ABCC11 expression is regulated by estrogen in MCF7 cells, correlated with estrogen receptor α expression in postmenopausal breast tumors and overexpressed in tamoxifen-resistant breast cancer cells

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M Honorat and A Mesnier contributed equally to this work

Abstract

ABCC11 (Multidrug resistance protein 8; MRP8), a plasma membrane ATP-binding cassette transporter, has been implicated in drug resistance of breast cancer by virtue of its ability to confer resistance to fluoropyrimidines and to efflux methotrexate, and by its expression in this tumor. Expression of ABCC11 in breast, a hormonally regulated tissue, as well as the pump's ability to transport estrogen conjugates, suggest the possibility that expression of ABCC11 may be susceptible to regulation by estrogen. However, nothing is currently known about regulation of this gene. In this study, estradiol (E2) treatment reduced expression of ABCC11 mRNA in estrogen receptor (ER)-α-positive MCF7 cells, and E2 antagonists such as ICI 182 780 and tamoxifen (TAM) abrogated E2-mediated downregulation. ABCC11 expression was positively correlated with ER-α expression in both breast cell lines, and two independent series of tumors from postmenopausal patients. In addition, expression of ABCC11 was upregulated in MCF7 cells exposed to TAM for 72 h, and was overexpressed in TAM-resistant cell lines. Drug sensitivity analysis of the TAM-resistant cells indicated that they were also resistant to 5-fluorouracil (5-FU), consistent with the reported ability of ABCC11 to confer resistance to this agent. These studies indicate that ABCC11 expression is negatively regulated by E2, but that ABCC11 expression is high in high-expressing ER-α breast cancers. Our findings support the notion that expression of ABCC11 in ER-α-positive breast cancers may contribute to decreased sensitivity to chemotherapy combinations that include 5-FU. ABCC11 may be a potential predictive tool in the choice of anticancer therapies in ER-positive breast cancers resistant to TAM.

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**Introduction**

Breast cancer is a complex and heterogeneous disease, with a range of overlapping clinical phenotypes with significant variations in prognosis and outcome. Histological type, grade, lymph node involvement, and estrogen receptor (ER) status influence prognosis and the probability of response to therapies. ER-positive breast cancers are responsive to endocrine therapies, such as the selective ER modulator (SERM), OH-tamoxifen (TAM), and the pure antagonist ICI 182 720 (fulvestrant; Howell 2006). However, almost all responding patients acquire resistance to TAM. Several mechanisms for this phenomenon have been suggested, including alteration of the availability or metabolism of TAM, alterations in the function of the ER-α and in the ER-α signaling cascade, and alterations in growth factor signaling pathways (MAPK, AKT; Dorssers et al. 2001, Acconcia & Kumar 2006, Girault et al. 2006, Milano et al. 2006). Treatment with cytotoxic chemotherapy is employed for patients with ER-negative and ER-positive hormone-refractory breast cancers. Standard agents include cyclophosphamide, methotrexate, vinca alkaloids anthracyclines, taxanes, and 5-fluorouracil (5-FU), with the latter agent recently assuming a larger role as a result of the availability of congeners that can be orally administered.

One of the mechanisms of drug resistance in cancer cells is increased efflux mediated by members of the ATP-binding cassette (ABC) family. Numerous studies have been conducted on expression of ABCB1 (multidrug resistance, MDR1/P-glycoprotein (P-gp)), ABCC1 (Multidrug resistance associated protein 1 MRPI), and ABCG2 (breast cancer resistance protein; BCRP) in breast cancers (Burger et al. 2003, Larkin et al. 2004). Recently, another ABC transporter, ABCC11 (Multidrug resistance protein 8; MRP8) has been implicated as a resistance factor in breast cancer (Park et al. 2006, Kruh et al. 2007). ABCC11 transcript has been reported to be expressed in normal mammary tissue, in breast cancer cell lines and in breast tumors, as well as in liver and brain (Bera et al. 2001, Anneraux et al. 2004, Bieche et al. 2004, Bortfeld et al. 2006). In addition, ectopic expression of ABCC11 is in vitro able to confer resistance to fluoropyrimidines, a class of agents that is widely employed in treating breast cancer (Guo et al. 2003, Oguri et al. 2007). This capability is attributable to ABCC11-mediated efflux of 5-FdUMP, the toxic intracellular metabolite of fluoropyrimidines. ABCC11 is also able to transport methotrexate (Chen et al. 2005), another agent that is employed in the treatment of breast cancer. The involvement of ABCC11 may also extend to physiological compounds that effluxed by this transporter in that notable physiological substrates of the pump include glucuronate and sulfate steroid conjugates, such as estradiol-17β-D-glucoronide (E217βG), desulfate estrone 1-sulfate (E1S), and dehydroepiandrosterone sulfate (DHEAS) (Chen et al. 2005). These resistance and transport activities, in combination with expression of ABCC11 in breast, a hormonally regulated tissue, suggest the possibility that expression of ABCC11 may be susceptible to regulation by estrogen. Were this the case, it could have important implications for treating breast cancer. However, nothing is currently known about regulation of expression of this gene. Here, we analyzed in vitro the effect of estrogen on ABCC11 expression and evaluated the relationship between ABCC11 expression levels and ER-α expression in breast cancer cell lines and tumor samples from postmenopausal patients.

Our results show that ABCC11 is negatively regulated by estradiol (E2) and that ABCC11 mRNA levels are increased in high-expressing ER-α breast cancers from independent series of tumors. In a first step, we used series A of tumors (‘training set’) including 60 primary tumors collected from postmenopausal women at the Pathology Department of Val d’Aurelle Cancer Center (Montpellier, France). Our independent collaborator (Dr I Bieche, Paris) validated our observations, in two independent series B and C (‘validation sets’) obtained from women at a second institution (Centre René Huguenin, St-Cloud, France).

In addition, ABCC11 is overexpressed in a cell line made resistant to TAM, and this overexpression is associated with increased resistance to 5-FU. These findings suggest that, in ER-positive breast cancers, ABCC11 may contribute to decreased sensitivity to chemotherapy combinations containing 5-FU and that its expression may be a predictive tool in the choice of anticancer therapies.

**Materials and methods**

**Materials and chemicals**

5-FU, 5-FdURD, E2, MK571, and TAM were purchased from Sigma. 7a,17b-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]-nonyl]-estra-1,3,5(10)-triene-3,17-diol (ICI 182 780) from Tocris Cookson Inc. (Ellisville, MO, USA) was kindly provided by Pr P Cohen (UMR 590 Lyon). HCl was purchased from Merck. TRIZol RNA extraction kit, murine Moloney leukemia virus reverse transcriptase (MMLV), Taq DNA polymerase and complete high-glucose Dulbecco’s modified Eagle’s medium (DMEM), L-glutamine, and
penicillin–streptomycin were manufactured by Gibco, and fetal bovine sera (FBS) from PAN Biotech GmbH (Aidenbach, Germany).

Cell culture

The six ER-α-positive (MCF7, ZR 7530, UACC 818, ZR 751, T47D, and BT474) and the six ER-negative (MDA MB 453, BT20, SK BR3, MDA MB 435, MDA MB 175, and MDA MB 231) breast cell lines were grown according to the ATCC’s (Type Culture Collection) recommendations. breast cell lines were grown according to the ATCC’s (Type Culture Collection) recommendations.

To avoid interferences by steroids present in classical media, cell lines were first purged for 4 days in DMEM without phenol red and supplemented with 3% steroid-depleted, dextran-coated charcoal-treated fetal calf serum (DCC medium). Then to study the response of MCF7 cells to E2, TAM, and ICI 182 780, the cells were supplemented with specific steroids under the following pharmacological conditions: steroid-depleted medium (vehicle), $10^{-9}$ M E2, $2 \times 10^{-7}$ M TAM, or $10^{-7}$ M ICI 182 780. No effects of the vehicle on cell viability and ABCC11 mRNA expression were observed at these concentrations.

MVLN cells are an ER-α-positive cells derived from MCF7 cells (Demirpence et al. 1993). Isolation of clones from TAM-treated MVLN cells was done after treatment with $10^{-7}$ M TAM in DCC medium (Badia et al. 2000). Using a cell growth assay, the DNA content of $10^{-7}$ M TAM- and $10^{-9}$ M E2-treated-resistant clones was two and fourfold higher (Badia et al. 2000) respectively than that of cells grown in DCC medium. Conversely, in parental MVLN cells, TAM inhibited cell proliferation by half (Badia et al. 2000). Furthermore, Vendrell et al. (2005) carried out TAM dose–response experiments to measure the concentration giving rise to a 50% reduction in cellular viability (IC50 values). These data have been demonstrated on several TAM-resistant cellular clones selected from MVLN. These TAM-resistant cells are kindly provided by Prof P Cohen (Inserm, U590, Lyon F-69008, France).

Breast samples

All experimental procedures complied with French laws and regulations and were approved by the National Ethics Committee. Sixty primary tumors from patients with no therapy prior to surgery were collected from postmenopausal women at the Pathology Department of Val d’Aurelle Cancer Center (Montpellier, France; series A). The malignancy of infiltrating carcinomas was scored according to the histoprognostic system of Scarff–Bloom–Richardson (Bloom & Richardson 1957). The high expression of ER-α was determined by a real-time quantitative PCR (QRT-PCR) assay and confirmed in most of the case at the protein level by a radioligand-binding assay (with a cut-off level for positively set at 10 fmole/mg protein according to the WHO typing system). For RNA extraction, breast tumors were disrupted with a tissue grinder under liquid nitrogen.

To confirm the inter-relationship between mRNA level of ABCC11 and ER-α expression levels, we also analyzed an independent set of tumors from an independent series of 42 primary breast tumors (series B; 21 ER-negative and 21 ER-positive tumors) excised from women at a second institution (Centre René Huguenin, St-Cloud, France).

Finally, to investigate the relationship between mRNA levels of ABCC11 during breast cancer progression, we analyzed tumor RNA of 10 normal breast tissues, 12 benign breast tumors, 11 invasive ductal grade I breast tumors, 30 invasive ductal grade II breast tumors, 32 invasive ductal grade III breast tumors, and 14 distant metastasis (series C).

Quantitative real-time (QRT)-PCR

Total mRNA was extracted using TRIZol method (Jordheim et al. 2004) and QRT-PCR was performed in a Lightcycler (Roche) in combination with the LightCycler Faststart DNA Master Sybr Green I (Roche). cDNA reverse transcription was performed on 1 μg total RNA with MMLV reverse transcriptase and 0.5 μg hexamer according to the manufacturer’s instructions. The primer pairs for ABCC11 were 5’-GTCTGGGTCTCATTCCACATCC-3’ (forward), 5’-CCAGAGCTTTGCTGGGGTTGTA-3’ (reverse). The thermal cycling conditions comprised an initial denaturation step at 95°C for 8 min, and 50 cycles at 95°C for 15 s and 63°C for 7 s and 72°C for 15 s. A specific set of gene primers including PNR2/PS2 (estrogen inducible protein) and ESR1/ER-α (estrogen receptor alpha) primers was described in Vendrell et al. (2005). For each set of primers, a standard curve was made with serial dilutions from a control cDNA sample in order to evaluate the efficiency of the primers and to relatively quantify the expression level of each sample. All measurements were normalized to the expression of the 18S or 28S ribosomal genes, considered as stable housekeeping genes (Applied Biosystems).

For the independent series B and C, ABCC11 expression was quantified using a validated and independent method developed by Dr I Bieche (Tozlu et al. 2006). The increase in fluorescent signal associated with exponential growth of PCR products is detected by the laser detector of the ABI Prism 7900.
Sequence Detection System (Perkin–Elmer Applied Biosystems, Foster City, CA, USA), using PE Biosystems analysis software according to the manufacturer’s manuals. All PCRs were performed using an ABI Prism 7900 Sequence Detection System, the SYBR Green PCR Core Reagents kit (Perkin–Elmer Applied Biosystems; Biocch et al. 2004). The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min, and 50 cycles at 95 °C for 15 s, and 65 °C for 1 min. Results, expressed as n-fold differences in target gene expression relative to the TATA box-binding protein (TBP) gene (termed ‘Ntarget’), were determined by the formula:

\[ N_{\text{target}} = 2^{\Delta C_t \text{ sample}} \]

where the \( \Delta C_t \) value of the sample was determined by subtracting the average \( C_t \) value of the target gene from the average \( C_t \) value of the TBP gene. The \( N_{\text{target}} \) values of the samples were subsequently normalized such that the mean of the \( N_{\text{target}} \) values of the normal breast samples would equal a value of 1.

Immunoblotting

SDS-PAGE of crude membrane proteins was carried out using 8% acrylamide gels. Proteins (150 μg) were transferred to poly vinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using 25 mM Tris-base, 192 mM glycine, and 20% methanol buffer. ABCC11 polypeptides were detected using an enhanced chemiluminescence kit (Amersham) and the ABCC11 polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany). Various polypeptide amounts were estimated by comparison with cell-expressed positive and negative control cell lines (pCDNA3 HEK293T and ABCC11 HEK293T cells) loaded using the same conditions. Equal protein loading was confirmed by α-tubulin immunoblotting of the PVDF membrane. Densitometry of the film images was performed using ImageJ software (National Institutes of Health, http://rsb.info.nih.gov/ij/). The relative protein expression levels were calculated by dividing the integrated densitometry values obtained for 150 μg total membrane protein from E2-treated cells by the integrated densitometry value obtained for the comparable amounts of total membrane proteins from vehicle-treated cells. Each comparison was performed three times in independent experiments.

Cytotoxicity assays

MCF7 cells were plated at 5000 cells per well in 96-well plates (Becton Dickinson, San Jose, CA, USA) in a volume of 100 μl and incubated for 24 h at 37 °C before 100 μl medium containing different drug concentrations was added. The cytotoxicity assay was carried out as described in Jordheim et al. (2004).

5-FdUMP efflux assays

Based on specific ABCC11 capability to transport 5-FdUMP and endogenous nucleosides (AMPc and GMPc), we developed an assay for the quantification of 5-FdUMP using high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS/MS). This approach has two great advantages: 1) it quantified specifically and sensitively various metabolites of 5-FU and 2) in contrast to several fluorescent substrates which are substrate of various ABCC proteins, 5-FdUMP is restricted substrate to ABCC5 and ABCC11 (Guo et al. 2003, Pratt et al. 2005). Analysis of 5-FdUMP efflux has, however, been shown to permit evaluation of ABCC11 activity in transfected cells with ABCC11 cDNA that do not overexpress other MDR transporter in comparison with parental cell line (Guo et al. 2003, Oguri et al. 2007).

Cells were first loaded with 100 μM 5-FdURD in the DMEM (without FBS) for 15 min at 37 °C. During this uptake period, 5-FdURD, a metabolite of 5-FU, is metabolized into 5-FdUMP by thymidylate kinase into cells (Parker & Cheng 1990). After washing three times with ice-cold PBS, conditions which inhibit all active transport, intracellular 5-FdUMP accumulation was quantified by HPLC MS/MS in an aliquot of cells.

To quantify 5-FdUMP efflux, cells were then re-incubated at 37 °C in 5-FdURD-free medium for 15 min. The representative transport function of ABCC11 is shown in aliquots of efflux medium, thus making it possible to directly investigate secretion of 5-FdUMP in the medium in the presence or absence of specific and well-known inhibitor of ABCC11 transport activity (MK571). To carry out HPLC analysis, these cells were lysed in 500 μl of pure methanol, then the samples were centrifuged at 20000 g (14 000 rpm). for 2 min. The supernatants were evaporated under a stream of nitrogen and residues were resuspended in water solution before injection in HPLC device. 5-chlorouracil was used as internal standard. Results were normalized to cellular protein content (Bradford assay) determined in parallel using Bio-Rad assay.

Statistical analysis

Data were analyzed for statistical significance using Mann–Whitney’s test, Spearman’s test, or Student’s
Results

ABCC11 expression is regulated by estrogen

The effect of estrogen on ABCC11 expression in ER-α-positive MCF7 cells was analyzed by exposing cells to 1 nM E2 and measuring ABCC11 transcript by QRT-PCR. As a positive control for these experiments, PNR2/PS2, a gene whose expression is known to be upregulated by E2, was used (Berry et al. 1989). PNR2/PS2 mRNA levels were increased approximately fourfold, with maximal induction observed as early as 6 h (Fig. 1). By contrast with PNR2/PS2, ABCC11 expression levels decreased as a result of treatment with E2. ABCC11 expression was not altered after 6 h exposure to E2 (Fig. 1), whereas maximal repression was achieved after 24 h of exposure. In contrast to the effect of E2 on ABCC11 expression in MCF7 cells, estrogen treatment did not affect expression of ABCC11 in ER-α-negative MDA MB 231 cells (Fig. 1).

In breast tissue, TAM and ICI 182 780 antagonize E2-mediated gene regulation by binding to ER-α. As expected, PNR2/PS2 upregulation by E2 was abrogated by 100 nM ICI 182 780 and 200 nM TAM (Fig. 2). Under the same conditions, these agents abrogated E2-mediated downregulation of ABCC11 expression (Fig. 2).

Crude membrane fractions were further carried out from MCF7 cells exposed to 1 nM E2 for 72 h (Fig. 3). The expression of the ABCC11 protein was analyzed by immunoblotting. The affinity-purified ABCC11 antibody, which is directed against amino acids 929–1144 of the human ABCC11 protein, detected a glycoprotein of ~150 kDa in both samples (Fig. 3). Under our experimental conditions, additional bands below 150 kDa, probably due to proteolytic degradation of ABCC11, were also detected in HEK293T cells transfected with pCDNA3 ABCC11 vector (Fig. 3 A). Similar observations had been described by Bortfeld et al. (2006). Analysis of the immunoblots revealed that in MCF7 cells, E2 slightly decreased ABCC11 protein expression compared with the vehicle control (Fig. 3B).

Expression of ABCC11 mRNA is correlated with expression of ER-α and ERBB2

Based upon the observation that ABCC11 expression is repressed in vitro by E2, we next evaluated whether there was a correlation between ABCC11 and ER-α.

Figure 1 Effect of E2 on expression of ABCC11 transcript in MCF7 (ER-α-positive) and MDA MB 231 (ER-α-negative) cell lines. Cells were exposed to 1 nM E2 for 6–72 h, and ABCC11 transcript levels were measured by QRT-PCR at various time points. As a positive control for E2 effect, PNR2/PS2 transcript levels were also measured. Fold change (mean values were indicated below the figure) of mRNA levels was quantitated as described in Materials and methods. The QRT-PCR values indicated below are means ± S.E.M. of at least three independent experiments. **P < 0.01; Student's t-test.

Figure 2 Effect of estrogen receptor antagonists on E2-mediated repression of ABCC11 transcript. MCF7 cells were exposed to 1 nM E2 for 24 h, and ABCC11 transcript levels were measured by QRT-PCR. As a positive control for antagonist effect, PNR2/PS2 transcript levels were also measured. Fold change (mean values were indicated below the figure) of mRNA levels was quantitated as described in Materials and methods. The QRT-PCR values indicated below are means ± S.E.M. of at least three independent experiments. *P < 0.05; **P < 0.01; Student's t-test.
expression in breast cancer cell lines. ABCC11 expression was analyzed in six ER-α-positive (MCF7, ZR 7530, UACC 818, ZR 751, T47D, and BT474) and six ER-α-negative (MDA MB 453, BT20, SK BR3, MDA MB 435, MDA MB 175, and MDA MB 231) cancer cells. We applied the non-parametric Mann–Whitney’s test, the raw data from ER-α-negative cell lines and ER-α-positive cell lines, were first be combined into a set of N cell lines, which were then ranked from ER-α expression status. ABCC11 mRNA expression was significantly higher ($P = 0.0021$; Mann–Whitney’s test) in ER-α-positive cells than in ER-α-negative cells (Fig. 4).

We next sought to determine whether there was a correlation between ABCC11 and ER-α in breast cancer samples. To accomplish this, ABCC11 expression was analyzed in primary breast tumors excised from 60 postmenopausal women. The samples were classified into subgroups according to gene expression levels (ER-α, ERBB2, PNR2/PS2, and ER-β), age, tumor size, and histological grade. ER-α expression was measured by QRT-PCR, and the cut off was the median of the total population. Significantly higher levels of ABCC11 mRNA in ER-α high-expressing tumors compared with those in ER-α low-expressing cancer samples were observed ($P = 0.003$; Mann–Whitney’s test; Table 1). The expression levels of ABCC11 in the ER high-expressing group were approximately sevenfold higher than in ER low-expressing group. We observed a significant positive correlation between the mRNA levels of ABCC11 and ER-α levels (Spearman’s rank correlation coefficient $= 0.341$; $P = 0.007$; Table 1). ABCC11 expression also correlated with high PNR2/PS2 and ERBB2 expression levels while it did not correlate with expression of ER-β (Table 1).

Furthermore, in tumors with low ERBB2 expression ($n = 47$, the chosen cut off was 2.5-fold the median value, this group represented 73% total tumors), ABCC11 expression levels correlated with ER-α expression levels (Spearman’s rank correlation coefficient $= 0.46$; $P = 0.0011$). In ERBB2 low-expressing group, the correlation between ABCC11 and ER-α was likely independent of ERBB2 expression. A high correlation between ERBB2 and ABCC11 expression was also found in the low ER-α expressing group ($n = 30$, Spearman’s rank correlation coefficient $= 0.676$; $P = 0.00004$). We observed no correlation between ER-α and ERBB2 expression in the small size of our set of tumors A ($n = 60$; data not shown).

In an independent set of 42 patients (tumor series B), included ER-α-positive and -negative tumors, we found a significant positive correlation between ABCC11 and ER-α status ($n = 32$, $P < 10^{-5}$ with Mann–Whitney’s test, Spearman’s rank correlation coefficient $= 0.57$; $P = 0.0005$) and ABCC11 and ERBB2 correlation was also confirmed in patient
with low-expressing ER-α \((n = 21, \text{ Spearman’s rank correlation coefficient } = 0.66; \text{ } P = 0.0009)\).

In tumor series B, we compared ABCC11 levels between various tumors with normal tissues. The median of ABCC11 expression was 1.0 (range 0.14–10.87), 0.06 (range 0.01–186.33), and 3.55 (range 0.05–42.42) respectively in 6 normal breast tissues, 21 ER-negative tumors, and 21 ER-positive tumors. There was a trend towards respectively lower and higher ABCC11 levels in ER-α-negative and α-positive breast tumors, in comparison with normal breast samples.

Furthermore, in tumor series A, we also observed that ABCC11 expression levels were significantly lower in tumors with histological grade III compared with grade II \((P = 0.003 \text{ with Mann–Whitney’s test; Table 2})\) and large tumor size \((P = 0.005 \text{ with Mann–Whitney’s test; Table 2})\). We suggest that the correlation was directly related to the high proportion of tumors with ER-α high-expressing tumors in grade II subgroup compared the weak proportion of ER-α high-expressing tumors in grade III subgroup. A positive correlation was also found for tumor size (Spearman’s rank correlation coefficient = 0.507; \(P = 0.013; \text{ Table 1})\). However, tumor size information was only available for 23 patients, essentially with high expression levels of ER-α. No correlation was found with age or the presence or absence of nodal involvement (Table 2).

To further evaluate the role of ABCC11 during the breast cancer progression, the expression of ABCC11 was analyzed in normal breast tissues, benign breast tumors, invasive ductal grade I breast tumors, invasive ductal grade II breast tumors, invasive ductal grade III breast tumors, and distant metastasis (series C). ABCC11 expression increased gradually from normal breast tissue to benign tumors, grade I and II invasive ductal tumors; then decreased in the grade III tumors (twofold in comparison with histological grade II tumors) and in metastatic tumors (30-fold decrease; Table 2). The expression level of ABCC11 was respectively increased (by ~14-fold) and slightly decreased in grade III tumors and metastatic tumors compared with normal tissue (Table 2). In this independent series C, a trend towards increased ABCC11 expression in grade II tumors compared with
grade III was confirmed. The strong decrease of ABCC11 expression in metastatic tissue may be partially due to the weaker proportion of ER-α-positive tumors compared with ER-α-negative tumors (data not shown).

**ABCC11 upregulation by TAM in MCF7 cells and ABCC11 overexpression in TAM-resistant breast cancer cells**

As patients with ER-α-positive breast tumors are typically treated with ER antagonists such as TAM, we studied the effect of this agent on ABCC11 expression levels in MCF7 cells. Interestingly, treatment with 200 nM TAM for 72 h increased ABCC11 mRNA levels by twofold (no effect was observed after 24 h; Fig. 5A).

To explore the possible correlation between TAM resistance and expression of ABCC11 in ER-α-positive cells, we analyzed ABCC11 levels in CL6.7 ER-α-positive TAM-resistant cell line derived by growth of MVLN cells for 6 months (Badia *et al.* 2000, Vendrell *et al.* 2005) and described in Materials and methods. Significant increase in ABCC11 expression was observed in CL6.7 cells compared with MVLN parental cells. In contrast, the expression of ABCC5, another MRP family member that is able to confer resistance to 5-FU was not increased (Fig. 5B).

<table>
<thead>
<tr>
<th>(A)</th>
<th>Number of patients (n=)</th>
<th>Median</th>
<th>P value</th>
<th>ABCC11 mRNA expressiona</th>
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<tr>
<td>ER-α expressiona,c</td>
<td>&gt; 4.1</td>
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<td>15.59 range (0.59–289.69)</td>
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<td>&lt;4.1</td>
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<td>2.22 range (0.12–354.45)</td>
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<tr>
<td>ER-β expressiona,c</td>
<td>&gt; 0.66</td>
<td>30</td>
<td>3.43 range (0.13–289.69)</td>
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<td>ERBB2 expressiona,d</td>
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<td>&lt;2.5</td>
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<td>5.58 range (0.12–289.69)</td>
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<tr>
<th>(B)</th>
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<td>Age (56–86)e</td>
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<td>−1.017</td>
<td>0.31 (NS)</td>
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<td>Tumor sizef</td>
<td>23</td>
<td>0.507</td>
<td>2.697</td>
<td>0.013</td>
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P<0.05 was considered significant (Spearman’s test).
aQRT-PCR expression levels.
bP values were considered statistically significant if P<0.05.
cThe median value was chosen as the cut off.
d2.5-X median value was selected as the cut off.
eInformation only available for 25 patients.
fInformation only available for 23 patients.
extracellular medium and is responsible for resistance to 5-FU (Guo et al. 2003, Oguri et al. 2007). We evaluated whether the overexpression of ABCC11 in CL6.7 cells was correlated to decreased sensitivity to 5-FU and 5-FdURD pro-drugs. Then, 5-FdURD or 5-FU dose–response experiments (MTT cytotoxicity assay) were performed to measure the concentration giving rise to a 50% reduction in cellular viability (IC50 values). The 5-FU IC50 values (means ± S.D.) from four independent experiments were 84.50 ± 13.70 and 225.00 ± 127.67 nmol/ml in the MVLN and CL6.7 cell lines respectively. The 5-FdURD IC50 values (means ± S.D.) from four independent experiments were 88.20 ± 67.96 and 321.25 ± 237.08 nmol/ml in the MVLN and CL6.7 cell lines respectively. The CL6.7 cell line displayed a 2.6- and 3.6-fold relative resistance to 5-FU and 5-FdURD respectively compared with MVLN (Mann–Whitney’s test; P < 0.05; Table 3).

The difference in extracellular 5-FdUMP concentrations in the presence and absence of MK571 was used as a measure of MK571 inhibitable 5-FdUMP efflux activity of the CL6.7 and MVLN cells. At the end of the uptake period, similar intracellular amounts of 5-FdUMP were observed in the two cell lines (CL6.7 and MVLN) and were estimated by HPLC MS/MS at 6.5 ng/mg total protein. At the end of efflux period, extracellular concentrations of 5-FdUMP were quantified in aliquots of efflux medium, thus making it possible to directly investigate efflux of 5-FdUMP in the medium. 5-FdUMP concentrations were estimated at 0.862 and 0.954 ng/mg total protein for CL6.7 and MVLN cells respectively (Table 3). We observed that 15 μM MK571 markedly blocked by approximately twofold the efflux of 5-FdUMP in CL6.7 cells while it had a very weak inhibitory effect on MVLN cells (Table 3).

Discussion

The aim of this work was to explore molecular mechanisms of ABCC11 regulation. In MCF7 cells,
estrogen downregulation was blocked by coexposure to TAM or ICI 182 780. In contrast, no effect was observed in MDA MB 231 ER-negative cells. These data indicate the involvement of ER-dependant pathways in the regulation of ABCC11 expression. Several studies have demonstrated that coactivators and corepressors form complexes with ERs which are involved in various regulating estrogen pathways (Smith & O'Malley 2004, Acconcia & Kumar 2006, Girault et al. 2006, Howell 2006). At the molecular level, ER activation appears to result in altered transcriptional activity and expression profiles of various target genes (Vendrell et al. 2004). Besides classical interaction with the ERE motifs, ligand-activated ER also regulates gene expression by interacting directly with the AP-1 protein complex, the Sp1 protein, and the NF-κB protein (Paech et al. 1997, Biswas et al. 2005). To support our findings, we carried out an in silico analysis of the human ABCC11 promoter region (−5000 pb chr 16: 38685979) using Genomatix software (GEMS Launcher software, München, Germany). This analysis revealed at least five ERE-, two Sp1-, five AP-1-, and four NF-κB-binding sites. The presence of these ERE-binding sites in the ABCC11 promoter is supported by a recently reported genome wide analysis of promoter occupancy by the ER (Laganiere et al. 2005). ERE responsive elements have also been identified in the promoters of other ABC transporters, such as ABCC3, ABCC5, and ABCG2, and expression of these transporters are downregulated by E2 exposure (Ee et al. 2004a, Vendrell et al. 2004, Imai et al. 2005, Wang et al. 2005).

In Szakacs et al. (2004) study, ABCC11 level was shown to be differently expressed in breast cancer cell lines. For the first time, we demonstrated that endogenous expression levels of ABCC11 in breast cell lines were directly correlated with ER-α status (Fig. 4). Since ER status influences prognosis in breast cancer and probability response to systemic therapies, we evaluated the potential relationship between ABCC11 expression and clinical status in series A and an independent series B of tumors. We found that ABCC11 expression was positively correlated with ER-α and ERBB2. We found similar observations in macro-array database of Richardson Breast 2 (Oncomine web site: http://www.oncomine.org/main/index.jsp). Breast cancers with high expression level of ER-α respond better to endocrine therapies and consequently ER-α predicts better outcome (predictive value; Kathleen 2002) while the evidence that ERBB2 abnormalities predict resistance to TAM therapy and relative sensitivity to chemotherapy regimens including adriamycin is presented in Ross & Fletcher (1998). We also noticed that in ERBB2 low-expressing group, the correlation between ABCC11 and ER-α was independent of ERBB2 expression.

In tumor series A, the relatively high level of expression of ABCC11 in grade II group compared with grade III group, might be related to the high expression of ER-α (70% of grade II tumors were ER-α high-expressing tumors while only 23% grade III tumors were ER-α high-expressing tumors). In Bieche’s independent series C, ABCC11 expression was strongly decreased in metastatic tumors in comparison with histological grade II tumors likely due to the weaker proportion of ER-α-positive tumors in this subgroup. Metastatic cells are typically more undifferentiated and
aggressive cells, whereas ER-positive tumors are more differentiated and have lower metastatic potential than ER-negative tumors (McGuire 1986, Garcia et al. 1992).

We also noticed a trend towards increased ABCC11 expression in grade III compared with metastatic (Table 2). However, the set of tumors was small and these results should be confirmed on a larger cohort.

Surprisingly, while ABCC11 expression positively correlated with ER expression levels in breast tumors, we are the first to find that ABCC11 expression is reduced in vitro by estrogen exposure. In the literature, it has been reported that ABCG2, another ABC transporter, highly involved in MDR mechanisms (MDR phenotype) of various cancers, was also downregulated in vitro by estrogens at physiological levels in the estrogen responsive MCF7 cells, but not in estrogen non-responsive human cancer cells (post-transcriptional mechanism; Imai et al. 2005). In addition, in another ER-positive cell line (T47D:A18), it had been described that ERE functional motif in ABCG2 promoter lead to an upregulation of ABCG2 mRNA (Ee et al. 2004b). In the human choriocarcinoma placental BeWo cells, E2 by itself likely downregulated ABCG2 expression through an ER pathway (Wang et al. 2005). In this same cell line (BeWo cells), controversial data were reported by Ee et al. (2004a), since these authors reported an upregulation of ABCG2 expression. Furthermore using the Oncomine web site, various meta-analyses reported correlations of biological parameters in breast cancer samples. For example, in the Chin Breast analysis and the Wang Breast analysis, we found a positive correlation between ABCG2 and ER-α expression. In complementary, in another meta-analysis (Lin et al. 2004), they compared the expression profiles of genes in response with E2 and plotted the average relative expression ratios of each gene (ER-positive/ER-negative) across all samples from the breast cancer studies. There was strong concordance between the estrogen responsive genes identified in T47D cells and genes differentially expressed in breast tumors. Nevertheless, they noted a subset (29.5%; 13/44) of genes that exhibited opposite responses following estrogen treatment in vitro as compared with the ER-status-associated expression in tumors (Lin et al. 2004). Taken altogether, these data about ABCG2 expression regulation, strongly suggested that various regulatory pathways can lead to regulation of ABC genes. Even if in vitro E2 reduced strongly ABCC11 expression in MCF7 cells, in vivo it had been associated with ER expression. However, it is well known that regulation of gene expression by estrogen is strongly dependent of ‘cellular context’ (McKenna & O’Malley 2002, Acconcia & Kumar 2006). Indeed, the discovery of regulator molecules has been the key to understanding of estrogen regulation of gene expression in spatial and temporal contexts (McKenna & O’Malley 2002). The transcriptional activities of the ER are regulated by a vast array of cellular proteins. The observation that these transcriptional activities are manifested in a tissue-selective

### Table 3

(A) Sensitivity of MVLN parental and tamoxifen (TAM)-resistant CL6.7 cells to 5-fluorouracil (5-FU) and 5-FdURD. IC50 values were determined by the MTT assay, and resistance ratios were calculated as described in Materials and methods (n=4). (B) Modulation of 5-FdUMP efflux by MK571 in CL6.7 and MVLN cells. Concentrations of 5-FdUMP (ng/ml) were normalized to cellular protein content

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Cell lines</th>
<th>IC50 (nmol/ml)</th>
<th>s.d.</th>
<th>Resistance ratio</th>
<th>P value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>CL6.7</td>
<td>225.00</td>
<td>±127.67</td>
<td>2.61</td>
<td>0.0286</td>
</tr>
<tr>
<td></td>
<td>MVLN</td>
<td>84.50</td>
<td>±13.70</td>
<td>3.47</td>
<td>0.0317</td>
</tr>
<tr>
<td>5-FdURD</td>
<td>CL6.7</td>
<td>321.25</td>
<td>±237.08</td>
<td>3.47</td>
<td>0.0317</td>
</tr>
<tr>
<td></td>
<td>MVLN</td>
<td>88.20</td>
<td>±67.96</td>
<td>3.47</td>
<td>0.0317</td>
</tr>
</tbody>
</table>

(B) Modulation of 5-FdUMP efflux by MK571 in CL6.7 and MVLN cells. Concentrations of 5-FdUMP (ng/ml) were normalized to cellular protein content

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>(5-FdUMP)</th>
<th>s.d.</th>
<th>Efflux factor</th>
<th>P value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL6.7</td>
<td>MK571</td>
<td>0.453</td>
<td>±0.148</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>0.862</td>
<td>±0.351</td>
<td>1.9</td>
</tr>
<tr>
<td>MVLN</td>
<td>MK571</td>
<td>0.816</td>
<td>±0.169</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>0.954</td>
<td>±0.335</td>
<td>1.2</td>
</tr>
</tbody>
</table>

aP<0.05 with Mann–Whitney’s t-test. The values are the mean ± s.d. of at least four independent experiments. bP<0.05 with Student’s t-test by bilateral and by pairs.
manner suggests that the receptor does not function in isolation, but rather, requires specific cellular factors for maximal responses. The complex network of coactivators and corepressors provides for balanced, sensitive control of ER target gene expression (Hall & McDonnell 2005). In addition to genomic regulatory pathway, other mechanisms including phosphorylation, acetylation, and ubiquitination modifications had been described to influence ER action (McKenna & O’Malley 2002, Smith & O’Malley 2004). Overall, post-translational modification of cofactors appears to provide a mechanism to integrate extracellular signaling pathways, regulate assembly and dissociation of coregulators, and enhance or decrease the transcriptional efficacy of ER cofactor complexes. Taken altogether, these in vitro validated estrogen responsive genes were also differentially expressed in ER-positive primary tumors, and may therefore have direct biological and clinical significance.

ER-positive breast cancer patients benefit from endocrine therapy, including the SERM TAM and the pure antagonist ICI 182 780 (Howell 2006). Nevertheless, almost all responding patients acquire resistance to the action of TAM over time and the disease progresses. Recently, Ma et al. (2004) identified gene expression patterns in hormone receptor positive, early stage invasive breast cancers that might predict response to therapy. They performed microarray gene expression analysis of tumors from 60 women uniformly treated with adjuvant TAM alone. Within this cohort, 46% women developed distant metastasis with a median time to recurrence of 4 years (‘TAM recurrences’) and 54% women remained disease-free with median follow-up of 10 years (‘TAM non-recurrences’). Very interestingly, among the identification of 19 differentially expressed genes, we found an approximately threefold increase of ABCC11 expression in non-responder patients. These data strongly suggested that ABCC11 might be highly expressed in TAM resistance cells. But no data were available to determine whether ABCC11 overexpression was associated with increased resistance levels to ABCC11 substrates. Consequently, we explored the possible association between ABCC11 expression and 5-FU resistance phenotype in various TAM-resistant models. As observed for basal expression of ABCG2 in ICI 182 780-resistant cells (Liu et al. 2006), we found for the first time an increase of ABCC11 levels by 4.485-fold (Fig. 5B) and 7.5-fold (data not shown) in CL6.7 and CL6.32 cells respectively compared with that observed in parental MVLN cells. Liu et al. (2006) related ABCG2 overexpression to down-expression of ER-α. Although, a decrease of ER-α expression by approximately threefold was observed in CL6.7 cells compared with parental MVLN cells (Badia et al. 2000), molecular mechanisms involved in ABCC11 overexpression in TAM-resistant cells remain to be clarified. To confirm these data, a 2.7-fold increase of ABCC11 mRNA levels was found in another cell line (VP267) derived from a patient suffering from breast cancer resistant to TAM compared with VP229 (data not shown). VP229 is a TAM-sensitive cell line established from a primary breast tumor removed before any pharmacological treatment, whereas VP267 was derived from the same patient after a local recurrence following TAM treatment (McCallum & Lowther 1996).

As reported in Ma’s study concerning increase of ABCC11 expression levels in TAM-resistant tumors from breast cancer patients, we found in vitro models (sensitive MVLN, and CL6.7 and CL6.32 TAM-resistant cells and sensitive VP267 and TAM-resistant VP229 cells) which are able to potentially reproduce ABCC11 overexpression in TAM-resistant cells. As it is difficult to evaluate directly drug efficiency of 5-FU therapy in isolated primary breast cancer cells, we decided to evaluate in vitro the drug sensibility to 5-FU of CL6.7 TAM-resistant cell model in comparison with MVLN-sensitive parental cell line. This in vitro tool allows the exploration of overexpression ABCC11 consequences on 5-FU resistance levels in TAM-resistant cells. Since chemotherapy combinations including 5-FU are the treatment of choice for patients with hormone-refractory breast cancer and 5-FdUMP, the active metabolite of 5-FU, is a substrate of ABCC11 and ABCC5 (Guo et al. 2003, Pratt et al. 2005), we studied the effect of prolonged 5-FU exposure on cell proliferation. Since ABCC5 levels were similar in CL6.7 and MVLN cells, we suggest that the variation of resistance levels to 5-FU was likely related to ABCC11 in CL6.7 cells (Table 3). Previously, an increase of resistance level to 5-FU was specifically demonstrated in LLC-PK1 cells stably expressing ABCC11 (Guo et al. 2003). In addition, the MK571 inhibitable 5-FdUMP efflux activity, observed in CL6.7 cells, could also be attributed to ABCC11. Taken altogether, these data strongly suggested that ABCC11 is functional in CL6.7 cells and related to the increase of 5-FU resistance level in CL6.7 cells. These data strongly strengthen the hypothesis that prolonged exposure of breast cancer cells to TAM is involved in an upregulation of ABCC11 and may have deleterious consequences on 5-FU therapies of these breast cancers.

In conclusion, ABCC11 expression is regulated by estrogen and ABCC11 endogenous levels is increased in ER-positive breast cancers. The latter finding strengthens the hypothesis that high expression levels of ABCC11 in the ER-positive breast cancers may contribute to decreased sensitivity to chemotherapy.
combinations containing 5-FU and possibly methotrexate. These data also suggest that ABCC11 may be a potential predictive tool in the choice of 5-FU containing treatments in ER-positive breast cancers resistant to TAM.

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