Pathol. Oncol. Res. (2012) 18:331–342
DOI 10.1007/s12253-011-9449-9

RESEARCH

ABCC2 (MRP2, cMOAT) Localized in the Nuclear Envelope of Breast Carcinoma Cells Correlates with Poor Clinical Outcome

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Received: 19 February 2011 / Accepted: 1 August 2011 / Published online: 11 October 2011

Abstract Nuclear expression of ABCC2 can be specific for lower differentiated cells and stem cells. The study aimed at examination of ABCC2 expression in breast cancers. The immunohistochemical analyses were performed on 70 samples of breast cancer. We have also studied prognostic value of the ABCC2 mRNA expression using the KM plotter which assessed the effect of 22,277 genes on survival in 1809 breast cancer patients. Immunohistochemical studies demonstrated that ABCC2 expression may be manifested in nuclear envelope of neoplastic cells (ABCC2n) as well as in their cell membrane and cytoplasm (ABCC2c). The univariate and multivariate analyses showed that higher expression of ABCC2n and ABCC2c was typical for cases of a shorter overall survival time. Higher ABBC2n expression was also typical for cases of a shorter disease-free survival and a shorter progression-free time. The KM plotter analysis of the prognostic value of ABCC2 mRNA expression showed that elevated ABCC2 expression was specific for cases of a shorter relapse-free survival only in the estrogen receptor-negative subgroup. The study demonstrated hat breast cancers manifest ABCC2 expression and that it is linked to a less favourable prognosis. Our results suggested that immunohistochemical tests represent a reliable way to detect prognostic value of ABCC2 expression, allowing to demonstrate differences related to subcellular localization of the protein. Cases with nuclear expression of ABCC2 manifested a more aggressive clinical course, which might reflect a less advanced differentiation of neplastic cells, resistance to the applied cytostatic drugs and tamoxifen.

Keywords ABCC2 · Breast cancer · Immunohistochemistry · Prognosis

Abbreviations

ABCC2c	cytoplasmic ABCC2 expression
ABCC2n	nuclear ABCC2 expression
IRS	immunoreacive score
DFS	disease-free survival
OS	overall survival

Introduction

Breast cancer represents the most frequent malignant tumour in women and the main cause of death due to

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malignant tumours all over the world. In the middle of 1990-ties a general tendency was noted for a decrease in breast cancer caused deaths, particularly among young women, due to early detection and more effective treatment of the disease [1, 2].

In the recent almost 40 years local techniques of treatment have been supplemented by a post-operative systemic treatment, encompassing chemotherapy, hormonotherapy or their combinations. Favourable results of the adjuvant treatment (employing both/either chemotherapy and hormonotherapy) in recent years altered the scope of patients in whom the therapeutic forms are applied. In the first years of applying them, they were suggested to be used, first of all, in cases of high risk patients while in the subsequent years the indications were gradually broadened. In selection of adjuvant therapy one should take into account not only prognostic factors but also efficiency of the patient and predictive factors, defining the probability that a given patient will respond to a specific therapy.

It is now well established that several members of the superfamily of adenosine triphosphate binding cassette (ABC)-transporters play an important role in drug resistance and MDR in tumour cell models as well as in the clinic [3, 4]. ABC-transporters are found in all cells of all species from the most primitive microorganism to man and, accordingly, they play central roles in various physiological systems.

One of the 48 human ABC-transporters involves ABCC2, also called the multidrug resistance-associated protein-2 (MRP2) or the canalicular multiple organic anion transporter (cMOAT). The *ABCC2* gene maps to chromosome 10q24 and is usually expressed in the apical membranes of canalicular cells in the liver [5]. Besides the expression in hepatocytes, it could be demonstrated that ABCC2 is also localized in the apical membranes of renal proximal tubules, epithelial cells of gall bladder, small intestine, colon, and lung [6].

Our previous studies have demonstrated that silencing of ABCC2 expression is linked to increased sensitivity of tumour cells to cisplatin [7, 8]. In our previous study we have demonstrated also that ABCC2 may be present in nuclear envelope of neoplastic cells resistant to cisplatin [9]. In clinical cases of ovarian cancer we have shown that expression of ABCC2 in nuclear envelope is linked to shorter overall survival time and relapse-free survival . We have shown also that in normal tissue the protein is manifested in nuclear envelope of stem cells. In normal tissues membranous expression was demonstrated in highly differentiated, polarised cells. Taking into account the fact that ABCC2 may represent a parameter characterizing not only resistance to cytostatic agents but also the extent of differentiation of neoplastic cells, an immunohistochemical determination of subcellular localization of the protein may provide a significant prognostic tool, permitting to distinguish patients with a potentially more aggressive tumour and a tumour more difficult to treat using chemotherapy [10, 11].

The study aimed at examination of ABCC2 expression in breast tumours and at defining a relationship between subcellular localization of the protein and the clinical course.

Materials and Methods

Patients

Immunohistochemical examination was performed retrospectively on tissue samples taken for routine diagnostic purposes. Seventy patients treated in 1999–2002 due to breast cancer in Lower Silesian Centre of Oncology in Wrocław, Poland were qualified for the studies. The cases were selected based on availability of tissue and were not stratified for known preoperative or pathological prognostic factors. The study was approved by an Institutional Review Board (IRB) and the patients gave their informed consent before their inclusion into the study. Following the surgery, all the patients were treated in line with the binding principles of chemotherapy (CMF: Cyclophosphamide, Methotrexate and 5-Fluorouracil) or hormonotherapy (Tamoxifen) and radiotherapy (Table 1). The patients were monitored by periodic medical check-ups, mammography, ultrasonographic and radiological examinations. During the follow-up period, 16 patients (22.8%) had a recurrent disease and 11 patients (14.3%) died of the disease. The mean relapse-free survival time was 47 months (range: 3 to 61 months), while the mean overall -free survival time was 50 months (range: 6 to 62 months).

Fragments sampled from studied tumours were fixed in 10% buffered formalin and, then, embedded in paraffin. In each case, hematoxylin- and eosin-stained preparations were subjected to histopathological evaluation by two pathologists. Stage of the tumours was assessed according to the AJCC [12]. Tumours were graded according to the Silverberg grading system [13].

Immunohistochemistry

Immunohistochemistry was performed as previously described [13, 14]. Formalin-fixed, paraffin embedded tissue was freshly cut (4 μm). The sections were mounted on Superfrost slides (Menzel Gläser, Germany), dewaxed with xylene, and gradually rehydrated. Activity of endogenous peroxidase was blocked by 30 min incubation in 1% H_2O_2. The sections were boiled for 15 min in a microwave oven, in Antigen Retrieval Solution (DakoCytomation, Denmark)

Table 1 Patient and tumour characteristics

Characteristics	No. (%)[a]
All patients	70 (100)
Age (mean)[a]	
≤50	29 (41)
>50	41 (59)
T	
1	37 (53)
2	20 (29)
3	6 (8)
4	4 (6)
No data	3 (4)
N	
0	30 (43)
1	33 (47)
2	3 (4)
No data	4 (6)
Stage	
I	25 (36)
IIA	16 (23)
IIB	15 (21)
III	7 (10)
IIIB	3 (4)
No data	4 (6)
Estrogen receptor status	
+	38 (54)
–	31 (44)
No data	1
Progesterone receptor status	
+	9 (12)
–	14 (20)
No data	47 (68)
Type of surgery	
Mammectomy	34 (49)
BCT	36 (51)
Systemic therapy	
Chemotherapy only	15 (22)
Hormonotherapy only	22 (31)
Chemotherapy and hormonotherapy	26 (37)
w/o	7 (10)

[a] Differences in the sum to 100% in groups are due to rounding up of the numbers.

at 250 W. This was followed by immunohistochemical reactions using the monoclonal mouse antibody against MRP2 (clone M2I-4; Monosan, Uden, the Netherlands). The antibodies were diluted 1:100 in the Antibody Diluent, Background Reducing (DakoCytomation, Denmark). The tested sections were incubated with antibodies for 1 h at room temperature. Subsequent incubations involved biotinylated antibodies (15 min, room temperature) and streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB+, HRP, DakoCytomation, Denmark). DAB liquid+ (DakoCytomation, Denmark) was used as a chromogen (7 min, at room temperature). All the sections were counterstained with Meyer's hematoxylin. In every case, control reactions were included, in which specific antibody was substituted by the Primary Mouse Negative Control (DakoCytomation, Denmark).

Control Reactions

Control reactions included: positive control involving sections of human healthy liver, control reactions on Tissue Microarrays (Oligene GmbH, Berlin, Germany) with healthy human tissues, RT-PCR reactions, prediction of nuclear localization signal (NLS) in ABCC2 using the software "PredictNLS Online" (Version Jun 7, 2000) (http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl). They were performed and described in detail previously [9].

Scoring of Immunostaining Results

The intensity of the immunohistochemical reactions was appraised using the semi-quantitative immunoreactive score [15], in which the intensity of the reaction and the percentage of positive cells were taken into account. The final result represented a product of scores given for individual traits and ranged from 0 to 12. Intensity of the reactions was evaluated independently by two pathologists. In case of divergences, the evaluation was repeated using a double-headed microscope (Table 2).

KM Plotter Online Survival Analysis

The KM plotter was capable to assess the effect of 22,277 genes on survival in 1809 breast cancer patients [16]. A background database was established using gene expression data and survival information of 1809 patients downloaded from GEO (Affymetrix HGU133A and HGU133+2 microarrays). The median relapse-free survival was 6.43 years, 968 patients being estrogen-receptor (ER) positive (for 1231 patients data on ER expression were available) and 190 lymph-node positive (for 1369 patients data on lymph node involvement were available). After quality control and normalization only probes present on both Affymetrix platforms were retained (n=22,277). The background database was handled by a MySQL server, which integrates gene expression and clinical data simultaneously. In order to analyze the prognostic value of a particular gene, the samples were split into two groups according to the median (or upper / lower quartile) expression of the gene. The two

Table 2 Evaluation criteria of ABCC2 expression using IRS (ImmunoReactive Score) score

Percentage of positive cells	Points	Intensity of reaction	Points
No positive cells	0	No reaction	0
<10% positive cells	1	Weak reaction	1
10–50% positive cells	2	Moderate reaction	2
51–80% positive cells	3	Intense reaction	3
>80% positive cells	4		

groups could be compared in terms of relapse free survival, overall survival and distant metastasis free survival.

Statistics

Statistical analysis of the results was performed using *Statistica 2007 PL* software [17]. Kaplan-Meier statistics, log-rank test and F Cox'es test were performed to estimate the significance of differences in survival times. In order to examine relationships between individual variables ANOVA rank test of Kruskal-Wallis and Spearman's rank correlation were used. The length of survival was defined as the time between the primary surgical treatment and diagnosis of a recurrent tumour or death due to the neoplastic disease. P values <0.05 were considered to indicate significant differences.

Results

ABCC2 Immunostaining in Breast Cancers

The performed immunohistochemical reactions demonstrated the following distribution of the reactions:

- in fragments of a healthy mammary gland no reaction could be detected in the myoepithelial cells while epithelial cells formed two subpopulations: cells manifesting nuclear expression of ABCC2 (ABCC2n) only and the subpopulation less intense nuclear reaction, a positive cytoplasmic reaction and expression of ABCC2 in the cell membrane (ABCC2c) (Fig. 1a);
- in cases with co-existing DCIS component of invasive cancer no reaction was detected in the myoepithelial cells and a strong (IRS=12) nuclear expression of the ABCC2 in the preinvasive cancer cells (Fig. 1b);
- in cells of invasive cancers in various cases a reaction of variable intensity was demonstrated of both nuclear localization and localization in cytoplasm and cell membrane (Fig. 1c). Mean overall immunoreactivity score of ABCC2n expression amounted to±4.51 SD (min 0 , max 12) and of ABCC2c to±2.6 SD (min 0 , max 8) (Table 3).

Using the ANOVA rank test of Kruskal-Wallis, the relationships were examined between overall immunoreactivity score of ABCC2 expression on one hand and pT, pN, ER status, PgR status and type of the therapy on the other. No significant relationships were detected (Table 4).

Using the Spearman's rank correlation, the relationships were examined between overall immunoreactivity score of ABCC2 expression on one hand and patients age on the other (Table 4). No significant relationships were detected.

Also using Spearman's rank correlation, relationships were examined between overall immunoreactivity score of ABCC2n expression on one hand and overall immunoreactivity score of ABCC2c expression on the other. The analysis demonstrated a weak (R=0.24) significant positive correlation (P=0.047).

Control Reactions; KM Plotter Online Survival Analysis

Using KM Plotter online survival analysis of the prognostic value of ABCC2 mRNA (Affy id.: 206155_at) we demonstrated that elevated mRNA of ABCC2 expression had a negative impact of patient's survival only in the group of ER negative patients (Fig. 2), (Table 5).

ABCC2 Expression and Patient Survival

At the first stage of statistical analysis related to relationships between ABCC2 expression and survival of the patients, log-rank test and Kaplan-Meier's analysis were used. The overall survival time and progression-free time were compared between groups showing lower (IRS 0–4)

Fig. 1 Immunohistochemical location of ABCC2 expression in: **a** ▶ Healthy breast epithelium. The myoepithelial cells are ABCC2 negative. Epithelial cells show distinct reaction patterns. A1. The arrow points to an epithelial cell with strong nuclear and cytoplasmic ABCC2 expression; A2. The arrow points to an epithelial cell with strong nuclear reaction and no cytoplasmic reaction; A3. The arrow points to the epithelial cell with a very weak nuclear reaction and strong cytoplasmic reaction. **b** DCIS components of the IDC. The myoepithelial cells are ABCC2 negative. Almost all DCIS cells show a pronounced nuclear reaction. **c** Invasive ductal carcinoma. C1. Pronounced nuclear reaction in the cancer cells; C2. Pronounced nuclear and cytoplasmic reaction in all cancer cells; C3. About half of the cancer cells show a pronounced cytoplasmic and no nuclear reaction, the other half—a pronounced nuclear and no cytoplasmic reaction

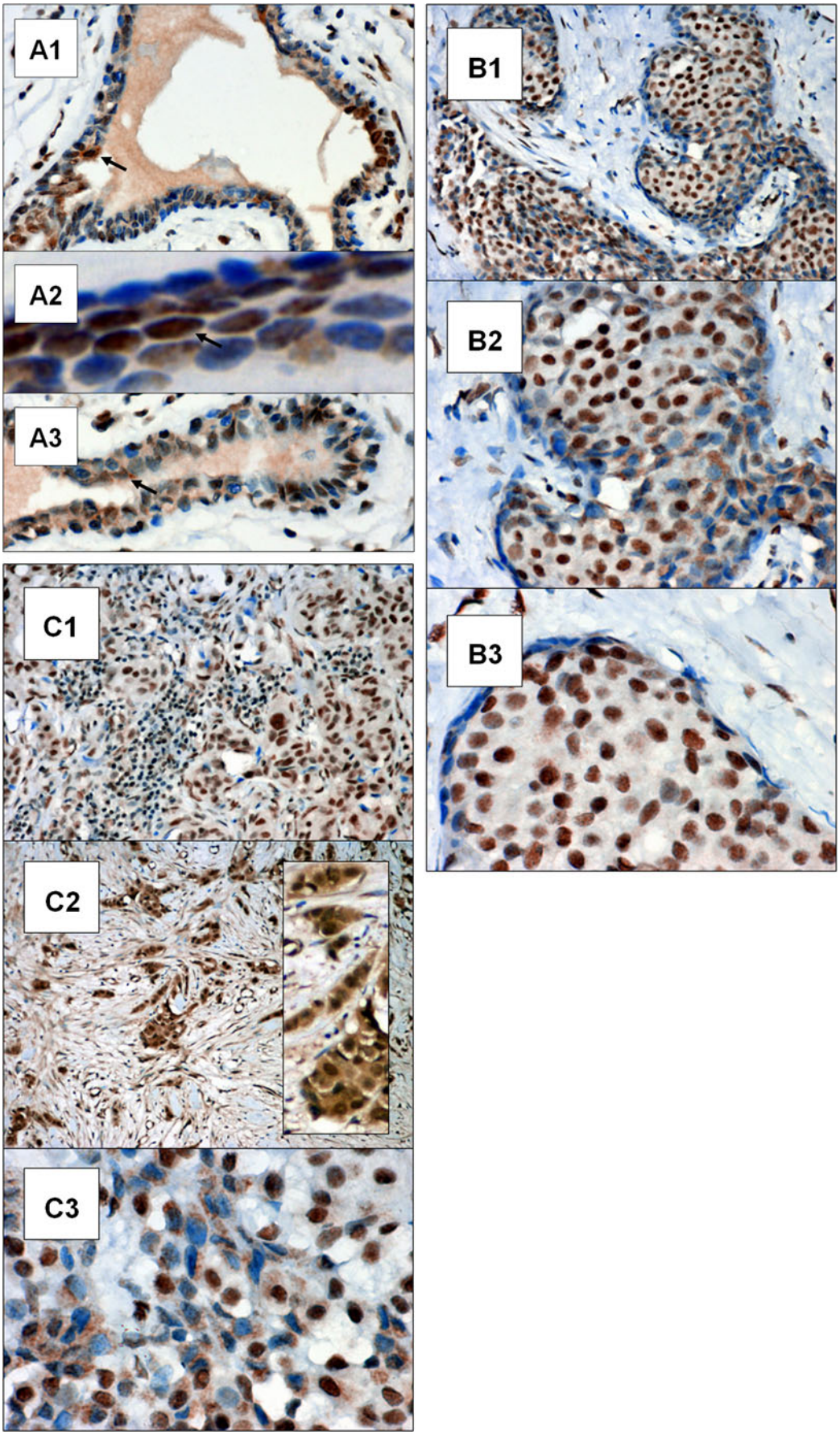
A1
A2
A3
B1
B2
B3
C1
C2
C3

Table 3 ABCC2 expression and selected clinical data of the studied patients

ABCC2n	ABCC2n	death	relapse	pT	pN	M	age	ER	mastectomy	quadrantectomy	chemotherapy
0	2	0	0	2	1	0	68	1	1	0	0
0	4	0	0	1	0	0	45	1	0	1	0
0	0	0	0	1	0	0	48	1	0	1	0
0	4	0	0	2	0	0	48	0	1	0	1
0	0	0	0	2	1	0	59	0	1	0	1
0	0	0	0	2	1	0	66	1	1	0	1
0	4	0	0	2	0	0	46	0	0	1	1
1	1	0	0	1	1	0	42	0	1	0	1
2	2	0	0	2	1	0	70	1	1	0	0
2	3	0	0	1	0	0	49	0	0	1	0
2	6	0	0	1	0	0	58	0	0	1	0
2	2	0	0	1	1	0	58	1	0	1	0
2	2	0	0	1	0	0	61	1	0	1	0
2	4	0	0	1	0	0	53		0	1	0
2	0	0	0	3	2	0	41	0	1	0	1
2	2	0	0	2	1	0	45	0	1	0	1
3	0	0	0	2	0	0	53	0	1	0	0
3	4	0	0	1	0	0	56	0	0	1	0
3	2	0	0	1	0	0	49	1	0	1	0
3	6	0	0	1	0	0	55	1	0	1	0
3	2	0	0	2	1	0	62	0	1	0	1
3	2	0	1	3	1	0	77	0	1	0	1
3	0	0	1	3	1	0	64	1	1	0	1
4	2	0	0	1	0	0	49	0	0	1	0
4	3	0	0	1	0	0	53	0	0	1	0
4	4	0	0	1	1	0	53	0	0	1	0
4	0	0	0	1	0	0	54	0	0	1	0
4	2	0	0	1	0	0	36	1	0	1	0
4	4	0	0	1	0	0	48	1	0	1	0
4	2	0	0	1	0	0	54	1	0	1	0
4	0	0	0	1	1	0	58	1	0	1	0
4	0	0	0	2	1	0	33	0	1	0	1
4	0	0	0	2	0	0	44	0	1	0	1
4	3	0	1			0	46	0	1	0	1
4	2	0	0	4	1	0	50	0	1	0	1
4	0	0	1	1	0	0	53	0	1	0	1
4	0	1	1			0	54	0	1	0	1
4	0	1	1	3	1	0	66	0	1	0	1
4	2	0	0	1	1	0	31	1	1	0	1
4	4	0	0	1	1	0	42	1	1	0	1
4	3	0	0	2	1	0	54	1	1	0	1
4	4	0	0	2	1	0	57	1	1	0	1
4	2	0	0	2	1	0	59	1	1	0	1
4	6	0	0	1	0	0	29	0	0	1	1
4	3	0	0	2	0	0	37	0	0	1	1
4	2	0	0	1	1	0	51	1	0	1	1
4	0	0	0	1	1	0	53	1	0	1	1
6	0	0	0	1	1	0	64	1	1	0	0
6	0	0	0	1	0	0	52	0	0	1	0

Table 3 (continued)

ABCC2n	ABCC2n	death	relapse	pT	pN	M	age	ER	mastectomy	quadrantectomy	chemotherapy
6	4	0	0	1	0	0	55	0	0	1	0
6	2	0	0	1	0	0	51	1	0	1	0
6	2	0	0	1	0	0	54	1	0	1	0
6	2	0	0	2	1	0	56	0	1	0	1
6	2	0	0	1	1	0	53	1	1	0	1
6	3	0	0	1	1	0	58	1	1	0	1
6	0	0	0	1	2	0	43	0	0	1	1
6	4	0	0	1	0	0	48	1	0	1	1
8	0	0	0	1	0	0	51	1	0	1	0
8	6	0	0	1	0	0	54	1	0	1	0
8	6	0	0	1	0	0	59	1	0	1	0
8	6	1	1	4b		0	33	0	0	0	1
8	8	1	1	4	2	0	44	0	1	0	1
8	3	1	1	4	1	0	47	0	1	0	1
8	6	1	1	3	1	0	51	0	1	0	1
9	4	1	1	2	1	0	29	0	0	0	1
9	2	1	1	3	1	0	64	0	1	0	1
12	8	1	1	2	1	0	48	0	1	0	1
12	8	1	1	2	1	0	57	0	1	0	1
12	6	1	1			0	58	0	1	0	1
12	0	0	1	2	1	0	47	1	1	0	1

or higher (IRS 6–12) overall immunoreactivity score of ABCC2n and ABCC2c expression. The computations demonstrated that higher expression of ABCC2n and ABCC2c was typical of cases manifesting shorter overall survival time and progression-free time (Table 6) (Fig. 3). Then, we analysed the prognostic value of ABCC2n and ABCC2c expression in following subgroups: estrogen receptor-positive patients, estrogen receptor-negative patients, patients not treated with tamoxifen, those treated with tamoxifen, patients not treated with chemotherapy and those treated with chemotherapy. The studies demonstrated that cytoplasmic and nuclear expression of ABCC2 manifested no relationships with OS and/or DFS in subgroups of ER-positive, ER-negative patients, patients treated with tamoxifen and patients not treated with tamoxifen. In the subgroup of patients treated with chemotherapy and also treated with tamoxifen a significantly shorter DFS and OS was detected among patients with tumours with high ABCC2n expression. In this subgroup, ABCC2c expression was higher in patients with low OS (Table 6).

Discussion

In our previous work we have demonstrated that ABCC2 may be demonstrated not only in cell membrane but also in nuclear envelope. In cases of ovarian cancer expression of

Table 4 Relationships between ABCC2 nuclear (ABCC2n) and ABCC2 cytoplasmic (ABCC2c) expressions and various clinico-pathologic factors in studied breast cancer cases (ANOVA rank test of Kruskal-Wallis)

Studied parameter	ABCC2n *P* value	ABCC2c *P* value
Age[a]	0.3508	0.2724
pT	0.3005	0.3138
pN	0.4765	0.3361
ER status	0.6207	0.3996
PgR status	0.1263	0.3335
Patey's mastectomy Yes / no	0.7911	0.1114
Quadrantectomy Yes / no	0.3491	0.2981
CMF chemotherapy Yes / no	0.1042	0.9171
Tamoxifen Yes / no	0.3309	0.6636

[a]Spearman's rank correlation

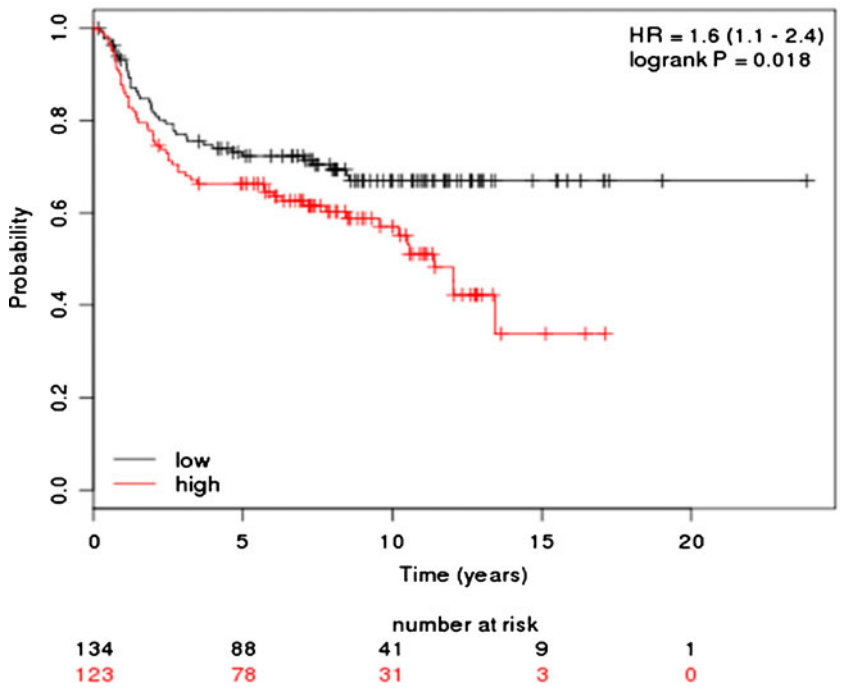

Fig. 2 KM Plotter online survival analysis of the prognostic value of ABCC2 mRNA expression in estrogen receptor-negative patients. Log rank test has shown that patients with higher ABCC2 mRNA expression have a significantly shorter overall survival time (P=0.018)

ABCC2 in nuclear envelope (ABCC2n) has been shown to be typical of cisplatin-resistant cells. In clinical cases an increased expression of ABCC2n has been typical of ovarian cancer cases with a significantly shorter overall survival time [9]. The unfavourable prognostic significance of ABCC2 expression has been confirmed also in cases of non-Hodgkin's lymphomas [18]. Our studies have demonstrated also that expression of ABCC2n in healthy tissues is typical of poorly differentiated cells, e.g. cells of basal layer in squamous epithelium, in crypts of large intestine, etc. [9]. The phenomenon suggests that ABCC2 may represent an exponent of not only lower sensitivity to anti-neoplastic drugs but also of a less advanced cell differentiation.

Investigators used to describe ABCC2 localization in the plasma membrane. Thus, ABCC2 is predominantly localized in the plasma membrane in cells involved in the transport of metabolites or in barrier cells. A further interesting observation is that ABCC2 is localized in cytoplasmic membranes of keratinocytes in the granular layer of the skin but it is localized in the nuclear envelopes of poorly differentiated cells in the basal layer. On several occasions, localization of ABCC2 was observed in nuclear envelope in cells representing stem cells of a given tissue. In poorly differentiated, more intensely dividing cells, ABCC2 is predominantly localized in the nuclear envelope. This interpretation is in line with the observation that alternative ABC transporters involved in drug resistance are components of poorly differentiated stem cells [9, 19].

Thus, our studies have shown that ABCC2 expression in plasma membrane is typical of highly differentiated polar cells, whereas its expression in the nuclear membrane is typical of poorly differentiated cells, most probably including stem cells. In fact, expression of ABC transporters was already described in stem cells [19, 20]. The

Table 5 KM Plotter analysis of prognostic significance of the ABCC2 mRNA expression

Subgroup	Relapse-free survival HR value Log rank P value	Overall survival HR value Log rank P value	Distant metastases-free survival HR value Log rank P value
All the group	0.73 (0.62–0.84) 2.9e-05	1.38 (0.92–2.07) 0.12	1.02 (0.77–1.37) 0.87
ER positive	0.81 (0.65–1.00) 0.051	1.17 (0.58–2.38) 0.66	1.02 (0.69–1.50) 0.93
ER negative	1.6 (1.1–2.4) 0.018	1.20 (0.55–2.64) 0.65	1.04 (0.54–1.98) 0.91
Lymph node positive	0.96 (0.64–1.45) 0.86	0.87 (0.71–1.08) 0.22	1.33 (0.68–2.58) 0.4
Lymph node negative	0.87 (0.71–1.08) 0.22	1.2 (0.7–2.0) 0.54	1.00 (0.72–1.37) 0.99
G1	0.81 (0.41–1.58) 0.53	2.23 (0.48–10.34) 0.29	0.40 (0.14–1.13) 0.073
G2	0.97 (0.72–1.31) 0.86	1.67 (0.87–3.21) 0.12	1.27 (0.85–1.91) 0.24
G3	0.75 (0.52–1.08) 0.12	1.1 (0.6–1.9) 0.8	0.89 (0.54–1.47) 0.66

Table 6 Survival analysis of the ABCC2 nuclear (ABCC2n) and ABCC2 cytoplasmic (ABCC2c) expressions in all the studied groups and the subgroups

Studied parameter	Log rank *P* value	F Cox'es test *P* value OST	Log rank *P* value PFT
The entire studied group			
ABCC2n	0.00029	0.00000	0.00783
ABCC2c	0.02585	0.00066	0.19916
Estrogen receptor-positive group			
ABCC2n	0.3349	–	0.3663
ABCC2c	0.4721	–	0.5059
Estrogen receptor-negative group			
ABCC2n	0.8025	–	0.7400
ABCC2c	0.2387	–	0.2438
Group not treated with tamoxifen			
ABCC2n	0.13719	–	0.21270
ABCC2c	0.70369	–	0.94443
Group treated with tamoxifen			
ABCC2n	–[a]	–	0.01723
ABCC2c	0.01688	–	0.14757
Group treated with CMF chemotherapy			
ABCC2n	0.00077	–	0.01352
ABCC2c	0.01710	–	0.10631
Group not treated with CMF chemotherapy[b]			
ABCC2n	–	–	–
ABCC2c	–	–	–

[a]The analysis could not be performed because all patients with higher expression died

[b]The analysis could not be performed because the studied group was too small

OST overall survival time; *PFT* progression-free time

transporters are supposed to protect stem cells and to inhibit apoptosis until the cells receive differentiation-promoting signals. Expression of the ABC transporters in cancer stem cells is also thought to provide reasons for drug resistance of the relapsed tumour.

On the other hand, ABCC2 may be involved in transport of all the drugs used in the studied group of patients. Among 51 tag-SNPs of transporter-coding genes, a significant relationship was demonstrated between polymorphism of *ABCC2* gene (at rs3740065 in *ABCC2*) and survival time of breast cancer patients treated with tamoxifen [21]. Authors of the paper suggested that ABCC2 may be involved in manifestation of resistance to tamoxifen. Involvement of ABCC2 was demonstrated in membranous transport of cyclophosphamide [22], methothrexate [23–25], and 5-fluorouracil [26, 27]. However, till now only in the case of cisplatin we have been able to demonstrate that expression of ABCC2 in nuclear envelope is typical for cells resistant to the drug. As a rule, methods of the above quoted studies did not permit to define localization of ABCC2 expression.

In this study, we have examined ABCC2 expression in breast cancers. In the healthy epithelium of mammary gland the demonstrated categories of epithelial cells have included the cells with strong nuclear reaction and no cytoplasmic reaction, the cells with strong nuclear and cytoplasmic ABCC2 expression, and the cells with very weak nuclear reaction or no nuclear reaction but with a strong cytoplasmic reaction. Relating the results to our earlier investigations it may be suggested that the cells with strong nuclear reaction and no cytoplasmic reaction may correspond to stem cells of lactigenous epithelium while the cells with very weak nuclear reaction or no nuclear reaction and strong cytoplasmic reaction may correspond to differentiated cells, capable of fulfilling secretory functions. The cells with strong nuclear and cytoplasmic ABCC2 expression probably represent an intermediate maturation stage of cells in lactigenous epithelium [9].

In the case of DCIS component we have shown that >90% cells demonstrate a strong ABCC2n reaction. The phenomenon suggests that at this stage of carcinogenesis poorly differentiated (stem?) cells may play a key role.

In the invasive part of the studies on breast cancers we have demonstrated both nuclear and cytoplasmic expression of ABCC2. Both in cases of ABCC2n and in those of ABCC2c a more pronounced expression has been found to be linked to cases of shorter overall survival time and shorter progression-free time. The results obtained in breast cancers differ from those obtained in ovarian cancers. In the latter, the unfavourable prognostic significance was detected only for ABCC2n. In breast cancers the conducted studies have shown that the unfavourable significance can be ascribed to expression of ABCC2 both in cell membrane and in nuclear envelope [9]. Probably, this is linked to

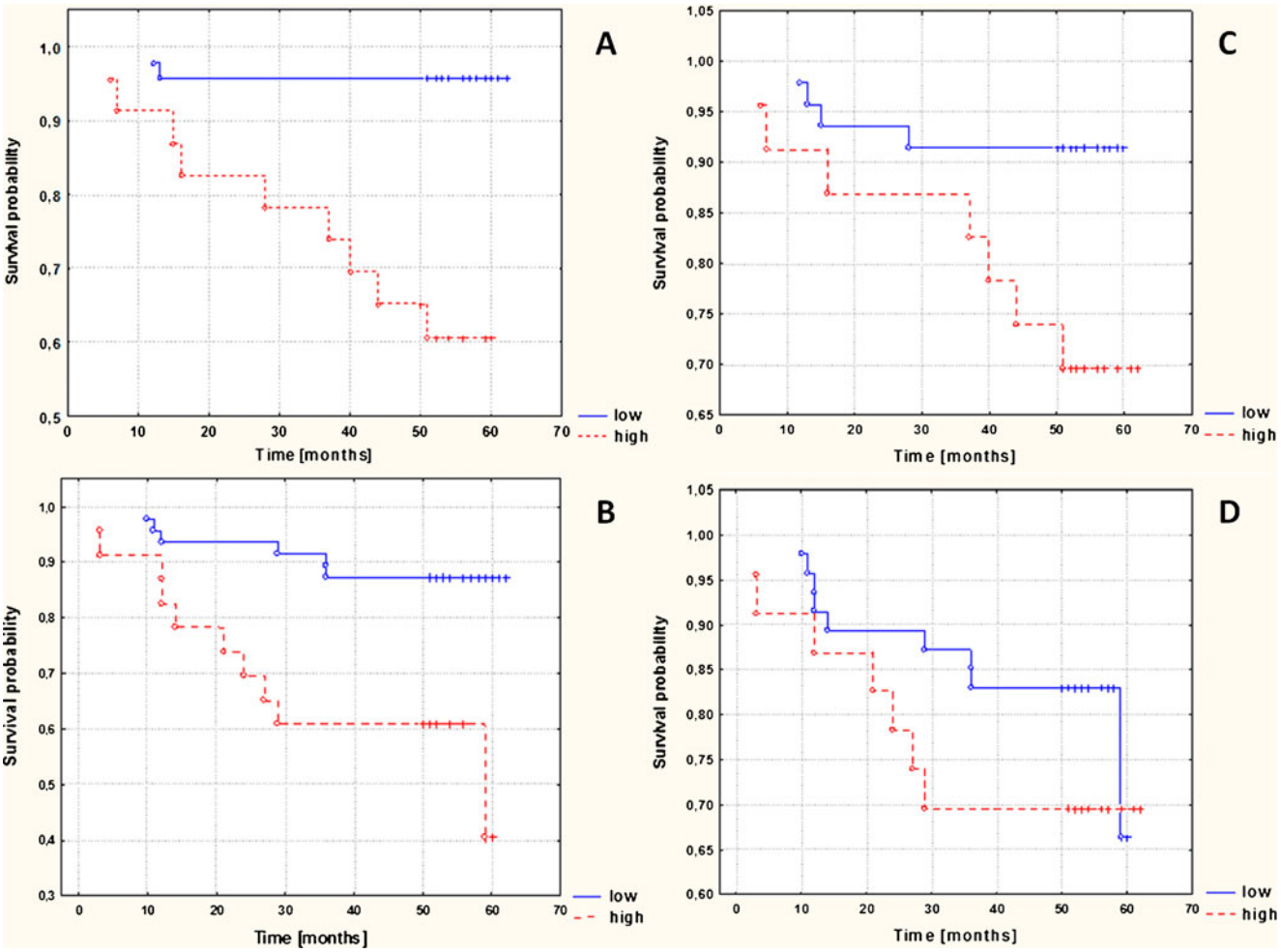

Fig. 3 Kaplan-Meier curves for survival and expression of ABCC2 in the studied group of 70 breast cancer patients: nuclear ABCC2 expression and **(a)** overall survival and **(b)** progression-free survival; cytoplasmic ABCC2 expression and **(c)** overall survival and **(d)** progression free survival

completely distinct mechanisms: in the case of ABCC2n it probably reflects just lower differentiation of neoplastic cells, linked to a more aggressive course, shorter survival times and progression-free times. In the case of ABCC2c expression, most probably typical of higher differentiated cells, manifesting apical pole and ability to excrete, the unfavourable prognostic significance is linked rather to transporting potential of ABCC2, or less advantageous response to the applied anti-cancer agents. Considering the fact that a potentially unfavourable significance of ABCC2 expression was reported in the course of therapy with tamoxifen, cyclophosphamide, methotrexate and 5-fluorouracil, it may be expected that expression of ABCC2c in the studied group of patients influenced the overall survival and progression-free time. In the case of ABCC2n expression we suggest that a less advanced differentiation of neoplastic cells might affect survival of the patients. It cannot be excluded that, similarly as it was in the case of cisplatin, expression of ABCC2n might have been involved in resistance to CMF and tamoxifen. It is worth noting that probability coefficients P in the case of ABCC2n have been always lower than those for ABCC2c. Thus, mechanisms linked to nuclear expression of ABCC2 exert a more pronounced influence on patient survival, whatever could be mechanisms of the phenomena.

An increasingly high number of authors stress a hierarchic structure of neoplastic tumours and an immense role of neoplastic stem cell activity in initiation and development of the lesions. Even if they comprise just an insignificant proportion of all tumour cells, they are decisive for growth and metastatic potential of the tumour. Several investigations prove that just this group of cells is more resistant to irradiation and chemotherapy. The presented by us results point to less advantageous results of treatment in patients with high expression of ABCC2c and, first of all, ABCC2n, or in a clinical situation in which high proliferative activity and a significant potential of resistance to chemotherapy and radiotherapy was disclosed.

Such a situation is typical of cells with uncontrolled growth or of tumour stem cells [28].

Analysis of prognostic value manifested by mRNA for ABCC2, conducted using the KM plotter on 1809 breast cancer patients, has shown that elevated expression of ABCC2 mRNA carries an unfavourable prognostic significance only in the group of estrogen receptor negative patients. Taking into account the fact demonstrated by us in earlier studies that, using our methodology, intensities of ABCC2 expressions on the level of protein (immunohistochemistry) and the level of mRNA (RT-PCR, Real-time RT-PCR) manifest a pronounced positive correlation, the results obtained at present are surprising. Using immunohistochemistry we have demonstrated no relationship between ABCC2 expression in ER- negative group, but we have shown a negative prognostic value of ABCC2, independently of its localization in the entire studied group and in the group treated with chemotherapy [9, 29]. The divergences may be related to distinct numerical forces of studied groups and to differences in expression evaluation using RT-PCR and DNA microarrays. The result may also suggest that expression of ABCC2 manifests a more pronounced clinical significance through the phenomenon of resistance to cytostatic drugs as compared to resistance to tamoxifen.

The performed studies have suggested that immunohistochemistry represents the optimum technique to evaluate prognostic value of ABCC2 expression, permitting to evaluate differences related to subcellular localization of the protein. Cases manifesting nuclear expression of ABCC2 may manifest a more aggressive clinical course, reflecting less advanced differentiation of the cells and potential resistance to some drugs while in cases manifesting expression of ABCC2 in cell membrane the aggressive course would reflect first of all resistance to therapy.

It seems worth while to continue the investigations in order to recognize detailed characteristics of tumour cells responsible for progression of the neoplastic process and for resistance to currently applied therapy. Immunohistochemical analysis of ABCC2 activity, particularly of the activity located in nuclear envelope, seems to provide a useful test , allowing not only prognostic evaluation of the tumour cells but also pointing to the need of implementing a more intense therapy. A relationship should also be examined between ABCC2 polymorphism on one hand and resistance to cytostatic drugs and subcellular localization of the protein on the other.

Acknowledgments The study was supported by the grant No. 0638/B/P01/2008/35 from the Ministry of Science and Higher Education (Poland).

Conflict of interest statement We declare that we have no conflict of interest.

References

1. Steward BW, Kleihues P (2003) World cancer report. IARC Press, Lyon
2. Tabar L, Yen MF, Vitak B et al (2003) Mammography service screening and mortality in breast cancer patients: 20-year follow-up before and after introduction of screening. Lancet 361:1405–1410
3. Gottesman MM, Fojo T, Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. Nature 2:48–58
4. Lage H (2003) ABC-transporters: implications on drug resistance from microorganisms to human cancers. Int J Antimicrob Agents 22:188–199
5. Wada M, Toh S, Taniguchi K et al (1998) Mutations in the canalicular multispecific organic anion transporter (cMOAT) gene, a novel ABC transporter, in patients with hyperbilirubinemia II/Dubin-Johnson syndrome. Hum Mol Genet 7:203–207
6. Taniguchi K, Wada M, Kohno K et al (1996) A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. Cancer Res 56:4124–4129
7. Materna V, Stege A, Surowiak P et al (2006) RNA interference-triggered reversal of ABCC2-dependent cisplatin resistance in human cancer cells. Biochem Biophys Res Com 348:153–157
8. Kowalski P, Surowiak P, Lage H (2005) Reversal of drug-resistant phenotypes by an autocatalytic multitarget multiribozyme directed against the transcripts of the ABC transporters MDR1/P-gp, MRP2, and BCRP. Mol Ther 11:508–522
9. Surowiak P, Materna V, Kaplenko I et al (2006) ABCC2 (MRP2, cMOAT) can be localized in the nuclear membrane of ovarian carcinomas and correlates with resistance to cisplatin and clinical outcome. Clin Cancer Res 12(23):7149–7158
10. Surowiak P, Materna V, Maciejczyk A et al (2007) Nuclear metallothionein expression correlates with cisplatin resistance of ovarian cancer cells and poor clinical outcome. Virchows Arch 450(3):279–285
11. Materna V, Surowiak P, Kaplenko I et al (2007) Taxol-resistance-associated gene-3 (TRAG-3/CSAG2) expression is predictive for clinical outcome in ovarian carcinoma patients. Virchows Arch 450(2):187–194
12. Sobin LH, Wittekind C (2002) TNM classification of malignant tumors. AJCC, Wiley-Liss, NewYork
13. Remmele W, Stegner HE (1987) Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue (Article in German). Pathologe 8:138–140
14. Surowiak P, Materna V, Matkowski R et al (2005) Relationship between cyclooxygenase 2 and MDR1/P-glycoprotein expressions in invasive breast cancers and their prognostic significance. Breast Cancer Res 7:862–870
15. http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl
16. Gyorffy B, Lanczky A, Eklund AC et al (2010) An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1809 patients. Breast Cancer Res Treat 123(3):725–731
17. Statistica PL (2007) (Statsoft, Poland).
18. Szczuraszek K, Materna V, Halon A et al (2009) Positive correlation between cyclooxygenase-2 and ABC-transporter expression in non-Hodgkin's lymphomas. Oncol Rep 22(6):1315–1323
19. Dean M, Fojo T, Bates S (2005) Tumour stem cells and drug resistance. Nat Rev Cancer 5:275–284
20. Donnenberg VS, Donnenberg AD (2005) Multiple drug resistance in cancer revisited: the cancer stem cell hypothesis. J Clin Pharmacol 45:872–877

21. Kiyotani K, Mushiroda T, Imamura CK et al (2010) Significant effect of polymorphisms in CYP2D6 and ABCC2 on clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients. J Clin Oncol 28(8):1287–1293
22. Qiu R, Kalhorn TF, Slattery JT (2004) ABCC2-mediated biliary transport of 4-glutathionylcyclophosphamide and its contribution to elimination of 4-hydroxycyclophosphamide in rat. J Pharmacol Exp Ther 308(3):1204–1212
23. Rau T, Erney B, Göres R et al (2006) High-dose methotrexate in pediatric acute lymphoblastic leukemia: impact of ABCC2 polymorphisms on plasma concentrations. Clin Pharmacol Ther 80 (5):468–476
24. Vlaming ML, van Esch A, Pala Z et al (2009) Abcc2 (Mrp2), Abcc3 (Mrp3), and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo. Mol Cancer Ther 8(12):3350–3359
25. Hulot JS, Villard E, Maguy A et al (2005) A mutation in the drug transporter gene ABCC2 associated with impaired methotrexate elimination. Pharmacogenet Genomics 15(5):277–285
26. Hinoshita E, Uchiumi T, Taguchi K et al (2000) Increased expression of an ATP-binding cassette superfamily transporter, multidrug resistance protein 2, in human colorectal carcinomas. Clin Cancer Res 6(6):2401–2407
27. Fujita K, Nagashima F, Yamamoto W et al (2008) Association of ATP-binding cassette, sub-family C, number 2 (ABCC2) genotype with pharmacokinetics of irinotecan in Japanese patients with metastatic colorectal cancer treated with irinotecan plus infusional 5-fluorouracil/leucovorin (FOLFIRI). Biol Pharm Bull 31(11):2137–2142
28. O'Brian CA, Kreso A, Dick JE (2009) Cancer stem cells in solid tumors: an overview. Semin Radiat Oncol 19(2):71–77
29. Materna V, Stege A, Surowiak P et al (2006) RNA interference-triggered reversal of ABCC2-dependent cisplatin resistance in human cancer cells. Biochem Biophys Res Com 348(1):153–157