Amplification of the ATP-Binding Cassette 2 Transporter Gene Is Functionally Linked with Enhanced Efflux of Estramustine in Ovarian Carcinoma Cells


Abstract

An estramustine-resistant human ovarian carcinoma cell line, SKEM, was generated to explore resistance mechanisms associated with this agent. Cytogenetic analysis revealed that SKEM cells have a homogeneously staining region (hsr) at chromosome 9q34. Microdissection of the hsr, followed by fluorescence in situ hybridization to SKEM and normal metaphase spreads, confirmed that the amplified region was derived from sequences from 9q34. In situ hybridization with a probe specific for ABC2, a gene located at 9q34 that encodes an ATP-binding cassette 2 (ABC2) transporter, indicated that this gene is amplified ~6-fold in the estramustine-resistant cells. Southern analysis confirmed that ABC2 was amplified in SKEM, and Northern analysis indicated that the ABC2 transcript was overexpressed ~5-fold. The ABC1 gene located at 9q22–31 was not amplified in the resistant cells, and mRNA levels of several other ABC transporter genes were unaltered. Consistent with the concept that increased ABC2 expression contributes to the resistant phenotype, we observed that the rate of efflux of dansylated estramustine was increased in SKEM compared with control cells. In addition, antisense-directed toward ABC2 mRNA sensitized the resistant cells to estramustine. Together, these results suggest that amplification and overexpression of ABC2 contributes to estramustine resistance and provides the first indication of a potential cellular function for this product.

Introduction

EM4, a conjugate of nor-nitrogen mustard and estradiol, is an effective chemotherapeutic agent that is presently used to treat metastatic, hormone-refractory prostate cancer (1). The cytotoxic activity of EM relates to its ability to induce metaphase arrest by interfering with microtubule structure and function (2). Biochemical studies have shown that EM binds to microtubule-associated proteins and tubulin (3, 4), the consequence of which is depolymerization of microtubules.

To understand mechanisms associated with resistance to this agent, we have previously characterized EM-resistant tumor cell lines and studied the effects of EM on MDR cell lines. These studies demonstrated that alterations in the expression of the β-subunit of the target protein tubulin are associated with resistance to EM and other anti-microtubule agents. In addition, increased expression of the β1 null isoform of tubulin was observed in EM-resistant prostate carcinoma cells (5). The subsequent observation that this isotype has reduced affinity for EM (6) suggests a mechanism by which its overexpression contributes to EM resistance. We have also detected enhanced efflux of EM in an EM-resistant cell line, suggesting that increased expression of a transporter could also contribute to EM resistance.

Although EM can bind to pgp and modulate its transport activity (7, 8), pgp-overexpressing cells do not display cross-resistance to EM. In addition, overexpression of pgp is not detected in any EM-resistant cell lines studied to date (9). These observations suggest that a transporter distinct from pgp may contribute to EM resistance.

To explore further EM resistance mechanisms, we characterized SKEM in this study, an EM-resistant human ovarian carcinoma cell line (SKOV3). Cytogenetic analysis revealed an hsr at chromosome 9q34, the location of ABC2, which encodes a partially characterized ABC transporter (10). Further analysis revealed that ABC2 is amplified and overexpressed in SKEM, that antisense-mediated down-regulation of ABC2 expression sensitized the cell line to EM, and that SKEM displays enhanced efflux of EM. This study thus suggests that overexpression of ABC2 contributes to EM resistance by functioning as an efflux pump of the drug. These data provide the first evidence for a critical cellular role for this new ABC transporter family member.

Materials and Methods

Development of Estramustine-resistant Cell Lines. SKOV3 ovarian carcinoma cells were maintained in α-MEM medium and cloned, and then one clone was used for all subsequent experiments. Resistant cells were developed by exposure to EM at concentrations starting at 1 μM with a gradual increase to 15 μM over a 6-month period. The resulting SKEM cells were maintained in 15 μM EM.

Chromosome Microdissection and DNA Amplification. Metaphase tumor cells of SKEM were harvested by overnight exposure to Colcemid (0.01 μg/ml). Metaphase spreads were prepared on 24 × 60-mm coverslips, air-dried, and banded with trypsin-Giemsa. A single copy of the hsr was dissected under a Zeiss Axiovert inverted microscope with UV light-stereilized glass needles controlled by an Eppendorf Micromanipulator 5171, as described in detail previously (11). Briefly, needle tips containing dissected chrosomosomal material were broken off directly into a 0.5-ml thin-walled Eppendorf tube. Microdissected DNA was PCR-amplified by the sequence-independent amplification method of Bohlander et al. (12). As a positive control, 20 pg of human placental DNA was amplified simultaneously. A negative control without DNA template was also included. A 10-μl sample of each reaction was visualized by ethidium bromide staining following electrophoresis through a 2% agarose gel.

Microdissection-FISH. Metaphase spreads from SKEM cells and phytohemagglutinin-stimulated lymphocytes from human peripheral blood were prepared according to the method of Fan et al. (13). Cultures were synchronized by treatment with 5-bromodeoxyuridine (0.18 μg/ml; Sigma Chemical Co.) for 16 h, followed by release from the block by incubation in fresh medium containing thymidine (2.5 μg/ml) for 6 h. Metaphase cells were harvested, and chromosome spreads were prepared according to standard procedures. A 1-μl aliquot of the primary PCR was labeled with biotin-16-dUTP (Enzo Diagnostics) in a secondary PCR reaction (14), and 200–400 ng (5–10 μl) of
this DNA were hybridized to metaphase spreads from both SKEM cells and normal blood lymphocytes.

The ABC2 probe was a 1.75-kb purified PCR product of human ABC2 cDNA. The primers used to generate the fragment were chosen from the sequence of an expressed sequence tag representing part of the human ABC2 cDNA (EST0600) and from a 500-bp sequence from the PCR analysis of ABC2 message levels. The 1.75-kb fragment was gel purified, cloned into the TA vector (Clontech), and sequenced. The sequence exhibited nearly >90% homology with the murine ABC2 sequence. The purified probe was labeled by nick translation with biotin-16-dUTP (Boehringer Mannheim) and mapped to normal human chromosomes.

FISH and detection of immunofluorescence were carried out essentially as described previously (15). Biotinylated probes were denatured, preannealed with excess Cot-1 DNA, and hybridized overnight at 37°C to metaphase spreads on a 24 × 60-mm slide. Hybridization sites were detected with fluorescein-labeled avidin (Oncor) and amplified by the addition of anti-avidin antibody (Oncor) and a second layer of fluorescein-labeled avidin. The chromosome preparations were counterstained with DAPI and observed with a Zeiss Axiohot epifluorescence microscope equipped with a cooled charge couple device camera (Photometrics, Tucson AZ) operated by a Macintosh computer workstation. Digitized images of DAPI staining and fluorescein signals were captured, pseudo-colored, and merged using Oncor version 1.6 software.

Southern Analysis. DNA was isolated with a Puregene kit (Genta Systems) and treated with RNase (1 h at 37°C). Aliquots of DNA (25 µg) were then cut with one of four restriction enzymes (12 h at 37°C), separated on an agarose gel, and transferred to a nylon membrane. Hybridization of the 32P-labeled 1.75-kb ABC2 probe to the blot was performed with RapidHyb (Amersham) according to the manufacturer's instructions.

Northern Analysis. RNA was isolated with the RNeasy kit (Quiagen). The RNA was then treated with DNase and purified with the RNeasy kit. RNA samples were separated by electrophoresis through a 1% agarose-2.2 M formaldehyde gel and transferred to Magna NT membrane (Micron Separations, Westborough, MA). The membranes were hybridized with 32P-labeled probes and imaged as described for the Southern analysis. For the analysis of both ABC1 and ABC2 mRNA levels, primers were chosen from the published murine sequences and were used to amplify the corresponding sequences from human cDNA. The resulting PCR products were sequenced, found to exhibit >93% (ABC1) and >90% (ABC2) homology with their murine counterparts, and then labeled with 32P.

RT-PCR Analysis. cDNA synthesis and RT-PCR were performed as described (16). Primers for RT-PCR analysis were derived from the published coding sequences as follows: MDR1, nucleotides 1325–1347, 1502–1523; MRP, 4208–4229, 4414–4435; murine ABC2 (EMBL accession no. X75927), 2503–2522, 3004–3025; ABC C (EMBL accession no. X97187), 21–39, 459–479; and cMOAT (GenBank accession no. U63970), 1–21, 516–536. Primers for 18S rRNA were as described (16). The predicted target size for each of the primer pairs was 198 bp (MDR1), 229 bp (MRP), 523 bp (ABC2), 459 bp (ABC C), 536 bp (cMOAT), and 311 bp (18S RNA). PCR cycling conditions were 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C.

Dansylated EM Fluorescence. Cells were plated on glass coverslips and grown to 50% confluency. Coverslips were incubated at 37°C for 1 h in α-MEM with 10 µM dansylated EM (Pharmacia, Helsingborg, Sweden). Coverslips were then washed, placed in fresh α-MEM, and returned to the incubator. At various times following removal of the drug, cells were imaged by a Quantix cooled charge coupled device camera coupled to a Nikon TE300 microscope, and the images were analyzed by ISEE analysis software (Innovation). Excitation wavelength was 360 nm, and the emission was recorded with a broad spectrum emission filter.

Daunorubicin Fluorescence. Cells on coverslips were treated with 10 µM daunorubicin (Sigma) for 2 h. Cells were washed and imaged after various time intervals using the 568-nm excitation line of a Bio-Rad MRC-600 laser scanning confocal microscope.

Results

ABC2 Is Amplified in the EM-resistant Cell Line. EM-resistant ovarian carcinoma cells were generated by exposing sensitive cells to gradually increasing concentrations of the drug. The resulting cells (designated SKEM) exhibited a 5–6-fold level of resistance to EM compared with the SKOV3 parental cells. Because drug-resistant cells often display newly acquired chromosomal abnormalities, karyotypic analysis of parental SKOV3 and SKEM cells was performed. The Giemsa-banded metaphase chromosomes from SKEM cells showed the presence of an abnormal chromosome 9 not present in the parental cells. This aberrant chromosome 9 contained an hsr located at band 9q34 (Fig. 1a). We used chromosome microdissection techniques to characterize the amplicon of the hsr. Several copies of the hsr from SKEM cells were microdissected (Fig. 1b), pooled, and PCR amplified. To confirm that the amplified DNA came from within the hsr, the PCR products were hybridized back to SKEM metaphase spreads. As anticipated, the resulting fluorescence was localized to the hsr region in chromosome 9q34 (Fig. 1c, arrow; for discussion of Fig. 1d, see below). Hybridization to a normal copy of chromosome 9 in SKEM was also observed (Fig. 1c, arrowhead). FISH analysis of the micro-
dissected DNA probe to normal lymphocyte metaphase spreads also indicated that the hsr sequences were from 9q34 (data not shown). In addition, strong hybridization was also observed over the telomeric region of an unidentified marker chromosome present in SKEM cells but not parental SKOV3.

A recently identified ABC cassette transporter gene, ABC2, was previously localized to this 9q34 (14). To determine whether the ABC2 gene was amplified within the hsr of the EM-resistant cells, a human ABC2 probe was synthesized based upon the published murine sequence. FISH analysis of human metaphase chromosomes with a murine probe suggested that the human homologue of murine ABC2 was localized to 9q34 (10). To confirm that the human homologue of ABC2 is located at 9q34, human ABC2 probes were generated. Although the sequence of the human ABC2 cDNA is not known, primers based on the published murine ABC2 sequence produced a 523-bp PCR product using human cDNA as template. From this PCR product, a second human ABC2 cDNA fragment, 1750 bp in length, was made by performing PCR using primers from within the 523-bp fragment and from a human expressed sequence tag (EST0600). The 1750-bp fragment was sequenced and found to be >90% homologous to the mouse sequence and was used in FISH analysis of chromosomes from the EM-resistant cells. Hybridization of this probe to SKEM metaphase spreads revealed multiple signals distributed throughout the hsr present in this cell line (Fig. 1d), confirming that the human ABC2 was present and amplified with the hsr. Hybridization signals were also observed at 9q34 of the normal homologue within SKEM. In addition, a marker chromosome that showed strong hybridization to the PCR products of the microdissected hsr also hybridized to the human ABC2 probe.

To estimate the number of copies of the ABC2 locus present in the hsr, additional FISH analysis of SKEM cells were performed using the ABC2 cDNA probe. Fluorescent signals were detected over the hsr in all 19 spreads examined. Hybridization to the normal chromosome 9 homologue was observed in 12 of 18 metaphases containing this chromosome. In a single metaphase, a normal homologue of chromosome 9 was not present. A total of 107 signals was observed over the 19 hsr scored. On average, 5.6 signals were distributed over each hsr (median, 6; range, 2-12). The mean number of signals on the normal chromosome 9 was 0.9 (median, 1; range, 0-2). On this basis, it is estimated that the hsr contains approximately six copies of the ABC2 gene. Additional copies of ABC2 were also present in the unidentified marker chromosome, but this was not quantitated because the chromosome could not be reliably scored in all cells.

Amplification of the ABC2 gene was confirmed by Southern analysis of DNA isolated from parental SKOV3 cells and SKEM cells (Fig. 2a, Lanes 1 and 2). Quantitation of the ABC2 signals indicated ~8-fold amplification of the ABC2 gene in resistant cells, a value in approximate agreement with the FISH data. The ABC2 gene encodes a protein closely related to ABC2 and is located on chromosome 9q22-31. ABC1 was not amplified in the SKEM cells, as shown in Fig. 2a (Lanes 3 and 4). These results indicate that the amplicon of the hsr is derived from the 9q34 region and contains the ABC2 gene but not ABC1.

ABC2 Transcript Is Overexpressed in SKEM Cells. To determine if ABC2 transcript is overexpressed in SKEM cells, RNA blot analysis was performed using a 523-bp human ABC2 probe. As shown in Fig. 2b (Lanes 1 and 2), ABC2 transcript levels in resistant cells are increased ~5-fold compared with parental cells. Northern analysis of the levels of ABC2 mRNA was also performed and showed no differences in the two cell lines (Fig. 2b, Lanes 3 and 4). Equivalent β-actin mRNA levels indicated equivalent RNA loading (data not shown). RT-PCR analysis was performed to assess the possibility that other ABC transporters may contribute to the EM-resistant phenotype in these cells. The RT-PCR results for ABC2 confirm that the message for this transporter is overexpressed in the resistant cells (Fig. 2c, Lanes 2–5). The mRNA levels were detectable and unchanged for ABC-C, MRP, and cMOAT (Fig. 2c, Lanes 6–11). Analysis of pgp mRNA levels by RT-PCR revealed undetectable levels in both cell lines. The lack of pgp mRNA expression corresponds with the cytogenetic observation of the apparent loss of both copies of the long arm of 7q, where the MDR1 gene is localized, in SKOV3 cells (data not shown).

SKEM Cells Display Increased Efflux of EM. The observation that ABC2 is overexpressed in SKEM raised the possibility that it might contribute to EM resistance by functioning as an efflux pump for EM. To assess this possibility, the cellular kinetics of EM were characterized using dansyl-EM, a fluorescent analogue of EM (18). Both wild-type and resistant cells were loaded with dansyl-EM, washed, and imaged at various times afterward. In both cell lines, dansyl-EM was localized primarily in the cytoplasm, with little fluorescence observed in the nucleus. The amount of dansyl-EM in the
Fig. 3.  

**a.** dansyl-EM fluorescence in SKOV3 and SKEM cells. Cells were incubated in media with 25 μM dansyl-EM for 2 h, washed, and imaged at various time points. The SKEM cells efflux dansyl-EM at an increased rate compared with the sensitive SKOV3 cells. Dansyl-EM appears mainly in the cytoplasm, with little observed in the nucleus. 

**b.** daunomycin fluorescence in SKOV3 and SKEM cells. No differences in the rate of daunomycin efflux were observed. Over the time course of the experiment, daunomycin became localized to the perinuclear region. Little efflux occurred in either of the cell lines over the 2-h period.
ABC2 functions as a transporter of the estradiol-mustard conjugate, and these studies demonstrate the value of combined chromosome microdissection-FISH to identify the chromosomal location of genes contributing to drug resistance. The microdissection of an hsr acquired during the development of resistance to EM combined with the mapping of the native location of the hsr sequence provided an entry point for further molecular analyses using a positional candidate gene approach. A similar strategy has been used by others to identify amplified putative oncogenes associated with tumorigenesis (18).

In contradistinction to the high levels of resistance associated with the pgp-mediated MDR phenotype, our previous experience suggests that EM resistance ratios of 6–8-fold are maximally attained (5, 9). Although EM photoaffinity analogues will bind pgp, this protein does not provide protection against the cytotoxic effects of the drug. As a consequence, EM resistance is distinct from the MDR phenotype, and pgp-overexpressing cells are sensitive to EM (9). The relatively low resistance ratio in the SKEM cells is quantitatively consistent with the levels of ABC2 overexpression. It is also feasible that the affinity of EM for ABC2 is low and that this is also a contributory factor to the low-level resistance.

EM binds to tubulin and microtubule-associated proteins (3, 4, 6), causes aberrant spindle formation in dividing cells (2), and alters the kinetics of tubule cycling (19). Each, or all, of these contribute to the antimitotic cytotoxic properties of the drug. It has been shown that EM has different binding affinities for individual β-tubulin isotypes (6). Thus, DU145 cells that overexpress β2 tubulin express resistance to EM and some other antimicrotubule agents (5). In addition, we have found that the SKEM cells overexpress the β2 tubulin isotype compared to wild-type, and this may also be a contributory factor in the overall resistant phenotype. In light of the important selective advantage of survival in response to toxic drug stress, the presence of multiple, complementary resistance mechanisms is not surprising. In the context of the present investigation, the relative importance of altered tubulin and transporter expression is difficult to assess accurately. However, the ABC2 antisense data would support in quantitative terms the more critical involvement of this transporter in determining resistance in SKEM cells.

Little is known about the normal function of the ABC2 transporter. It is expressed in several murine tissues and in a wide variety of tumor cell lines at detectable levels (10). The fact that ABC2 is expressed at high levels in the mouse brain suggests that, like pgp, it may play a role in the maintenance of the blood-brain barrier. ABC2 mRNA is expressed in the murine uterus and is found at higher levels in the pregnant uterus, tissues that are highly responsive to estrogens.

Although EM has an antimicrotubule mechanism of action, structurally the drug maintains the crystal structure of estradiol (20) and has estrogenic properties when administered in vivo (21). EM is not a natural product, and it is unlikely that ABC2 evolved in any natural setting to deal specifically with the toxic consequences of the drug. We are presently investigating the possible role of ABC2 in hormone transport. Because glucuronides of various steroids have been shown to be substrates for MRP (22) and recent studies have shown that the yeast ABC transporters PDR5 and SNQ2 can transport estradiol (23), it remains feasible that ABC2 may be a mammalian counterpart, evolving as an endogenous transporter of steroid hormones and their metabolites.

**Acknowledgments**

We gratefully acknowledge Drs. Takahiro Taguchi and Ze Min Liu for assistance with chromosome dissection and karyotype analysis.

**References**


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