Loss of B-cell translocation gene 2 expression in estrogen receptor-positive breast cancer predicts tamoxifen resistance

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B-cell translocation gene 2 (BTG2), a gene suppressed in a subset of aggressive breast cancer, is repressed by estrogen. BTG2 inhibits the expression of HER ligands and promotes AKT activation, which plays an essential role in the tamoxifen resistance of estrogen receptor (ER)-positive breast cancer. To determine if BTG2 expression modifies tamoxifen efficacy, a cohort of 60 patients treated with adjuvant tamoxifen monotherapy was analyzed. We found that increased BTG2 expression showed better clinical survival and was the only independent prognostic factor for disease-free survival (hazard ratio, 0.691; 95% confidence interval, 0.495–0.963; $P = 0.029$). Tamoxifen suppressed the human epidermal growth factor receptor 2 (HER2)-Akt signaling in BTG2 expressing ER-positive breast cancer cells with a correlated increase in sensitivity, whereas BTG2 knockdown abrogated this sensitivity. Consistent with this observation, tamoxifen significantly suppressed the growth ratio, tumor weight and Ki-67 expression in BTG2 expressing breast cancer xenografts in mice. These studies demonstrate that BTG2 is a significant factor in tamoxifen response, acting through modification of AKT activation in ER-positive/HER2-negative breast cancer.

The B-cell translocation gene 2 (BTG2) is a member of the antiproliferative protein family. The BTG/Tob family of proteins contain two highly conserved domains known as BTG boxes A and B separated by a spacer sequence of 20–25 non-conserved amino acids.[1–3] BTG2 is expressed in the epithelium of the normal mammary gland[4] where its expression sharply declines to induce cyclinD1 expression during pregnancy.[5]

In breast tumors expressing the estrogen receptor (ER), suppression of BTG2 expression is associated with increasing tumor grade, size and overexpression of the cyclinD1 protein.[6] Loss of BTG2 in fibroblasts mediates p53-dependent cellular transformation by oncogenic Ras,[7] indicating that BTG2 might play an important role in the tumorigenic process.

Most patients with ER- or progesterone receptor (PR)-positive breast cancers are suitable for systemic hormonal therapy, including selective ER modulators (SERM). These SERM behave primarily as competitive inhibitors of estrogen binding to ER. Furthermore, the levels of ER expression, as well as those of PR, are currently the most important predictors of tamoxifen responses in the clinic.[8,9] However, the subset of patients whose prognoses are considered relatively good may still develop recurrence following curative surgery and adju-

vant hormonal therapy,[10] suggesting that the outcome of this therapy may be limited by acquired drug resistance.

Suppression of BTG2 has been shown to strongly induce activation of the HER pathway via stabilization of the mRNA of HER ligands, which play a critical role in the progression of breast cancer.[11] The activation of epidermal growth factor receptor and human epidermal growth factor receptor 2 (HER2) can further extend the role of ER in the proliferation of breast cancer cells[2,12] and is demonstrated to be associated with tamoxifen resistance.[2,13,14] Consistent with these observations, decreased BTG2 expression was associated with recurrence in breast cancer patients undergoing tamoxifen treatment.[11]

In the present study, we evaluated whether suppression of BTG2 expression in breast cancer would be associated with decreased responsiveness to tamoxifen. Our data demonstrate a correlation between the efficacy of tamoxifen treatment and the level of BTG2 expression and that AKT activation plays a major role in tamoxifen resistance.

Materials and Methods

Cell culture, treatments and proliferation assays. The non-tumorigenic human breast epithelial cell line, MCF10A, and several human breast cancer cell lines (MCF7, T47D,

MDA-MB231, HCC1500 and MDA-MB468) were obtained from the American Type Cell Culture Collection (ATCC; Rockville, MD, USA). The culture conditions used for the maintenance of cell lines have been described previously.[15] In brief, human breast cancer cell lines were maintained in Dulbecco's modified Eagle medium (Wako, Osaka, Japan). The MCF-10A cells were maintained in DMEM/F12 (Invitrogen, Carlsbad, CA, USA). All cell culture media were supplemented with 10% fetal bovine serum (BioWest, Nuaillé, France) and 1% penicillin-streptomycin (Invitrogen) and cells were grown in an incubator infused with 5% CO_2 at 37°C.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay is based on the cleavage of the tetrazolium salt MTT to purple formazan crystals by metabolically active cells. In brief, the cell lines were rinsed with phosphate-buffered saline, trypsinized and seeded at a density of 3×10^3 cells per well in 96-well plates. The cells were allowed to adhere overnight and serial dilutions of tamoxifen (Sigma-Aldrich, St Louis, MO, USA) were added to the wells at concentrations ranging from 0.01 to 10 μM, either with or without a 48 h pretreatment with 1 μg/mL of tetracycline. The cells were then exposed to the drug for 7 days. The drug-containing medium was then removed and 0.2 mL MTT solution (final concentration, 0.5 mg /mL MTT; Sigma-Aldrich) was added. The plates were incubated for 3 h before the medium was removed and 0.1 mL DMSO (Sigma-Aldrich) was added. Plates were agitated for 15 min before reading absorbance using a microplate reader (Sunrise Rainbow RC-R, Tecan Austria GmbH, Grödig, Austria) at 570 nm. All experiments were repeated at least twice. The IC_{50} value was determined as the concentration at which a 50% loss of viability occurred relative to untreated cells.

Antibodies. The following antibodies were used in the present study: BTG2 (Abcam, Cambridge, MA, USA); GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA); AKT and P-AKT (Cell Signaling Technology, Danvers, MA, USA); HER2 and P-HER2 (Abcam); anti-V5 (Invitrogen); epiregulin (LifeSpan BioSciences, Seattle, WA, USA); and Ki-67 (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Establishment of tetracycline-inducible cells. The tetracycline-inducible BTG2 expression model was developed using the T-REx system (Invitrogen).[16] The BTG2-expressing vector was constructed using the Virapower Lentiviral gene expression system (Invitrogen) according to the manufacturer's instructions. In brief, BTG2 cDNA was cloned into the plenti4/TO/V5-DEST vector. Either this construct or the pLenti6/TR (Tet repressor; Invitrogen) was transfected into the lentiviral packaging cell line 293FT together with ViraPower packaging mix (Invitrogen) using the Fugene HD transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA). The virus-containing culture medium was collected after 48 h and filtered through a 0.45-μm syringe filter. The viruses were mixed with 10 μg/mL polybrene (Sigma-Aldrich) and used to infect MCF7 cells. After 48 h post-infection, cells were maintained in a medium containing 5 μg/mL of blasticidin and 100 μg of Zeocin. The pLenti4 /TO/V5-GW/lacZ plasmid was used as a control. The expression of BTG2 mRNA was detected using PCR.

Gene silencing. Lentiviruses carrying the sequences 5′-CAA GAACTACGTGATGGCAGT-3′ and 5′-CGTGAGCGAGCAG AGGCTTAA-3′ targeting the coding sequence of BTG2 were obtained from the RNAi Consortium shRNA Library,[17] the Broad Institute (Cambridge, MA, USA), and were used to knockdown BTG2 expression in HCC1500, as described previously.[11,15]

Quantitative real-time PCR analysis. First-strand cDNA were synthesized using the PrimeScript RT Reagent Kit (Takara

Bio, Shiga, Japan) for reverse transcriptase-PCR. Quantitative real-time PCR analysis was performed using the SYBR Premix Ex Taq and the Thermal Cycler Dice Real Time System (Takara Bio).

The following primer sequences were used: BTG2: forward, 5′-GCGAGCAGAGGCTTAAGGTCTTC-3′ and reverse, 5′-AT GCGAATGCAGCGGTAGC-3′; and GAPDH: forward, 5′-AT CATCCCTGCCTCTACTGG-3′ and reverse, 5′-TTTCTAGAC GGCAGGTCAGGT-3′. Human GAPDH was used for normalization. All experiments were performed in duplicate.

Animal experiments. All animals were cared for and experiments were performed using protocols approved by the institutional review board and the institutional animal care and use committee of Keio University. MCF7 cells expressing activated H-Ras^{V12}-expressing lentivirus and either tet-BTG2 or tet-LacZ were generated using the above protocols. Xenografts were established by injecting 5×10^6 cells in 100 μL of phosphate-buffered saline into the mammary fat pad of 6-week-old female, BALB/c, nude mice (Sankyo Labo Service, Tokyo, Japan). Each group consisted of 8–11 mice. Tumor volumes were measured once every two days using the formula length $\times$ (width)^2. To determine the effect of tamoxifen on tumor growth, these tumor-bearing mice were administered water containing tetracycline or tamoxifen pellets (21-day time-release containing 1.5 mg tamoxifen; Innovative Research of America, Sarasota, FL, USA) from the day when the xenografted tumor grew to be over 125 mm^3 and then killed after 3 weeks of treatment.

Immunohistochemistry. Paraffin-embedded sections were cut into 4-μm sections, mounted onto slides, dewaxed in xylene and then rehydrated in alcohol. Endogenous peroxidase activity was then blocked using peroxidase-blocking reagent (0.03% hydrogen peroxide containing sodium azide; DAKO, Carpinteria, CA, USA) for 10 min. After the sections were autoclaved in target-retrieval solution (pH 6.0; DAKO) for 10 min, they were blocked with bovine serum albumin at a dilution of 1:100. Samples were incubated with anti-V5-specific antibody at a dilution of 1:250 at 4°C for 12 h to detect the V5-tagged BTG2, followed by treatment with a secondary antibody (peroxidase-labeled polymer; DAKO) for 30 min. DAB substrate buffer (substrate buffer solution, pH 7.5; DAKO) mixed with DAB chromogen (3,3′-diaminobenzidine chromogen solution; DAKO) was then added for 1 min. Between these steps, the slides were rinsed for 5 min in PBS three times. Sections were counterstained with hematoxylin, dehydrated and mounted.

To assess cell proliferation in tumors, anti-human Ki-67-specific antibody was used at a dilution of 1:200. The Ki-67-labeling index was determined in the tumor areas showing the highest labeling density, as determined by an initial scan at low magnification. The Ki-67-labeling index is a ratio of positively stained tumor cells to all tumor cells and is expressed as a percentage. In all, 1000 individual tumor cell nuclei were counted in each case.

Gene expression data. For primary analysis of gene expression profiles in clinical samples, we collected microarray data from 60 breast cancer patients using Gene Expression Omnibus (GEO) datasets (accession no. GDS807). These datasets were generated using a Arcturus 22k human oligonucleotide microarray.[18] The data were standardized by centering and scaling and patients were dichotomized into low- and high-expressing groups. Clinical features, including tumor size, histological grade, lymph node status, age, hormonal status and HER2 status, were previously compiled from the supplementary information in a previously published study.[18] Since all

the data came from publicly available resources, ethical approval was not required to conduct this part of the study.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Disease-free survival (DFS) curves were drawn using Kaplan–Meier estimates and compared using log-rank tests. Multivariate analysis of DFS, with stepwise variable selection, was conducted using Cox's proportional hazards regression models. We also examined the correlation between IC_{50} and the relative expression levels of BTG2 to internal control in each subcloned MCF7 cell line using a Spearman correlation coefficient. Statistical differences were determined for *in vitro* and *in vivo* assays using the Student's *t*-test. We considered values of $P < 0.05$ to be statistically significant and values of $P < 0.01$ highly significant.

Results

Prognostic significance of BTG2 expression in breast cancer. A previous expression analysis study in human breast carcinoma showed a loss of nuclear expression of BTG2 protein in 46% of tumors.[6] Analysis of the Oncomine data set also revealed a strong correlation between decreased BTG2 expression and recurrence in patients undergoing tamoxifen treatment.[11] To further validate the ability of BTG2 loss to predict the response to tamoxifen monotherapy, we analyzed gene expression data generated from a collection of laser-microdissected breast cancer samples ($n = 60$) from subjects who received tamoxifen alone as adjuvant systemic therapy (GEO database accession no. GDS807). According to the original study, patient inclusion criteria was defined as women diagnosed at the Massachusetts General Hospital between 1987 and 2000 with hormone receptor-positive breast cancer, treated with standard breast surgery, followed by 5 years of systemic adjuvant tamoxifen. No patient who received chemotherapy prior to recurrence was allowed.[18] The list of patient and tumor characteristics (tumor size, histological grade, lymph node status, age and status of ER, PR and HER2) is available in a previous publication.[18] Statistical analyses revealed that higher histological grade ($P = 0.007$) and lymph node negativity ($P = 0.043$) were significantly associated with BTG2 suppression (Table 1). We also conducted stepwise variable selection of the Cox proportional hazards regression model to identify clinical variables and BTG2 expression that were significant predictors of DFS. The results identified increased BTG2 expression as the only independent prognostic factor for DFS (hazard ratio, 0.691; 95% confidence interval, 0.495–0.963; $P = 0.029$). The DFS curve was drawn using Kaplan–Meier estimates and compared using log-rank tests. It revealed that patients with tumors displaying increased BTG2 expression showed good clinical outcomes with a statistically significant difference (Fig. 1).

These results raised the possibility that BTG2 could serve as a predictive marker of tumor responses to adjuvant tamoxifen alone. Although the loss of BTG2 may contribute to breast cancer progression, we hypothesized that it may also influence the efficacy of tamoxifen in treating breast cancer.

Tamoxifen efficiently suppresses the proliferation of cells expressing BTG2. To determine the functional consequence of BTG2 expression in regulating tamoxifen sensitivity, a panel of human breast cancer cell lines was screened for endogenous BTG2 expression. MCF7, an ER-positive breast cancer cell line, showed decreased expression of BTG2 compared with MCF10A, which is an immortalized human mammary

Table 1. Patient and tumor characteristics

|	BTG2 low ($n = 12$)	BTG2 high ($n = 48$)	*P*-value
Size (cm)			
Mean	2.5	2.0	0.89
Range	1.0–4.0	0.8–5.5	
Grade			
1	0 (0)	3 (6.3)	0.007
2	4 (33.3)	35 (73.0)	
3	8 (66.7)	10 (20.8)	
Nodes†			
–	9 (81.8)	19 (45.2)	0.043
+	2 (18.2)	23 (54.8)	
Age (years)			
Mean	64.6	67.9	0.25
Range	53–81	48–85	
Receptor status			
ER (+)	11 (91.7)	48 (100)	0.20
PR (+)	10 (83.3)	40 (83.3)	0.68
HER2 (+)	2 (16.7)	1 (2.1)	0.10

†Cases of missing data omitted. –, Negative; +, positive; DFS, disease-free survival; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.
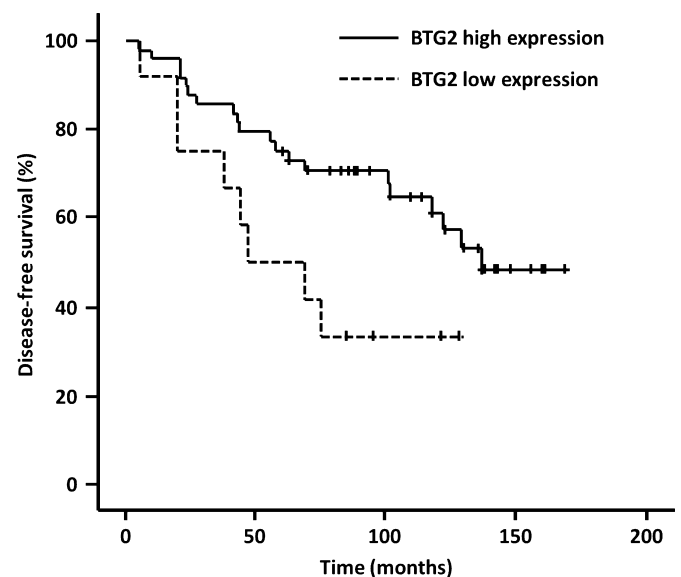
Fig. 1. Association of B-cell translocation gene 2 (BTG2) expression with disease-free survival (DFS) in patients who received tamoxifen alone as adjuvant therapy. A cohort of 60 breast cancer tumors compiled from the GEO datasets GDS807[18] was dichotomized into groups expressing either high or low levels of BTG2 and Kaplan–Meier curves were constructed for the probability of DFS in each group (log-rank test, $P = 0.029$).

epithelial cell line whereas T47D and HCC1500 cell lines, both ER positive, showed increased expression (Fig. 2a). Two ER-negative breast cancer cell lines, MDA-MB231 and MDA-MB468, demonstrated a 10-fold reduction in BTG2 expression compared with MCF10A (Fig. 2a). An *in vitro* cytotoxicity assay showed that T47D and HCC1500 expressing the highest levels of endogenous BTG2 had more drug sensitivity than MCF7 (MCF7: IC_{50}, 4.48 ± 0.21 μM; T47D: IC_{50}, 1.31 ± 0.13 μM; HCC1500: IC_{50}, 0.19 ± 0.15 μM). ER-negative MDA-MB468 was not responsive to tamoxifen ($IC_{50} > 25$ μM; Fig. 2b).

To further validate the relationship between BTG2 expression and tamoxifen efficacy *in vitro*, we selected the MCF7
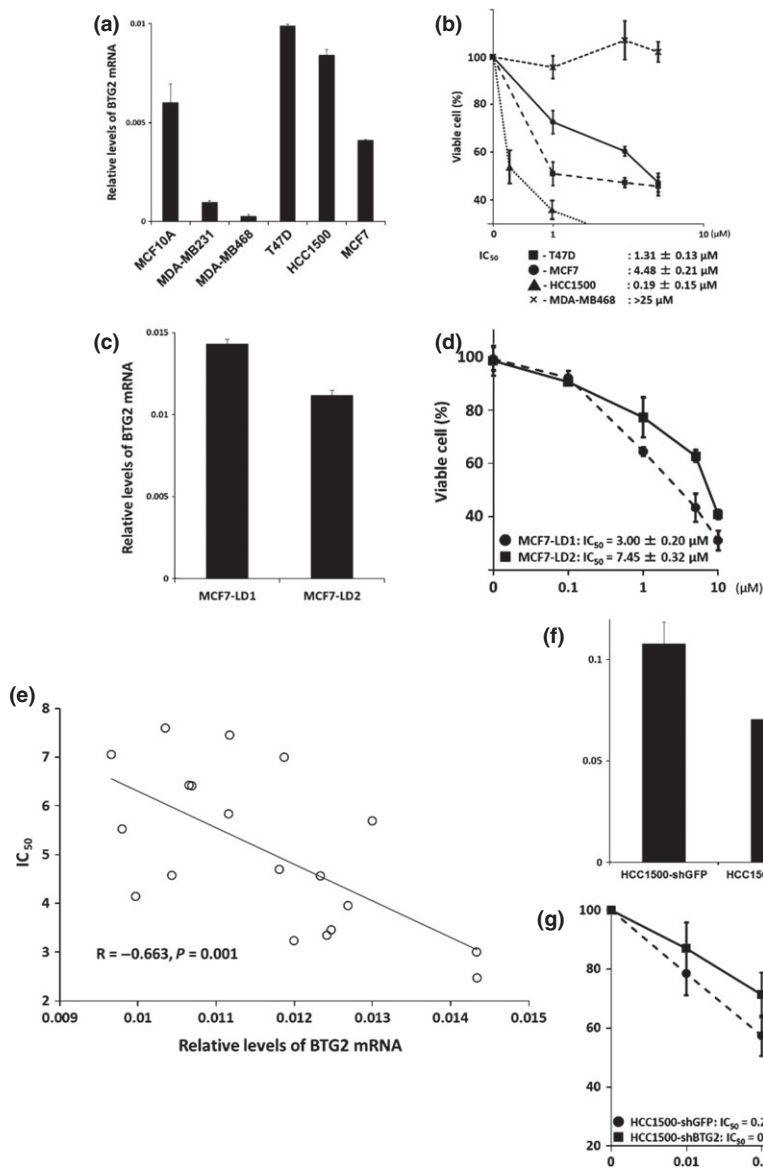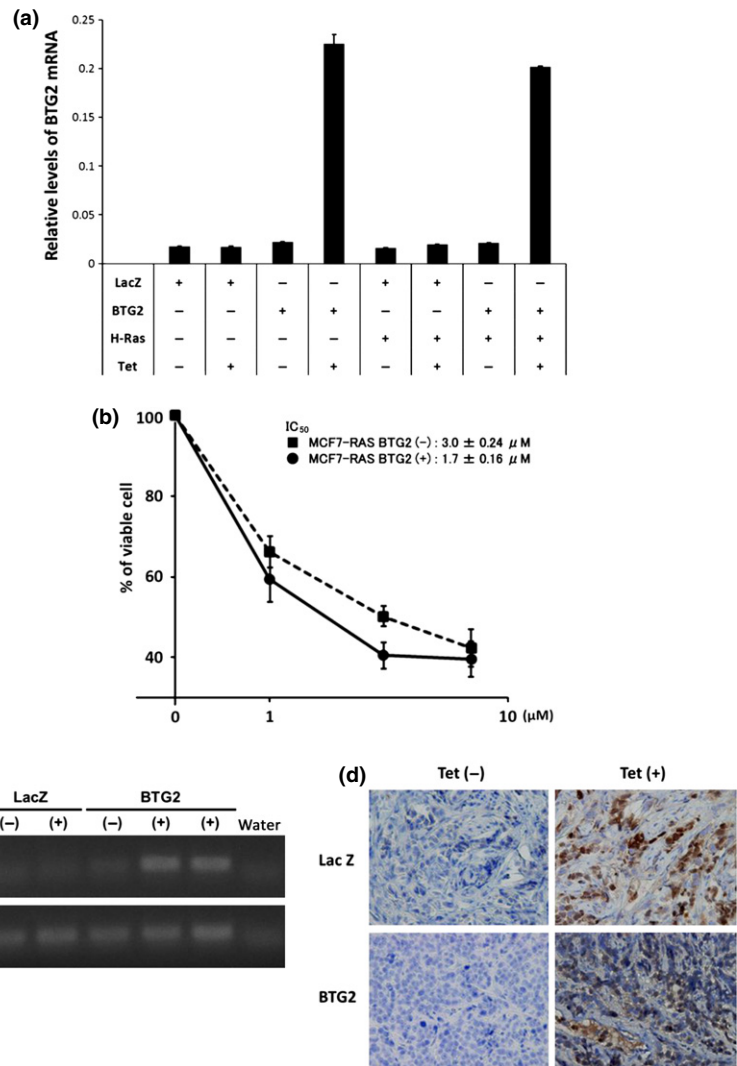
Fig. 2. Association of B-cell translocation gene 2 (BTG2) expression and tamoxifen effect in breast cancer cell lines. (a) Quantitative PCR analysis (qPCR) of BTG2 expression in an immortalized mammary epithelial cell line, MCF10A, and breast cancer cell lines MDA-MB231, MDA-MB468, T47D, HCC1500 and MCF7. The fold-change in expression relative to GAPDH is shown. (b) Inhibition curve of MCF7, T47D, HCC1500 and MDA-MB231 (negative control) treated with several different concentrations of tamoxifen. The IC_{50} value was calculated as the concentration at which a 50% loss of viability occurred relative to the untreated cells. (c–e) A single MCF7 cell was plated onto 96-well plates for limiting dilution and 20 subclones were established. An *in vitro* cytotoxic assay and assessment of BTG2 expression using quantitative RT-PCR analysis were conducted. Each subclone expressed a different amount of BTG2 mRNA and different IC_{50} against tamoxifen. Relative expression levels of BTG2 on the *x*-axis and IC_{50} values on the *y*-axis were plotted for each subcloned cell in the correlation diagrams (Spearman correlation coefficient, $R = -0.663$; $P = 0.001$). (e) RNA from HCC1500 cells infected with shGFP and shBTG2 was analyzed using qPCR to determine the expression of BTG2. (f) The inhibition curve of HCC1500 infected with either shBTG2 or shGFP and treated with several different concentrations of tamoxifen.

cell line for a cell-based screen. This line is commonly used to assess therapies that target ER activity and displays a heterogeneous response to hormonal manipulation, resulting in limited but not complete cell killing after tamoxifen treatment.[19] Therefore, limiting dilution was carried out to separate 20 subcloned cell lines that showed a range of sensitivities against tamoxifen and a range of BTG2 expression levels. MCF7-LD1 expressing a higher level of BTG2 showed more sensitivity to tamoxifen treatment (IC_{50}, 3.00 ± 0.20 μM) than MCF7-LD2 expressing a lower level of BTG2 (IC_{50}, 7.45 ± 0.32 μM; $P < 0.001$; Fig. 2c,d). An *in vitro* cytotoxicity assay and assessment of BTG2 expression using quantitative RT-PCR analysis were conducted with all of the 20 subcloned cell lines. There was a linear relationship (Fig. 2e; Spearman correlation coefficient, $R = -0.663$; $P = 0.001$) between the relative expression levels of BTG2 and IC_{50} values, although the variance increased with increasing IC_{50} values. This result suggests that tumor cells with lower BTG2 expression would be less sensitive to tamoxifen treatment, a finding consistent with the clinical evaluation data.

To determine whether the loss of BTG2 is sufficient to increase tamoxifen sensitivity, we selected HCC1500 for further study, given its high level of BTG2 expression. Short hairpin (sh) BTG2 lentiviral constructs capable of reducing endogenous BTG2 levels by approximately 65% were identified (Fig. 2f). We found that HCC1500 cells infected with shBTG2 exhibited significantly increased IC_{50} values (0.56 ± 0.18 μM) compared with those infected with shGFP (0.22 ± 0.12 μM; $P = 0.034$; Fig. 2g).

BTG2 expression promotes tamoxifen efficacy. To evaluate the relationship between BTG2 expression and tamoxifen efficacy *in vivo*, oncogenic H-RAS[V12] was introduced into the MCF7 cells. This was done because wild-type MCF7 could not constitute a xenograft tumor without additional estrogen management in an immunodeficient mouse; however, estrogen treatment during the experiment would strongly influence tamoxifen efficacy. MCF7-RAS[V12] maintains characteristics of hormone-sensitive breast cancer and forms tumors in immunodeficient mice without estrogen treatment.[20,21] Oncogenic H-RAS[V12] transformation of MCF7 had no effect on the

Fig. 3. *In vitro* and *in vivo* conditional expression model of B-cell translocation gene 2 (BTG2) using the tetracycline inducible system. (a) BTG2 was induced by treating MCF7/tet-BTG2 and MCF7-RAS^{V12}/tet-BTG2 cells with tetracycline for 48 h and BTG2 mRNA expression was assessed using qPCR. Additionally, MCF7/tet-LacZ and MCF7-RAS^{V12}/tet-LacZ cells were analyzed as controls. (b) Several different concentrations of tamoxifen were added to MCF7-RAS^{V12}/tet-BTG2 cells to draw inhibition curves for cells with or without tetracycline treatment. (c) MCF7-RAS^{V12}/tet-BTG2 cells were injected into the mammary fat pad of immunodeficient mice to form tumors. These tumor-bearing mice were administered water containing tetracycline. To confirm the BTG2 expression, mRNA was extracted from the harvested tumors and the expression was analyzed using semi-quantitative RT-PCR. (d) The resected tumors were also immunostained with anti-V5 antibody to confirm the nuclear expression of BTG2 in the tumor cells. MCF7-RAS^{V12}/tet-LacZ cells were also injected to create tumors as negative controls.

endogenous level of BTG2 expression (Fig. 3a). To assess the cytotoxicity of tamoxifen treatment on BTG2 overexpression, a tetracycline-inducible BTG2 expression model was developed in the MCF7-RAS^{V12} cells. The addition of low concentrations of tetracycline sharply induced BTG2 mRNA expression in both MCF7/tet-BTG2 and MCF7-RAS^{V12}/tet-BTG2 cells (Fig. 3a). As expected, MCF7-RAS^{V12}/tet-BTG2 tumors without BTG2 expression showed less sensitivity to tamoxifen treatment (IC$_{50}$, 3.0 ± 0.24 μM) than tumors with induced BTG2 expression with statistical significance (IC$_{50}$, 1.70 ± 0.16 μM; $P = 0.043$; Fig 3b) .

Since induction of BTG2 increases tamoxifen efficacy *in vitro*, we tested whether treatment of MCF7-RAS^{V12}/tet-BTG2 tumor-bearing mice with tamoxifen would efficiently interfere with tumor progression *in vivo*. We found that the inoculation of MCF7-RAS^{V12}/tet-BTG2 and MCF7-RAS^{V12}/tet-LacZ (negative control) into the mammary fat pads of nude mice consistently created a tumor, without the need for estrogen treatment. When the tumors reached >125 mm^3, the tumor-bearing mice were administered water containing a tetracycline and/or tamoxifen pellet (release time, 21 days). After 3 weeks of treatment, BTG2 transgene expression in harvested tumors was examined using both RT-PCR and immunohistochemistry,

confirming strong BTG2 expression in the nucleus of the tumor cells in tetracycline-administered mice (Fig. 3c,d).

After 21 days of treatment there was no significant difference in tumor volumes between treated and untreated tumors in animals bearing control tumors ($n = 11$) or mice treated with tetracycline alone ($n = 10$), suggesting that BTG2 expression did not affect tumor growth in this xenograft model (Fig 4a). Tamoxifen treatment alone, without tetracycline ($n = 10$), significantly decreased the tumor growth ratio compared with the negative control ($P = 0.009$). Remarkably, the tumor growth ratio was strongly suppressed, to a statistically significant level, with concomitant administration of tetracycline and tamoxifen ($n = 8$) in comparison with tamoxifen monotherapy ($P = 0.044$; Fig 4a). Correspondingly, tamoxifen treatment in the context of BTG2 expression significantly reduced the tumor weight compared with tamoxifen treatment alone ($P = 0.034$; Fig 4b). Concomitant administration of tamoxifen and tetracycline led to decreased Ki-67 expression compared with the control group ($P = 0.009$) or the group treated with tamoxifen alone ($P = 0.039$; Fig. 4c, d).

BTG2 expression modifies HER2 and AKT activation. Clinical and laboratory evidence supports an essential role for crosstalk
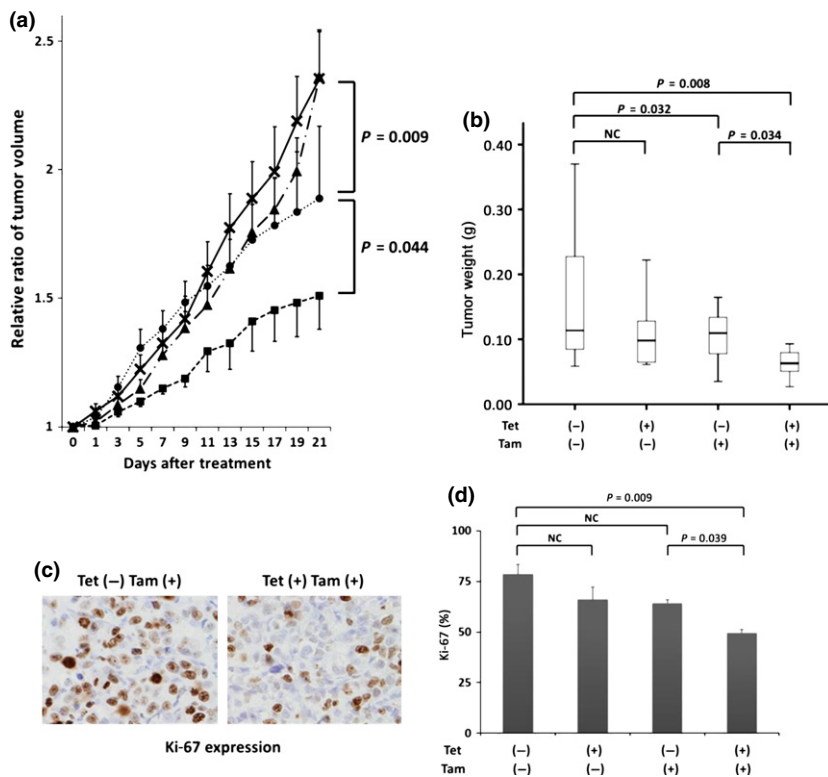
Fig. 4. Tamoxifen treatment effectively inhibits tumor progression with B-cell translocation gene 2 (BTG2) expression. (a) MCF7-RAS^{V12}/tet-BTG2 cells were inoculated into the mammary fat pad of 6-week-old female BALB/c nude mice. After the tumors grew to a volume of more than 125 mm^3, mice harboring these tumors were administered water containing tetracycline (Tet) and/or tamoxifen (Tam) pellets for 3 weeks. Tumor growth ratios were measured in all four groups ([×]: Tet(−)Tam(−), $n = 11$; [▲]: Tet(+)Tam(−), $n = 10$; [●]: Tet(−)Tam(+), $n = 11$; [■]: Tet(+)Tam(+), $n = 8$). Data are shown as averages ± SE. Tumor growth ratio was significantly suppressed with the concomitant administration of tetracycline and tamoxifen in comparison with the animals treated with tamoxifen alone ($P = 0.044$, Tet[−]Tam[+] vs Tet[+]Tam[+]; Student's t-test). (b) Tumors were harvested at 21 days after treatment to be weighed. Tumor weight was also significantly suppressed with the concomitant administration of tetracycline and tamoxifen in comparison with the animals treated with tamoxifen alone ($P = 0.034$, Tet[-]Tam[+] vs Tet[+]Tam[+]; Student's t-test). (c) Immunohistochemistry of Ki-67 in tumor samples from Tet(−)Tam(+) and Tet(+)Tam(+) mice. (d) The number of portions stained by Ki-67. The Ki-67 ratios were significantly different between Tet(−)Tam(+) and Tet(+)Tam(+) mice ($P = 0.039$; Student's t-test). The average number of stains was counted by studying three portions in each slide.

between the ER and HER2 signaling pathways in resistance to hormone therapies.[13,14,22] As has been shown previously, both estrogen and tamoxifen can activate the HER2 signaling pathway and stimulate tumor growth via non-genomic ER activity.[13,23] In addition, suppression of BTG2 strongly induces the activation of HER2 and HER3 phosphorylation, which is reduced by the restoration of BTG2 expression due to neuregulin-1, and epiregulin (EREG) was induced via transcript stabilization on knockdown of BTG2.[11] In the immunohistochemical staining of tumors consisted of MCF7-RAS^{V12}/-tet-BTG2 cells, EREG expression was observed under the condition of loss of BTG2. In contrast, EREG expression was reduced with BTG2 overexpression, which is consistent with the results of a previous study[11] (Fig. 5a).

To determine the molecular mechanism that decreases tamoxifen sensitivity in BTG2 repressed cells, we tested the activation of HER2 and AKT in MCF7-RAS^{V12}/tet-BTG2 cells, which were used in in vitro and in vivo drug sensitivity assays, with or without tamoxifen and/or tetracycline. Although the expression levels of total HER2 and AKT were not altered, consistent with previous studies,[13,23] phosphorylation of HER2 was slightly increased by tamoxifen treatment alone (Fig. 5b). Induction of BTG2 either with or without tamoxifen treatment reduced phosphorylation of HER2 and AKT (Fig. 5b). Additionally, BTG2 expression decreased the phosphorylation of IGF1R, another tyrosine kinase upstream of AKT. These results suggest that BTG2 is able to modify the HER2-AKT axis to reinforce the efficiency of tamoxifen treatment.

Discussion

We demonstrated that BTG2 expression modulates tamoxifen responsiveness in ER-positive/HER2-negative breast cancer

cells both in vitro and in mouse breast tumor xenograft models. This was further validated in human breast cancer samples where BTG2 expression was the single predictor of survival following tamoxifen treatment.

The ER signaling pathway has been shown to interact with HER2 signaling and tamoxifen-resistant breast tumors are characterized by HER2 activation.[24] ER-positive cell lines have also been shown to acquire HER2 overexpression resulting in tamoxifen resistance.[25] Moreover, PAX2 co-recruitment with ER-alpha to the HER2-regulatory element plays an essential role as a transcriptional repressor inhibiting HER2 expression in breast cancer cells. Therefore, loss of PAX2 expression leads to HER2 expression and confers tamoxifen resistance.[26]

Expression of BTG2 does not alter the HER2 protein level, but suppresses HER2 phosphorylation levels leading to increased sensitivity to tamoxifen treatment. Loss of BTG2 has been shown to stabilize the HER ligands neuregulin-1 and EREG resulting in activation of HER2 and HER3 receptors and AKT phosphorylation. Conversely, restoration of BTG2 reduces the phosphorylation of HER2, HER3 and AKT, and the expression of neuregulin-1.[11] In the present study, immunohistochemical analysis revealed that EREG expression was increased under the condition of loss of BTG2 in a mouse xenograft model (Fig. 5a) Previous studies have shown that tamoxifen treatment increased HER2 phosphorylation via crosstalk between ER and HER signaling.[13,23] However, these were not phosphorylated under the condition of high BTG2 expression with tamoxifen treatment, instead leading to the interruption of signal transduction for cell proliferation. BTG2 did not alter ER itself or several ER co-activators, such as AIB1, PIN1 and SRC1, as well as PAX2 (data not shown), suggesting that the contribution of BTG2 to tamoxifen sensitivity might be due mainly to suppression of HER signaling.
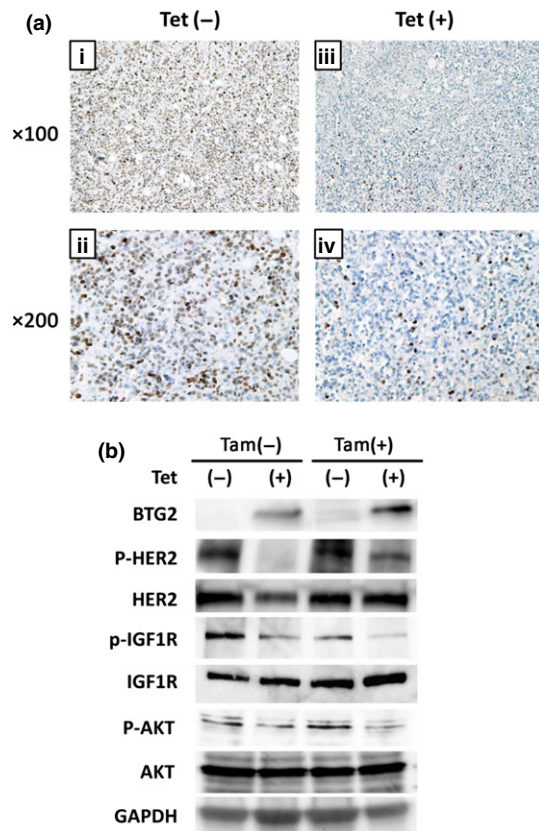
Fig. 5. (a) Immunohistochemistry of epiregulin (EREG) in tumor samples from (i,ii) tetracycline (Tet)(−) and (iii,iv) Tet(+); original magnification: (i,iii) ×100; (ii,iv) ×200. (b) Crosstalk between estrogen receptor (ER) and the human epidermal growth factor receptor 2 (HER2)-AKT pathway was modified by B-cell translocation gene 2 (BTG2) expression. BTG2 was induced for 48 h by 1 μg/mL tetracycline treatment of MCF7/tet-BTG2 cells. These cells were serum starved for 6 h and then treated either with or without 1 μM tamoxifen (Tam) for 24 h. Western blotting of total cell lysates was used to monitor the expression of BTG2 and the kinetics of HER2, IGF1R and AKT phosphorylation.

BTG2 induction even in the absence of tamoxifen reduced phospho-HER2 but did not change AKT phosphorylation. It is possible that the other tyrosine kinase might maintain the activation of AKT. Phosphorylation of IGF1R was also reduced by induction of BTG2. This may also contribute to tamoxifen efficacy; however, the relationship between BTG2 and IGF1R has not yet been studied. This unknown mechanism should be explored in future experiments.

Loss of BTG2 protein in breast cancer is associated with higher tumor grade and size and overexpression of the cyclinD1 protein,[5,6] increased cell migration *in vitro* and enhancement of tumorigenesis and metastasis *in vivo*. Breast tumor progression promoted by loss of BTG2 can be abrogated by treatment with the tyrosine kinase inhibitor lapatinib. These results indicate a critical role of the HER2 pathway in breast cancer progression induced by loss of BTG2,[11] suggesting that loss of BTG2 may serve as a prognostic factor in breast cancer patients. BTG2 is abundantly expressed in the epithelium of the normal mammary gland;[4] however, expression analysis of BTG2 protein in human breast cancer ($n = 148$) revealed the loss of nuclear expression in 40.4% of ER-positive tumors.[6] This suggests that loss of BTG2 can occur in the transformation process leading to intrinsic resistance against tamoxifen. In parallel, epigenetic mechanisms might play a critical role in regulating the levels of BTG2 in breast cancer cells; additionally, several microRNA (such as mir-21, which is overexpressed in breast cancer cells) potentially target BTG2 mRNA.[27–29] This regulation can occur not only at the time of carcinogenesis, but also at the time of tamoxifen treatment, resulting in acquired tamoxifen resistance. This should also be studied from both clinical and basic perspectives in future research.

In summary, our findings suggest that BTG2 is a significant element in tamoxifen response, acting through modification of AKT activation in ER-positive/HER2-negative breast cancer. The understanding of resistance to endocrine therapies in hormone receptor-positive breast cancer previously suggested new therapeutic strategies that would enhance efficacy.[30] Therefore, extensive studies investigating the loss of BTG2 might lead to clinical applications of novel strategies. Simultaneously, the identification of a subgroup of patients with poor outcome using this biomarker should facilitate clinical trial designs.

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Disclosure Statement

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