Effect of Wnt5a on drug resistance in estrogen receptor-positive breast cancer

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Abstract

Background: It was previously reported by us that Wnt5a-positive breast cancer can be classified as estrogen receptor (ER)-positive breast cancer and its prognosis is worse than that of Wnt5a-negative breast cancer. Herein, the molecular mechanisms underlying the poor prognosis of Wnt5a-positive breast cancer patients were examined.

Methods: A total of 151 consecutive ER-positive breast cancer patients who underwent resection between January 2011 and February 2014 were enrolled. DNA microarray and pathway analyses were conducted using MCF-7 cells stably expressing Wnt5a (MCF-7/Wnt5a(+)). Based on the results, cell viability and drug sensitivity assays as well as mutation analysis were performed using culture cells and breast cancer tissue. The relationship between Wnt5a and the PI3K–AKT–mTOR signaling pathway was examined.

Results: The relapse-free survival rate in patients with Wnt5a-positive breast cancer was significantly lower than that in patients with Wnt5a-negative breast cancer ($P = 0.047$). DNA microarray data indicated that only the cytochrome P450 (CYP) pathway was significantly upregulated in MCF-7/Wnt5a(+) cells ($P = 0.0440$). MCF-7/Wnt5a(+) cells showed reduced sensitivity to the metabolic substrates of CYP,
tamoxifen \((P < 0.001)\), and paclitaxel \((P < 0.001)\). \textit{PIK3CA} mutations were unrelated to Wnt5a expression in breast cancer tissue and culture cells.

\textbf{Conclusions:} In ER-positive breast cancer, Wnt5a upregulated the CYP metabolic pathway; additionally, it inhibited the sensitivity to tamoxifen and paclitaxel, which constitute the standard treatment options for ER-positive breast cancer. Wnt5a could be involved in the poor prognosis of ER-positive breast cancer independently of the PI3K–AKT–mTOR signaling pathway.

\textbf{Keywords:} Wnt5a, estrogen receptor-positive breast cancer, cytochrome P450, pathway analysis, drug resistance, PI3K–AKT–mTOR signaling pathway
Background

The Wnt pathway is classified into the β-catenin-dependent and -independent pathways [1]. Wnt5a is a typical ligand of the β-catenin-independent pathway and modulates cell polarity and cell migration via the PCP/CE and Ca^{2+} pathways [1, 2]. The Wnt5a signaling pathway is involved in the progression of several cancers [1, 3-7]. Wnt5a acts as a tumor-promoting factor in gastric, lung, and prostate cancers [3-5]; conversely, it acts as a tumor suppressor in thyroid and ovarian cancers [6, 7]. It was previously reported by us that Wnt5a is overexpressed in approximately 30% of all breast cancer cases, and most Wnt5a-positive breast cancers are estrogen receptor (ER)-positive breast cancers. The 5-year relapse-free survival (RFS) rate was found to be significantly lower in Wnt5a-positive breast cancer than in Wnt5a-negative breast cancer [8].

Regarding the relationship between Wnt5a and drug sensitivity, ovarian cancer cells overexpressing Wnt5a show low chemosensitivity to paclitaxel and epirubicin [9]. In breast cancers, relatively few studies have examined the association between Wnt5a expression and drug sensitivity. Therefore, it is significant to investigate the drug sensitivity in Wnt5a-positive breast cancer.

The upregulated PI3K–AKT–mTOR signaling pathway is also associated with the poor prognosis of ER-positive breast cancer [10]. Mutations in PIK3CA are generally found
in approximately 40% of the ER-positive breast cancer cases [11]; furthermore, it is believed that PI3K–AKT–mTOR signaling promotes resistance to hormone therapy in ER-positive breast cancer [12]. Currently, the PI3Kα-specific inhibitor alpelisib [13] and the mTOR inhibitor everolimus [14] are clinically approved for the treatment of PIK3CA mutation-positive hormone receptor-positive advanced breast cancer and ER-positive breast cancer, respectively.

Wnt5a expression is reported to be augmented in ER-positive breast cancers with PIK3CA mutation [15], indicating a close relationship between PI3K signaling and the Wnt5a pathway in ER-positive breast cancer. In the present study, the signaling pathways associated with Wnt5a were investigated to analyze the molecular mechanisms underlying Wnt5a-mediated poor prognosis in Wnt5a-positive breast cancer.

Methods

Patients and breast cancer tissues (RFS-tracked cases)

The present study was approved by the institutional review board of Hiroshima University Hospital (No. 926) and compliant with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Consecutive breast cancer tissues
resected between 2011 and 2014 were reviewed as previously described [8]. In total, 151 cases were reviewed; two cases with distant metastasis diagnosed preoperatively were excluded. The prognostic data were updated until December 2019 for the present study.

Cell culture, transfection, and small interfering (si) RNA

The breast cancer cell line MCF-7 (RRID: CVCL_0031) was obtained from ATCC (catalog #HTB-22, Manassas, VA, USA) and was confirmed not to be listed in the ICLAC Register of Misidentified Cell Lines (version 10). This cell line was cultured using RPMI-1640 medium with added 10% fetal bovine serum (Fisher Scientific, Göteborg, Sweden) and 50 µg/mL penicillin/streptomycin/glutamine (Gibco, Invitrogen, Paisley, UK) in a 5% CO₂ cell culture incubator at 37 °C. The pPGK-neo/Wnt5a was transfected into MCF-7 cells using Lipofectamine LTX with PLUS reagent (Life Technologies, Carlsbad, CA, USA). The successfully transfected cells were selectively isolated by G418 administration and they formed colonies. The colonies were screened for Wnt5a expression by western blotting. Thereafter, certain MCF-7 cells stably expressing Wnt5a, MCF-7/Wnt5a(+), as well as those not expressing Wnt5a,
MCF-7/Wnt5a(−), were established. The suppression of Wnt5a via siRNA was conducted as previously described [8].

**Gene microarray analysis**

The Oligo DNA microarray analysis was performed using 3D-Gene Human Oligo chip 25 k (Toray Industries, Tokyo, Japan) as previously described [8]. In all three types of MCF-7/Wnt5a(+), genes for which the expression level was elevated by two-fold or more and genes for which the expression level was attenuated to 1/2 or less were selected. Gene ontology and pathway analyses for the selected genes were performed using the DAVID online tool (Version 6.8, https://david.ncifcrf.gov/).

**Cell viability assay**

(Z)-4-hydroxytamoxifen, paclitaxel, and epirubicin (hydrochloride) were obtained from Sigma-Aldrich (catalog #H7904; St. Louis, MO, USA), FUJIFILM Wako Pure Chemical Corporation (catalog #33069-62-4; Osaka, Japan), and Cayman Chemical (catalog #56390-09-1; Ann Arbor, MI, USA), respectively. After the cells had grown to 80% confluency, 6×10³ MCF-7 normal cells, MCF-7/Wnt5a(+) cells, and MCF-7/Wnt5a(-) cells were seeded into a 96-well plate in triplicate. At 24, 48, and 72 h
after the administration of 15 µM tamoxifen, 200 nM paclitaxel, and 800 nM epirubicin, 20 µL CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI, USA) was added into each well and the plate was incubated at 37°C. After a 2-h incubation period in a humidified, 5% CO₂ incubator, the absorbance of the cells was determined at OD = 490 nm using Varioskan Flash (Thermo Fisher Scientific, Rockford, IL, USA).

**Western blot analysis**

For immunoblot analysis, MCF-7 normal, MCF-7/Wnt5a(+), and MCF-7/Wnt5a(-) cells were washed with PBS and lysed with lysis buffer containing a Phosphatase Inhibitor Cocktail (Nacalai Tesque Inc., Kyoto, Japan). Proteins were separated on an SDS-PAGE gel. The proteins were transferred onto a nitrocellulose membrane (Amersham Protran Premium, GE Healthcare, Buckinghamshire, UK) after electrophoresis. The membranes were probed with various primary and secondary antibodies and visualized with enhanced chemiluminescence detection reagents (Amersham ECL Select, GE Healthcare, Buckinghamshire, UK). All western blotting experiments were performed in triplicate. Anti-Wnt5a/b rabbit monoclonal antibody (mAb) (Cat #2530), anti-phospho-AKT (Ser473) antibody (Cat #9271), anti-phospho-AKT (Thr308) rabbit mAb (Cat #4056), and anti-phospho-SAPK/JNK
antibody (Cat # 9251) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-ER (Cat #ab16660) and anti-HER2 antibodies (Cat #ab214275) were procured from Abcam (Cambridge, UK). Anti-β-actin antibody was purchased from Sigma-Aldrich (Cat #A5441, St Louis, MO, USA).

Detection of PIK3CA mutant variants

Among 151 cases showing immunoreactivity for Wnt5a, PIK3CA mutations were evaluated in cases with tumor size equal to or more than 1 cm in diameter. The QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany) was employed to extract DNA from formalin-fixed paraffin-embedded (FFPE) tissue. Mutations in PIK3CA (E542K, E545D/K, and H1047R/L) were detected by direct sequencing. The following primers were used: 5′-GCTAGAGACAATGAATTAAGGGAAAA-3′ (forward) and 5′-CTCCATTTTAGCACTTACCTGTGAC-3′ (reverse) for E542K and E545D/K; 5′-GCAAGAGGGCTTTGGAGTATTT-3′ (forward) and 5′-GTGTGGAAGATCCAATCCATTT-3′ (reverse) for H1047R/L.

Quantitative examination of mRNA in Wnt5a
RNA was extracted from tumor cells using NucleoSpin total RNA FFPE (Takara Bio, Shiga, Japan). Tissues were sliced from the FFPE block, including tumor component only; alternatively, the tumor component was dissected from FFPE slices attached to slides for laser microdissection (MembraneSlide 1.0PEN®, 145190-9041-000, Carl Zeiss, Cambridge, UK). Synthesized cDNA was obtained by reverse-transcription using the PrimeScript II High Fidelity RT-PCR Kit (Takara Bio). Wnt5a expression was quantitatively analyzed by real-time polymerase chain reaction (PCR) using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) and CFX96 real-time PCR detecting system (Bio-Rad). Wnt5a expression was quantified using Delta Ct value. The following primers were used: 5′-AAGTTGGTGACAGGTCAACAGCCGCT-3′ (forward) and 5′-CACATGAGCTCGCAGGCATCCATCCATG-3′ (reverse) for Wnt5a; 5′-TGAGCGCGGCTACAGCTT-3′ (forward) and 5′-TCCTTAATGTACGCAGCGATTT-3′ (reverse) for β-actin.

**Statistical analysis**

Statistical analysis was performed using the EZR (Version 1.50, Saitama Medical Center, Jichi Medical University, Saitama, Japan) [16] and SPSS (Version 20.0, Chicago, IL, USA) software packages. Student’s t-test was used to compare the age and
cell viability between Wnt5a-negative and -positive patients. Clinicopathologic characteristics were analyzed using the Chi-square test. The significance between RFS curves was analyzed using the generalized Wilcoxon test. The frequency of Wnt5a positivity and the expression level of Wnt5a mRNA were compared between PIK3CA mutation-negative and -positive cases using the Chi-square test and Welch's t-test, respectively. \( P \) values < 0.05 were defined as statistically significant.

**Results**

**The prognosis of Wnt5a-positive breast cancer patients was poor**

A total of 151 patients were enrolled. The median ages (ranges) of the Wnt5a-negative and -positive patients were 63.0 (47 to 69) years and 57.5 (46 to 68) years, respectively. Wnt5a-positive patients manifested a greater degree of lymph-node metastasis \( (P < 0.001) \). Tumor sizes of Wnt5a-positive breast cancers tended to be larger than those of Wnt5a-negative breast cancers \( (P = 0.059) \). There were no differences with respect to HER2 status or the frequency of receiving hormonal therapy. Wnt5a-positive patients received chemotherapy more significantly, probably because of the malignancy grade and progression of the tumors \( (P = 0.016) \) (Table 1). Most patients were treated with hormonal therapy (93%); specifically, 27% were receiving TAM, 55% AI, and 11%
TAM and AI sequentially. Approximately 30% of all patients also received adjuvant chemotherapy, and the most common regimen was taxane/cyclophosphamide (TC) (19%). Apart from this, the 5-fluorouracil + epirubicin + cyclophosphamide (FEC) and FEC-taxane sequential regimens were also provided (Table 1). The median (range) follow-up period was 73.2 months (11.7 to 102) for all patients. The RFS rate of Wnt5a-positive breast cancer patients was lower than that of Wnt5a-negative patients (8-year RFS rate: 91.9% (95% CI = 85.1-98.7) vs 98.6%, (95% CI = 96.0-100.0), $P = 0.047$; using the generalized Wilcoxon test) (Figure 1). The postoperative treatment regimen for recurrent patients of ER-positive breast cancer is shown in Supplementary Table S1.

The CYP pathway was upregulated in MCF-7 cells expressing Wnt5a

To investigate pathways related to the recurrence of Wnt5a-positive breast cancer, MCF-7 cells stably expressing Wnt5a were established and DNA microarray analysis was performed. A total of 176 candidate genes, for which the expression level varied more than 2-fold or less than 1/2 between MCF-7/Wnt5a(+) and MCF-7/Wnt5a(-) cells, were identified. Of these, 76 unique genes were upregulated and 100 unique genes were downregulated (Supplementary Table S2). $P$-value was calculated using the DAVID
database from 176 analysis results (Table 2A, 2B). By Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the 176 genes, physiological pathways that directly or indirectly influence Wnt5a expression were identified. Only the cytochrome P450 (CYP) pathway, which is involved in drug metabolism [17], was identified on the upregulated genes ($P = 0.0440$) (Table 2A). Conversely, certain virus-related pathways were identified on the downregulated genes (Table 2B). As a signaling pathway associated with cancer progression, the JAK–STAT signaling pathway was attenuated in the MCF-7/Wnt5a(+) cells (Table 2B).

To identify factors associated with a particular biological or molecular process, the 176 candidates were annotated and categorized by their function. The upregulated genes were primarily involved in the oxidation–reduction process, response to calcium ions, retinoid metabolic process, response to hypoxia and retinal metabolic process, ATPase binding, and oxidoreductase activity (Table 2A). Among the downregulated genes, genes related to viruses and interferons accounted for the majority (Table 2B).

**Wnt5a expression decreased sensitivity to tamoxifen and paclitaxel**

Tamoxifen and paclitaxel, but not epirubicin, are metabolized by CYP [18-20]. The sensitivity to these three drugs, which serve as the standard treatment option for
ER-positive breast cancer, was examined. The MCF-7/Wnt5a(+) cells were shown to exhibit significantly lower sensitivity to tamoxifen than the MCF-7/Wnt5a(-) cells at 48 h and 72 h ($P < 0.001$) (Figure 2A). Similarly, MCF-7/Wnt5a(+) cells showed lower sensitivity to paclitaxel at 48 h and 72 h ($P < 0.001$) (Figure 2B). In contrast, no difference was found in the sensitivity to epirubicin ($P = 0.12$) (Figure 2C).

**The PI3K signaling pathway was not correlated with Wnt5a expression**

The relationship between Wnt5a expression and the PI3K and JNK signaling pathways was examined. Western blotting was performed using MCF-7/Wnt5a(+) and MCF-7/Wnt5a(-) cells. The expression of phosphorylated JNK, which is reported to be located downstream of the Wnt5a signaling pathway [2], remained unaltered despite variation in Wnt5a expression as well as with Wnt5a knockdown (Figure 3). There was no difference in the expression level of phosphorylated AKT due to Wnt5a expression (Figure 4).

$PIK3CA$ mutations in 40 cases of ER-positive breast cancers were examined (Table 3); three principal mutation sites were identified in $PIK3CA$: E542K, E545K, and H1047R [21] (Figure 5). $PIK3CA$ mutations were detected in 19 cases (Table 4A). As a result, $PIK3CA$ mutations were observed in 8 cases of Wnt5a-positive breast cancers and in 11
cases of Wnt5a-negative breast cancers. However, there was no significant difference in the frequency of PIK3CA mutations due to Wnt5a expression ($P = 0.73$) (Table 4A). In addition, no difference in Wnt5a expression was observed depending on the mutation site (Table 4B).

Next, the expression of Wnt5a mRNA according to PIK3CA mutations was investigated. The median (range) expression of Wnt5a mRNA was 1.7 (0.94 to 3.9) in PIK3CA mutation-negative cancer and 2.5 (range, 0.83 to 5.1) in PIK3CA mutation-positive cancer. There was a trend that PIK3CA mutation-positive cancers have higher Wnt5a level, however, no significant difference was found between the two groups ($P = 0.92$) (Figure 6).

**Discussion**

The recurrence rate of Wnt5a-positive breast cancer patients was significantly higher than that of Wnt5a-negative breast cancer patients. Thereby, the association between Wnt5a expression and malignancy grade and prognosis was analyzed. Pathway analysis revealed that the CYP metabolic pathway was upregulated by Wnt5a expression.

CYP is a key enzyme that oxidizes various substrates and mainly metabolizes drugs in the liver [16]. In our study, the upregulation of CYP by Wnt5a expression reduced
sensitivity to tamoxifen and paclitaxel, both of which are metabolized by CYP [17, 18]. Conversely, epirubicin is not metabolized by CYP, and there was no significant difference in the sensitivity to epirubicin due to Wnt5a expression. From these results, it is anticipated that Wnt5a expression enhances the metabolism of tamoxifen and paclitaxel via CYP, thus decreasing the drug concentration in the cells. The termination of adjuvant drug therapy for breast cancer without completion of the tumor-suppressive effect of the drug may lead to a high recurrence rate of Wnt5a-positive breast cancer.

A recent study on the effect of Wnt5a on drug sensitivity was conducted in ovarian cancer [8]; however, such studies are rare for breast cancer. As no prior study exists showing the association between Wnt5a and CYP in any cancer research area, determining these associations is a novel strategy.

Comprehensive somatic mutation analysis of breast cancer revealed that ER-positive breast cancer cells harbored PIK3CA (45%) mutations, and the frequency of PIK3CA is the highest [22]. Mutations in PIK3CA enhance the PI3K–AKT–mTOR pathway and accelerate malignancy [23]. Thus, the PI3K–AKT–mTOR pathway was involved in poor prognosis [24]. Crosstalk between the JAK–STAT pathway and the PI3K–AKT–mTOR signaling pathway has been reported [25]. Together with previous studies, it was hypothesized herein that there is some association between Wnt5a
expression and the PI3K–AKT–mTOR signaling pathway. *PIK3CA* mutations and the activation of the PI3K signaling pathway, the signal transduction pathway that is most involved in causing drug resistance in ER-positive breast cancer [12], were examined. However, unexpectedly, no correlation was found between them. The results indicated the involvement of pathways other than PI3K in the recurrence of Wnt5a-positive breast cancer.

The present study indicated that Wnt5a may serve as a marker of drug resistance in ER-positive breast cancer. In the future, the possibility of developing new drugs targeting drug resistance can be explored. In addition, it is necessary to develop a therapeutic treatment for Wnt5a-positive breast cancer together with anti-Wnt5a antibody treatment, which is being advanced in Wnt5a-positive gastric cancer [26]. Furthermore, in addition to our *in vitro* experiments conducted in the present study, *in vivo* experiments using mice and further verification of CYP-related drug metabolism are required in future research.

**Conclusions**

Wnt5a upregulated CYP expression and diminished drug sensitivity to key drugs used for treatment of ER-positive breast cancer, including tamoxifen and paclitaxel. Wnt5a
may be involved in the poor prognosis of ER-positive breast cancer independently of the PI3K–AKT–mTOR signaling pathway.

**Abbreviations**

ER: estrogen receptor

PI3K: phosphoinositide 3-kinase

PIK3CA: the phosphatidylinositol-4,5-bisphophonate 3-kinase, catalytic subunit alpha polypeptide gene

AKT: protein kinase B

mTOR: mammalian target of rapamycin

CYP: cytochrome P450

RFS: relapse-free survival

siRNA: small interfering RNA

FFPE: formalin-fixed paraffin-embedded

PCR: polymerase chain reaction
Declarations

Ethics approval

The study was approved by the institutional review board of Hiroshima University Hospital (No. 926) and was performed in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Patients signed informed consent regarding publishing their data and photographs.

Availability of data and materials

Data sets analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.
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**Authors’ Contributions**

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by AA, TK, and MI. The first draft of the manuscript was written by AA and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figure legends

Fig. 1 Prognosis of Wnt5a in ER-positive breast cancer was estimated using a Kaplan–Meier plot (n = 151). Patients with Wnt5a-positive breast cancer (n = 68) demonstrated lower 8-year RFS probability over time. * \( P = 0.047 \) generalized Wilcoxon test; RFS, relapse-free survival

Fig. 2 Wnt5a expression diminishes drug sensitivity to tamoxifen and paclitaxel. a. MCF-7 normal, MCF-7/Wnt5a (+), and MCF-7/Wnt5a (−) cells were exposed to 15 \( \mu \)M tamoxifen. b. MCF-7 normal, MCF-7/Wnt5a (+), and MCF-7/Wnt5a (−) cells were exposed to 200 nM paclitaxel. c. MCF-7 normal, MCF-7/Wnt5a (+), and MCF-7/Wnt5a (−) cells were exposed to 800 nM epirubicin. Each error bar represents the mean ± S.D. of 6 measurements. * \( P < 0.001 \) student’s t-test; n.s., not significant

Fig. 3 Influence of Wnt5a expression on the expression of breast cancer-related signaling molecules. The expression of phosphorylated JNK (indicated by arrows) located downstream of AKT was not altered by Wnt5a.
**Fig. 4** The expression level of phosphorylated AKT (p-AKT) was not altered by Wnt5a.

ER, estrogen receptor

**Fig. 5** Occurrence of mutations within exons 9 and 20 of PIK3CA.

**Fig. 6** Wnt5a mRNA expression according to the mutation status of PIK3CA (n = 40). Wnt5a mRNA expression was examined in the PIK3CA mutation-positive and -negative cancers. Median Wnt5a mRNA expression was 1.7 in PIK3CA mutation-negative cancers and 2.5 in PIK3CA mutation-positive cancers. * $P = 0.92$ Welch’s t-test