

# The Association of Estrogen Receptor Activity, Interferon Signaling, and MHC Class I Expression in Breast Cancer

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# Abstract

**Background:** The expression of major histocompatibility complex class I (MHC I) has previously been reported to be negatively associated with estrogen receptor (ER) expression. Furthermore, the expression of MHC I, the level of tumor-infiltrating lymphocytes (TILs), and the expression of interferon (IFN) mediator MxA are positively associated with one another in human breast cancers. This study aimed to investigate the mechanisms of association of MHC I with estrogen and IFN signaling.

**Methods:** The human leukocyte antigen (HLA)-ABC protein expression was analyzed in breast cancer cell lines. The expressions of *HLA-A* and *MxA* mRNAs were analyzed in MCF-7 cells in Gene Expression Omnibus (GEO) data. ER and HLA-ABC expressions and TIL levels in tumor tissue were also analyzed in ER+/ human epidermal growth factor receptor 2 (HER2)- breast cancer patients who randomly received either neoadjuvant chemotherapy or estrogen modulator treatment following surgical resection.

**Results:** HLA-ABC protein expression was decreased after  $\beta$ -estradiol treatment or hESR-GFP transfection and increased after fulvestrant or IFN- $\gamma$  treatment in breast cancer cell lines. In GEO data, *HLA-A* and *MxA* expression was increased after *ESR1* shRNA transfection. When comparing the two patient groups, ER Allred score was significantly lower and the HLA-ABC expression and TIL levels were significantly higher in the estrogen modulator treated group than the chemotherapy treated group.

**Conclusion:** MHC I expression and TIL levels might be affected by ER pathway modulation and IFN treatment. Further studies elucidating the mechanism of MHC I regulation could suggest a way to boost TIL influx in cancer in a clinical setting.

## Introduction

Tumor-infiltrating lymphocytes (TILs) have consistently been reported to play an important role in breast cancer.<sup>1-7</sup> TILs have a strong prognostic and predictive significance, particularly in triple-negative breast cancer (TNBC). CD8 + cytotoxic TILs are activated by the T cell receptor-recognition of a specific peptide, which is generally generated from endogenous proteins, and are presented by a major histocompatibility complex class I (MHC I) on the surface of tumor cells.<sup>8</sup> The recognition of these peptides by cytotoxic CD8 + TILs triggers a series of events that can result in tumor cell lysis. A better understanding of TILs and related features could facilitate the development of efficient immunotherapeutic approaches in breast cancer.

MHC I proteins are membrane proteins that are expressed on almost all nucleated cells and are encoded by human leukocyte antigen (HLA)-A, -B, and -C genes.<sup>9</sup> The expression of HLAs varies from tissue to tissue and is largely stimulated by interferon (IFN) signaling.<sup>10 11</sup> The downregulation of HLAs is frequently observed in tumors and is reported to be correlated with disease progression.<sup>10</sup> Aberrant HLA expression in tumor cells might be caused by alterations in *HLA* gene transcription, the translation of *HLA* mRNA, or post-translational modifications. Torigoe *et al.*<sup>12</sup> established a monoclonal anti-pan HLA class I

antibody suitable for the immunostaining of formalin-fixed tissue and found a high rate (85%, 35 out of 41 cases) of HLA downregulation in breast cancer compared with other malignancies (20–42%). Since HLA expression on tumor cells is important for the function of TILs, the downregulation of HLA might compromise the effective immune response in patients with breast cancer. Moreover, recent studies have reported increased IFN signaling in cancer cells and their association with a good response to anthracycline-based chemotherapy in breast cancer.<sup>13</sup> However, HLA expression, the level of IFN signaling activation, and their relationship in normal breast tissue and various subtypes of breast cancer have not been extensively studied.

We previously demonstrated the differential expression of HLA-ABC in breast cancer. HLA-ABC protein expression was negatively correlated with estrogen receptor (ER) protein expression but was not significantly correlated with human epidermal growth factor receptor 2 (HER2) protein expression.<sup>14 15</sup> HLA-ABC expression was higher in TNBC and hormone receptor (HR)-/HER2 + breast cancers than in HR + breast cancers, was positively correlated with TILs, and was associated with better clinical outcomes in breast cancer patients.<sup>1 14 15</sup> In The Cancer Genome Atlas (TCGA) data analysis, *HLA-A* gene expression was positively correlated with *CD8B* gene expression but was not significantly correlated with the total number of mutations. Instead, *HLAs*, *CD3*, and *CD8* gene expression were positively correlated with IFN receptor genes and the IFN-inducible *MxA* gene.<sup>14</sup> Additionally, MxA protein expression was higher in TNBC than in other types of breast cancer, was positively correlated with TIL levels, and was associated with better clinical outcomes.<sup>16</sup> Therefore, it can be hypothesized that ER activity, IFN signaling, and MHC I expression regulate one another and influence TIL influx.

The current study aimed to clarify the mechanisms of the association of MHC I with estrogen and IFN signaling.

## Materials And Methods

### Cell lines, cultures, drug treatments, and plasmid transfections

This study used breast cancer cell lines obtained from ATCC, including ER $\alpha$ + (MCF-7 and T47D) and ER $\alpha$ - (MDA-MB-231). The MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (Cat No. 11995, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Cat No. 16000, Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Cat No. 15140, Invitrogen). The T47D and MDA-MB-231 cells were grown in Roswell Park Memorial Institute 1640 (Gibco, El Paso, TX) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were cultured at 37°C in the presence of 5% CO<sub>2</sub>. The cells were starved for 24 hours and treated with ICI (fulvestrant, Sigma-Aldrich, St. Louis, MO, 1 to 10  $\mu$ M), IFN- $\gamma$  (R&D Systems, Minneapolis, MN, 100 units/ml), or  $\beta$ -estradiol (Sigma-Aldrich, 1 nM) in 2 ml of medium for an appropriate time. The cells were then used in the protein expression assays.

For the *ESR1* plasmid transfection, ER $\alpha$ -cells were plated and cultured in a 6-well plate at 90% confluency and transfected with 2.5  $\mu$ g of hESR-GFP (Cat No. #28230, Addgene, Cambridge, MA) using 3.75  $\mu$ l of

Lipofectamine® 3000 reagent (Life Technologies) and 5 µl of P3000™ reagent (Life Technologies) per well according to the manufacturer's protocol.

### **Protein isolation and Western blotting**

The cells were lysed with RIPA buffer, and the Pierce BCA Protein Assay Reagent Kit (Cat No. 23225, Thermo Fisher, Waltham, MA) was used to measure the protein concentration. Approximately 10 µg of protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). Table 1 summarizes the antibodies used for protein detection. Equal loading of the protein samples was verified with an antibody to β-actin. Immunoreactive signals were detected with the Promega Western Blot Detection System (Cat No. W1008).

### **GEO data analysis**

We analyzed one dataset from the GEO database that included Affymetrix RNA microarray analysis data from MCF-7 breast cancer cells that were transfected with *ESR1* shRNA (5'-GCTTCAGGCTACCATTATGttcaagagacataATGGTAGCCTGAAGCttttttacgcgt -3') (accession number: GDS4061).<sup>17</sup> The fold changes of *ESR1*, *HLA-A*, and *MxA* mRNA expressions were calculated.

### **Patients and tissue specimens**

A total of 126 patients who were diagnosed with ER+/HER2- invasive ductal carcinoma were randomized to receive either estrogen modulator treatment or chemotherapy for 24 weeks as their neoadjuvant systemic therapy.<sup>18</sup> We analyzed the clinicopathologic data of the patients and the HLA-ABC and ER protein expressions and TIL levels in the pre-neoadjuvant biopsy tissues and the post-neoadjuvant resected tissues.

### **Histological evaluation**

The histologic type was defined based on the 2012 WHO classification criteria, and the histologic grade was assessed using the modified Bloom–Richardson classification.<sup>19</sup> The hematoxylin and eosin (H&E)-stained slides were histopathologically analyzed for TILs (defined as the percentage of the invasive carcinoma's stroma that was infiltrated by lymphocytes in 10% increments, if less than 10% of the stroma was infiltrated by TILs, 1% or 5% criteria were used, all available full sections were evaluated), histological subtype and grade, tumor size, pT stage, pN stage, and lymphovascular invasion.<sup>1 20</sup> The tumor response to neoadjuvant systemic therapy was evaluated based on the Miller-Payne grade (1, no change, 2, up to 30% reduction, 3, 30–90% reduction, 4, more than 90% reduction, 5, no residual malignant cells).<sup>21</sup> A pathologic complete response (pCR) was defined as the absence of residual invasive cancer cells in the breast and lymph nodes.<sup>22</sup>

### **Tissue microarray construction and immunohistochemical evaluation**

Among the 126 patients, fifty-six were available for formalin-fixed, paraffin-embedded (FFPE) tissue blocks of both biopsies and resected specimens. Each resected tissue sample was arrayed in three 1-mm diameter cores to minimize tissue loss and overcome tumor heterogeneity. Full sections of biopsy tissues and tissue microarray sections of the resected tissues were stained with an automatic immunohistochemical staining device (Benchmark XT, Ventana Medical Systems, Tucson, AZ). Table 2 summarizes the antibodies used for the immunohistochemical staining.

ER and progesterone receptor (PR) levels were regarded as positive if there was at least 1% positive tumor nuclei staining.<sup>23</sup> Additionally, the Allred score, which is the sum of the intensity score (0–3) and the proportion score (0–5), was calculated for the ER and PR.<sup>24</sup> HR+ tumors were defined as those determined to be ER-positive and/or PR-positive. HER2-overexpressing tumors were defined as those with scores of 3+ according to the immunohistochemistry or gene amplification by silver in situ hybridization.<sup>25</sup> The HLA-ABC staining was semi-quantitatively evaluated as the H-score, which is the product of the actual percentage of positive-stained cells and the intensity score (0–3), the H-score can range from 0–300. We then categorized HLA-ABC expression in tumor cells as one of two levels (negative, H-score < 50, positive, H-score  $\geq$  50).

## Statistical analysis

All statistical analyses were performed using R version 3.2.3.<sup>26</sup> The Wilcoxon rank-sum test, chi-square test, Fisher's exact test, and log-rank test were used as appropriate. All tests were two-sided, and statistical significance was set at 5%.

## Results

### The expression of HLA-ABC in breast cancer cell lines treated with ICI or IFN- $\gamma$

We evaluated the expression of the HLA-ABC protein in the ER-positive cell lines (MCF-7 and T47D) and in an ER-negative cell line (MDA-MB-231) by Western blot analysis. The baseline HLA-ABC protein expression was higher in the MDA-MB-231 cell line than in the MCF-7 and T47D cell lines (Figure 1A). Next, we treated the MCF-7 cells with 1 to 10  $\mu$ M ICI, which is an ER downregulator, for 24 or 48 hours. The ER protein expression decreased and HLA-ABC increased with ICI treatment under all conditions (Figure 1B). When the MCF-7 cells were treated with 100 units/ml of IFN- $\gamma$  with or without 1 nM of estradiol for 48 hours, the HLA-ABC protein expression was markedly increased (Figure 1C).

### The expression of HLA-ABC in the ER-negative breast cancer cell line transfected with hESR-GFP

We also observed changes in the HLA-ABC protein expression in the ER-negative MDA-MB-231 breast cell line. Twenty-four or forty-eight hours after transfection with the hESR-GFP plasmid, the MDA-MB-231 cells showed ER expression and decreased HLA-ABC protein levels (Figure 2A). When the MDA-MB-231 cells were treated with 100 units/ml of IFN- $\gamma$  with or without 1 nM of estradiol for 48 hours, HLA-ABC protein expression was markedly increased (Figure 2B).

## The change of *HLA-A* and *MxA* mRNA expressions in breast cancer cell lines in the GEO data

We also analyzed the *HLA-A* and *MxA* mRNA expressions in breast cancer cells from the GEO database. When the MCF-7 cells were transfected with *ESR1* shRNA, the *ESR1* mRNA expression decreased, and the *HLA-A* and *MxA* mRNA expressions increased (Figure 3). The fold changes were 0.360, 1.225, and 1.156 for *ESR1*, *HLA-A*, and *MxA*, respectively.

## The changes of ER and HLA-ABC expressions and TIL levels after neoadjuvant chemotherapy or estrogen modulators in breast cancer patients

The clinicopathologic characteristics of the 126 patients who randomly received chemotherapy (Adriamycin and cyclophosphamide) or estrogen modulator treatment (tamoxifen and goserelin) as their neoadjuvant systemic therapy and following surgical resection were analyzed (Table 3). Before the neoadjuvant therapy, there were no differences between the two groups in patient age, cT and cN stages, ER Allred score, and TIL levels in biopsy specimens. After neoadjuvant therapy, no significant difference was observed in the pCR ratio ( $P = 0.058$ ). However, the chemotherapy group showed significantly lower ypT and ypN stages ( $P < 0.001$  and  $P = 0.016$ , respectively) and a more reduced tumor burden according to the Miller-Payne grade ( $P < 0.001$ ). The estrogen modulator group showed more frequent lymphovascular invasion ( $P = 0.046$ ), a lower ER Allred score ( $P = 0.027$ ), and higher TIL levels ( $P < 0.001$ ) in resected specimens than the neoadjuvant chemotherapy group (Figures 4 and 5).

Next, we evaluated HLA-ABC expression in both the biopsy and resection tissues of fifty-six patients by immunohistochemistry (Table 3). Before neoadjuvant therapy, the HLA-ABC positivity in tumor cells did not differ significantly between the two groups ( $P = 0.243$ ). After neoadjuvant systemic therapy, however, the estrogen modulator group showed higher HLA-ABC positivity than the chemotherapy group ( $P = 0.038$ ).

The overall survival and recurrence-free survival between the two groups did not differ significantly ( $P = 0.396$  and  $0.758$ , respectively) (Figure 6).

## Discussion

Recently, the significance of TILs, particularly that of CD8 + cytotoxic T cells, in breast cancer has been revealed.<sup>4 27–29</sup> The expression of MHC I proteins on the tumor cell surface is essential for CD8 + T cells to act, and several reports have documented the positive relationship between TIL levels and MHC I expression. We previously reported that TILs were more abundant in tumors with a stronger expression of HLA-ABC, and abundant TILs was an independent prognostic factor for better disease-free survival in HER2-positive breast cancer patients treated with chemotherapy and trastuzumab.<sup>15</sup> We also reported that the HLA-ABC expression of the tumor cells was positively correlated with TIL levels in consecutive breast cancer and TNBC cohorts.<sup>14</sup> Although some may think that high HLA-ABC expression is due to a high mutation rate and more immunogenic mutations, we previously revealed that the total number of mutations was not associated with *HLA-A* expression in the tumor,<sup>14</sup> and Spranger *et al.* also reported

that the density of nonsynonymous somatic mutations is not significantly associated with T cell related gene expression.<sup>30</sup>

Instead, some evidence has indicated that MHC I expression is related to ER expression and IFN signaling. In our previous study, HLA-ABC expression was negatively associated with ER expression in a consecutive breast cancer cohort and normal breast tissue, and *HLA* mRNA expression was positively correlated with IFN-associated gene expression in a TCGA and Cancer Cell Line Encyclopedia data analysis.<sup>14</sup> We also reported that the high expression of IFN-mediator *MxA* in the tumor cells was positively associated with TIL levels, CD8 + cell number, and stronger HLA-ABC expression and was an independent prognostic factor for better disease-free survival in breast cancer.<sup>16</sup> Although one article has reported that ER $\alpha$  signaling modulates IFN- $\gamma$  inducible MHC II expression through class II transactivator in breast cancer cells, the mechanism of the relationship between HLA-ABC expression, ER activity, and IFN signaling in breast cancers has never been reported.<sup>31</sup>

In the current study, we revealed that the expressions of HLA-ABC protein and *HLA-A* mRNA in breast cancer cells are negatively affected by ER signaling *in vitro*. We insist that this is a novel and important finding in addition to previously known tumor-intrinsic oncogenic pathways that have been suggested to be associated with the reduction of immune reactions, such as the WNT/ $\beta$ -catenin pathway, the mitogen-activated protein kinase pathway, the PI3K/AKT pathway, MYC upregulation, and CDK4/6 activation.<sup>32</sup> The baseline HLA-ABC protein expression was higher in ER-negative cells than in ER-positive cells, and HLA-ABC protein expression was increased after ICI treatment and decreased after ER overexpression. *HLA-A* mRNA expression was also increased after *ESR1* shRNA transfection. We also revealed a positive association between HLA-ABC and IFN signaling and a negative association between ER and IFN signaling. Breast cancer cells showed increased HLA-ABC protein expression after IFN treatment and decreased *MxA* mRNA expression after *ESR1* shRNA transfection. This is the first study that analyzed the relationship between HLA-ABC expression, ER activity, and IFN signaling in breast cancer *in vitro*.

We also analyzed the breast cancer tissues of patients who received either neoadjuvant chemotherapy or estrogen modulator therapy. We revealed that the HLA-ABC expression in breast cancer cells increased and ER expression decreased after neoadjuvant estrogen modulator treatment compared with neoadjuvant chemotherapy. Estrogen modulator treatment is known to negatively regulate ER signaling in breast cancer.<sup>33</sup> By combining the results of the *in vitro* experiments, our data suggested that ER signaling was downregulated by neoadjuvant estrogen modulator treatment, and this caused the increase of HLA-ABC expression in tumor cells. We also analyzed the TIL levels in biopsies and resection tissues from the two groups. TIL levels were significantly decreased in resected tissue after neoadjuvant chemotherapy compared with biopsy tissue, which is concordant with a previous publication.<sup>34</sup> This might be explained by the fact that conventional chemotherapeutic agents are generally toxic to proliferative cells.<sup>35</sup> Although the HLA-ABC expression in tumor cells was increased in the estrogen modulator treatment group, we did not identify a significant change in TIL levels in the estrogen

modulator treatment group. This is possibly due to the insufficient duration of neoadjuvant estrogen modulator therapy. Further studies with a longer duration of neoadjuvant treatment are necessary.

This study has several limitations. First, our data do not include *in vitro* results with estrogen treatment, the key molecule in ER signaling, or with tamoxifen treatment, an important selective ER modulator drug. Second, molecular mechanisms and mediators on how HLA-ABC expression and IFN signaling are regulated by ER signaling must be identified. Therefore, further experiments are necessary to more precisely define these molecular mechanisms. We suggest that more significant results might be obtained by future studies with more patient samples and a longer follow-up duration.

## Conclusions

This study demonstrated that MHC I expression and TIL levels were affected by ER pathway modulation and IFN treatment. Further studies elucidating the mechanism of MHC I regulation could suggest a way to boost TIL influx in cancer in a clinical setting.

## Abbreviations

ER, estrogen receptor

FFPE, formalin-fixed, paraffin-embedded

GEO, Gene Expression Omnibus

H&E, hematoxylin and eosin

HER2, human epidermal growth factor receptor 2

HLA, human leukocyte antigen

HR, hormone receptor

IFN, interferon

MHC I, major histocompatibility complex class I

pCR, pathologic complete response

PR, progesterone receptor

TCGA, The Cancer Genome Atlas

TIL, tumor-infiltrating lymphocyte

TNBC, triple-negative breast cancer

# Declarations

## Ethics approval

This study was approved by the Institutional Review Board of the Asan Medical Center.

## Consent for publication

Not applicable

## Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

## Competing interest

The authors declare that they have no conflicts of interest.

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## Authors' contributions

Conceptualization: HJL, GG

Design of the work: HJK, SHA, HJL, GG

Data acquisition: IHS, YK, SH, WSB, HSP

Data interpretation: IHS, HJL, GG

Writing – original draft preparation: IHS

Writing – review and editing: All authors

Approval of final manuscript: All authors

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## Tables

Table 1. Experimental conditions for Western blotting.

Primary antibody	Provider	Dilution
β-actin	Abcam, Cambridge, UK	1:3000
ERα	Novus Biologicals, Littleton, CO	1:200
HLA-ABC	Abcam, Cambridge, UK	1:3000

Abbreviations: ER, estrogen receptor, HLA, human leukocyte antigen.

Table 2. List of antibodies for immunohistochemistry.

Primary antibody	Provider	Dilution
ER	Leica Biosystems, Newcastle, UK	1:200
PR	Leica Biosystems	1:200
HER2	Ventana Medical Systems, Tucson, AZ	1:8

Abbreviations: ER, estrogen receptor, PR, progesterone receptor, HER2, human epidermal growth factor receptor 2, HLA, human leukocyte antigen.

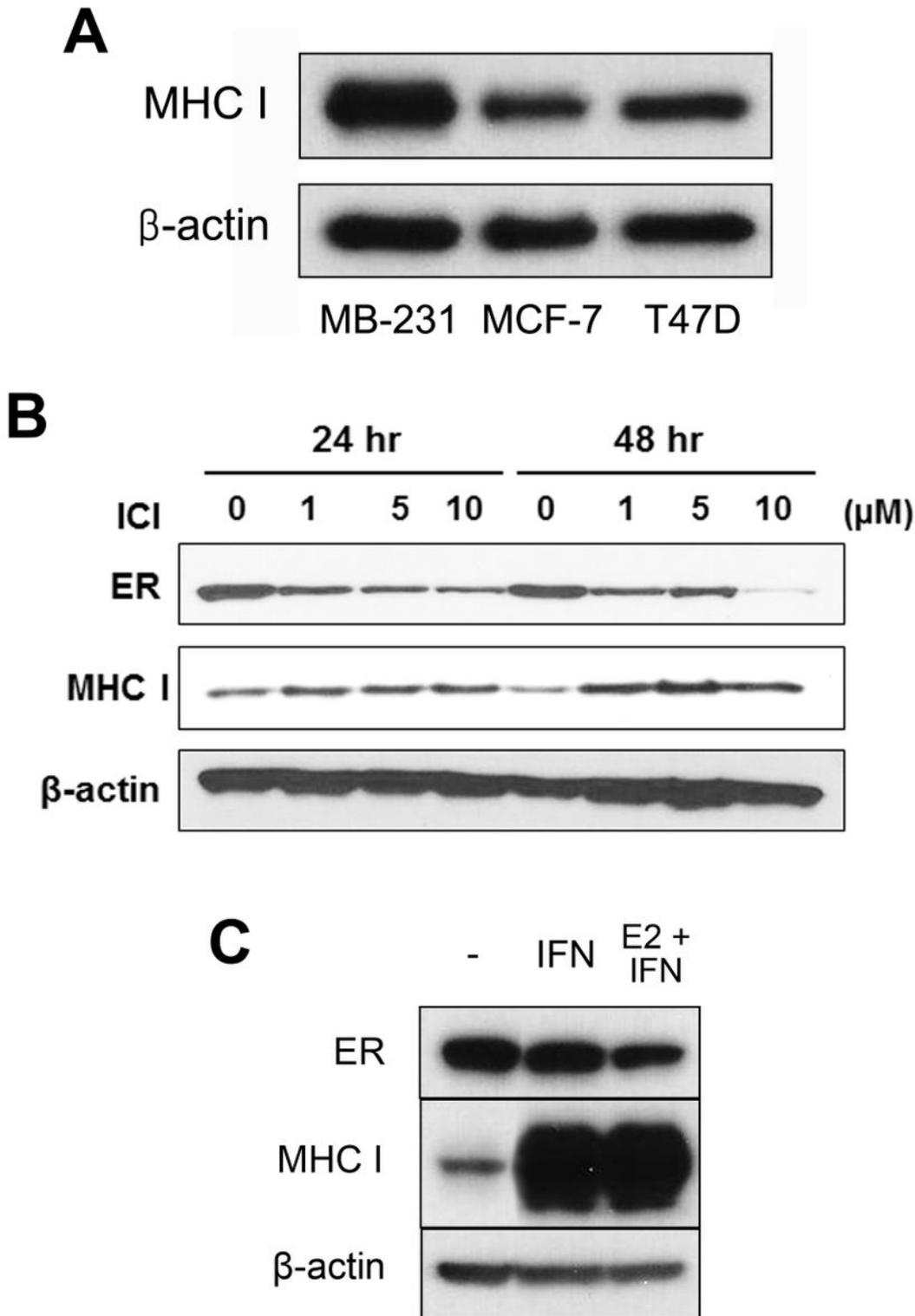
Table 3. Comparison of clinicopathologic variables according to neoadjuvant systemic therapy in breast cancer patients.

Variables	Neoadjuvant systemic therapy		p value
	Chemotherapy (n = 65)	Estrogen modulator (n = 61)	
Pre-neoadjuvant			
Age at diagnosis (years), Median (IQR)	43 (38–46)	42 (38–46)	0.784
cT			0.522
1	12 (18.6)	7 (11.5)	
2	42 (64.6)	42 (68.9)	
3	10 (15.4)	12 (19.7)	
4	1 (1.5)	0 (0.00)	
cN			1
0	1 (1.5)	0 (0.00)	
1	54 (83.1)	52 (85.2)	
2	4 (6.2)	4 (6.6)	
3	6 (9.2)	5 (8.2)	
ER Allred score in biopsy, Median (IQR)	8 (8–8)	8 (8–8)	0.467
HLA expression in biopsy			0.243
Negative	17 (65.4)	24 (80.0)	
Positive	9 (34.6)	6 (20.0)	
TIL level in biopsy, Median (IQR)	10 (0–12.5)	10 (0–20)	0.514
Post-neoadjuvant			
Histologic grade			0.358
1	4 (6.9)	3 (5.0)	
2	46 (79.3)	53 (88.3)	
3	8 (13.8)	4 (6.7)	
ypT			<b>&lt;0.001</b>
0	7 (10.8)	1 (1.6)	

1	32 (49.2)	12 (19.7)	
2	20 (30.8)	37 (60.7)	
3	6 (9.2)	9 (14.8)	
4	0 (0.0)	2 (3.3)	
ypN			<b>0.016</b>
0	8 (12.3)	2 (3.3)	
1	39 (60.0)	27 (44.3)	
2	15 (23.1)	23 (37.7)	
3	3 (4.6)	9 (14.8)	
Miller-Payne grade			<b>&lt;0.001</b>
1	2 (3.1)	20 (32.8)	
2	10 (15.4)	24 (39.3)	
3	36 (55.4)	14 (23.0)	
4	10 (15.4)	2 (3.3)	
5	7 (10.8)	1 (1.6)	
Pathologic complete response			0.058
No	60 (92.3)	61 (100.0)	
Yes	5 (7.7)	0 (0.0)	
LVI			<b>0.046</b>
Absent	36 (55.4)	22 (36.1)	
Present	29 (44.6)	39 (63.9)	
HLA expression in resected tissue			<b>0.038</b>
Negative	17 (68.0)	10 (35.7)	
Positive	8 (32.0)	18 (64.3)	
TIL in resected tissue,			<b>&lt;0.001</b>
Median (IQR)	0 (0–10)	10 (10–20)	

Abbreviations: AC, adriamycin + cyclophosphamide, ER, estrogen receptor, IQR, interquartile range, LVI, lymphovascular invasion, TIL, tumor-infiltrating lymphocyte.

## Figures



**Figure 1**

The effect of estrogen signaling and interferon signaling on HLA-ABC expression. (A) The baseline HLA-ABC protein expression is higher in ER-negative MDA-MB-231 cell lines than in ER-positive MCF-7 and T47D cell lines. (B) After 24 or 48 hours of ICI treatment (1 to 10  $\mu\text{M}$ ), the ER protein expression decreased, and HLA-ABC protein increased in MCF-7 cells. (C) The HLA-ABC protein expression increased in MCF-7 cells 48 hours after 100 units/ml of IFN- $\gamma$  treatment.

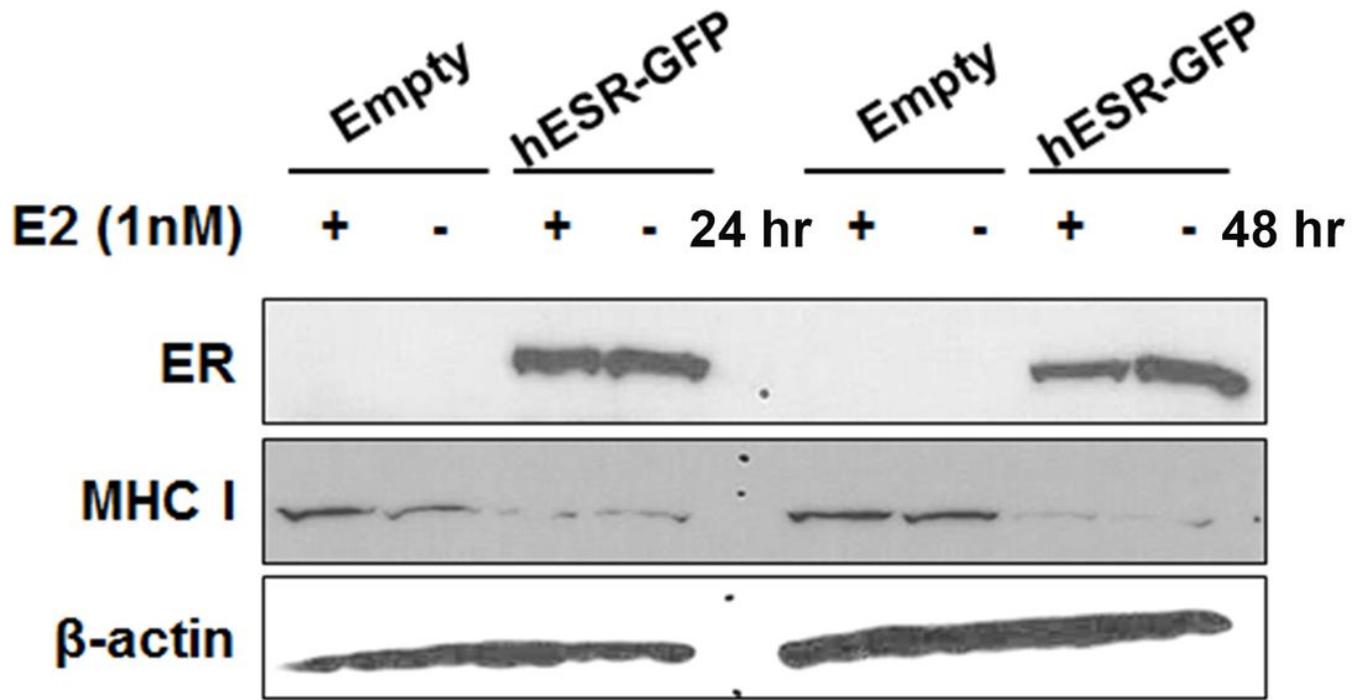
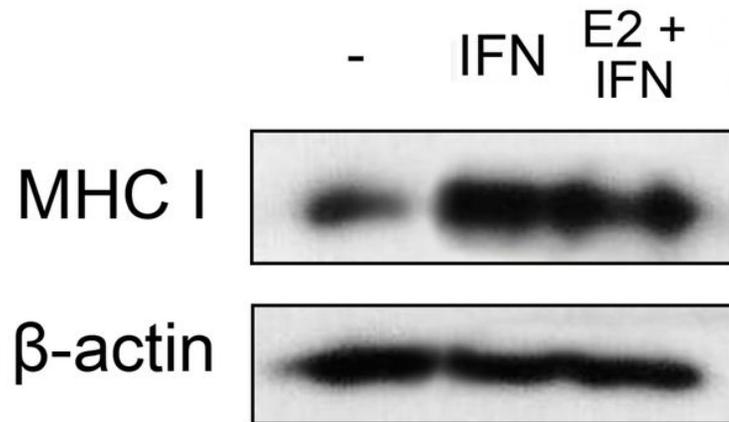
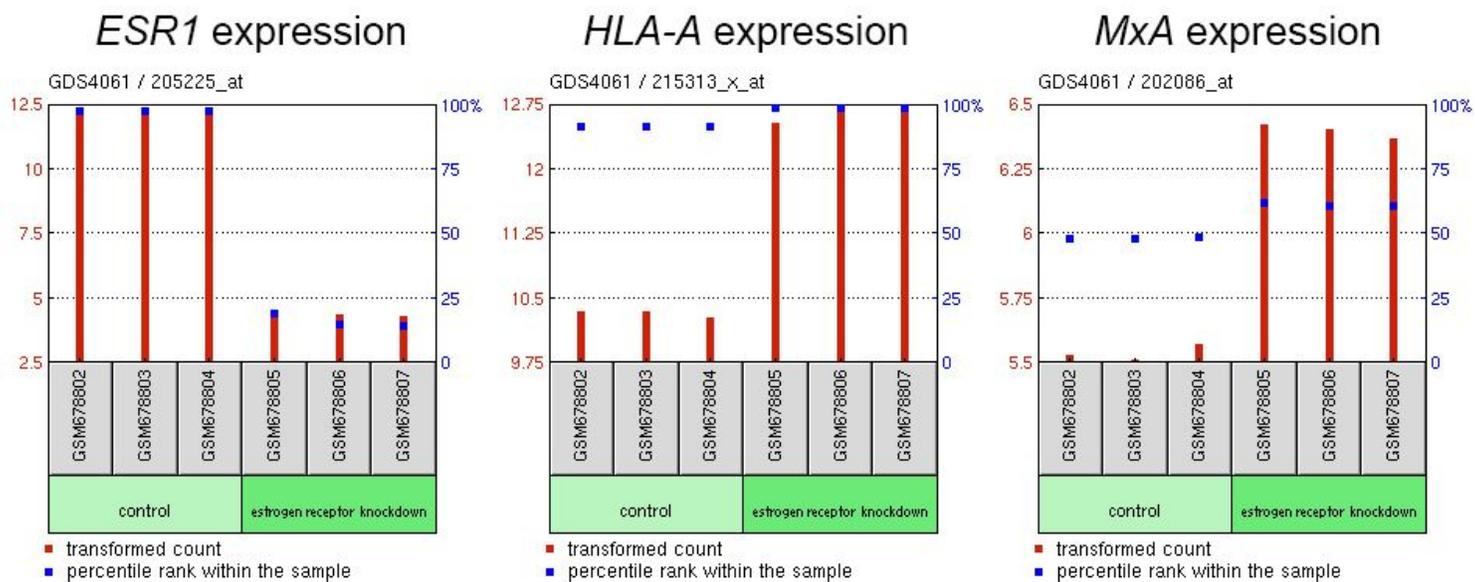
**A****B**

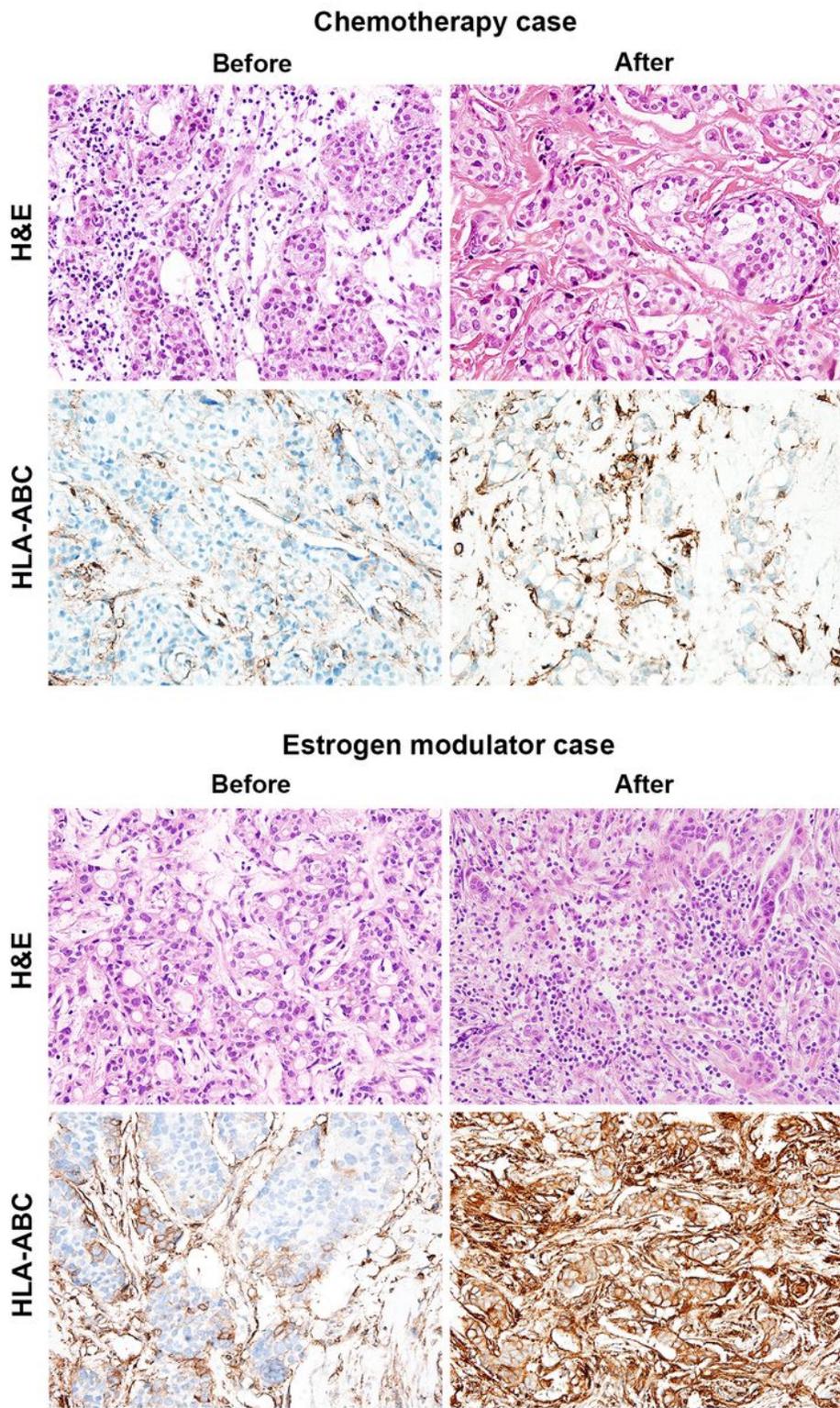
Figure 2

HLA-ABC protein expression in the MDA-MB-231 cells. (A) Twenty-four or forty-eight hours after transfection with the hESR-GFP plasmid, MDA-MB-231 cells show ER expression and decreased HLA-ABC protein levels. (B) The HLA-ABC protein expression was markedly increased 48 hours after 100 units/ml of IFN- $\gamma$  treatment in MDA-MB-231 cells.



**Figure 3**

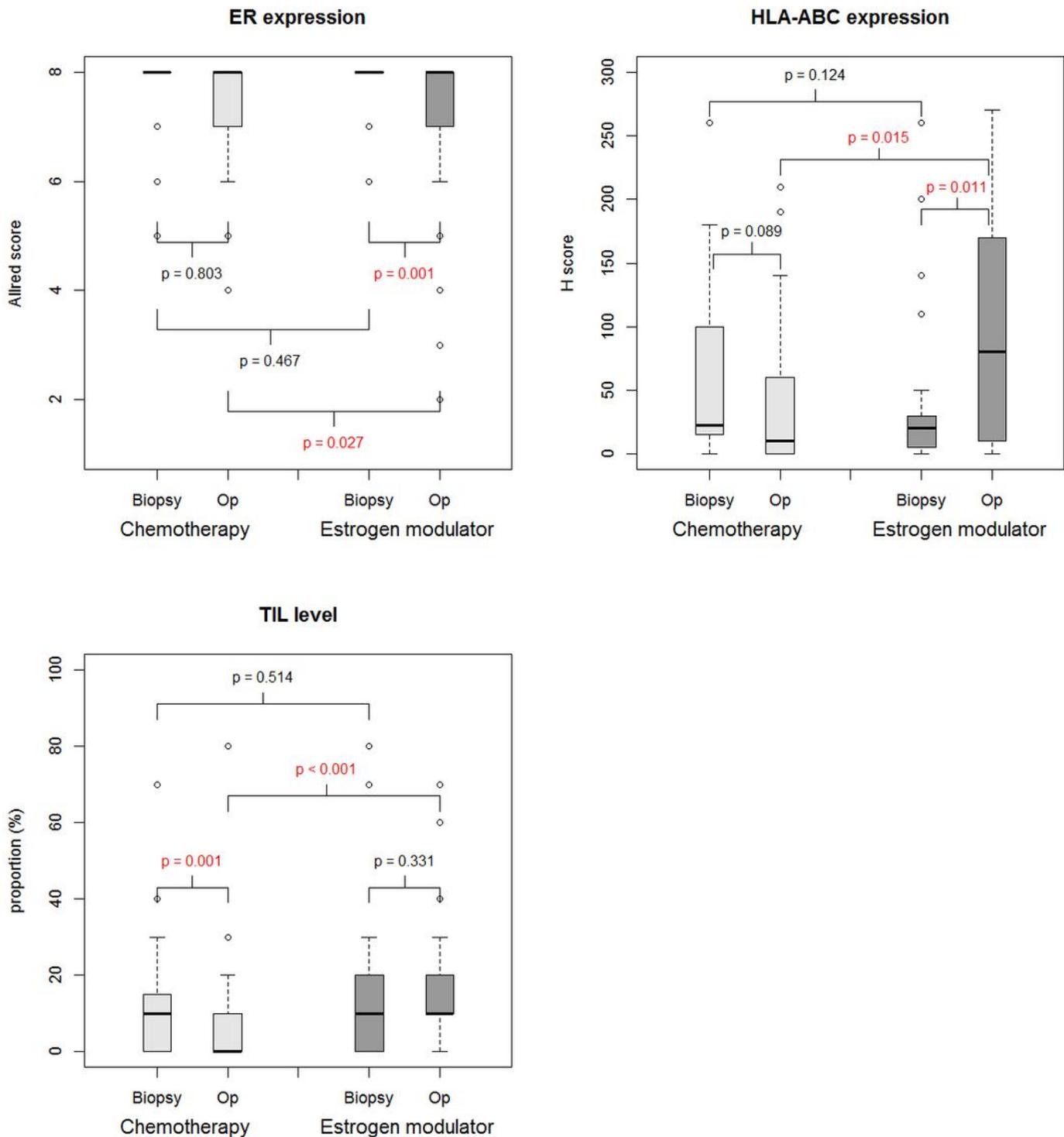
The changes of mRNA expression in MCF-7 breast cancer cells after ESR1 shRNA transfection. ESR1 expression decreased after transfection (left), and HLA-A and MxA expressions increased after transfection (middle and right).



**Figure 4**

The microscopic images of breast tissue before and after chemotherapy or estrogen modulator treatment. In the patient who received neoadjuvant chemotherapy (the upper images), the quantity of tumor-infiltrating lymphocytes (TILs) decreased and HLA-ABC expression was similar in the resection specimen compared with the pre-neoadjuvant biopsy specimens. By contrast, the TIL levels and HLA-ABC

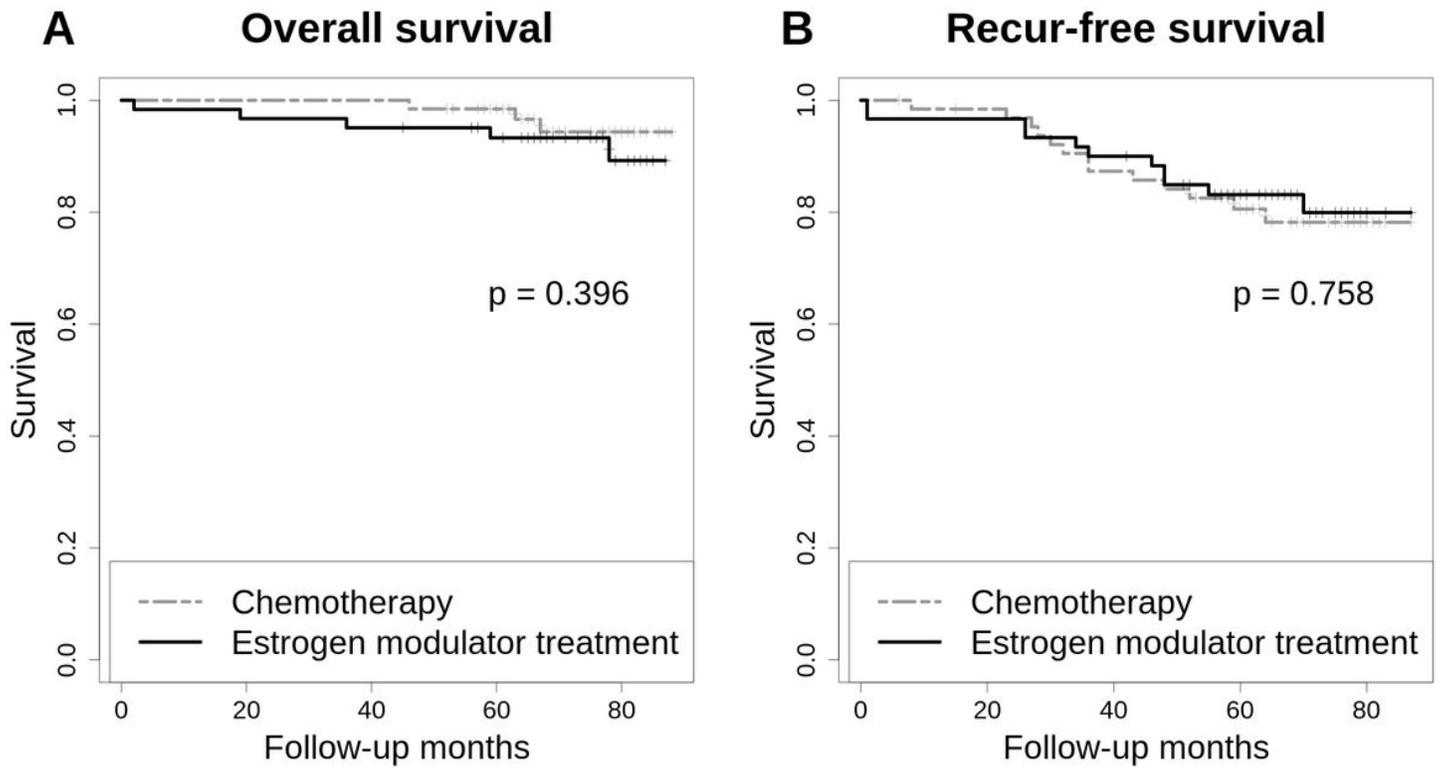
expression increased after estrogen modulator treatment in the patient who received estrogen modulator treatment (Nolvadex and Zoladex, the lower images) ( $\times 400$ ).



**Figure 5**

The changes of ER and human leukocyte antigen (HLA)-ABC expression and tumor-infiltrating lymphocyte (TIL) levels. The TIL levels significantly decreased after chemotherapy, while the expression

of ER decreased after estrogen modulator treatment. Compared with the chemotherapy group, HLA-ABC expression increased significantly after estrogen modulator treatment (Op, operation).



**Figure 6**

The overall and recurrence-free survival between the chemotherapy group and the estrogen modulator treatment group.