

HMGB1 is a key factor for tamoxifen resistance and has the potential to predict the efficacy of CDK4/6 inhibitors in breast cancer

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Funding information

National Natural Science Foundation of China, Grant/Award Number: 81730074

Abstract

Breast cancer is the leading cause of cancer death in women. Hormone-receptor-positive breast cancer (HR + BC) is the most common pathological type of breast cancer, of which the main treatment method is endocrine therapy. Unfortunately, primary or acquired drug resistance greatly limits its efficacy. In recent years, the newly launched CDK4/6 inhibitors could effectively reverse endocrine resistance in breast cancer. However, considering their expensive price and side effects, it is particularly important to find out effective biomarkers and screen sensitive patients. Here, we found through bioinformatics analysis that high mobility group box 1 (HMGB1) expression increased in endocrine-resistant HR + BC. In clinical specimens, the higher expression of HMGB1 was associated with shorter progression-free survival (PFS) for HR + BC patients with endocrine therapy after surgery. For endocrine-resistant breast cancer, compared with HMGB1-negative patients, HMGB1-positive patients who received CDK4/6 inhibitors treatment benefited more in PFS. Moreover, we demonstrated that HMGB1 promoted tamoxifen resistance by combining with the Toll-like receptor 4 (TLR4) and activating nuclear factor kappa B (NF- κ B) pathway. CDK4/6 inhibitors could downregulate the expression of HMGB1 and suppress the TLR4-NF- κ B pathway, and in turn reverse tamoxifen resistance. These results illuminated the critical role of HMGB1 in the process of tamoxifen resistance, explained the mechanism of CDK4/6 inhibitors reversing tamoxifen resistance, and suggested the feasibility of HMGB1 as a potential biomarker for screening sensitive patients receiving CDK4/6 inhibitors.

KEYWORDS

breast cancer, CDK4/6 inhibitors, HMGB1, tamoxifen resistance, TLR4

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1 | INTRODUCTION

Breast cancer is the malignant tumor with the highest incidence and is the leading cause of cancer death in women.¹ Up to 70% of breast cancer is HR + BC, while endocrine therapy is the most effective treatment for it.² Although endocrine therapy has made a rapid development in recent years, its efficacy is still greatly limited by the occurrence of primary or acquired drug resistance.³ Previous studies have suggested that multiple mechanisms are responsible for endocrine resistance, including estrogen receptor (ER) loss or mutation, alteration of the ER pathway, deregulation of cell cycle signaling molecules and activation of various escape pathways.^{4,5} However, the currently known mechanisms of endocrine resistance are still not comprehensive and need to be explored. Despite the marked improvement in treatment of advanced HR + BC, a large proportion of patients' therapeutic effects are still unsatisfactory.

In recent years, multiple clinical trials have established the efficacy of CDK4/6 kinase inhibitors palbociclib, ribociclib, and abemaciclib in the treatment of advanced HR + BC.⁶⁻¹⁰ CDK4/6 inhibitors are small-molecule inhibitors specially targeted to CDK4/CDK6, which can block cell cycle progression in G0-G1 phase and suppress the development of tumor cells.¹¹ Recent studies have reported that CDK4/6 inhibitors also have other anti-tumor functions beyond blocking cell cycle progression.^{12,13} For example, CDK4/6 inhibitors can augment anti-tumor immunity by enhancing T-cell activation.¹⁴ Combining with anti-PD-L1 therapy, CDK4/6 inhibitors lead to immunological memory and tumor regression.¹⁵ Although CDK4/6 inhibitors are effective in clinical trials, c. 16% of HR + breast cancer patients have shown primary resistance to them, and c. 50% of initially sensitive patients have developed secondary resistance.⁶⁻¹⁰ Various adverse events occur during treatment, including neutropenia, fatigue, pulmonary embolism, back pain, and diarrhea, among which neutropenia is a dose-limiting event in treatment.¹⁶ It has important clinical significance of screening sensitive patients to avoid over-treatment. Currently in the clinical treatment of breast cancer, ER status is the only viable biomarker for CDK4/6 inhibitors.¹⁷ There is still a lack of reliable biomarkers that can predict the therapeutic effect of CDK4/6 inhibitors.¹⁸ Screening new predictive markers is helpful to accurately predict curative effects, and is of great significance to realize individualized treatment and precision medicine.

As a protein that is located in the nucleus and can be released into extracellular matrix, HMGB1 has many biological functions inside and outside the cell.¹⁹ Initially, HMGB1 was thought to be only an important regulatory factor for inflammatory diseases, but recent studies have shown that HMGB1 also played a crucial role in cancer.²⁰ HMGB1 can activate its downstream pathways, promote the

release of proinflammatory cytokines, and consequently sustain the inflammatory microenvironment.^{21,22} Moreover, HMGB1 can promote the apoptosis of immune cells and the inhibition of anti-tumor immunity.²³

Here, we found through bioinformatics analysis that HMGB1 expression increased in endocrine-resistant HR + BC. In clinical specimens, the higher expression of HMGB1 was associated with shorter PFS for HR + BC patients with endocrine therapy after surgery. For endocrine-resistant breast cancer, compared with HMGB1-negative patients, HMGB1-positive patients who received CDK4/6 inhibitor treatment benefited more in PFS. Moreover, we found that HMGB1 promoted endocrine resistance by binding to the TLR4 receptor on cell membranes and activating the NF- κ B pathway. CDK4/6 inhibitors could downregulate the expression of HMGB1 and suppress the TLR4-NF- κ B pathway, and in turn reverse tamoxifen resistance. These results illustrated the critical role of HMGB1 in the process of tamoxifen resistance, explained the mechanism of CDK4/6 inhibitors reversing tamoxifen resistance, and suggested the feasibility of HMGB1 to serve as a potential biomarker for screening sensitive patients treated with CDK4/6 inhibitors.

2 | MATERIALS AND METHODS

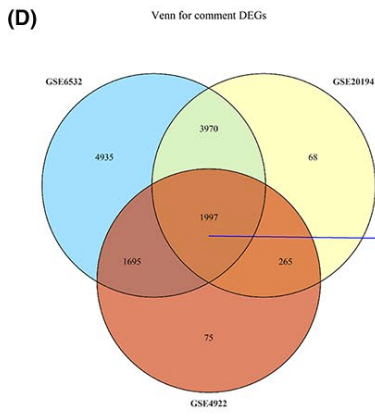
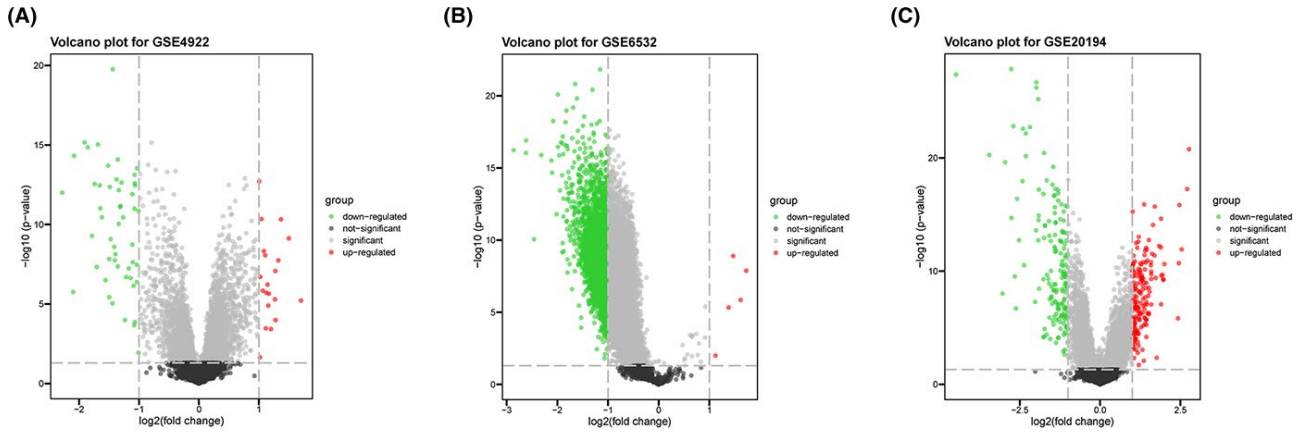
2.1 | mRNA expression profiles

Gene expression datasets (GSE4922, GSE6532, GSE20194, GSE26459, GSE98987) were downloaded from the Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>). The breast cancer patients in GSE4922, GSE6532 and GSE20194 datasets were grouped into ER+ and ER- groups, patients in GSE26459 datasets were grouped into tamoxifen-resistant and control groups, while patients in GSE98987 datasets were grouped into CDK4/6 inhibitors treatment and control groups. R limma was used for differential expression analysis, taking the genes with $P < .05$ as the differentially expressed genes (DEGs).

2.2 | Patients samples

Breast cancer cohort in Table 2 consisted of 130 HR + BC patients who accepted endocrine therapy (letrozole 2.5 mg/d, anastrozole 1 mg/d or tamoxifen 20 mg/d) after surgery between July 2011 and August 2019. The breast cancer cohort in Table 3 consisted of 83 endocrine-resistant breast cancer patients who accepted second-line endocrine therapy or second-line endocrine therapy

FIGURE 1 Identification of differentially expressed genes (DEGs) in HR + BC. A, Volcano map of DEGs between ER+ and ER-BC, data from GSE4922 datasets. DEGs were selected with a fold change > 2 and P -value $< .05$ among the mRNA expression profiling. B, Volcano map of DEGs between ER+ and ER-BC, data from GSE6532 datasets. C, Volcano map of DEGs between ER+ and ER-BC, data from GSE20194 datasets. D, Intersection of DEGs in 3 datasets. E, Volcano map of DEGs between tamoxifen-resistant HR + BC and normal HR + BC, data from GSE26459 datasets. F, Heatmap of DEGs between tamoxifen-resistant HR + BC (TR) and normal HR + BC (control). G, Expression of HMGB1 in tamoxifen-resistant HR + BC (TR) and normal HR + BC (control)



NDUFAF4, STUB1, ADAMTS3, CDC45, CEP170P1, SLC1A3, FBXL5, MRPL24, APOBEC3B, ZNF729, SIRT3, RIN2, LDHC, CTDSP1, CXCL11, KIF20A, CD177, TYMP, CITED1, PKIG, XPO1, WDR62, CLIC3, KRT6A, TGS1, CRABP1, LRPAP1, IL1R2, TRIM22, MARCH8SOX11, ECI2, GCC2, EGOT, TBCC, EEF1A2, GALNT14, NIN, S100A6, PRRG1, EBF2, MAGEA2B, ENTPD1, C9orf116, NCK1, ATP6V0A4, RAX, TKFC, EPM2AIP1, HIST1H2BK, NAMPT, FABP6, NUP205, COG2, MSL3, CSNK2B, CRYAB, RSAD2, HARS2, RAB11FIP5, AIM2, CHI3L2, AXL, ENTPD1-AS1, RTN1, LOC100996693, **HMGB1**, OIP5, PTPRK, SPAG5, XIST, MIR4640, FUZ, TFPC2L1, FBXO16, IL7R, MAGEA6, PSTPIP2, SPR, GTSE1, CYP27B1, FAM134ACDCA3, HECTD4, SLC2A6, MYOZ3, NDUFAF3, PFN1, MFAP1, TBX21, CRIP2, HILPDA, GSTA4, GALNS, LMNB2, HACD3,

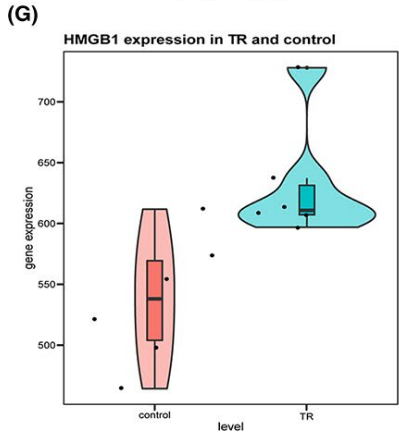
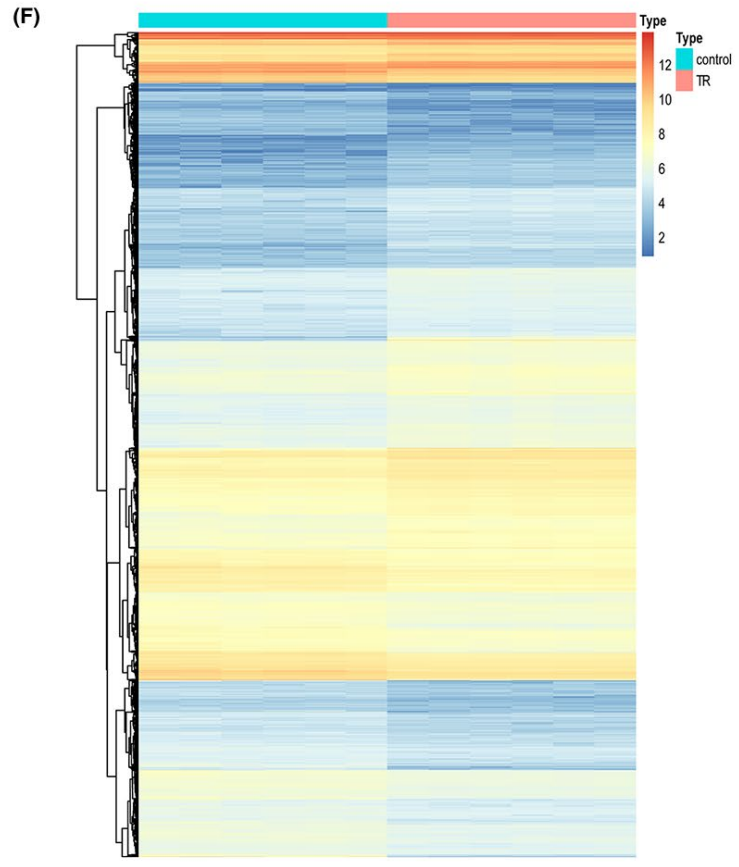
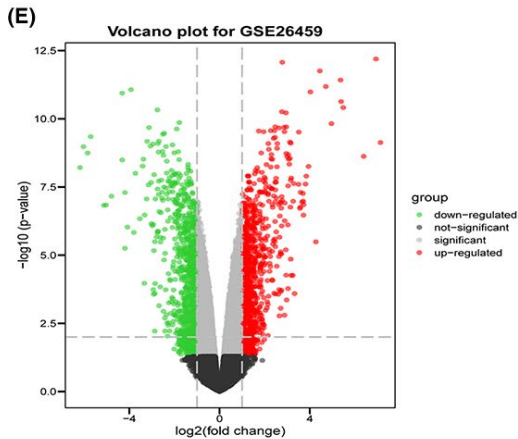


TABLE 1 The number of breast cancer patients in 3 groups in the NCBI GEO database

Id	ER+	ER-
GSE6532	45	200
GSE20194	154	68
GSE4922	211	34

Abbreviation: ER, estrogen receptor.

TABLE 2 PFS of patients with different HMGB1 status receiving first-line endocrine therapy

	HMGB1 positive group (n, %)	HMGB1 negative group (n, %)	P
Treatment			
Tamoxifen	55.2 (41.32%)	64.0 (26.20%)	<.001
AI	56.1 (38.29%)	65.8 (25.19%)	<.001

Abbreviation: AI, aromatase inhibitor.

TABLE 3 PFS of endocrine therapy resistance patients with different HMGB1 status receiving advanced first-line treatment of CDK4/6 inhibitors combined with AI or AI

Treatment	CDK4/6i + AI (n, %)	AI (n, %)	P
HMGB1-positive group	21.7 (35.42%)	10.1 (25.30%)	.001
HMGB1-negative group	18.4 (13.16%)	12.2 (10.12%)	.013

Abbreviations: AI, aromatase inhibitor; CDK4/6i, CDK4/6 inhibitors.

plus CDK4/6 inhibitors after the progression of disease between September 2015 and November 2019. All of the patients' specimens were obtained from Harbin Medical University Cancer Hospital.

2.3 | Reagents and cell culture

Antibodies against HMGB1 were purchased from Abcam. Antibodies against TLR4 and NF- κ B (RelA) were purchased from Boster. CDK4/6 inhibitor abemaciclib was purchased from Selleck. HMGB1 human recombinant protein (for cell culture) was purchased from Boster. 4-Hydroxytamoxifen (4-OH Tam) was purchased from Sigma-Aldrich. HMGB1 inhibitor glycyrrhizic acid (GA) and NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC) were purchased from Selleck. MCF-7 and T47D cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences and maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM, 4500 mg/L glucose, Gibco) with 10% heat-inactivated fetal bovine serum (FBS, ScienCell). Tamoxifen-resistant MCF-7 (MCF-7R) cells were obtained by exposing MCF-7 cells to 4-OH Tam whose concentration was increased up to 1000 nmol/L for 6 mo. Tamoxifen-resistant T47D (T47DR) cells were obtained by exposing T47D cells to 4-OH Tam whose concentration was increased up to 1000 nmol/L

for 12 mo. MCF-7R and T47DR cells were maintained in DMEM with 5% FBS plus 1000 nmol/L 4-OH Tam.

2.4 | Immunohistochemistry

Pathological sections were incubated with anti-HMGB1 antibody (1:400 dilution). Five pathological fields were selected randomly in each section. HMGB1 staining of the sections was scored based on the following standards: the percentage of positive staining was classified as 0 (<5%), 1 (5%-25%), 2 (26%-50%) or 3 (>50%), and the staining intensity was classified as 0 (no staining), 1 (light yellow), 2 (brown yellow), or 3 (dark brown). Then the total score of each pathological section was calculated. HMGB1-negative group's total score was 0-3 while HMGB1-positive group's total score was more than 3.

2.5 | Western blotting

RIPA buffer was used to lyse tumor cells. Protein samples were sonicated followed by centrifugation at 17 153 g for 15 min. Supernatants were collected and protein concentrations were determined using the Bradford Assay (Bio-Rad). Protein lysates were then subjected to 4%-20% Tris-glycine SDS-PAGE, and transferred onto PVDF membranes in accordance with the manufacturer's instructions (Invitrogen). The membranes were blocked in 5% milk-Tris-buffered saline with 0.1% Tween (TBS-T) at 23°C for 1 h, followed by incubation with HMGB1 (1:1000 dilution), TLR4 (1:1000 dilution), or NF- κ B (RelA) (1:1000 dilution) antibodies at 4°C overnight. On the following day, the membranes were washed with TBS-T 3 times before incubation with HRP-conjugated secondary antibodies (Cell Signaling) at 23°C for 1 h. Protein expression was visualized by ECL chemiluminescence (Promega) and quantified by ImageJ software (National Cancer Institute).

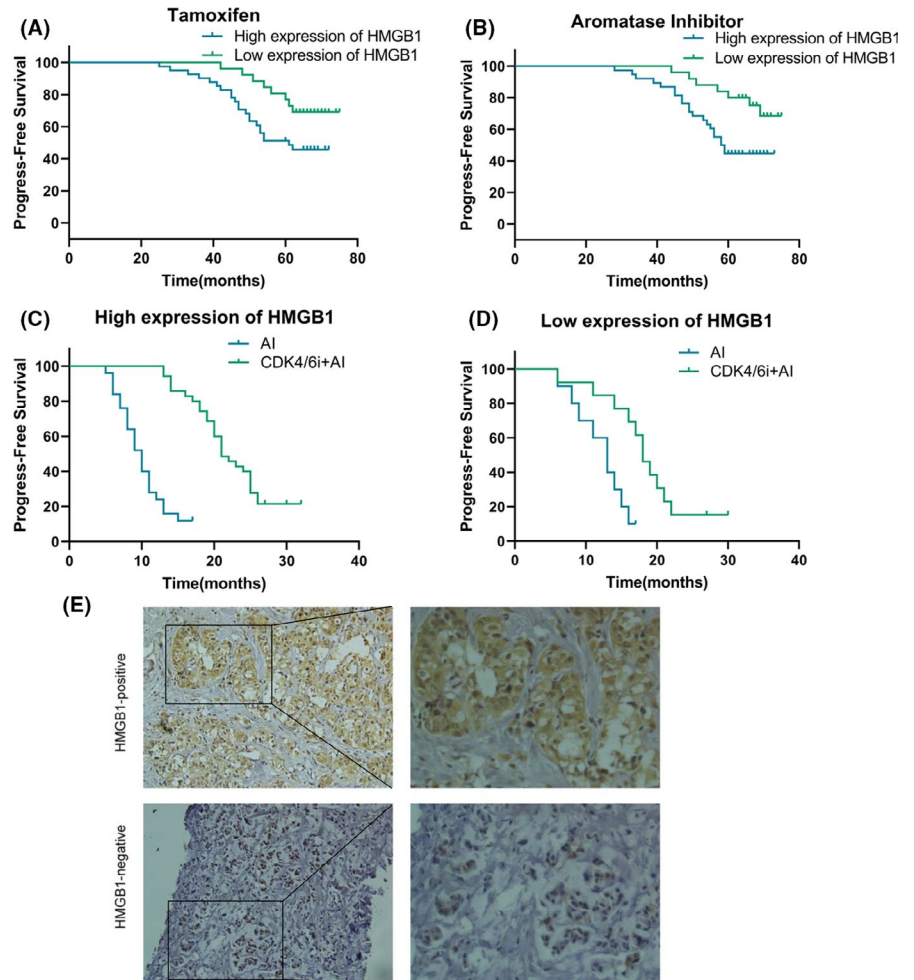
2.6 | Cell viability assay

Cell Counting Kit-8 (CCK-8, Vazyme) was used to assess cell proliferation. Firstly, cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated with 5 μ mol/L tamoxifen or 1000 nmol/L abemaciclib for 0, 24, 48, and 72 h. Then the cells were incubated in medium with 10% CCK-8 reagent for 2 h. At last, the absorbance of the sample at 450 nm was measured in a microplate reader (Tecan). The experiments were repeated 3 times.

2.7 | Real-time quantitative PCR

By using TRIzol reagent (Invitrogen), total RNA from MCF-7R, T47DR cells, and MCF-7R, T47DR cells treated with 1000 nmol/L abemaciclib

FIGURE 2 Correlation of the efficacy of HR + BC endocrine therapy and CDK4/6 inhibitor with HMGB1 expression in clinical specimens. A, Kaplan-Meier progression-free survival (PFS) curves of HR + BC patients with negative ($n = 26$) and positive ($n = 41$) HMGB1 expression who received tamoxifen treatment. B, Kaplan-Meier PFS curves of HR + BC patients with negative ($n = 25$) and positive ($n = 38$) HMGB1 expression who received aromatase inhibitor (AI) treatment. C, Kaplan-Meier PFS curves of advanced HR + BC patients with positive HMGB1 expression who received AI ($n = 25$) or AI + CDK4/6 inhibitor ($n = 35$) treatment. D, Kaplan-Meier PFS curves of advanced HR + BC patients with negative HMGB1 expression who received AI ($n = 10$) or AI + CDK4/6 inhibitor ($n = 13$) treatment. E, Representative images of immunohistochemical staining for HMGB1 in tumor tissues of Tables 1 and 2. The staining results were observed under high-power lens ($\times 100$, $\times 400$). Score > 3 was defined as positive HMGB1 staining in tumor tissues, score ≤ 3 was defined as negative HMGB1 staining in tumor tissues



were isolated. RNA samples were then reverse transcribed into cDNA using reverse transcriptase (Vazyme). cDNA was amplified by real-time quantitative PCR (RT-PCR) with specific primers for HMGB1 (upper 5'-TCAAAGGAGAACATCCTGGCCTGT-3', lower 5'-CTGCTTGTCATCTGCAGCAGTGT-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; upper 5'-GGTGAAG TCGGAGTCAACGG-3', lower 5'-GGTCATGAGTCCTCCACGATAC C-3'). RT-PCR was conducted by using the SYBR Green I Real-Time Detection kit (CWBio) on the CFX96 Detection System (Bio-Rad). HMGB1 gene expression was normalized to GAPDH expression, and the control groups (MCF-7R, T47DR) were set as 1.

2.8 | Functional and pathway enrichment analysis

To extract the protein-protein interaction (PPI) network related to the HMGB1 gene, biological analyses were performed using the STRING online database. Then, Cytoscape was imported for visualization, and MCODE was used to identify functional sub-modules in PPI. The DAVID database was used for Gene Ontology (GO) function annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the genes in the functional submodule.

2.9 | Statistical analysis

Statistical analyses were carried out using GraphPad Prism software (version 6.0) and SPSS 19.0. The Kaplan-Meier method was used to plot survival curves. Log-rank test was used to compare each group. P -values $< .05$ were considered statistically significant (* $P < .05$, ** $P < .01$, *** $P < .001$).

3 | RESULTS

3.1 | Identification of DEGs in HR + BC

To verify the difference in gene expression of HR-positive breast cancer compared with other types of breast cancer, we obtained the gene expression profile data from 3 groups of breast cancer patients in the NCBI GEO database. The breast cancer patients in 3 datasets were grouped into ER+ and ER- groups, and R package limma was used for differential expression analysis ($P < .05$) (Table 1). Analysis results are shown as volcano plots (Figure 1A-C). In total, 1997 DEGs were identified, among which the expression of HMGB1 was significantly different (Figure 1D). We analyzed DEGs in tamoxifen-resistant HR + BC patients compared with normal HR + BC patients

(Figure 1E,F). In total, 11 108 DEGs were identified, consisting of 1009 downregulated genes and 1104 upregulated genes (Tables S1-S3), among which the expression of HMGB1 was significantly up-regulated. Thus, we particularly analyzed the difference in HMGB1 expression between the tamoxifen-resistant group and the control group. The results showed that HMGB1 expression was significantly increased in the tamoxifen-resistant group (Figure 1G).

3.2 | Correlation of HMGB1 expression with HR + BC endocrine resistance and CDK4/6 inhibitor efficacy in clinical specimens

To investigate the relationship between HMGB1 and endocrine resistance in HR + BC, we obtained 130 tumor tissues from HR + BC patients who accepted endocrine therapy (tamoxifen or aromatase inhibitor) after surgery (Table 2). We analyzed the expression of HMGB1 by immunohistochemical (IHC) (Figure 2E). Compared with the HMGB1-positive group, patients' PFS was found to be prominently longer in HMGB1-negative group (Figure 2A,B). Then we obtained 83 tumor tissues from endocrine-resistant breast cancer

patients who accepted second-line endocrine therapy or second-line endocrine therapy plus CDK4/6 inhibitors after the progression of disease (Table 3). We found that patients who received CDK4/6 inhibitors plus endocrine therapy in the HMGB1-positive group benefited more in PFS compared with those in the HMGB1-negative group (Figure 2C,D).

3.3 | HMGB1 promotes HR + BC cells tamoxifen resistance

To examine the expression of HMGB1 in tamoxifen-resistant BC, HR + BC cell lines MCF-7 and T47D were selected out and treated to be resistant to tamoxifen. Western blot (WB) was carried out on tamoxifen-sensitive MCF-7 cells (MCF-7), T47D cells (T47D) and tamoxifen-resistant MCF-7 cells (MCF-7R), and T47D cells (T47DR). Results showed that the expression of HMGB1 in MCF-7R and T47DR was distinctly higher compared with in MCF-7 and T47D (Figure 3A,B). To make sure that the sensitivity of tumor cells to tamoxifen was relative to the HMGB1 expression, HMGB1 inhibitor GA, 200 μ mol/L) was added to MCF-7, MCF-7R, T47D and T47DR

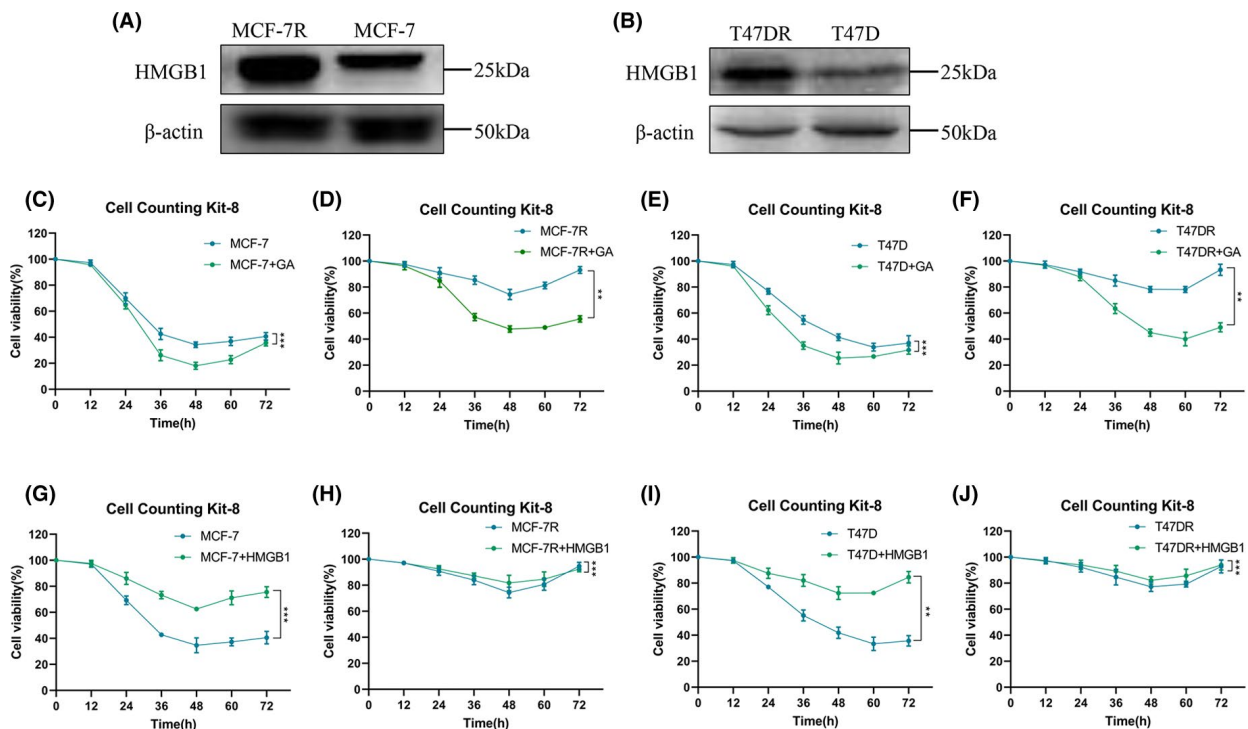


FIGURE 3 HMGB1 promotes MCF-7 and T47D tamoxifen resistance. A, Western blot analysis of HMGB1 expression in MCF-7 and MCF-7R cells. B, Western blot analysis of HMGB1 expression in T47D and T47D R cells. C, Relative viability of MCF-7 treated with 5 μ mol/L tamoxifen and 5 μ mol/L tamoxifen plus 200 μ mol/L HMGB1 inhibitor glycyrrhizic acid (GA). *** P < .001. D, Relative viability of MCF-7R treated with 5 μ mol/L tamoxifen and 5 μ mol/L tamoxifen plus 200 μ mol/L GA. ** P < .01. E, Relative viability of T47D treated with 5 μ mol/L tamoxifen and 5 μ mol/L tamoxifen plus 200 μ mol/L GA. *** P < .001. F, Relative viability of T47DR treated with 5 μ mol/L tamoxifen and 5 μ mol/L tamoxifen plus 200 μ mol/L GA. ** P < .01. G, Relative viability of MCF-7 treated with 5 μ mol/L tamoxifen and 5 μ mol/L tamoxifen plus 100 μ g/L HMGB1 recombinant protein. *** P < .001. H, Relative viability of MCF-7R treated with 5 μ mol/L tamoxifen and 5 μ mol/L tamoxifen plus 100 μ g/L HMGB1 recombinant protein. *** P < .001. I, Relative viability of T47D treated with 5 μ mol/L tamoxifen and 5 μ mol/L tamoxifen plus 100 μ g/L HMGB1 recombinant protein. ** P < .01. J, Relative viability of T47DR treated with 5 μ mol/L tamoxifen and 5 μ mol/L tamoxifen plus 100 μ g/L HMGB1 recombinant protein. *** P < .001

expression of TLR4 and NF- κ B in 4 cell lines (MCF-7, MCF-7R, T47D, T47DR) by WB (Figure 5B,C). Compared with MCF-7 and T47D, TLR4 and NF- κ B expression were found to be dramatically increased in MCF-7R and T47DR. Then MCF-7R and T47DR were treated with 200 μ mol/L GA, which significantly reduced the expression of TLR4 and NF- κ B (Figure 5D,E). To make sure that HMGB1 promoted tamoxifen resistance by activating the TLR4-NF- κ B pathway, we treated MCF-7 and T47D with 100 μ g/L HMGB1 recombinant protein and 100 μ g/L HMGB1 recombinant protein plus NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC, 100 μ mol/L), respectively, while MCF-7R and T47DR were treated with 100 μ mol/L PDTC at the same time. CCK8 was used to examine the sensitivity of each cell line (MCF-7 + HMGB1, MCF-7 + HMGB1+PDTC, T47D + HMGB1, T47D + HMGB1+PDTC, MCF-7R, MCF-7R + PDTC, T47DR, T47DR + PDTC) to tamoxifen (5 μ mol/L) (Figure 5F-I). We found that PDTC significantly increased the sensitivity of HMGB1-treated tumor cells to tamoxifen. The same result was observed in tamoxifen-resistant cells. These observations indicated that the promotion of HMGB1 to tamoxifen resistance relied on the TLR4-NF- κ B pathway.

3.6 | CDK4/6 inhibitor abemaciclib reverses tamoxifen resistance by suppressing HMGB1 expression

Datasets GSE98987²⁵ were used to analyze the effect of CDK4/6 inhibitors on the expression of HMGB1 in BC patients. The datasets included 6 CDK4/6 inhibitors treatment groups and 5 control groups. Results showed that the expression of HMGB1 in BC patients decreased after treated by CDK4/6 inhibitors (Figure 6A). To verify the effect of CDK4/6 inhibitor on the expression of HMGB1, TLR4 and NF- κ B in tamoxifen-resistant breast cancer cells, MCF-7R and T47DR were treated by CDK4/6 inhibitor abemaciclib with different concentrations (0, 100, 500 and 1000 nmol/L) for 72 h. Then we measured the levels of HMGB1, TLR4 and NF- κ B expression in 2 cell lines by WB (Figure 6B,C). Compared with 1000 nmol/L abemaciclib group, the levels of HMGB1, TLR4 and NF- κ B expression were significant higher in other groups. These results indicated that abemaciclib could restrain the expression of HMGB1, TLR4, and NF- κ B. Figure 6D,E showed how HMGB1 expression changed over time (0, 24, 48, 72 h) in MCF-7R and T47DR during the treatment

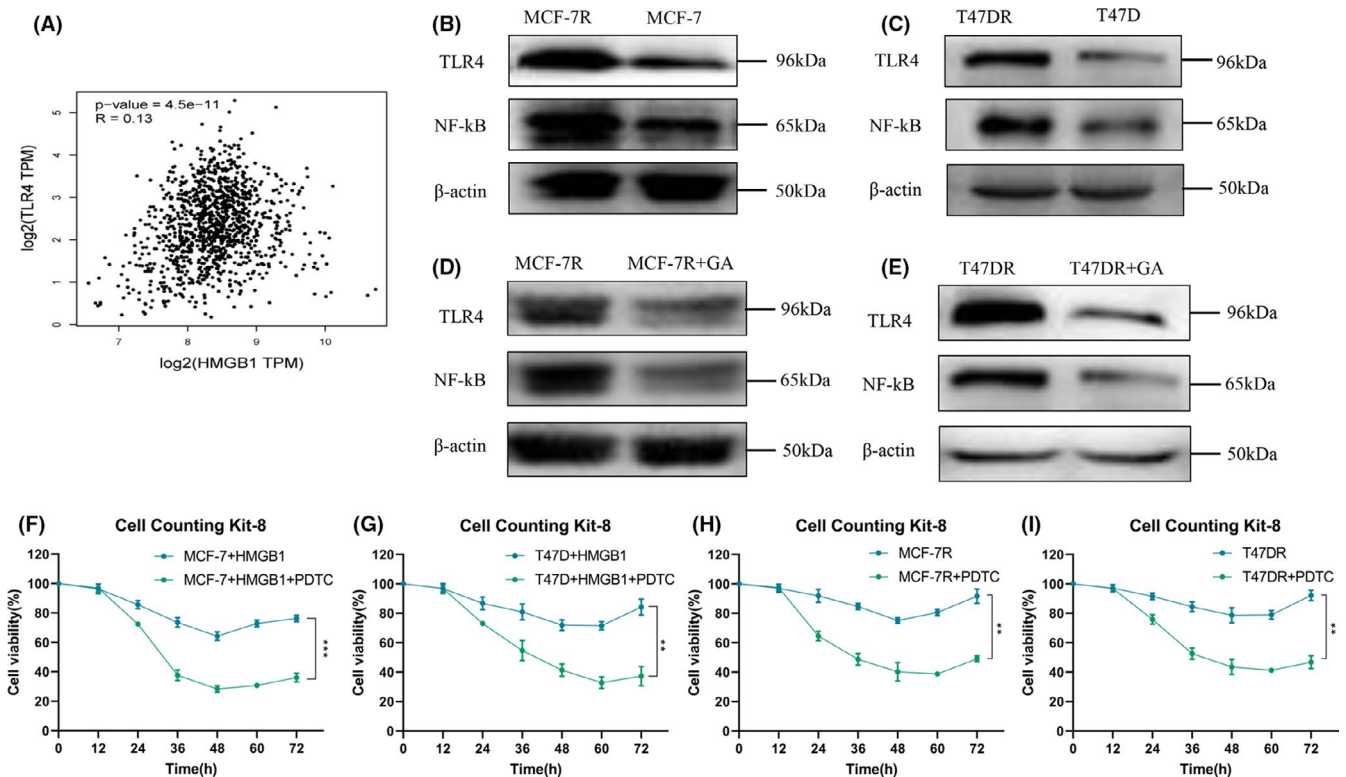


FIGURE 5 HMGB1 activates TLR4-NF- κ B pathway and promotes tamoxifen resistance in MCF-7 and T47D. A, Correlation of HMGB1 and TLR4 in breast cancer tissue specimens. B, Western blot analysis of TLR4 and NF- κ B expression in MCF-7 and MCF-7R cells. C, Western blot analysis of TLR4 and NF- κ B in T47D and T47DR cells. D, Western blot analysis of TLR4 and NF- κ B expression in MCF-7R and MCF-7R treated with 200 μ mol/L GA. E, Western blot analysis of TLR4 and NF- κ B expression in T47DR and T47DR treated with 200 μ mol/L GA. F, MCF-7 was treated with 100 μ g/L HMGB1 recombinant protein and 100 μ g/L HMGB1 recombinant protein plus 100 μ mol/L NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC). Relative viability of each cell line treated with 5 μ mol/L tamoxifen. *** P < .001. G, T47D was treated with 100 μ g/L HMGB1 recombinant protein and 100 μ g/L HMGB1 recombinant protein plus 100 μ mol/L PDTC. Relative viability of each cell line treated with 5 μ mol/L tamoxifen. ** P < .01. H, Relative viability of MCF-7R treated with 5 μ mol/L tamoxifen and 5 μ mol/L tamoxifen plus 100 μ mol/L PDTC. ** P < .01. I, Relative viability of T47DR treated with 5 μ mol/L tamoxifen and 5 μ mol/L tamoxifen plus 100 μ mol/L PDTC. ** P < .01

with 1000 nmol/L abemaciclib. Furthermore, RT-PCR results demonstrated that abemaciclib's inhibitory effect on HMGB1 expression originated at the genetic level (Figure 6F,G). Afterwards, we treated MCF-7R and T47DR with abemaciclib (1000 nmol/L), abemaciclib plus HMGB1 recombinant protein (100 μ g/L), and abemaciclib plus GA (100 μ mol/L) separately. As shown in Figure 6H,I, compared with abemaciclib plus HMGB1, abemaciclib could significantly suppress the growth of MCF-7R and T47DR. Although compared with abemaciclib, abemaciclib plus GA did not significantly enhance the growth inhibitory effect on MCF-7R and T47DR. These results showed that the reversion of abemaciclib to tamoxifen resistance relied on the inhibition of HMGB1-TLR4-NF- κ B pathway. HMGB1 was expected to be a potential biomarker for predicting abemaciclib efficacy.

4 | DISCUSSION

In the present study, we discussed the significance of HMGB1 in the process of tamoxifen resistance, providing evidence that high expression of HMGB1 could promote tamoxifen resistance in breast cancer by activating the TLR4-NF- κ B pathway and CDK4/6

inhibitors could reverse tamoxifen resistance by suppressing the HMGB1-TLR4-NF- κ B pathway. These findings suggested the feasibility of HMGB1 as a potential biomarker for screening sensitive patients of CDK4/6 inhibitors.

HMGB1 was initially thought to be an inflammatory factor, but later studies showed that it also played an important role in the development of tumors.²⁶ Nowadays, it is believed that HMGB1 has both the roles of promoting and inhibiting tumors, while most of the time it promotes tumors.²⁷ Considering the results of bioinformatics analysis and our clinical data, we speculated that HMGB1 might play an important role in the promotion of HR + BC tamoxifen resistance. We validated this conclusion on HR + BC cell lines MCF-7 and T47D. Meanwhile, we found that the inhibition of HMGB1 increased the sensitivity of MCF-7R and T47DR to tamoxifen.

Recent studies have reported that HMGB1 could activate several downstream pathways by binding with cell surface receptors such as CCD24, CXCL4, RAGE and Toll-like receptors (TLRs, such as TLR2, TLR4, and TLR9).^{28,29} Through PPI network analysis and Spearman correlation coefficient calculation, we found that TLR4 and NF- κ B were significantly correlated with HMGB1 in breast cancer tissue specimens. In our previous study, we demonstrated that compared with MCF-7, MCF-7R were more likely to induce macrophages M2

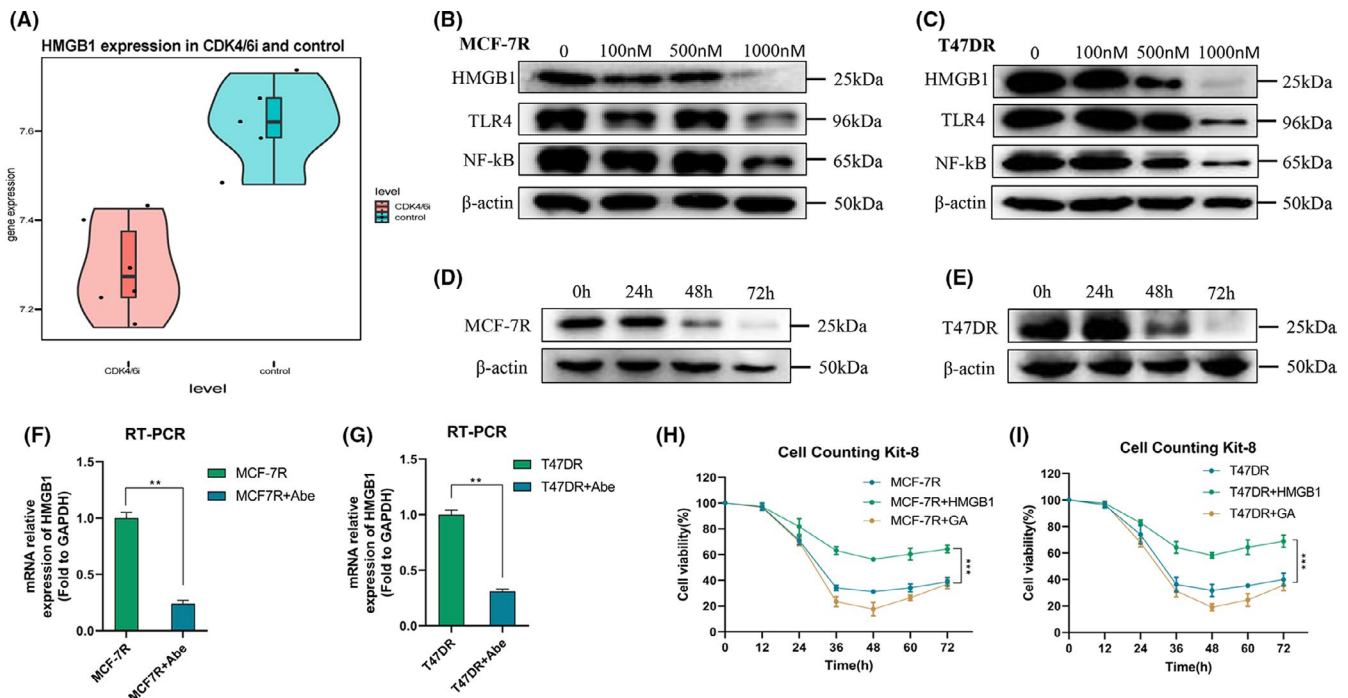


FIGURE 6 CDK4/6 inhibitor abemaciclib reverses tamoxifen resistance by suppressing the expression of HMGB1 and TLR4-NF- κ B pathway. A, Expression of HMGB1 in CDK4/6 inhibitor treatment groups and control groups. Patients were selected from GSE98987 dataset. B, Western blot analysis of HMGB1, TLR4 and NF- κ B expression in MCF-7R and MCF-7R treated with 0, 100, 500, 1000 nmol/L abemaciclib for 72 h. C, Western blot analysis of HMGB1, TLR4 and NF- κ B expression in T47DR and T47DR treated with 0, 100, 500, 1000 nmol/L abemaciclib for 72 h. D, Western blot analysis of HMGB1 expression over time (0, 24, 48, 72 h) in MCF-7R treated with 1000 nmol/L abemaciclib for 72 h. E, Western blot analysis of HMGB1 expression over time (0, 24, 48, 72 h) in T47DR treated with 1000 nmol/L abemaciclib for 72 h. F, RT-PCR analysis of HMGB1 mRNA expression in MCF-7R and MCF-7R treated with 1000 nmol/L abemaciclib (Abe) for 72 h. G, RT-PCR analysis of HMGB1 mRNA expression in T47DR and T47DR treated with 1000 nmol/L abemaciclib (Abe) for 72 h. H, Relative viability of MCF-7R treated with 1000 nmol/L abemaciclib, 1000 nmol/L abemaciclib plus 100 μ g/L HMGB1 and 1000 nmol/L abemaciclib plus 200 μ mol/L GA. *** P < .001. I, Relative viability of T47DR treated with 1000 nmol/L abemaciclib, 1000 nmol/L abemaciclib plus 100 μ g/L HMGB1 and 1000 nmol/L abemaciclib plus 200 μ mol/L GA. *** P < .001

polarization and up-regulate the expression of NF- κ B.³⁰ Several studies demonstrated that by binding with TLR4, HMGB1 could activate NF- κ B pathway and induce macrophage M2 polarization.²⁰ The activation of TLR4/STAT3/NF- κ B and TLR4/PI3K/AKT/NF- κ B signal pathway promoted breast cancer proliferation and metastasis.^{31,32} All of these suggested that NF- κ B might play an important role in the process of HMGB1 promoting tamoxifen resistance. Consistent with these studies, we showed that TLR4-NF- κ B was the key pathway for HMGB1 to promote tamoxifen resistance, and that the inhibition of NF- κ B could weaken the promotion of HMGB1 to tamoxifen resistance.

As a kind of cell cycle inhibitors, CDK4/6 inhibitors have significant efficacy in endocrine-resistant HR + BC patients, which greatly prolong their survival.³³ Since palbociclib was approved for the treatment of recurrent metastatic breast cancer by the United States Food and Drug Administration (FDA) in 2015, 3 kinds of CDK4/6 inhibitors combined with endocrine therapy have been approved for marketing, which changed the history of endocrine therapy for advanced breast cancer with their excellent efficacy.³⁴⁻³⁹ Numerous studies have shown that CDK4/6 inhibitors have many other anti-tumor effects besides cell cycle inhibition.⁴⁰⁻⁴³ However, although they can eliminate tumors through multiple mechanisms, CDK4/6 inhibitors are not effective for all patients. Acquired and intrinsic drug resistance greatly limits their efficacy. In addition, expensive prices and side effects bring a great burden in unsuitable patients. ER status is the only viable biomarker for CDK4/6 inhibitors in the current,¹⁷ but it is not enough to meet clinical needs. To find out new biomarkers is of great significance for breast cancer patients. In our studies, through bioinformatics analysis of the GSE98987 dataset, we found that the treatment of CDK4/6 inhibitors in BC patients significantly inhibited HMGB1 expression. Applying CDK4/6 inhibitor abemaciclib to MCF-7R and T47DR could dramatically reduce the expression of TLR4 and NF- κ B. Moreover, abemaciclib's ability of reversing tamoxifen resistance could be weakened by HMGB1 recombinant protein. These results indicated that the reversion of abemaciclib to tamoxifen resistance partly relied on HMGB1 expression, and HMGB1 was the key factor for abemaciclib efficacy.

The mechanism of CDK4/6 inhibitors inhibiting HMGB1 expression is unclear at this time. Previous studies have shown that the HMG box-containing transcription factor UBF is the primary target for retinoblastoma protein(Rb)-induced transcriptional repression.⁴⁴ As we know, CDK4/6 inhibitors can relieve the phosphorylation of cyclin D1 and CDK4/6 on Rb. We speculated that dephosphorylation Rb inhibited the activity of the RNA polymerase/transcription factor UBF, which in turn repressed the expression of HMGB1. This may be the reason why CDK4/6 inhibitors inhibit HMGB1 expression, and we will verify this conclusion in follow-up experiments.

In this study, we are the first to verify high expression of HMGB1 leading to tamoxifen resistance in breast cancer. Moreover, we show for the first time that CDK4/6 inhibitors can limit HMGB1 expression. Reversion of CDK4/6 inhibitors to tamoxifen resistance partly relies on its function of suppressing the HMGB1-TLR4-NF- κ B

pathway. The in-depth analysis of this mechanism will help us to further understand CDK4/6 inhibitors' anti-tumor effects, and provide new ideas for the clinical application of related targets and medicines.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (Grant No. 81730074).

DISCLOSURE

The authors have declared that no competing interests exist.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zhang H, Wang J, Li J, et al. HMGB1 is a key factor for tamoxifen resistance and has the potential to predict the efficacy of CDK4/6 inhibitors in breast cancer. *Cancer Sci*. 2021;112:1603-1613. <https://doi.org/10.1111/cas.14813>