

ABCBI Regulates Immune Genes in Breast Cancer

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Background: Resistance to standard chemotherapy is a critical problem for breast cancer patients. The ATP-binding cassette (ABC) superfamily transporters actively pump out drugs and play an important role in chemoresistance. ABCB1 (ABC subfamily B, member 1, also named as multidrug resistance protein 1, MDR1) and suppressive myeloid-derived suppressor cells (MDSCs) potentially involve in chemoresistance of breast cancer. The relationship between ABCB1 and immune genes in breast cancer has not been widely studied.

Methods: Microarray and RNA sequencing data were obtained from The Cancer Genome Atlas Breast Invasive Carcinoma in Genomic Data Commons Data Portal and Gene Expression Omnibus database. A patient-derived xenograft (PDX) model of HER2⁺ breast cancer was established to investigate the association between *ABCB1* and immune genes in breast cancer.

Results: Expression of *ABCB1* increased in doxorubicin-selected MCF-7/ADR cells. High expression of *ABCB1* mRNA is correlated with lymph-node metastasis and worse overall survival in patients with breast cancer. *ABCB1* is positively correlated with *IL6*, *CSF1*, *CSF3*, and *PTGS2*. In the HER2⁺ stage IIA breast cancer PDX model, both doxorubicin and paclitaxel suppressed growth of P2 tumors. *IL6*, *CSF1*, *CSF3*, and *PTGS2* expression were suppressed by paclitaxel but not doxorubicin. Intrasplenic MDSCs, including CD11b⁺Ly6G⁺ and CD11b⁺Ly6C⁺ cells, were more abundant than intratumor MDSCs in PDX-carrying nude mice. Clinically, the patient developed cancer recurrence after adjuvant chemotherapy with doxorubicin-based regimen and was well controlled after paclitaxel-trastuzumab combined therapy.

Conclusion: ABCB1 was a poor predictor of HER2⁺ LN⁻ breast cancer. Regulation of immune genes by ABCB1 contributed to cancer recurrence and treatment effect. The PDX model was suitable for investigation the expression of target genes and expansion of immune cells.

Keywords: breast cancer, ABC transporters, ABCB1, myeloid-derived suppressor cells, patient-derived xenograft

Introduction

Chemoresistance is a major challenge for breast cancer treatment.¹ The mechanisms of chemoresistance are complex because of crosstalk between receptor tyrosine kinases and downstream pathways, deregulation of cell-cycle and apoptosis regulators, and modulation of tumor-infiltrating immune cells.² The ATP-binding cassette (ABC) superfamily is one of the largest families of membrane-bound transport proteins in humans. ABC transporters use the energy from ATP hydrolysis to actively pump out substrates through the cell membrane. ABC transporters also function in cell apoptosis, energy metabolism, and material transport. In cancer cells, ABC transporters participate in detoxification and drug resistance.¹ Multidrug resistance protein 1 (MDR1; gene: ABC subfamily B, member 1, *ABCB1*), multidrug resistance-associated protein 1 (MRP1; gene: ABC subfamily C, member 1, *ABCC1*), and breast cancer resistance protein (BCRP; gene: ABC subfamily G, membrane 2, *ABCG2*) have been studied in chemoresistant breast cancer.

Increased expression of ABCB1 has been reported in recurrent breast cancer, and patients with ABCB1-positive cancer fail to respond to chemotherapy.³ ABCB1 is also a metastatic marker for triple-negative breast cancer.⁴ ABCB1 is overexpressed in doxorubicin-resistant MCF7 breast cancer cells.⁵ Theoretically, inhibition of ABC transporters during cancer therapy should improve the sensitivity of cancer cells to cytotoxic agents. Several clinical trials of ABCB1-reversing agents combined with chemotherapeutic drugs are currently ongoing in cancer patients, but the results have not been satisfactory. No effective ABCB1-reversing drug without significant toxicity has been approved.^{6,7} Treatment with doxorubicin also induces *ABCB1* expression and enhances drug efflux potential through FOXO3a signaling in K562 leukemia cells.⁸ Non-synergistic effects of chemotherapy agents and ABC-reversing drugs are possible because the downstream pathway of ABCB1 is complicated. Further study of ABCB1 in cancer is needed to aid development of potential therapeutic agents.

The ability to avoid immune surveillance is important for cancer cells, which they achieve by secreting specific cytokines to recruit and activate suppressive immune cells in the tumor microenvironment, including regulatory T cells, myeloid-derived suppressor cells (MDSCs) and M2 tumor-associated macrophages.⁹ In the literature, 16 genes of immune factors are expressed by human solid-tumor cell lines, including transforming growth factor beta (gene *TGFBI*), interleukin-1 beta (IL-1 β , gene *IL1B*), IL-4, IL-6, and IL-10; granulocyte-macrophage colony-stimulating factor (gene *CSF2*); macrophage colony-stimulating factor (gene *CSF1*); indoleamine 2,3-dioxygenase (gene *IDO*); fms-related tyrosine kinase 3 ligand (gene *FLT3L*); c-kit ligand (gene *KITLG*); inducible NO synthase (gene *NOS2*); arginase-1 (gene *ARG1*); TNF-alpha (gene *TNF*); cyclooxygenase 2 (gene *PTGS2*); vascular endothelial growth factor (gene *VEGFA*), and granulocyte colony-stimulating factor (gene *CSF3*).^{10,11} Cancer cells may regulate suppressive MDSCs through these cytokines.

A patient-derived xenograft (PDX) is created by engrafting tumor tissues in immunodeficient mice. The tissue structure, cell morphology, genetic features, and molecular biology are similar to those of surgical specimens. Gradual infiltration of host immune cells has been detected after 3–5 passages.¹² Currently, the PDX model is used to identify therapeutic agents, study carcinogenesis, investigate cancer heterogeneity, or develop precision medicine.¹³ The engraftment rate of PDX depends on the tumor burden and cancer characteristics and ranges from 20% to 90%. Advanced cancers with malignant potential have the highest establishment rate; for example, colorectal, pancreatic, head and neck, and ovarian cancer. Breast cancer has the relatively lowest success rate of PDX engraftment (21–37%).¹⁴ Triple-negative breast cancer or HER2⁺ subtypes have higher success rates than estrogen receptor (ER) positive cancers.¹⁵ PDX tumors have comparable therapeutic responses to corresponding clinical observations.¹⁶ PDX models have shown superior predictive value in cell-line xenografts or genetically engineered mouse models.¹⁷

For the present study, we hypothesized that ABCB1 predicts poor prognosis of patients with breast cancer and regulates secretion of cytokines. To test this hypothesis, we studied the RNA sequencing data from The Cancer Genome Atlas Breast Invasive Carcinoma in Genomic Data Commons Data Portal (GDC TCGA-BRCA) and examined the gene expressions of ABC transporters and cytokine genes. In addition, a PDX model from one patient with ER⁻, progesterone receptor (PR)⁺, and HER2⁺ breast cancer was used to test ABCB1, immune genes, and tumor-infiltrating immune cells in breast cancer.

Methods

Bioinformatics

The Illumina platform was used to sequence the RNA of 1217 breast cancer samples, and raw data were downloaded from the GDC TCGA-BRCA.¹⁸ The latest Human Genome Assembly hg38 was used to re-analyze the results, which were re-organized by the University of California Santa Cruz Xena team.¹⁹ The upper quartile of the fragments per kilobase of transcript per million mapped reads (HTSeq-FPKM-UQ) was selected. The expressions of *ABCB1*, ABC transporters, and MDSC-associated genes were extracted for further analysis. Survival status was also obtained.

The cBioPortal platform collects multidimensional cancer genomics and datasets.^{20,21} A total of 15 breast cancer datasets with 10,928 samples were selected from cBioPortal, including those of primary and metastatic cancer. Breast fibroepithelial tumors, xenografts of breast cancer, metaplastic breast cancer, juvenile papillomatosis, or adenoid cystic

breast cancer were excluded because of different tumor pathophysiologies. Amplification, mutation, or deletion of *ABCB1* genes were explored.

Gene expression data on breast cancer were collected from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). Doxorubicin-selected MCF-7/ADR cells were compared with parental MCF-7 cells in the GSE24460 dataset to study ABC transporters in chemoresistant cells.²² The Affymetrix Human Genome U133A 2.0 Array was used to examine gene expression. Raw data were obtained and normalized according to the robust multichip average (RMA). The RMA signal was computed for gene-level probe set summaries using the Affymetrix Expression Console (version 1.3) (Affymetrix, Santa Clara, CA, USA) and R (version 3.2.0) (www.r-project.org). A heatmap of ABC transporters was drawn. *ABCB1*-overexpressing human mammary epithelial cells were compared with parental cells.²³ High-throughput RNA sequencing was performed (Illumina, San Diego, CA, USA), and 162 genes were differentially expressed. We uploaded the raw data into the Gene Set Enrichment Analysis platform and used the BIOCARTE pathways for analysis.²⁴ RNA sequencing data from HER2⁺ patients were download from the GSE43837 dataset. The levels of *ABCB1* and *ERBB2* mRNA were collected.

The Kaplan–Meier Plotter platform was used to correlate the results of the mRNA microarray and recurrence-free survival of patients with breast cancer.²⁵ The Affymetrix gene chip (Santa Clara, California, United States) using JetSet probe for *ABCB1* and 201873_s_at was used to examine mRNA expression. The cutoff point of low or high expression of *ABCB1* was based on the median mRNA level. Redundant samples and biased arrays were excluded. The Kaplan–Meier method was used to estimate survival curves and compared by performing the Log rank test. Hazard ratios with 95% confidence intervals (CIs) were used to measure the association between the expression of *ABCB1* and survival.

Patients and PDX

One PDX was obtained from a 67-year-old female patient with invasive ductal carcinoma after curative resection in 2018. The characteristics of the patient are presented in Table 1. Written informed consent was obtained from the patients, and the study was approved by the Institutional Review board of the National Cheng University Hospital (NCKUH IRB no. A-ER-106-157). The clinical outcomes were also recorded.

Animal procedures and experimental protocols, such as animal housing and care, were approved by the Laboratory Animal Center and Institutional Animal Care and Use Committee (IACUC, No. NLAC(TN)-104-M-028-R2/R3 & 107-

Table 1 Patient Characteristics

Age at surgery (years)	67
Sex (Male/Female)	Female
Diagnosis	Invasive ductal carcinoma
Operative methods	Total mastectomy and sentinel lymph-node biopsy
Tumor size	2.6 cm
Histology grade	Grade III
Extensive intraductal component	Present
Lymphatic tumor emboli	Absent
Tumor stage	pT2
Nodal stage	pN0
AJCC TNM stage	pT2N0, stage IIA
Subtype	HER2-positive
Estrogen receptor	Negative (<1%)
Progesterone receptor	Positive (5%)
Ki-67	20%
Recurrence at postoperative 23 months	Left-upper lung nodule, metastatic carcinoma of breast origin, negative ER, weak positive PR (~2%), HER2 3+ by IHC staining Right-lower lung nodule in chest computed tomography
Survival	Partial response 43 months after the first operation

Abbreviations: AJCC TNM stage, American Joint Committee on Cancer tumor–node–metastasis staging system; ER, estrogen receptor; IHC, immunohistochemistry; PR, progesterone receptor.

M-001-R1/R2, from Apr 13, 2016 to Apr 30, 2020) of the National Laboratory Animal Center of National Applied Research Laboratories, Tainan, Taiwan, and the Laboratory Animal Center (approval No. NCKU-107226 and 108114, from Aug 1, 2018 to Dec 31, 2022), College of Medicine, National Cheng Kung University. Animal studies and welfare were carried out following the Guide for the Care and Use of Laboratory Animals (eighth edition).²⁶ Fresh samples from 10 breast cancer patients were collected immediately after surgery. The cancer tissue was minced into small pieces and subcutaneously transplanted into the flanks of NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/YckNarl mice. These mice were maintained at the National Laboratory Animal Center, Tainan, Taiwan. Tumor growth was assessed by measuring two perpendicular diameters once a week. Tumor volumes were calculated as the length \times width² / 2. The relative tumor volume of each tumor was calculated as the ratio of the current volume in relation to the initial volume. The take rate was 10% in 10 breast cancer patients. The first passage (P1) of PDX was established in three mice. After tumor volumes approached 1000 mm³, the PDX cancer tissues were removed. The P1 tumor with highest growth rate was re-implanted into flanks of 20 NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/YckNarl mice to get P2 mice. Twenty P2 PDX mice were transported to the Laboratory Animal Center, College of Medicine, National Cheng Kung University. Next generation of PDX mice was established by implanting cancer tissue into the subcutaneous fat of non-obese diabetic/severe combined immunodeficiency disorder (NOD/SCID) mice. The PDX mice were maintained until passage 13.

Seventeen P2 PDX mice were treated intraperitoneally with normal saline (n = 3), doxorubicin (18 mg/kg, titrate to 12 mg/kg, n = 5), paclitaxel (10 mg/kg, n = 3), trastuzumab (30 mg/kg, n = 3), or AZD8055 (mTOR inhibitor, 20 mg/kg, n = 3) twice a week for four doses. Tumor size was recorded, and the mice were sacrificed at Day 12 after the first treatment dose. Cancer specimens were obtained for other experiments. Two passage 7 (P7) and two P9 PDXs were established in athymic nude mice. Athymic nude mice lack of T or B cells with preserved innate immunity. Tumor and spleen were collected and minced into small pieces. Intratumor and intrasplenic immune cells were isolated. Flow cytometry was performed to identify cell types. CD4⁺CD25⁺ and CD8⁺ lymphocytes were identified as negative control. Innate immunity cells were analyzed, including CD11b⁺Ly6C⁺ and CD11b⁺Ly6G⁺ monocytes, and F4/80⁺ macrophages.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

The fresh cancer tissues from P2 PDX mice after drug treatment were obtained. A RNeasy[®] Mini kit was used to extract total RNA according to the manufacturer's instructions. Single-stranded complementary DNA (cDNA) was synthesized from 2 μ g of total RNA using M-MuLV reverse transcriptase (Roche) and oligo-dT random primers. The cDNA was amplified with specific genes, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. Primers were designed to be specific for the following genes: *IL6* (NM_00600.5), colony-stimulating factor 1 (*CSF1*, NM_000757.6), *CSF3* (NM_000759.4), prostaglandin-endoperoxide synthase 2 (*PTGS2*, NM_000963.4), vascular endothelial growth factor A (*VEGFA*, NM_001025366.3), and *GAPDH* (NM_002046.3) (Table S1). SYBR green fluorescence was added. The PCR parameters were as follows: 95°C for 10 min; 95°C for 15s, 60°C for 1 min and total 40 cycles. Fluorescence increase of fluorescein was automatically measured during PCR. All samples were amplified in duplicate, and the C_T value was recorded. The 2-delta-delta C_T value was calculated following *GAPDH* normalization.

Flow Cytometry

Fresh cancer tissues and spleen from nude mice were finely minced into small pieces, then ground, filtered, and centrifugated. Detached cells were washed with PBS and incubated with commercially available anti-CD4, anti-CD25, anti-CD8, anti-CD11b, anti-Ly6G, or anti-Ly6C antibodies (BD Biosciences) under 4°C overnight. Then, 1 mL of staining buffer was added, and cells were analyzed by flow cytometry (BD Biosciences). Data analysis was performed using WinMDI 2.9.

Statistical Analysis

All statistical analyses were performed using STATA version 16.0 (StataCorp LLC, Texas, USA). Continuous variables with normal distribution were compared between two groups by performing Student's *t*-test. Results are expressed as the mean \pm standard deviation (SD). The nonparametric Wilcoxon rank-sum or Kruskal–Wallis test was performed for two or more groups of non-normally distributed continuous variables. Correlation between two continuous variables was

calculated by Spearman's method and expressed as rho (ρ) with a 95% confidence interval (95% CI). Survival curves were estimated using the Kaplan–Meier method and compared between groups by performing the Log rank test. The median split was used for turning a continuous variable into a binary variable to visualize the difference between groups in the Kaplan–Meier method. A P value <0.05 was considered to be indicative of statistical significance.

Results

ABC Transporters in Breast Cancer

There are a total of 48 ABC transporters in seven suprafamilies.¹ RNA sequencing data of the 48 ABC transporters were obtained from TCGA datasets and compared between survivors (censored) and non-survivors (expired). Lower expressions of *ABCA3*, *ABCA7*, *ABCB2*, *ABCB8*, *ABCF3*, and *ABCG1* were detected in non-survivors. In contrast, the expressions of *ABCA1*, *ABCA5*, *ABCA6*, *ABCA8*, *ABCA9*, *ABCA10*, *ABCB1*, *ABCB5*, *ABCB7*, *ABCC2*, *ABCC9*, *ABCD2*, *ABCE1*, *ABCG2*, and *ABCG4* were higher in non-survivors than in survivors (Figure S1). We also obtained consistent data and found that compared with the MCF-7 parental cells, *ABCB1* had the highest expression of ABC transporter genes in doxorubicin-selected MCF-7/ADR cells from the GSE24460 dataset (Figure S2).

In these ABC transporters, *ABCB1* is the most well-known transporter related to drug resistance. The genetic mutation rate of *ABCB1* is 1.7% in the cBioPortal database. Amplification of the *ABCB1* gene is the most common form of gene alteration in breast cancer (Figure S3). RNA sequencing data from 1217 breast cancer samples, and the corresponding patients' outcomes were obtained from the TCGA. The patients with lymph-node metastasis had a higher level of *ABCB1* mRNA (mean \pm SD = 13.9 ± 0.1 , median 13.9, range 8.0–17.6) than those without lymph-node metastasis (mean \pm SD = 13.7 ± 0.1 , median 13.8, range 7.6–17.4) (Figure 1A). Overall survival tended to be worse in the patients with higher *ABCB1* expression than in patients with lower *ABCB1* expression (Figure 1B). Details of clinical or pathological factors were absent from the TCGA database. Therefore, we used the Kaplan–Meier Plotter, a public database, to analyze survival of patients with breast cancer. The overall survival rates of patients with high or low mRNA levels of *ABCB1* were similar (Figure S4A). The patients with high *ABCB1* mRNA had worse post-progression survival ($P = 0.015$, Figure S4B). We selected patients with HER2⁺ and ESR1⁻ breast cancer according to the St. Gallen molecular subtypes. The overall survivals of high and low *ABCB1* were similar between the patients with HER2⁺ breast cancer and those receiving systemic treatment (Figure S4C and D). However, high *ABCB1* mRNA was correlated with worse prognosis of patients without lymph-node metastasis and with better prognosis in those with lymph-node metastasis (Figure S4E and F). *ABCB1* was a better predictor of poor prognosis for the HER2⁺ and ESR1⁻ patients without lymph-node metastasis. We further collected RNA sequencing data from HER2⁺ patients in the

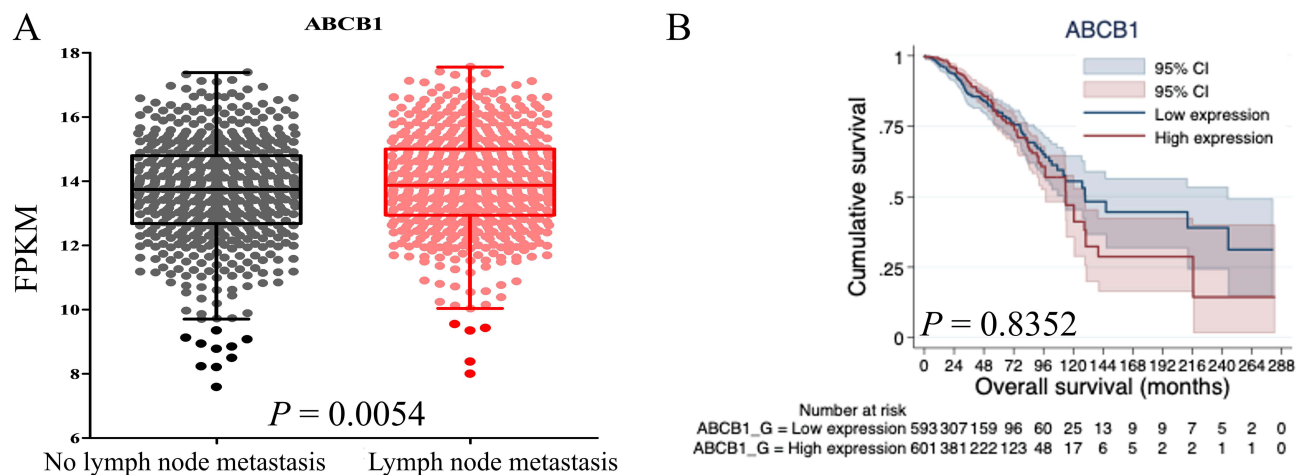


Figure 1 Expression of *ABCB1* mRNA in breast cancer samples. **(A)** Correlation of *ABCB1* expression with lymph-node metastasis of patients with breast cancer. **(B)** Overall survival of patients with high and low expression of *ABCB1* mRNA. The patients with lower expression of *ABCB1* tended to have better prognosis. *ABCB1*, ABC subfamily B, member 1; FPKM, fragments per kilobase of transcript per million mapped reads.

GSE43837 dataset. The expressions of *ABCB1* and *ERBB2* mRNA were compared, and positive correlation was showed in [Figure S5](#).

In summary, expression of *ABCB1* increased in non-survivors and in doxorubicin-selected MCF-7/ADR cells. Amplification of *ABCB1* genes was the most common type of genetic alteration and correlated with *ERBB2* level. High expression of *ABCB1* correlated with worse post-progression survival and overall survival in patients with HER2⁺, ESR1⁻, and LN⁻ breast cancer.

Expression of Immune Genes in *ABCB1*^{high} Breast Cancer

RNA sequencing data of *ABCB1* and immune genes were obtained from the TCGA dataset. Spearman's rank-order correlation coefficient (ρ) was calculated to express correlations. The results showed that *ABCB1* was positively correlated with *IL6* and *PTGS2* with stringent association by ρ values >0.4 ([Figures 2A](#) and [B](#)). Weak association between *CSF1/CSF3* and *ABCB1* was also detected by ρ values 0.3 – 0.4 ([Figures 2C](#) and [2D](#)). A negative correlation between *ABCB1* and *VEGFA* was found as shown by a ρ value <-0.3 ([Figure 2E](#)). *ABCB1* mRNA was not correlated with other cytokines (*TGFB1*, *IL1B*, *IL4*, *IL10*, *CSF2*, *FLT3*, *KITLG*, *NOS2*, *ARG1*, *IDO1*, and *TNF*) ([Figure 2F–P](#)).

Since *ABCB1* showed high expression in doxorubicin-selected breast MCF-7/ADR cells, we then performed BIOCARTA pathway analysis for the upregulated genes in *ABCB1*-overexpressing human mammary epithelial cells in the GSE173411 dataset. The data indicated that cytokines and inflammatory response-related genes, including *IL6*, *CSF1*, and *CSF3*, were significantly associated with *ABCB1*-overexpressing cells ([Figure S6](#)).

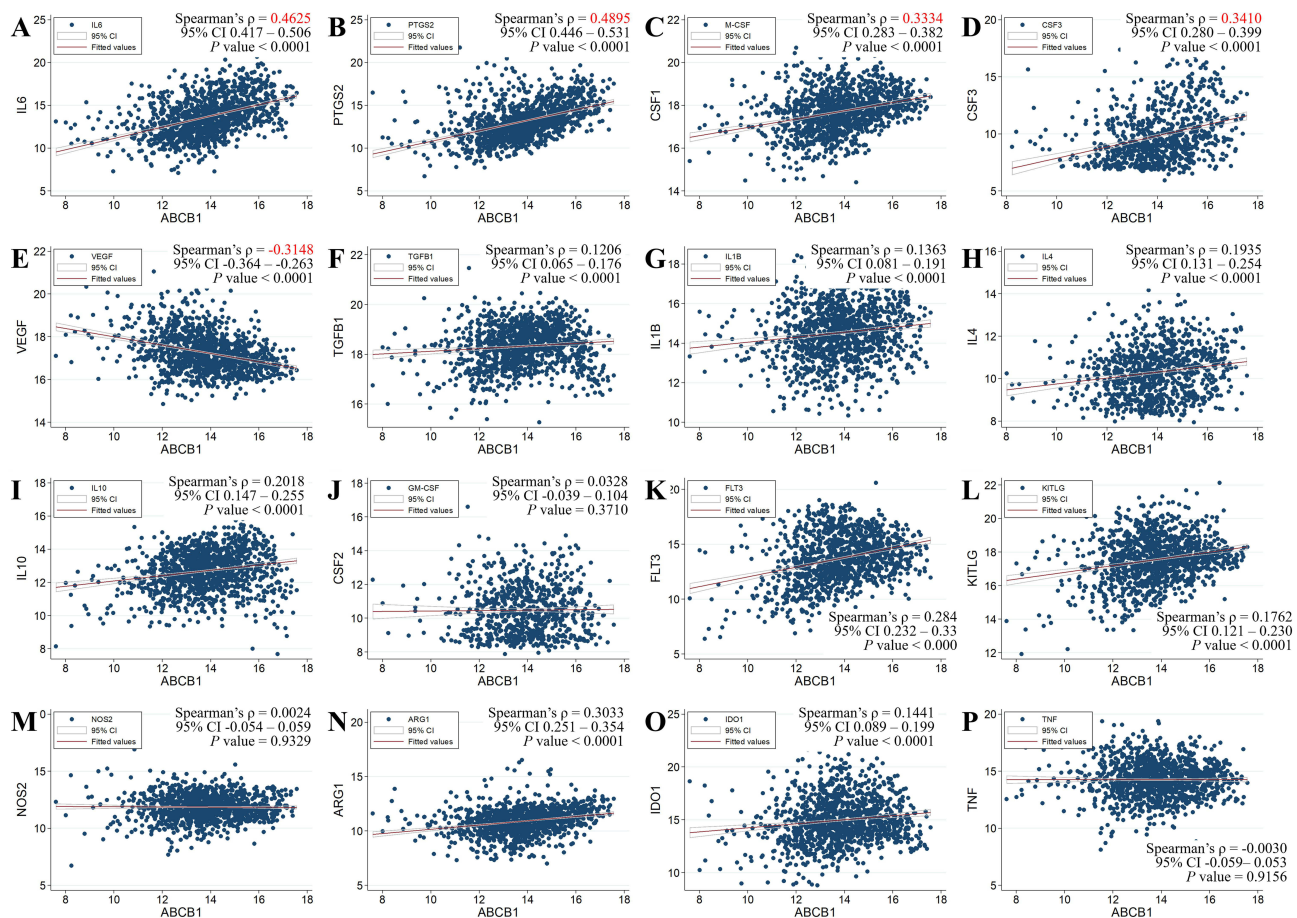


Figure 2 Correlation between the *ABCB1* gene and immune genes in 1217 breast cancer samples from the TCGA. (A) *IL6*, (B) *PTGS2*, (C) *CSF1* (M-CSF), (D) *CSF3*, (E) *VEGFA*, (F) *TGFB1*, (G) *IL1B*, (H) *IL4*, (I) *IL10*, (J) *CSF2* (GM-CSF), (K) *FLT3*, (L) *KITLG*, (M) *NOS2*, (N) *ARG1*, (O) *IDO1*, and (P) *TNF*. MDSC, myeloid-derived suppressor cell; TCGA, The Cancer Genome Atlas.

Clinical Course of a Human Patient and Establishment of a PDX Mouse Model

We used a PDX mouse model to assess the significance of *ABCBI* and immune genes in breast cancer. The postoperative pathological report of a 67-year-old female patient with invasive ductal carcinoma who underwent a total mastectomy and sentinel lymph-node biopsy in 2018 showed that she had ER⁻, PR⁺, and HER2⁺ stage IIA cancer without lymph-node metastasis. She was treated with adjuvant therapy with cyclophosphamide-epirubicin-5-fluorouracil (500–75–500 mg/m²) every 3 weeks for six courses, followed by adjuvant endocrine therapy with letrozole. After her mastectomy, a left-upper lung nodule was detected at 23 months, and a right-lower lung nodule was detected at 28 months by chest computed tomography. Bilateral lung nodules were resected by video-assisted thoracoscopic surgery. The pathological report confirmed a HER2-enriched metastatic carcinoma of breast origin. Salvage therapy with docetaxel–pertuzumab–trastuzumab (60 mg/m²–840 mg–6 mg/m²) every 3 weeks for six courses and pertuzumab–trastuzumab for a total of 1 year. The patient was maintained on trastuzumab therapy through ≥43 months after mastectomy with partial response (Table 1).

The take rate of 10 breast cancer samples was 10% in NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/YckNarl mice. The first passage (P1) of PDX was established in three mice at Day 50 after transplantation (Table S2). The mice were maintained at the National Laboratory Animal Center, Tainan, Taiwan. The P1 tumor with highest growth rate was re-implanted into back of 20 NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/YckNarl mice to get P2 mice. Twenty P2 PDX mice were transferred to the Laboratory Animal Center, College of Medicine, National Cheng Kung University. Three P2 mice were maintained until Day 49 after implantation for further passage. Next, the mice were sacrificed, and cancer tissues were re-implanted into the subcutaneous fat of NOD/SCID mice to establish the next passage of PDX. The growth rate was better for P2–P6 than for P7–P12 PDX (Figure 3A).

Phosphoinositide 3-kinases/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling is downstream of HER2 signaling.²⁷ We treated the other 17 P2 mice with standard chemotherapy drugs (paclitaxel and doxorubicin) and anti-HER2 agents (trastuzumab and AZD8055) (Figure 3B). AZD8055 is a selective ATP-competitive mTOR kinase inhibitor. There was no effect on tumor growth by treatment with AZD8055 (blue line in Figure 3B) and only a minimal tumor suppressive effect by treatment with trastuzumab (green line in Figure 3B). Treatment with doxorubicin suppressed tumor growth after post-transplantation Day 43 (red line in Figure 3B). However, significant body weight loss (>10%) was recorded in the doxorubicin group, and survival was shorter. Treatment with paclitaxel successfully suppressed the P2 xenograft growth at post-implantation Day 43 and after Day 48 (orange line in Figure 3B). The mice were sacrificed, and the xenografts were removed at post-implantation Day 62 (12 days after the first dose). The cancer tissues were collected for qPCR analysis.

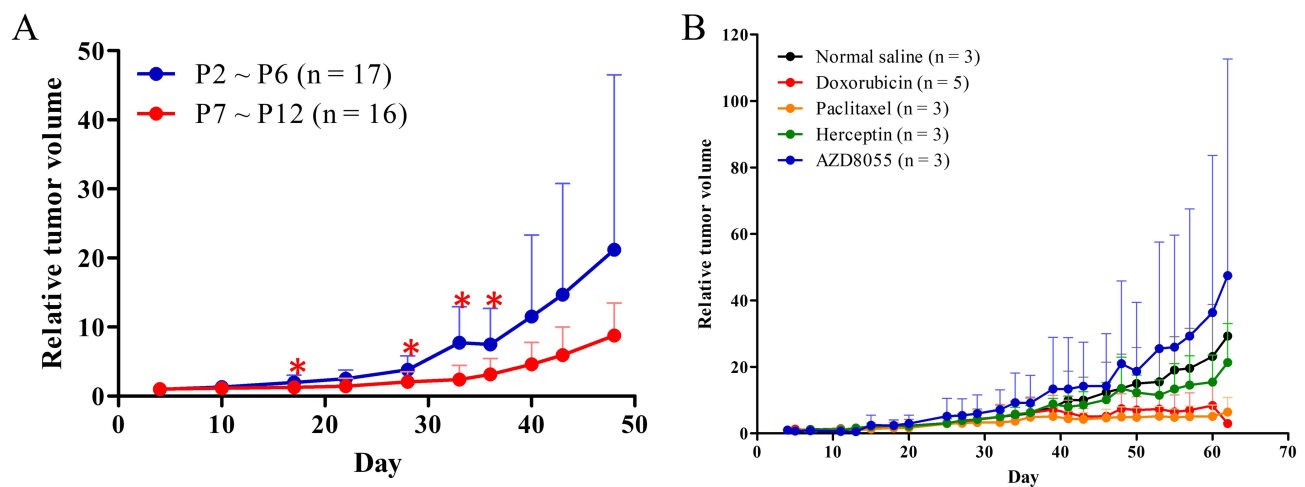


Figure 3 Relative tumor volume of PDX mice. **(A)** Serial passages of PDX compared between P2–P6 and P7–P12. The standard deviation is presented as upward error bars. The nonparametric Wilcoxon rank-sum test was used to analyze the difference between two groups. Statistically significant differences were detected on post-implantation days 17, 28, 33, and 36. **(B)** Relative tumor volume of P2 PDX mice after treatment with normal saline (black), doxorubicin (red), paclitaxel (Orange), trastuzumab (green), or AZD8055 (blue). * $P < 0.05$. PDX, patient-derived xenograft.

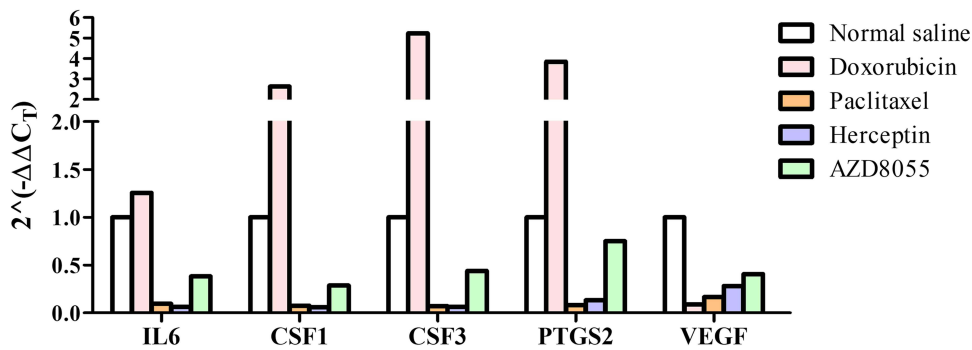


Figure 4 Expression of *ABCB1* in cancer samples from PDX mice after drug treatment. *ABCB1*, ABC subfamily B, member 1; PDX, patient-derived xenograft.

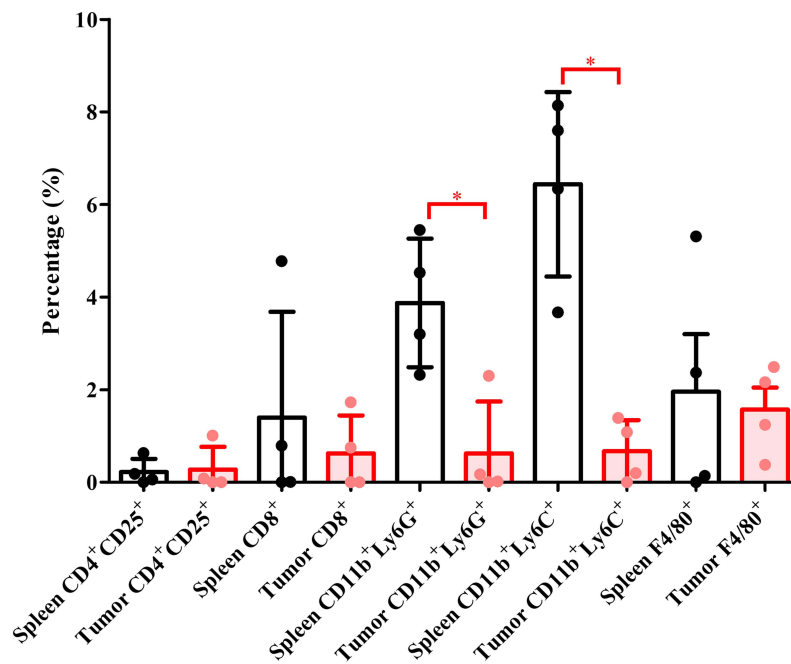


Figure 5 MDSCs in spleen and tumor samples from PDX-implanted nude mice. Intrasplenic immune cells are represented as circulatory immune cells, and intratumor immune cells correspond to the tumor microenvironment. * $P < 0.05$.

Abbreviations: MDSC, myeloid-derived suppressor cell; PDX, patient-derived xenograft.

The expressions of immune genes in the P2 xenografts were detected by qPCR and compared with the expression of *GAPDH*. The ΔC_T values were recorded and adjusted by subtracting those of the normal saline group. The expressions of *IL6*, *CSF1*, *CSF3*, and *PTGS2* were increased in the xenograft after treatment with doxorubicin but decreased after treatment with paclitaxel or trastuzumab. The expression of *VEGFA* was suppressed in all groups. Treatment with AZD8055 inhibited the expressions of *IL6*, *CSF1*, *CSF3*, and *VEGFA*, but not of *PTGS2* (Figure 4).

We also established a PDX model in nude mice to examine innate immunity in cancer-bearing mice. Intrasplenic and intratumor immune cells were analyzed by flow cytometry. The levels of CD4⁺CD25⁺, CD8⁺, and F4/80⁺ cells were low with large standard deviation in the spleen and xenograft (Figure 5). Mouse MDSCs include CD11b⁺Ly6G⁺ and CD11b⁺Ly6C⁺ cells. Intrasplenic CD11b⁺Ly6G⁺ and CD11b⁺Ly6C⁺ cells were higher than intratumor MDSCs (Figure 5).

Discussion

Early recurrence is one of the critical issues in treatment of patients with breast cancer. The PDX model was established to test drugs and study the mechanism of carcinogenesis or cancer heterogeneity. In our data, the expression of *ABCB1*

was correlated with lymph-node metastasis and poor survival in 1217 patients with breast cancer in the GDC TCGA-BRCA database. Increased expression of *ABCB1* gene was detected in doxorubicin-resistant MCF-7/ADR cells. High expression of *ABCB1* was correlated with increased *IL6*, *CSF1*, *CSF3*, and *PTGS2* levels and decreased *VEGFA* level. Cytokine- and inflammatory response-related genes, including *IL6*, *CSF1*, and *CSF3*, were significantly associated with *ABCB1*-overexpressing cells. We also used a PDX model to study the expressions of *ABCB1* and cytokine genes. Treatment with doxorubicin and paclitaxel suppressed growth of a PDX tumor; however, monotherapy with anti-HER2 monoclonal antibodies (trastuzumab) or mTOR inhibitor (AZD8055) failed to suppress tumor growth. The expression of *VEGFA* was repressed after treatment with doxorubicin, paclitaxel, trastuzumab, and AZD8055. The expressions of *IL6*, *CSF1*, *CSF3*, and *PTGS2* increased after treatment with doxorubicin, but decreased after treatment with paclitaxel or trastuzumab. Expansion of splenic MDSCs (CD11b⁺Ly6G⁺ and CD11b⁺Ly6C⁺ cells) was detected in PDX-implanted nude mice. The human donor for the PDX developed cancer recurrence and lung metastasis 23 months after curative resection and adjuvant chemotherapy with anthracycline-based regimen. The metastatic cancer was well controlled after a taxane-based regimen with anti-HER2 therapy, which was consistent with the results of suppression of *ABCB1* and immune genes in the PDX model.

The PDX model was initially established to study cancer heterogeneity and preclinical drug testing.²⁸ The successful rate of PDX engraftment in breast cancer ranges from 21% to 37%, which is lower than that in other kinds of cancer. The take rate is highest in TNBC (51.3%), followed by HER2⁺ cancer (26.5%).¹⁴ Our patient had pT2N0, stage IIA, HER2⁺ invasive ductal carcinoma. Cancer recurrence with lung metastasis developed after curative surgery and adjuvant anthracycline-based chemotherapy. The mechanism of drug resistance to anthracycline is complicated, and activation of MDSCs to inhibit cytotoxic T cells has been reported.²⁹ An increase in circulating MDSCs has been correlated with cancer stage and treatment with doxorubicin–cyclophosphamide. By contrast, the level of MDSCs has been shown to decrease to pretreatment status after paclitaxel.³⁰ Our findings are consistent with the change in MDSC level in a previous report. Immune genes were increased in the PDX tumors after treatment with doxorubicin and reduced after paclitaxel. Implantation of PDX in nude mice was also useful for studying immune cells in circulation or the tumor microenvironment; the formers were considered to be intrasplenic cells, and the latter were intratumor cells. The level of intrasplenic MDSCs (CD11b⁺Ly6G⁺ and CD11b⁺Ly6C⁺ cells) increased. These results confirmed the usefulness of the PDX model for studying MDSCs and cytokines.

Chemoresistance is a major challenge for the treatment of patients with breast cancer, and the mechanism is complex. Membranous transporters of the ABC superfamily use the energy from ATP hydrolysis to actively pump substrates out through the cell membrane. ABC transporters also function in cell apoptosis, energy metabolism, and material transport. In cancer cells, ABC transporters participate in detoxification and drug resistance.¹ MDR1 (gene: *ABCB1*, also named P-glycoprotein, P-gp) is important in breast cancer chemoresistance. Increased expression of *ABCB1* protein has been detected in recurrent breast cancer and patients with *ABCB1*-positive cancer who were unresponsive to chemotherapy.³ *ABCB1* genes are overexpressed in doxorubicin-resistant MCF7 breast cancer cells.⁴ Other studies have reported that loss of *ABCB1* protein in immunohistochemistry staining was correlated with triple-negative breast cancer, lymph-node metastasis, larger tumor size, and poor prognosis of patients.³¹ In our study, we collected RNA sequencing data from 1217 breast cancer samples, and *ABCB1* gene expression was higher in non-survivors and patients with lymph-node metastasis. High expression of *ABCB1* mRNA was correlated with post-progression survival of all patients with breast cancer and overall survival of HER2⁺, ESRI⁻, and LN⁻ patients. Based on our results, *ABCB1* performed as a tumor promoter in HER2⁺ breast cancer.

There is no direct evidence linking expression of *ABCB1* with secretion of cytokines except in several related reports. Increased expression of *PTGS2* genes has been detected by *ABCB1*-expressing normal human mammary epithelial cells, and *PTGS2* was found to encode cyclooxygenase-2 protein.²³ Treatment with paclitaxel has been shown to suppress secretion of VEGF from cancer cell lines.³² In our data, the expressions of *IL6*, *CSF1*, *CSF3*, and *PTGS2* increased in the PDX tumor after treatment with doxorubicin but decreased with treatment of paclitaxel or trastuzumab. Expression of *VEGFA* was suppressed in all groups. An increased number of intrasplenic MDSCs was also detected in PDX-implanted nude mice.

A limitation of this study was the small sample size. The successful rate of PDX engraftment was quite low, and we only had one PDX model. To solve this problem, we used RNA sequencing data from 1217 breast cancer samples to study the correlation between *ABCB1* and immune genes. High expression of *ABCB1* was correlated with increased levels of *IL6*, *CSF1*, *CSF3*, and *PTGS2* and a decreased level of *VEGFA*. Larger RNA sequencing datasets could provide useful evidence of correlations, and PDX is an operable model for assessing causal relationships. The two experimental methods are complementary.

In summary, we found that *ABCB1* mRNA expression was correlated with lymph-node metastasis and poor survival in patients with breast cancer. High expression of *ABCB1* was correlated with increased expressions of *IL6*, *CSF1*, *CSF3*, and *PTGS2* and decreased expression of *VEGFA*. Treatment with doxorubicin and paclitaxel suppressed growth of PDX tumors and repressed *ABCB1* and *VEGF* expression. The expression of *IL6*, *CSF1*, *CSF3*, and *PTGS2* increased after treatment with doxorubicin, but decreased after treatment with paclitaxel or trastuzumab. The PDX model reflected the clinical course of the donor patient. The combination of big data from RNA sequencing and experimental results from a PDX model represents a foundation for precision medicine.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval and Informed Consent

This study was approved by the Institutional Review Board of National Cheng Kung University Hospital (A-ER-106-157) and was performed according to the Declaration of Helsinki. Written informed consent was obtained from all participants. The animal study was approved by the National Laboratory Animal Center of National Applied Research Laboratories, Tainan, Taiwan (NLAC(TN)-104-M-028-R2/R3 & 107-M-001-R1/R2), and the Laboratory Animal Center, College of Medicine, National Cheng Kung University (NCKU-107226 and 108114).

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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