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Detection of Multidrug Resistance (*MDR*1) Gene RNA Expression in Human Tumors by a Sensitive Ribonuclease Protection Assay

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The human *MDR*1 gene encoding P-glycoprotein, an energy-dependent drug-efflux pump, was initially isolated from a multidrug-resistant KB carcinoma cell. When a 3 kb genomic sequence isolated from normal human tissue including the major downstream promoter and the first and second exons of the *MDR*1 gene was compared to the equivalent fragment from KB cells, the *MDR*1 gene from KB carcinoma cells was found to have a point mutation in the first exon. Although this mutation does not affect the downstream promoter sequence or the coding sequence of the *MDR*1 gene, it creates a single base mismatch between the 5′ KB genomic fragment previously used for RNase protection analysis of *MDR*1 RNA expression in normal tissues and thereby reduces the sensitivity of this assay. Using the DNA fragment from normal tissues rather than KB cells, we have reanalyzed *MDR*1 mRNA levels in 12 renal carcinomas and 4 colon adenocarcinomas. By this RNase protection assay, *MDR*1 RNA levels are as high in these tumors as in the multidrug-resistant cell line, KB-8-5. The ribonuclease protection assay indicated that the major downstream promoter was mainly used in these clinical samples including two samples of RNA from metastatic renal cancer. This assay appears to be a very sensitive and specific assay for detecting *MDR*1 mRNA levels and mRNA initiation sites in clinical samples.

Key words: *MDR*1 gene — Multidrug resistance — Ribonuclease protection assay

A 170 kDa transmembrane glycoprotein (P-glycoprotein), encoded by the *MDR*1 gene, works as an energy-dependent drug-efflux pump in multidrug-resistant cell lines.[1–9] The *MDR*1 gene is expressed in various normal human organs such as adrenal, liver, kidney and colon, and in cancers which originated from these organs.[10–14]

We have previously isolated a 1 kb genomic fragment (PV) containing the major transcription initiation sites for the human *MDR*1 gene from the vinblastine-selected multidrug-resistant human KB carcinoma mutant cell line KB-V1.[15] This fragment linked to the chloramphenicol acetyltransferase gene showed promoter activity, although its activity was low.[15] No differences were detected in the promoter activity of the cloned fragment measured as chloramphenicol acetyltransferase activity in drug-resistant and drug-sensitive KB cell lines. These results suggest that the cloned promoter sequence was insufficient to allow regulation of transcription after transfection or that the promoter itself might be altered in the drug-resistant cell lines.

To study the sequence of the promoter in the native *MDR*1 gene, we isolated a 3 kb genomic fragment from a phage library made from human normal tissue by Maniatis *et al.*[16] The sequence of the PV fragment was identical to that of the native *MDR*1 gene except for a point mutation in the first exon. In this report, we also show that a ribonuclease protection assay using the promoter sequence isolated from normal tissue is very sensitive and reliable for studying *MDR*1 mRNA expression in tumor samples.

MATERIALS AND METHODS

Materials Cytidine-5′[α-^{32}P]triphosphate (30 TBq/mmol) was purchased from Amersham. SP6 RNA polymerase was from Promega Biotec. Ribonucleases A and T1 were from Boehringer Mannheim and Bethesda Research Laboratories, respectively.

Specimens Twelve renal cell carcinoma (RCC) samples (9 primary and 3 metastatic lesions) and 4 colon adenocarcinomas were surgically obtained at Kyoto University Hospital and its satellite hospitals, Shiga University of Medical Science Hospital and Mie University Hospital. These patients had not been treated with any anti-cancer drugs prior to surgery.

Cell lines KB-3-1, a HeLa subline, is a drug-sensitive human carcinoma cell line. KB-8-5 and KB-C4 were multidrug-resistant cell lines selected in successive steps from KB-3-1 by using colchicine. Multidrug-resistant cell lines KB-V1 and KB-A1 were isolated from KB-3-1 by

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using vinblastine and adriamycin (doxorubicin), respectively. The details of isolation and characterization of the cell lines have been previously described.[17, 18]

Isolation of *MDR1* genomic fragment A human genomic library in a Charon 4A vector made by Maniatis *et al.*[16] was screened with the 5′ region of human *MDR1* cDNA as a probe. A phage clone was identified. A 3 kb *Eco*RI fragment was subcloned into pUC19. The nucleotide sequence of this fragment was determined by the dideoxy method of Sanger.

Ribonuclease protection assay The 1 kb *Pst*I fragment in the cloned fragment was inserted into pGEM3 (pMDR-P3). The uniformly labeled antisense RNA probe (785 nucleotides) was transcribed with SP6 RNA polymerase from *Pvu*II-digested plasmid pMDR-P3 according to the protocol supplied by Promega Biotec. Total RNA (10 μg or 20 μg) was hybridized with 2×10^6 cpm of probe at 45°C in hybridization buffer (80% formamide, 0.4 *M* NaCl, 40 m*M* PIPES, 1 m*M* EDTA) for 14 h. The probe was digested by ribonucleases A (49 μg/ml) and T1 (28 units/ml) at 30°C for 1 h. After proteinase K treatment, phenol/chloroform extraction and ethanol precipitation, the protected probe was analyzed on a 6% or 8% polyacrylamide, 7 *M* urea gel.

RESULTS

The 3 kb genomic fragment was isolated from the phage library made from human normal tissue by Maniatis *et al.*[16] This fragment contained the promoter region, the 134 bp first exon, the 562 bp first intron, the 74 bp second exon and a part of the second intron of the *MDR1* gene (Fig. 1). The nucleotide sequence analysis revealed that the genomic fragment (PV) previously isolated from vinblastine-selected multidrug-resistant KB cell line[10] had a point mutation at +98 in the first exon, which is a non-coding region (Fig. 2). The nucleotide sequence of the first and second exons determined from the normal human genomic *MDR1* gene fragment was identical with the nucleotide sequence of the full-length *MDR1* cDNA isolated from normal human adrenal.[19]

We have reported that normal human adrenal, colon and liver cells, the human hepatoma cell line HepG2, and vinblastine-selected human KB multidrug-resistant cells initiate transcription of the *MDR1* gene at the same site as detected by a ribonuclease protection assay using the PV fragment.[15] We compared the ribonuclease protection assay using the 1 kb *Pst*I fragment isolated in this

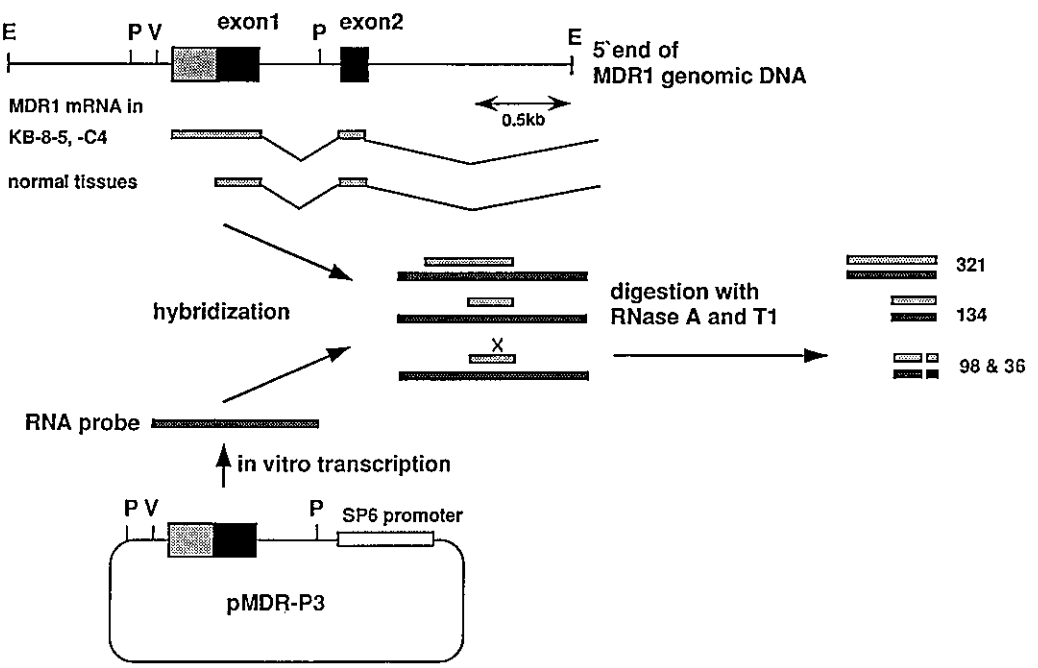

Fig. 1. Schematic diagram of ribonuclease protection assay for *MDR1* mRNA. The uniformly labeled antisense RNA probe was transcribed with SP6 RNA polymerase from *Pvu*II-digested plasmid pMDR-P3. Total RNA samples from KB cells, normal tissues and clinical samples were hybridized with the RNA probe and digested with ribonucleases A and T1. The protected probe was analyzed as described in "Materials and Methods" and in the text. The single base change at +98 in PV probe is indicated by X. Because of this base change, a fraction of the RNA probe was digested and produced two extra bands of about 98 nucleotides and 36 nucleotides.

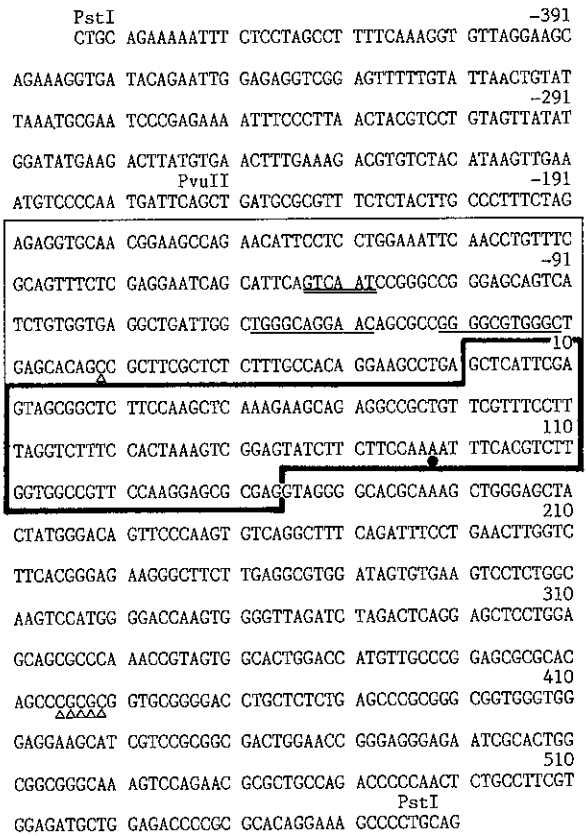

Fig. 2. Nucleotide sequence of the 1 kb genomic *Pst*I fragment isolated from human tissue. Exon 1 is enclosed with a bold line. Transcripts present in colchicine-selected multidrug-resistant KB cells initiating from the upstream promoter contain the exon enclosed by a thin line linked to exon 1. A CAAT box (double underlined) and GC box-like sequence (underlined) are shown. The A residue indicated by ● at +98 is the nucleotide which is changed to G in KB cells. The C residue at −32 and the CGCGC sequence from +356 to +369 indicated by △'s were mistakenly deleted or presented as G, respectively, in the previously published sequence.[15] The numbers are presented with the major transcription initiation site as +1.

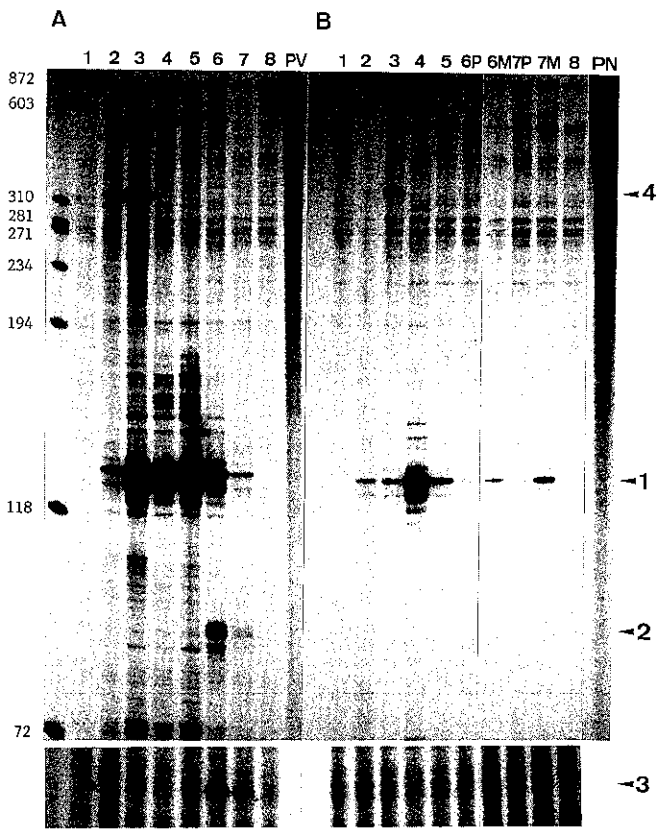

Fig. 3. Ribonuclease protection assay. The PV RNA probe (A) transcribed from pMDR-P2[15] or the PN RNA probe (B) transcribed from pMDR-P3 was hybridized with total RNA and analyzed as described in "Materials and Methods." A1 and B1, KB-3-1 (20 μg); A2 and B3, KB-8-5 (20 μg); B2, KB-8-5 (10 μg); A3, KB-C4 (5 μg); A4, KB-V1 (5 μg); A5, KB-A1 (5 μg); A6 and B4, adrenal (10 μg); A7 and B5, kidney (10 μg); B6 and B7, renal cell carcinomas (P, primary lesions; M, metastatic lesions). B6 and B7 correspond to B5 and B7 in Fig. 5. A8 and B8, tRNA (20 μg); PV, PV RNA probe; PN, PN RNA probe. Arrows 1 to 4 indicate protected RNA probes. The numbers indicate size markers in nucleotides.

report (PN) with that using the PV fragment (Fig. 3). Using the PV RNA probe, human adrenal RNA and kidney RNA produced three bands (arrows 1, 2 and 3) but RNAs from KB mutant cell lines did not produce band 2 or 3 (Fig. 3). Using the PN RNA probe, all RNA samples produced a clear major band (arrow 1) but did not produce band 2 or 3. In both cases, RNA from KB-8-5 and KB-C4 produced a fourth band, indicating the presence of a transcript from an upstream promoter. These results indicate that i) because of the single base change at +98, a fraction of the PV RNA probe was digested by ribonuclease A and produced two extra bands of about 98 nucleotides (arrow 2) and 36 nucleotides (arrow 3) as schematically shown in Fig. 1; ii) the uridine residue at +98 in the PN RNA probe base paired with the guanine residue in RNAs from KB mutant cell lines (Fig. 4) and was not digested by ribonuclease A or T1; and iii) RNAs from the KB mutant cell lines independently isolated by using colchicine (KB-8-5 and KB-C4), vinblastine (KB-V1) and adriamycin (KB-A1) did not produce band 2 or 3, suggesting that they have a point mutation at the same site, and that this point mutation was probably derived from the parent cell line KB-3-1.

The mismatch at the point mutation in the PV probe resulted in a reduction of the strength of the major signal (arrow 1) produced with RNAs from human adrenal and kidney (Fig. 3, A6 and A7) compared with the signal using the PN probe (Fig. 3, B4 and B5), whereas the strength of the band produced with RNA from KB-8-5 did not change (Fig. 3, A2 and B3). The ribonuclease protection assay using the PN RNA probe is 1.5- to 2-fold more sensitive for RNAs from human tissues than the assay using the PV RNA probe and would, therefore, be more sensitive and reliable for studying *MDR*1 mRNA expression level in clinical samples.

We measured *MDR*1 mRNA levels in 12 renal cell carcinoma samples (9 primary and 3 metastatic lesions) and 4 colon adenocarcinomas (Fig. 5) using the PV RNase protection probe. The RNA levels in many of these carcinoma cells were as high as the levels in the multidrug-resistant KB-8-5 cells. Elevated *MDR* 1 mRNA levels were also observed in 2 out of the 3 metastatic lesions. These two cases were confirmed by using the PN probe (Fig. 3). All of these samples produced band 2 with the PV RNA probe (Fig. 5), indicating that the nucleotide at position +98 in these RNA samples is adenosine (wild-type).

	+98
Normal RNA	–AAAAUU–
PV RNA probe	–UUCUAA–
	+98
KB RNA	–AAGAUU–
PN RNA probe	–UUUUAA–

Fig. 4. Nucleotide sequence (+96 to +101) of RNAs from human normal tissue and KB mutant cells hybridized with PV RNA probe and PN RNA probe, respectively.

DISCUSSION

The nucleotide sequence analysis of the 3 kb genomic fragment containing the promoter region, the first and second exons, and the first intron of the human *MDR*1 gene isolated from human normal tissue revealed that this gene from the multidrug-resistant KB mutant cells has a point mutation in the first exon. A ribonuclease protection assay of several independently isolated KB multidrug-resistant mutants suggested that this point mutation was derived from the drug-sensitive KB-3-1 parent cell, and that all the tested RNAs from human tissues and carcinoma cells were "wild type." It remains to be determined if this point mutation affects the regulation of *MDR*1 gene expresssion.

Recently, we isolated a full-length cDNA of the *MDR* 1 gene from normal human adrenal and found that the *MDR*1 cDNA previously isolated from a colchicine-selected multidrug-resistant KB cell has 9 point mutations.[19] These mutations include the first exon mutation noted in this work, as well as changes in the coding

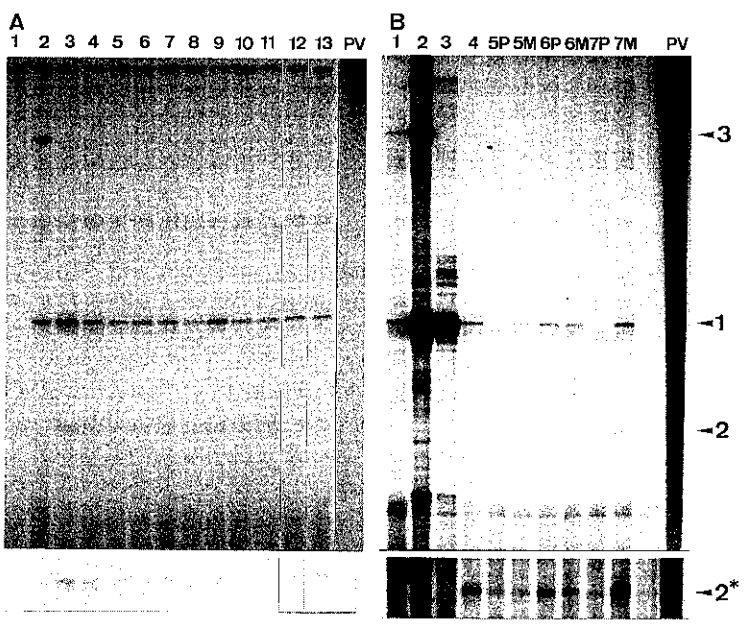

Fig. 5. Ribonuclease protection assay. The PV RNA probe was hybridized with 10 μg of total RNA from clinical samples. A: 1, tRNA; 2, KB-8-5; 3, normal kidney; 4–9, renal cell carcinomas; 10–13, colon adenocarcinomas. B: 1, KB-8-5; 2, KB-C4; 3, KB-V1 (5 μg); 4, normal kidney; 5–7, renal cell carcinomas (P, primary lesions; M, metastatic lesions); PV, PV RNA probe. Arrows 1–3 indicate protected RNA probes. Arrow 2* indicates the band 2 in a long-exposed autoradiograph.

sequence. It has previously been reported that mutations occur in the coding sequence of the *MDR*1 gene during the selection of resistant cells in colchicine.[20] Two point mutations resulting in a Gly-Val mutation at codon 185 alter the pattern of cross resistance.[19, 20] The other amino acid substitution, Ser-Ala at codon 893, probably reflects genetic polymorphism and may slightly alter the pattern of cross resistance.[19]

Whatever the functional significance of one mutation in the first exon, the nucleotide change produced a mismached nucleotide which caused partial digestion by ribonucleases as shown in Fig. 3. This may result in underestimation of the *MDR*1 mRNA level if the mutant PV probe is used with wild-type RNA. The RNA probe isolated in this report contains the wild-type *MDR*1 RNA sequence from the first non-coding exon. Therefore, the ribonuclease protection assay using this probe is expected to be very sensitive and specific.

Detection of *MDR*1 mRNA with DNA and RNA probes and of P-glycoprotein with monoclonal antibody revealed that the *MDR*1 gene is expressed in normal adrenal, kidney, liver and colon[10-12, 21] and in cancers which originated from these organs.[10-14] Occasional high expression of the *MDR*1 gene is also found in leukemias and lymphomas, and in a variety of other tumors.[22] *MDR*1 RNA levels are also higher in cancers, such as neuroblastomas and leukemias, relapsing after chemotherapy.[22]

We have reported that in colchicine-selected multidrug-resistant KB cell lines, transcription also started from another promoter upstream of the promoter used in normal tissues.[15] In all the clinical samples studied in this report including two metastatic RCC samples with high RNA levels, only the normal promoter was active. Although some clinical samples appear to use the upstream as well as downstream promoters,[22] further investigations are necessary to clarify the role of the upstream promoter in clinical samples.

In the study of *MDR*1 expression level in clinical samples, overestimation of *MDR*1 RNA levels may result from the cross-hybridization of DNA probes to the closely related *MDR*2 (*MDR*3) gene[23] which has not been shown to be associated with multidrug resistance. The ribonuclease protection assay using the PN RNA probe is specific for *MDR*1 mRNA, since the detection of the specific 134 nucleotide band eliminates problems of cross-hybridization and background. The degradation of RNA may also result in underestimation of *MDR*1 mRNA. Because the probe is at the 5′ end of the *MDR*1 mRNA, the 134 nucleotide band may be the last to be affected by degradation of RNA samples. This ribonuclease protection assay using the PN probe provides a sensitive and specific method to study *MDR*1 mRNA levels in clinical samples, and could be employed to measure the threshold level of *MDR*1 mRNA expression as an indicator of sensitivity to the standard *in vivo* chemotherapy.

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