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cDNA Cloning of a Short Type of Multidrug Resistance Protein Homologue, *SMRP,* from a Human Lung Cancer Cell Line

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Members of the ATP binding cassette (ABC) superfamily are involved in the energy-dependent transport of a wide variety of substrates including anticancer agents across the membranes. We have cloned a cDNA fragment including a novel ABC sequence from a cisplatin-resistant human lung adenocarcinoma cell line, PC-14/CDDP, by reverse transcription polymerase chain reaction (RT-PCR) using degenerate primers and screened a cDNA library using the cDNA fragment as a probe. A full-length cDNA clone, BM4.8, was obtained. Sequence analysis showed that the cDNA encoded a short type of multidrug resistance protein homologue, *SMRP,* by computed homology search. *SMRP* was composed of 946 amino acids and had two ABCs with walker A and B motifs. This gene was mapped on chromosome 3 at band q27 by fluorescence *in situ* hybridization (FISH) analysis and was found to be expressed in various tissues by Northern blot analysis.

Acquirement of drug resistance by tumor cells is frequently observed in cancer patients receiving chemotherapeutic treatment. It is therefore important to find molecules which can transport anticancer agents and to clarify the mechanisms in drug resistance. We have established and characterized cisplatin-resistant cell lines (1). One of the mechanisms of cisplatin resistance has been reported to be decreased intracellular accumulation of drugs caused by decreased influx and increased efflux of the drugs (1). An elevated glutathione (GSH) level has been achieved by transfection of γ-glutamylcysteine synthetase (γ-GCS) cDNA, which is known to be a rate limiting step enzyme on GSH synthesis, and increase of the cisplatin resistance and decrease of intracellular cisplatin accumulation have been observed in the γ-*GCS* transfected lung cancer cell line (2). Active transport of drugs across the cell membrane is mainly carried out by ATP-dependent transporters in cells. Some ATPases and ATP binding cassette (ABC) superfamily are reported to participate in this transport system.

Recently, the GS-X pump has been revealed to belong to the ABC superfamily (3, 4). The GS-X pump family includes multidrug resistance protein (MRP), canalicular multispecific organic anion transporter (cMOAT) and yeast cadmium factor (YCF1), which play an important role as active transporters for anticancer agents (5-10).

In this paper, we report the molecular cloning of a full-length cDNA of a novel ABC superfamily from a human lung cancer cell line resistant to cisplatin, PC-14/CDDP, by reverse transcription polymerase chain reaction (RT-PCR) using degenerate primers and initial characterization including mRNA expression on various tissues and chromosomal locations of this gene.

MATERIALS AND METHODS

Cell line. A cisplatin-resistant subline, PC-14/CDDP was established from parental human non-small cell lung cancer cell line, PC-14, by stepwise escalation of cisplatin concentration as described previously (1). PC-14/CDDP cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C.

RT-PCR for ABC region. cDNA fragments were amplified from mRNA of PC-14/CDDP cells by RT-PCR using degenerate primers

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constructed from the conserved sequence in the ABC region of human *MRP* (11). The sequences of the forward and reverse primers were

5′-dGAGAAGGTCGGCATCGTGGGNCGNACNGG-3′
5′-dGTCCACGGCTGCNGTNGCYTCRTC-3′.

Reverse transcriptase reaction and PCR were performed using RNA-PCR kit (Perkin Elmer, Foster City, CA). The amplified PCR products were subcloned into the TA cloning vector pCR2.1 (Invitrogen, San Diego, CA). Sequencing analysis was carried out by using THERMO Sequence (Amersham, Buckinghamshire, UK), and the product was analyzed on Genetic Analyze System model 310 (Perkin Elmer). Computed homology search was performed with GENETYX-MAC 8.5d program (Software Development, Tokyo, Japan).

Cloning of a full-length cDNA of the short type of multidrug resistance protein homologue (SMRP) gene. The oligo(dT)- and random-primed human bone marrow cDNA library (Clontech, Palo Alto, CA) was screened with the subcloned PCR fragment (E1) as a probe by standard procedures (12). The inserted DNAs were cleaved from the λ vector by *Eco*R I-digestion, and ligated into pBluescript SK$^-$ (Stratagene, La Jolla, CA). *E. coli* XL-1 Blue (Stratagene) cells were transformed by the ligation mixture. The plasmid was purified by the Plasmid Kit Mini (QUIAGEN, Hilden, Germany).

FISH analysis. Prometaphase chromosome preparations for high-resolution band analysis were obtained from peripheral lymphocyte cultures of healthy donors by the method of thymidine (300 μg/ml) synchronization followed by 5′-bromo-2′-deoxyuridine (30 μg/ml) incorporation (13, 14). A cDNA fragment was used as a probe for FISH. This DNA fragment was labeled by nick translation with biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany). Hybridization conditions were essentially the same as previously described (14). The biotin-labeled probe was detected using the anti-fluorescein isothiocyanate (FITC) conjugate. The FITC signals and chromsome G-banding patterns were observed with a Nikon Microphot FXA microscope using B-2A and UV-2A filters, respectively (14).

Northern blot analysis. Human normal multiple tissue blot (MTN-blot, Clontech) was prehybridized in 5×SSC [1×SSC: 0.15 M NaCl and 15 mM sodium citrate (pH7.0)], 5×Denhardt's solution, 0.1 % SDS, 0.1 mg/ml sonicated salmon sperm DNA and 50 % formamide at 42 °C for 3 hr. Hybridization was performed overnight in the same buffer containing 10 % dextran sulfate and ^{32}P-labeled cDNA fragment, which

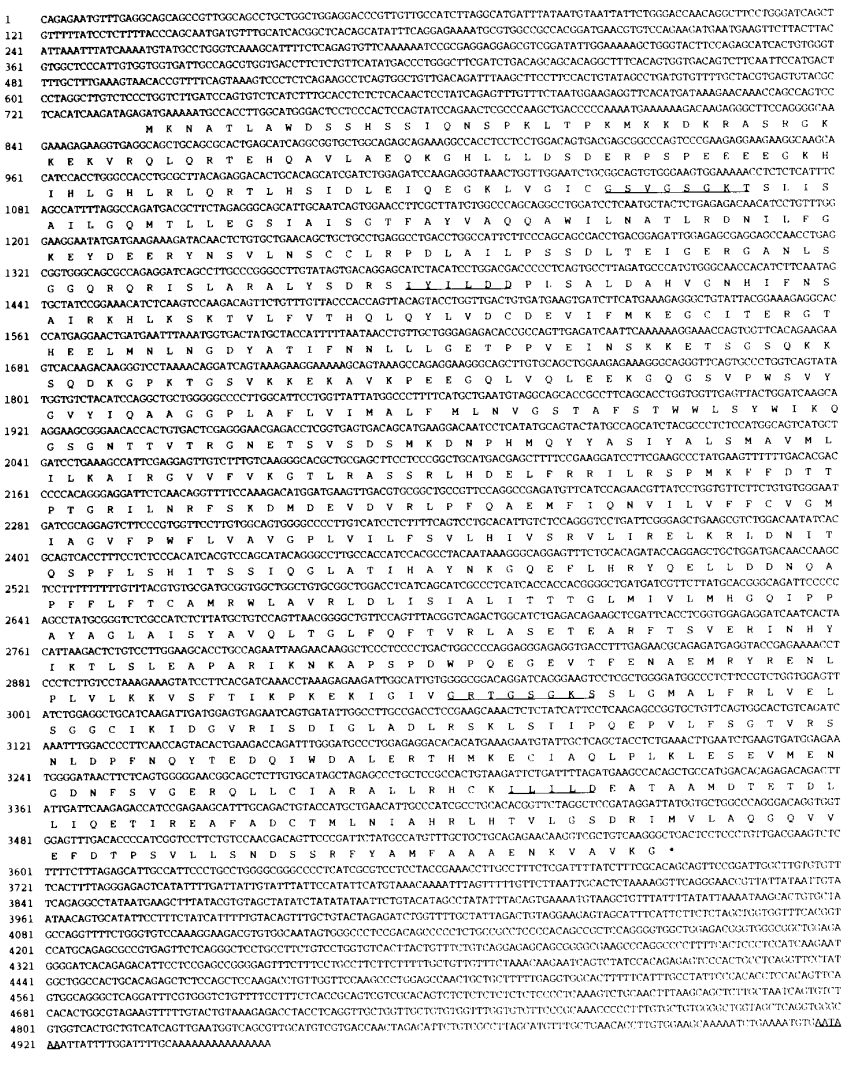

FIG. 1. cDNA and predicted amino acid sequence of *SMRP*. Walker A and B motifs are underlined, and a poly A additional signal is also underlined at the 3′ non-coding region.

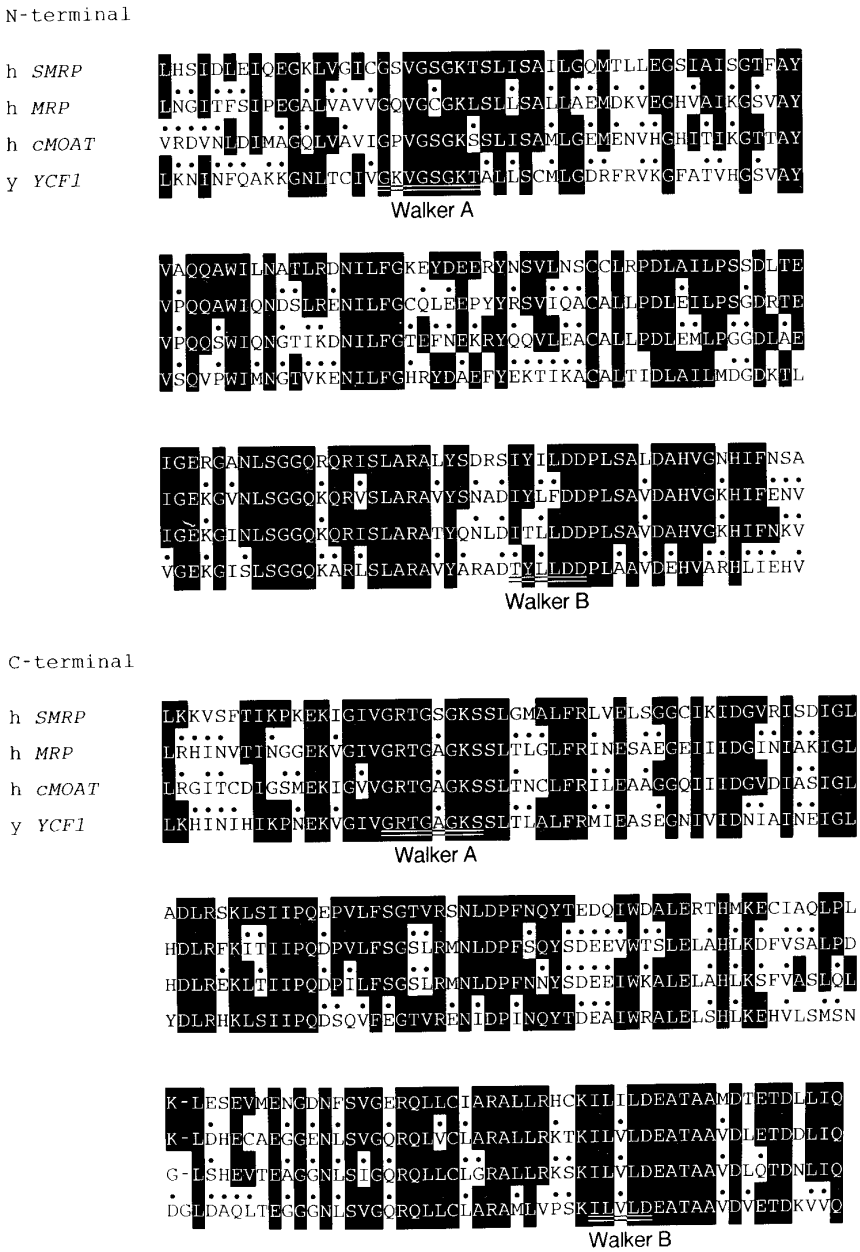

FIG. 2. Alignment region of human SMRP vs human MRP, human cMOAT of the extended ABC, and yeast YCF1. Shown are the N-terminal and C-terminal ABC regions. White type on black background indicates that positions are identical and dots represent conserved amino acids. The conserved motifs, Walker A and B, are underscored by a double line.

was prepared by a random primed labeling method (Rediprime, Amersham). The hybridized membrane was washed in 2×SSC, 0.1 % SDS at room temperature for 30 min and 15 min, followed by washing with 0.1×SSC, 0.1 % SDS at 55 °C for 10 min. Filters were exposed to a BioMAX MS film (Amersham) at −80 °C.

RESULTS

Using the RT-PCR method with degenerate primers prepared from the conserved sequence on the C-terminal ABC region, approximately 400 bp fragments were amplified from an RNA sample of PC-14/CDDP cells. By sequence analysis, a cDNA fragment, E-1, of 20 fragments exhibited significant homology with human *MRP* and human c*MOAT* genes. The homology scores were 62.6 % and 59.8 % at the nucleotide level, and 62.4 % and 63.8 % at the deduced amino acid level. To isolate a full-length cDNA clone of this gene, we screened the human bone marrow cDNA library with the E-1 fragment as a probe by standard procedures (12). A 2.4 kb cDNA clone, BM2.4, was obtained

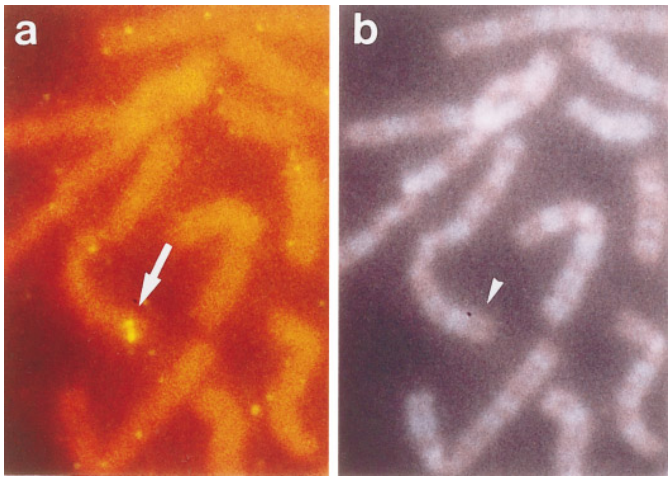

FIG. 3. Gene mapping by FISH. FISH analysis was performed using the BM4.4 clone as a probe, demonstrating fluorescent signals at chromosome band 3q27. (a) FITC signal (arrow) on chromosome 3, as detected using a Nikon B-2A filter; (b) G-banding patterns on the same chromosome (arrowhead) as seen in a, visualized with a Nikon UV-2A filter.

through the first screening. By the second screening using the BM2.4 clone, BM4.4 (4.4 kb) was obtained. A third hybridization was performed using the *Eco*R I-*Xho* I restriction fragment (1.2 kb) on the 5′ region of the BM4.4 clone. The third screening further led to the isolation of a full-length clone, BM4.8 (4.8 kb). BM4.8 encoded 946 amino acids. The nucleotide sequence and deduced amino acid sequence are shown in FIG. 1. The first in-frame ATG codon was at nucleotide position 737, and an in-frame terminator codon TGA was observed at 114 nucleotides upstream from the first in-frame ATG. The nucleotide sequence flanking the start methionine conforms to the Kozak consensus sequence for initiation of protein synthesis (15), and we found a polyA additional signal in the 3′ non-coding region of BM4.8. The predicted amino acid sequence from cDNA has two highly conserved ABC regions. The number of the predicted amino acid sequence of this new ABC superfamily gene was smaller than that of human MRP (1531 aa), human cMOAT (1545 aa) and yeast YCF1 (1515 aa). Thus, BM4.8 was designated as *SMRP* (a *s*hort type of *m*ultidrug *r*esistance *p*rotein homologue).

Similarity scores for the first ABC region relative to *SMRP* are as follows: human *MRP*, 60.4 %; human c*MOAT*, 62.2 %; yeast *YCF1*, 47.3 %, and for the second ABC region as follows: human *MRP*, 59.4 %; human c*MOAT;* 61.2 %; yeast *YCF1*, 58.4 %. The domains containing Walker motifs (16) are double underlined as shown in FIG. 2.

To determine chromosome localization of the *SMRP* gene, FISH analysis was performed using the BM4.4 clone as a probe, and the resulting hybridization signal was exclusively detected on chromosome 3 at band q27 (FIG. 3).

The expression level of the *SMRP* gene in PC-14/CDDP cells was compared with that in PC-14 cells by Northern blot analysis. An approximetely 5.0 kb of the *SMRP* mRNA was expressed slightly in the PC-14 cells and the PC-14/CDDP cells, and there was no difference of mRNA levels of the *SMRP* in both cell lines (data not shown). The expression levels of the *SMRP* gene were compared with those of the human *MRP* gene in normal tissues using MTN-blot. As shown in FIG. 4, *SMRP* mRNA was expressed in various tissues including heart, brain, skeletal muscle, spleen, thymus, prostate, testis, ovary, small intestine and peripheral blood leukocytes, and low expression was detected in placenta, lung, liver and colon. A relatively high expression of the *SMRP* gene compared with human *MRP* was observed in the brain (FIG. 4).

DISCUSSION

In a report on characterization of the human ABC superfamily using the expressed sequence tags database (17), we can find an identical amino acid sequence of EST277145 on the C-terminal region of the *SMRP* gene cloned. In this paper, we exhibited nucleotide sequence and deduced amino acid sequence of a full-length cDNA clone containing the EST sequence. As shown in FIG. 2, *SMRP* is composed of 946 amino acids containing two ABC regions with Walker motifs. The *SMRP* gene as well as *MRP* and c*MOAT* genes had about 40% homology with the yeast cadmium factor gene, *YCF1* (data not shown). These data suggest that this gene belongs to the GS-X pump family genes including *MRP*, c*MOAT*, and *YCF1* genes within the ABC transporter superfamily.

The human *MRP* and human c*MOAT* genes have been mapped to chromosome 16p13.1 and chromosome 10q24, respectively (5, 8). In contrast, the present FISH analysis revealed that the *SMRP* gene was located at chromosome band 3q27 (FIG. 3). These facts demonstrated that there is no cross hybridization among *MRP*, c*MOAT* and *SMRP* genes, suggesting that *SMRP* is a single gene on the human chromosome, and that there is no pseudogene.

Induction of mRNA expression of *MRP and γ-GCS* genes in cisplatin resistant HL-60 cells by addition of cadmium, zinc and arsenic has been reported (18). Investigations of resistance to cadmium on *SMRP* transfectant cells and induction of mRNA expression of *SMRP* by cadmium treatment were required. Although, by Northern blot analysis as shown in FIG. 4, not so many distinct differences of mRNA levels of *SMRP* on various tissues tested were observed compared with those of *MRP*, a relatively high expression of the *SMRP* gene compared with human *MRP* and *cMOAT* was observed in the brain. P-glycoprotein was reported to be expressed in the brain capillary endothelium rolled on blood brain barrier (19). Immunohisto-

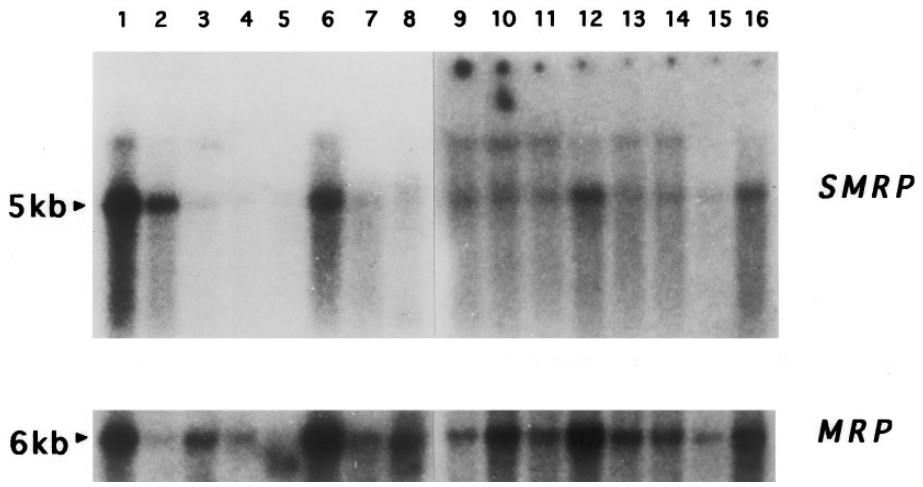

FIG. 4. Tissue distribution analysis. MTN-blot was hybridized with an ABC region fragment of human *SMRP* (E-1) as a probe. The blots were stripped and reprobed with an ABC region fragment of human *MRP*. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas; lane 9, spleen; lane 10, thymus; lane 11, prostate; lane 12, testis; lane 13, ovary; lane 14, small intestine; lane 15, colon; lane 16, peripheral blood leukocyte.

chemical analysis and/or RNA *in situ* hybridization analysis will be required for determination of localization of *SMRP* products in brain. However, *SMRP* encodes smaller protein than *MRP*, c*MOAT* and *YCF1* as shown in FIG. 1. Six hydrophobic transmembrane regions between the first ABC and the second ABC were conserved among SMRP and MRP, while 12 transmembrane regions on the N-terminal region of the first ABC observed in MRP (20) were completely deleted in SMRP. Therefore, functions of SMRP could be different from those of MRP, cMOAT, and YCF1.

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