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RNA interference-triggered reversal of ABCC2-dependent cisplatin resistance in human cancer cells

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Abstract

The adenosine triphosphate binding cassette (ABC)-transporter ABCC2 (MRP2/cMOAT) can mediate resistance against the commonly used anticancer drugs cisplatin and paclitaxel. To overcome the ABCC2-depending drug resistance, two specific anti-ABCC2 small interfering RNAs (siRNAs) were designed for transient triggering of the gene-silencing RNA interference (RNAi) pathway in the cisplatin-resistant human ovarian carcinoma cell line A2780RCIS. Since both siRNAs showed biological activity, for stable inhibition of ABCC2 a corresponding short hairpin RNA (shRNA)-encoding expression vector was designed. By treatment of A2780RCIS cells with this construct, the expressions of the targeted ABCC2 encoding mRNA and transport protein were inhibited. These effects were accompanied by reversal of resistance against cisplatin and paclitaxel. Thus, the data demonstrate the utility of the analyzed RNAs as powerful laboratory tools and indicate that siRNA- and shRNA-mediated RNAi-based gene therapeutic approaches may be applicable in preventing and reversing ABCC2-depending drug resistance.

Keywords: RNAi; ABCC2; MRP2; cMOAT; ABC-transporter; Cisplatin; Paclitaxel; Drug resistance

The adenosine triphosphate binding cassette (ABC)-transporter ABCC2, also designated MRP2 or cMOAT, was identified to confer cellular resistance of tumor cells to various anticancer drugs, e.g. platinum-containing drugs [1–4], taxanes [5], and vinca alkaloids [2,3,5]. These membrane-embedded transporter molecules can act as energy-dependent xenobiotic efflux pumps [6]. The transport activity results in decreased intracellular concentrations of anticancer drugs. Thus, the specific inhibition of ABC-transporter-depending drug extrusion results in a re-sensitization of tumor cells to treatment with anticancer agents, and therewith may allow a successful drug treatment of drug-resistant cancer cells.

Although a limited number of compounds with ABCC2-inhibiting features are available, none of these low molecular weight inhibitors has been clinically applied for reversal of ABCC2-mediated drug resistance. Furthermore, these compounds, e.g. MK-571 [7], can be toxic and they are not specific for ABCC2. Thus, these inhibitors are not satisfactory laboratory tools for studying ABCC2 biology. Consequently, it is necessary to develop alternative, less toxic, and more efficient strategies to inhibit ABCC2. Such an alternative procedure to circumvent drug resistance in cancer cells is to prevent the biosynthesis of the ABC-transporter by selectively blocking the expression of the corresponding mRNA by RNA technology-based gene therapeutic strategies, e.g. by hammerhead ribozymes or small interfering RNAs (siRNAs). This approach is aimed at increasing the efficiency and specificity of chemosensitization of drug-resistant cancer cells while at the same time reducing toxicity and undesirable side effects. The proof of principle, that this strategy can specifically inhibit the drug extrusion activity of a given ABC-transporter was already demonstrated for the classical multidrug resistance-associated transporter ABCB1 or MDR1/P-glycoprotein [8].

In the case of ABCC2, the RNAse H pathway-depending hammerhead ribozyme strategy was successfully applied to

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modulate cisplatin resistance in human cancer cells [4,9]. First studies using siRNAs against the rat homologue of ABCC2, Mrp2, for triggering the RNA interference (RNAi) pathway reported of altered carboxydichlorofluorescein transport [10]. However, data of RNAi-depending modulation of human ABCC2 expression and its influence on resistance to anticancer drugs are missing. In this study, the RNAi pathway was used for silencing the ABCC2 expression and therewith for modulation of the drug-resistant phenotype in the cisplatin-resistant human ovarian carcinoma cell line A2780RCIS. These experiments were performed transiently by using chemically synthesized siRNAs and stably by treatment of the cells with a short hairpin RNA (shRNA)-encoding expression vector.

Materials and methods

Cell lines and cell culture. The human ovarian carcinoma cell line A2780 and its cisplatin-resistant subline A2780RCIS were cultivated in supplemented Leibovitz L-15 medium as described previously [4]. To ensure maintenance of the cisplatin-resistant phenotype, medium of A2780RCIS contained 10 μg/ml cisplatin (GRY-Pharma, Kirchzarten, Germany). Medium used for cultivation of stably transfected cell clones of A2780RCIS contained 400 μg/ml zeocin (Invitrogen, Carlsbad, CA, USA).

Cell proliferation assay. Chemosensitivity was tested using proliferation assay based on sulphorhodamine B (SRB) staining, as described previously [4]. Briefly, cells were seeded in 96-well plates in triplicate. After 24 h attachment, cisplatin (GRY-Pharma, Kirchzarten, Germany) or paclitaxel (Mayne Pharma, Haar, Germany) was added in different concentrations for a 5-day incubation before SRB staining was performed. IC_{50} values were calculated from at least three independent experiments in triplicate for each cell line.

Design and application of anti-ABCC2 siRNAs. Two different ABCC2-specific target sequences were chosen according to general recommendations (http://www.ambion.com/techlib/misc/siRNA_finder.html) using the ABCC2 reference sequence (GenBank Accession No. NM_000392). The target sequences of ABCC2-A (5′-AAU UCC UCA UUC CUG GAC AGU-3′) and ABCC2-B (5′-AAG GGC ACC ACU GCC UAU GUC-3′) are homologous to nt 71–91 and 2129–2149 of the ABCC2-specific mRNA, respectively. Furthermore, the ABCC2-B targeting sequence was chosen to contain the well-assessable hammerhead ribozyme cleavage site GUC at nt 2147–2149, that was analyzed in detail previously [4,11]. The 21-nt siRNAs directed against ABCC2-A and ABCC2-B contained 3′-dTdT extensions and were commercially obtained from Dharmacon (Lafayette, CO, USA). Transfection of human ovarian carcinoma cells with siRNAs was performed using Oligofectamine Reagent (Invitrogen) as described previously [12].

Construction of shRNA expression vectors. Construction of an anti-ABCC2 shRNA expression vector, psiRNA/shABCC2-B, was performed as described previously [13,14]. The 5-nt (CCACC) loop-structure containing sequence of the shRNA was chosen to be homologous to the chemically synthesized siRNA ABCC2-B demonstrated for its more pronounced gene-silencing activity when compared to ABCC2-A. As control, a shRNA expression vector, psiRNA/shGFP, encoding a shRNA sequence (5′-TCC CAA CTA CCA GCA GAA CAC CCC CCA CCG GGG TGT TCT GCT GGT AGT TT-3′) directed against green fluorescent protein (GFP) that was demonstrated to be biologically active previously was designed [15]. The correct insertion of the specific shRNA-encoding DNA molecules was confirmed by sequencing.

Treatment of cisplatin-resistant carcinoma cells with shRNA-encoding expression vectors. For stable down-regulation of ABCC2, A2780RCIS cells were transfected with 1 μg plasmid DNA, psiRNA/shABCC2-B or psiRNA/shGFP, using DMRIE-C reagent (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's recommendations. Zeocin (400 μg/ml, Invitrogen) was used for selection of positively transfected cell clones, which were picked, expanded, and analyzed separately. Routine checks of the cell doubling time demonstrated that the viability of the clones was not impaired by the transfection procedure.

Quantitative RT-PCR. For quantitative mRNA expression analysis, a real-time RT-PCR protocol was applied using a LightCycler instrument and SYBR-green fluorescent dye (Roche Diagnostics, Mannheim, Germany) as described previously [4]. The copy number was measured by serial dilutions of cDNA fragments, cloned in the pCR2.1 vector (Invitrogen; 10^0–10^7 cDNA copies). Primer sequences, annealing temperatures (T_A), and temperatures for fluorescence measurement (T_M) are shown in Table 1. For mRNA quantification, samples were normalized against the expression of the GAPDH-encoding mRNA. Specificity of amplification products was confirmed by melting curve analysis. Each sample was measured at least three times.

Immunologic staining of ABCC2. Cells were seeded on slides, fixed in a methanol/acetone (1:1) mixture for 10 min at −20 °C, and air-dried. To block endogenous peroxidase, the slides were incubated in 3% H_2O_2 for 5 min. Immunologic staining reaction was performed using a mouse mAb directed against ABCC2 (clone M_2I-4, MONSAN, Uden, Netherlands) in a 1:100 dilution in Antibody Diluent, Background Reducing (DakoCytomation, Glostrup, Denmark), for 1 h at room temperature (RT). Subsequent incubations involved biotinylated antibodies (15 min, at RT) and streptavidin-biotinylated peroxidase complex (15 min, at RT) (LSAB+, HRP, DakoCytomation). NovaRed (Vector Laboratories, Peterborough, UK) was used as a chromogen (10 min, at RT). Control reactions were included, in which specific antibody was substituted by the Primary Mouse Negative Control (DakoCytomation). Preparations were counterstained with Mayer's hematoxylin (DakoCytomation), dehydrated, and mounted.

Table 1
Primer sequences used for quantitative real-time RT-PCR

Target	Sequence	T_A[c] (°C)	T_M[d] (°C)
GAPDH-fw[a]	5′-CAC CGT CAA GGC TGA GAA C-3′	55	87
GAPDH-rev[a]	5′-ACC ACT GAC ACG TTG GCA G-3′		
ABCB1-fw[b]	5′-GCC CTT GGA ATT ATT TCT TT-3′	58	84
ABCB1-rev[b]	5′-TGG GTG AAG GAA AAT GTA AT-3′		
ABCC1-fw	5′-AGA ACC TCA GTG TCG GGC AGC G-3′	68	87
ABCC1-rev	5′-TCG CAT CTC TGT CTC TCC TGG G-3′		
ABCC2-fw[a]	5′-GGA ACA ATT GTA GAG AAA GGA TC-3′	55	83
ABCC2-rev[a]	5′-CAC AAA CGC AAG GAT GAT GAA GAA-3′		

[a] According to [16].
[b] According to [12].
[c] T_A, annealing temperature.
[d] T_M, temperature during fluorescence measurement.

Statistical analysis. Levels of statistical significance were evaluated by calculation of the *P*-values performing the unpaired and two-tailed *t*-test using Microsoft Excel 2000 software.

Results and discussion

Modulation of ABCC2 mRNA expression by siRNAs

Two different siRNA constructs, ABCC2-A and ABCC2-B, were used to decrease the mRNA expression of ABCC2 in the cisplatin-resistant human ovarian cancer cell line A2780RCIS. ABCC2-A was designed according to general recommendations of siRNA selection, the target sequence of ABCC2-B was chosen to be homologous to a well-characterized hammerhead ribozyme cleavage site within the ABCC2-specific mRNA. Quantitative real-time RT-PCR experiments were performed to quantify ABCC2 mRNA expression values in a time-dependent manner (Fig. 1). Application of both siRNAs achieved a considerable decrease of the ABCC2 mRNA expression level. The specific gene silencing started after one day, continued for three days, and showed a peak of mRNA down-regulation after two days, 54% down-regulation using ABCC2-A, and 70% down-regulation with ABCC-B. During the complete time period, the gene silencing efficacy of ABCC2-B was stronger than that of ABCC2-A. Therewith, the data are in line with siRNA-mediated mRNA expression modulation of the alternative ABC-transporters ABCB1 [12] and ABCG2 [14]. As well duration and efficiency of gene silencing are comparable as the observation, that siRNAs directed against well-assessable hammerhead ribozyme target sites are more effective than that designed according to commonly available recommendations.

Modulation of ABCC2 mRNA expression by an shRNA expression vector

Due to the more pronounced activity of ABCC2-B, this sequence was chosen for construction of a stable shRNA-mediated ABCC2 gene-silencing system. For this approach, an H1 promoter-driven expression vector encoding the ABCC2-B shRNA was designed and used for treatment of cisplatin-resistant A2780RCIS cells.

Quantitative real-time RT-PCR demonstrated a stable 74% down-regulation of the ABCC2-specific mRNA (Fig. 2A). For analyzing potential anti-ABCC2 shRNA-depending effects on the mRNA expression levels of alternative ABC-transporters with particular features of

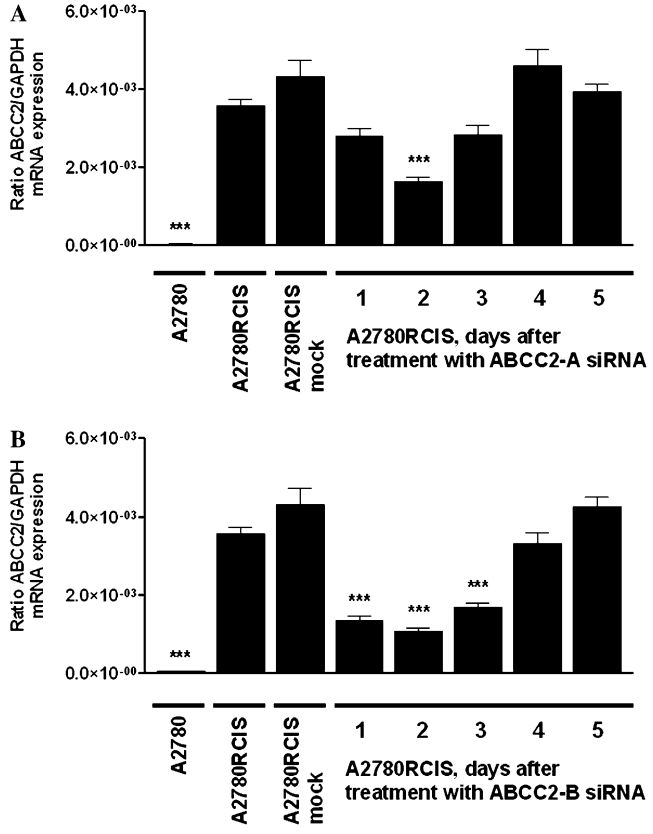

Fig. 1. Analysis of siRNA-mediated silencing of ABCC2 mRNA expression by quantitative real-time RT-PCR. ABCC2 mRNA expression levels were normalized to GAPDH mRNA expression in human cisplatin-resistant ovarian carcinoma cells A2780RCIS over time (day 1 to day 5). Expression analyses were performed using anti-ABCC2 SiRNAs (A) ABCC2-A, and (B) ABCC2-B. Control cells were treated with medium and transfection reagent at the same time. $^{***}P < 0.001$.

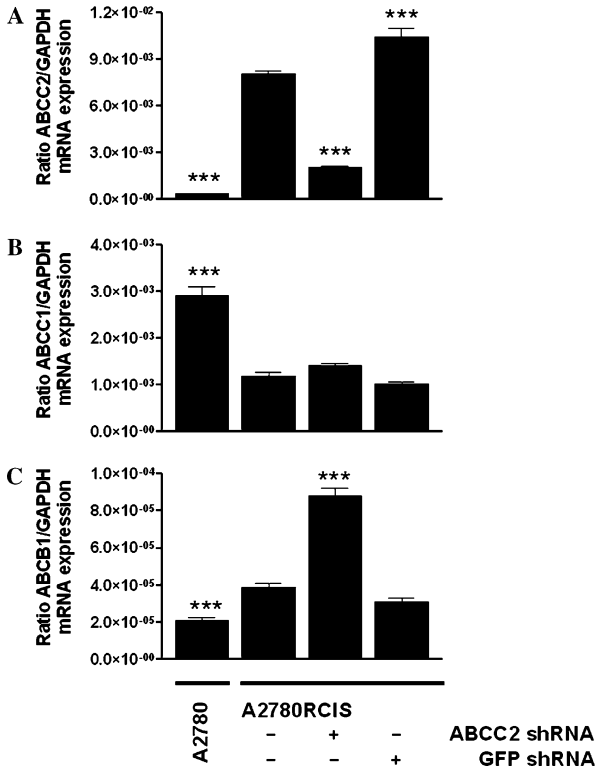

Fig. 2. Analyses of ABC-transporter mRNA expression levels in cisplatin-resistant A2780RCIS ovarian carcinoma cells treated with psiRNA/shABCC2-B by quantitative real-time RT-PCR. As control, A2780RCIS cells were treated with an anti-GFP (green fluorescent protein) shRNA-encoding expression vector. Expression analyses were performed for (A) ABCC2, (B) ABCC1, and (C) ABCB1 and normalized to GAPDH mRNA expression. $^{***}P < 0.001$.

cisplatin or paclitaxel transport, also the expression levels of the ABCB1 (MDR1/P-glycoprotein) and ABCC1 (MRP1)-specific mRNAs were measured. Fig. 2B demonstrated that no effect on the ABCC1 mRNA expression level could be observed. In contrast, in anti-ABCC2 shRNA-treated cells, the expression level of the ABCB1-encoding mRNA was 2-fold increased (Fig. 2C). This effect, that could not be observed using the anti-GFP shRNA expression vector, may be a cellular response triggered by ABCC2 down-regulation. As reported previously, drug treatment of cancer cells primarily induces the increased expression of various ABC-transporters, but in the end, merely a single extrusion pump will be dominant [17]. Thus, the specific inhibition of ABCC2 could facilitate the up-regulation of ABCB1 by a reduction of negative regulation of ABC-transporter expression. However, in general it has to be noticed that the expression levels of both alternative ABC-transporters, ABCB1 and ABCC1, are at very low levels. Furthermore, the expression of ABCG2 was analyzed. Since no specific ABCG2 signal could be detected in cisplatin-resistant A2780RCIS cells by RT-PCR, no real-time RT-PCR quantification was performed. Interestingly, the parental, non-resistant cell line A2780 displayed a weak ABCG2 mRNA expression, which was not further quantified (data not shown).

Modulation of ABCC2 protein expression by an shRNA expression vector

For analyzing the biological effects of the ABCC2-B shRNA on the cellular ABCC2 protein content, ABCC2 was immunologically stained. Fig. 3 demonstrates that treatment with the ABCC2-B shRNA strongly diminished the nuclear membranous ABCC2 protein expression in the cisplatin-resistant cell line A2780RCIS. As demonstrated previously, ABCC2 is embedded in the nuclear membrane of cisplatin-resistant A2780RCIS cells [9,18] and therewith, ABCC2 probably protects the nucleus from formation of platinum–DNA adducts [16,18].

Stable modulation of drug resistance by shRNA expression

Treatment of cisplatin-resistant A2780RCIS cells with the ABCC2-B shRNA reversed the cisplatin-resistant phenotype, in terms of IC_{50} values, for about 50% (Table 2), which is comparable to hammerhead ribozyme-mediated modulation of cisplatin resistance in the same cell model A2780RCIS [4,9]. Since previous investigations demonstrated that additional mechanisms than ABCC2 overexpression contribute to the cisplatin-resistant phenotype in

Table 2
Modulation of resistance against cisplatin and paclitaxel

Cell lines	IC_{50}[a] (RR)[b]	
	Cisplatin (μM)	Paclitaxel (nM)
A2780	8.7 ± 0.3 (1.0)[c]	5.74 ± 0.68 (1.0)
A2780RCIS	74.0 ± 3.1 (8.5)	2.60 ± 0.31 (0.5)
A2780RCIS/shABCC2-B	38.8 ± 2.1 (4.5)[c]	0.77 ± 0.11 (0.1)[c]
A2780RCIS/shGFP	95.6 ± 6.1 (11.0)	1.12 ± 0.14 (0.2)

[a] IC_{50} and SD values were calculated from three independent experiments in triplicate.
[b] RR, relative resistance (x-fold) compared to cell line A2780.
[c] Difference regarding untreated A2780RCIS was statistically significant with $P < 0.001$.

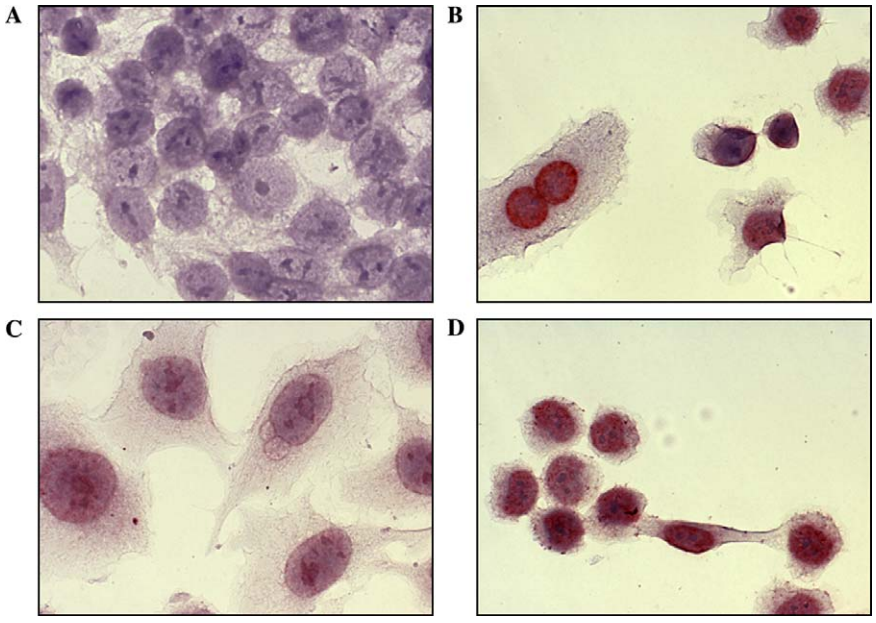

Fig. 3. Immunologic staining of ABCC2 expression in ovarian carcinoma cells (600×; counterstaining with hematoxylin). (A) Parental, drug-sensitive A2780 cells; (B) cisplatin-resistant A2780RCIS cells; (C) A2780RCIS stably transfected with psiRNA/shABCC2-B; (D) A2780RCIS stably transfected with psiRNA/shGFP.

A2780RCIS cells, it is not astonishing that the reversal of drug resistance is not complete. Thus, it was shown that A2780RCIS cells exhibit a distinct elevated cellular glutathione content compared to the drug-sensitive parental cells [4]. Increased levels of the cisplatin-conjugating peptide glutathione were already detected in various cisplatin-resistant cell lines [19]. Furthermore, two functionally relevant mutations within the p53-encoding gene could be found in A2780RCIS cells that could not be detected in parental A2780 cells (L111Q and K351N; unpublished data). Due to a mutated p53, the cellular capability to activate apoptotic pathways in response to cisplatin-dependent DNA damage can be diminished [20].

Likewise, the sensitivity against paclitaxel was negatively influenced, which was also shown by others previously [5]. It is important to note that already the cisplatin-resistant cell variant A2780RCIS is more sensitive against paclitaxel treatment than parental A2780 cells. Since the differences of the paclitaxel-specific IC_{50} values are very low, the demonstration of these differences is very difficult by using a cell proliferation assay. However, merely in the ABCC2-B shRNA-treated cells, a statistical significant alteration in paclitaxel resistance could be observed.

Conclusions

In conclusion, RNAi technology is effective to modulate the ABCC2 expression in human ovarian carcinoma cells. The RNAi technology showed a comparable gene-silencing efficiency compared to hammerhead ribozymes targeting the identical site of the ABCC2 mRNA sequence in these cells. Thus, the data demonstrate the utility of the analyzed siRNAs and shRNAs as powerful laboratory tools and indicate that siRNA- and shRNA-mediated RNAi-based gene therapeutic approaches may be applicable in preventing and reversing ABCC2-depending cisplatin resistance in cancer cells.

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