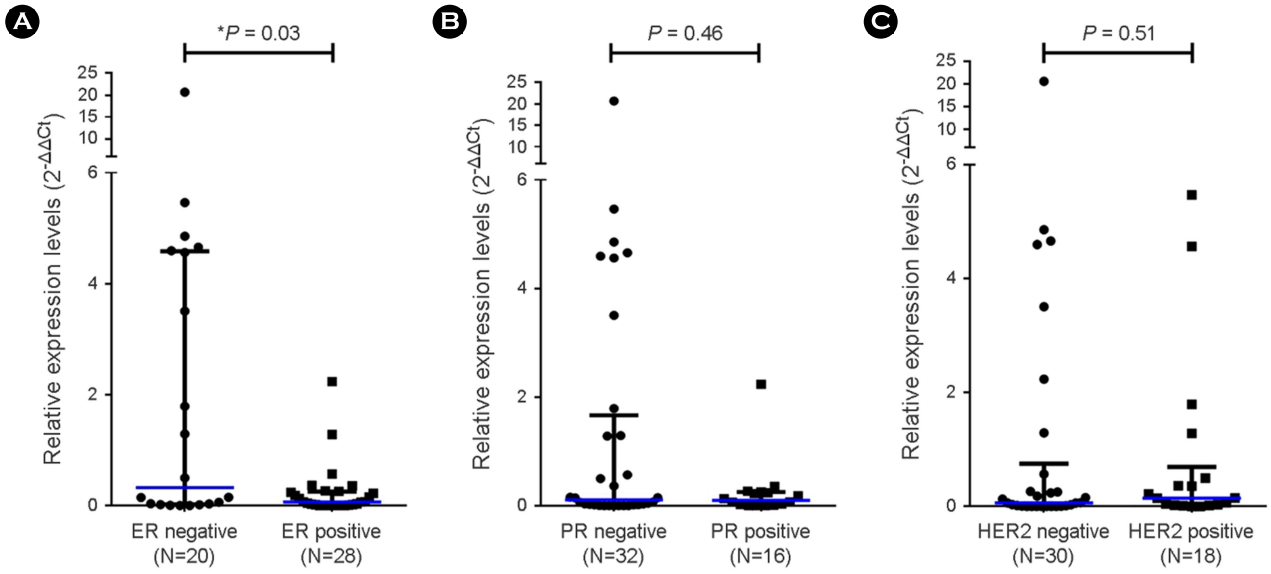


**Fig. 4.** Relative expression levels of *TWIST1* markers in the blood of patients with breast cancer according to IHC results. The relative expression levels of *TWIST1* in (A) estrogen receptor (ER) negative patients with breast cancer were higher than that in ER-positive patients ($P = 0.03$). However, there was no significant difference in the immunohistochemistry data for (B) progesterone receptor (PR) ($P = 0.46$), and (C) HER2 ($P = 0.51$).

IHC data. There were no significant differences of between patients who were PR-negative (mean ± SD, 1.7 ± 3.9) and those who were PR-positive patients (mean ± SD, 0.3 ± 0.5, $P = 0.46$). The relative expression levels of *TWIST1* was higher in patients who were HER2-negative (mean ± SD, 1.5 ± 3.9) than in those who were HER2-positive (mean ± SD, 0.8 ± 1.6), but this was not statistically significant ($P = 0.51$). However, ER-negative patients with breast cancer

showed higher relative expression of *TWIST1* (mean ± SD, 2.6 ± 4.7) than those who were ER-positive (mean ± SD, 0.2 ± 0.5, $P = 0.03$) (Fig. 4).

**Comparison of the relative expression levels of *CCND1* and *TWIST1* in patients with late-stage breast cancer grouped according to survival**

To confirm the association of *CCND1* and *TWIST1* with prognosis in patients with breast cancer, the relative expression levels of *CCND1* and *TWIST1* were analyzed by dividing the patient with late-stage breast cancer into two groups: patients who survived and those who died.

In the *CCND1* set, 8 of the 16 patients with late-stage breast cancer died. The relative expression levels of *CCND1* in the 8 patients who died (mean ± SD, 1.77 ± 1.22) was lower than that of the survivors (mean ± SD, 10.15 ± 11.56). However, there was no significant difference in the expression between the two groups ($P = 0.06$). In the *TWIST1* set, 8 of the 14 patients with late-stage cancer died. In contrast to *CCND1*, the relative expression levels of *TWIST1* was higher in the patients who died (mean ± SD, 2.49 ± 1.95) than in the survivors (mean ± SD, 0.71 ± 1.38). It is also showed no significant difference in expression between the two groups ($P = 0.08$).

## DISCUSSION

*CCND1* and *TWIST1* have been widely used as markers for the diagnosis and prognosis of breast cancer. However, the functions of these two markers, which have been associated with EMT or cell proliferation, have been studied using breast cancer cell lines. Also, studies that explored EMT-associated cancer metastasis at the animal model level predominate (Yang et al., 2004; Tian et al., 2007). However, gene expression levels of *TWIST1* and *CCND1* have rarely been applied to clinical samples of patients with breast cancer. In particular, there have been few studies using blood as a marker for detecting CTCs that are highly related to EMT and metastasis.

*CCND1* is overexpressed in 20% of the patients with

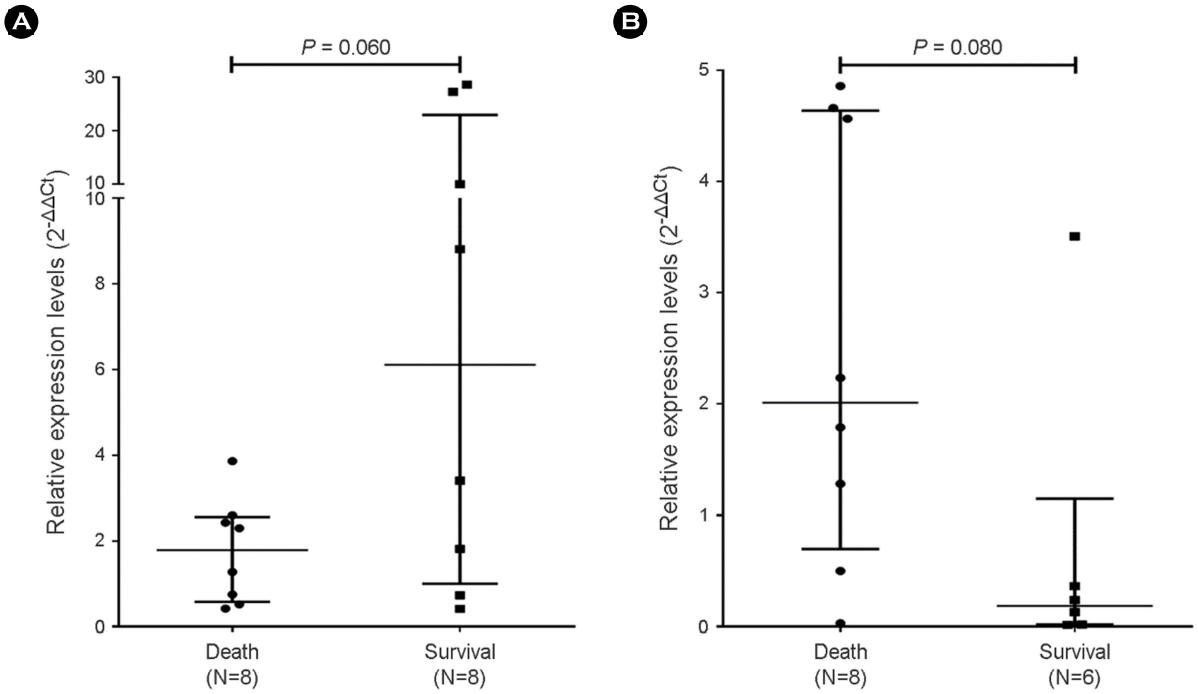


**Fig. 5.** Comparison of the relative expression levels of *CCND1* and *TWIST1* in breast cancer patients who survived and died. The relative expression level of (A) *CCND1* in the survivors was higher than that in breast cancer patients who died. The relative expression level of (B) *TWIST1* in the survivors was lower than that in breast cancer patients who died.

breast cancer, and Cyclin D1 has been reported to be elevated in 50% of patients with breast cancer (Reis-Filho et al., 2006). Cyclin D1, which is involved in the cell cycle has been reported to promote tumor cell proliferation when overexpressed (Bostner et al., 2007; Jares et al., 2007). A previous study reported that the increased expression of *CCND1* increased the proliferation of cancer but decreased migration (Lehn et al., 2010). Furthermore, it has been reported that when *CCND1* expression has decreased the proliferation of cancer cells decreased and their migration ability increased (Lehn et al., 2010). Another study reported that *CCND1*-negative patients with breast cancer had a lower survival rate than *CCND1*-positive patients with breast cancer (Barnes and Gillett, 1998). However, the results of this study showed that *CCND1* expression was higher in patients with late-stage breast cancer than in those with stage 0 breast cancer. To analyze the relationship between *CCND1* expression and mortality, we analyzed the survival of patients with late-stage breast cancer and the expression of *CCND1* in them. The expression levels of *CCND1* in patients who died was 5.7-fold lower than that of the surviving patients (Fig. 5, $P = 0.06$). Also, higher expression of *CCND1* in patients with stage II breast cancer and larger tumor sizes compared with that in patients with stage 0 breast cancer may be associated with tumor proliferation. Contrarily, the low expression levels of *CCND1* may be associated with the poor prognosis of breast cancer.

*TWIST1* has been known to be associated with tumor initiation, angiogenesis, invasion, metastasis, and drug resistance in a variety of cancers (Qin et al., 2012; Khan et al., 2013). In this study, the expression levels of *TWIST1* did not differ between healthy individuals and patients with breast cancer. However, a stage analysis of patients with breast cancer showed higher expression levels of *TWIST1* in patients with late-stage breast cancer than in those with early-stage breast cancer. Furthermore, the expression of *TWIST1* has been reported to be highly expressed in invasive carcinoma (Yang et al., 2004). Tumor cells overexpressing *TWIST1* in an animal model were more likely to have bone metastasis (Croset et al., 2014). Furthermore, patients with *TWIST1* overexpression have been reported to have a poorer prognosis (Martin et al., 2005). To confirm this, we compared the survival of 12 patients with late-stage breast cancer and the expression level of *TWIST1* in them, which showed that the relative expression of *TWIST1* was higher in the patients who died than in the survivors, but this was not statistically significant.

Furthermore, it has been reported that *CCND1* is highly expressed in the ER-positive patients with breast cancer (Elsheikh et al., 2008). The expression of *CCND1* has also been reported to be higher in the ER-positive patients than in the ER-negative patients. Additionally, compared with the ER-positive patients, both *CCND1* and ER-positive patients with breast cancer (71%) had a better response to tamoxifen (67%) (Barnes and Gillett, 1998). In this study, however, there was no significant difference in the relative expression level of *CCND1* between the ER-positive and ER-negative patients. Previous studies have analyzed the association of *CCND1* and ER in cell lines and tumor tissue, but this study used blood samples. This may be owing to changes in the characteristics of the tumor cells by EMT when entering the blood vessel. The association of *CCND1* with PR and HER2 has not been reported, and a statistical significance was not shown in this study.

Additionally, another previous study has demonstrated that *TWIST1* acts as a negative regulator of ER expression and contributes to resistance to hormone therapy (Vesuna et al., 2012). The relative expression level of *TWIST1* has been reported to be higher in PR-negative breast cancer patients than in those that were PR-positive (Zhang et al., 2015). Similar to the results of the previous study, the relative expression of *TWIST1* was significantly higher in the ER-negative patients than in the ER-positive patients. *TWIST1* expression was higher in the PR-negative group than in the PR-positive group, but not significantly. The total number of patients included in the *TWIST1* set was small, but the proportion of patients with late-stage breast cancer was high. In patients with late-stage breast cancer, including those with metastatic cancer, it has been reported that cancer cells can enter the blood vessels without EMT (Hou et al., 2012). These results suggest that tumor cells with the same characteristics of primary tumors are present in blood vessels, and therefore, show a significant correlation with the IHC results of primary tumors. The relative expression level of

*TWIST1* was similar in both HER2-negative and HER2-positive patients with breast cancer.

The results of this study suggest that *CCND1* and *TWIST1* are not valuable diagnostic markers for the diagnosis of breast cancer. However, the possible prognostic value of the two markers was confirmed, and these markers should be further validated in a large-scale study on patients with breast cancer who died.

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None.

**CONFLICT OF INTEREST**

The authors have no conflicts of interest to disclose.

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