Short Technical Report

Semi-Quantitative RT-PCR Method to Estimate Full-Length mRNA Levels of the Multidrug Resistance Gene

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ABSTRACT

*Expression levels of P-glycoprotein (P-gp), the transporter encoded by the human multidrug resistance gene (*MDR1*), may play an important role in drug disposition. The ability to quantitate full-length* MDR1 *mRNA levels may be predictive of P-gp expression and function. Therefore, a semi-quantitative RT-PCR assay was developed to assess full-length* MDR1 *mRNA levels. Levels of full-length 3.8-kb* MDR1 *mRNA were estimated by comparing PCR amplification of the RNA extract with that of an internal standard,* ΔMDR1*. The 2.9-kb* ΔMDR1 *competitor RNA standard was constructed by deleting 965 bp from the interior of* MDR1 *mRNA. The full-length* MDR1 *and* ΔMDR1 *share identical 5′ and 3′ primer binding sequences, allowing for their simultaneous amplification in the same RT-PCR. With this approach,* MDR1 *mRNA levels can be sensitively and reliably estimated with a detection limit of 2000 copies. Full-length* MDR1 *mRNA levels in various human cell lines and lymphocytes from leukemia patients varied over 100-fold, ranging from 0.3 to 36.5 × 10^5 copies/μg total RNA. The semi-quantitative full-length RT-PCR assay may be useful in estimating* MDR1 *mRNA levels to assess P-gp expression, which may be important in studying the role of P-gp in drug disposition and cancer chemotherapy efficacy.*

INTRODUCTION

P-glycoprotein (P-gp) is an ATP-dependent membrane transporter originally discovered in drug-resistant Chinese hamster ovary cell mutants (8). P-gp, the product of the human multidrug resistance gene (*MDR1*), acts to decrease drug accumulation, leading to cellular resistance to a variety of anticancer drugs. P-gp has also been found to mediate the energy-dependent efflux of xenobiotics in normal tissue barriers throughout the human body. P-gp expression has been described at the intestinal epithelium, biliary canalicular membrane of hepatocytes, kidney proximal tubules, blood-brain barrier, and placenta (19). Substrates of P-gp come from a variety of functionally and structurally diverse classes of drugs including antiarrythmics, antifungals, calcium-channel blockers, chemotherapeutic agents, hormones, immunosuppressants, and HIV-protease inhibitors (21). Therefore, P-gp may play a central role in drug disposition in vivo.

Substantial interindividual variability exists in the pharmacokinetics of many P-gp substrates (3,6,20). Variable P-gp tissue expression may be an important factor in explaining this variation. Because of the lack of purified P-gp protein standards, estimation of P-gp levels in tissues and cell lines by Western immunoblot analysis is difficult. Another strategy in estimating P-gp expression is based on quantitating full-length *MDR1* mRNA levels. Northern blot analysis is a common method used for mRNA quantitation; however, it requires a large amount of total RNA and may not be practical for RNA collected from tissues (5). Wang et al. (22) have shown that the amount of IL-1α mRNA quantitated by RT-PCR methods correlates with levels observed by Northern blot analysis. In addition, lower levels of the drug-metabolizing enzyme cytochrome P450 2C19 (*CYP2C19*) mRNA are quantifiable by full-length RT-PCR than by Northern blot detection (23).

A number of researchers have developed short fragment RT-PCR assays to measure the levels of *MDR1* mRNA (9,12,25). However, detection of short mRNA fragments may not provide an accurate estimate of the amount of full-length mRNA transcript; short fragment RT-PCR assays may overestimate full-length mRNA (4). RNA degradation or incomplete transcription could provide target fragments of mRNA detectable by short RT-PCR methods, which would not likely translate into functional protein. These smaller RNA fragments would not be detected by full-length mRNA analysis. Recently, Hu et al. (7) used a long RT-PCR method to detect, but not quantitate, the presence of full-length *MDR1* mRNA in cell lines, demonstrating the feasibility of RT-PCR to detect the 3.8-kb *MDR1* mRNA.

Previously, we have developed an RT-PCR method to quantitate full-length 1.5-kb mRNA transcripts of *CYP2C19* (23). This method was extended and optimized to quantitate 3.8-kb full-length *MDR1* message levels based on a competitive RT-PCR assay. The key feature of this RT-PCR approach is the use of a homologous RNA standard (Δ*MDR1*) sharing identical

primer binding sites as the native *MDR1* mRNA and containing a small deletion in the interior of the *MDR1* sequence. The benefit of using a homologous RNA standard, as opposed to an endogenous RNA standard, is that the internally deleted Δ*MDR1* standard will likely have similar RT and PCR efficiencies as the native *MDR1* mRNA (5).

MATERIALS AND METHODS

Materials

The kits used include Purescript® RNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA), QIAquick™ gel extraction kit (Qiagen, Valencia, CA, USA), RNA transcription kit (Stratagene, La Jolla, CA, USA), and Titan™ One-Tube PCR System (Roche Applied Science, Basel, Switzerland). Cell lines were purchased from ATCC (Manassas, VA, USA). The pCR3.1 TA cloning vector, T4 DNA ligase, and RNase-free DNase I were purchased from Invitrogen (Carlsbad, CA, USA).

Isolation of Total RNA

Total RNA was obtained from either human cell lines or lymphocytes from leukemia patients. RNA samples extracted from blood or bone marrow of leukemia patients were provided by our collaborator, Dr. Dan Wu, at the UWMC-VA hospital. Total RNA was extracted from human cell lines using the Purescript RNA Isolation Kit, according to the manufacturer's specifications. The concentration of RNA in extracted samples was estimated as the product of a conversion factor (40 µg/mL) and absorbance at λ_{260} measured with a spectrophotometer (model DU7400; Beckman Coulter, Fullerton, CA, USA).

Design and Selection of Primers

Primers were designed based on *MDR1* sequences available in the GenBank® database (accession no. M14758) with an online primer design software program (Primer 3). For full-length *MDR1* RT-PCR, primer sequences were 5′-ATGGATCTTGAAGGGGACCG-3′ (residues 1–20; forward primer) and 5′-TCACTCCGCCTTTGTTCCAGC-3′ (residues 3822–3842; reverse primer).

Design and Synthesis of an Internal Control RNA Competitor

The internally deleted RNA competitor design for *MDR1* mRNA was similar to that previously described for *CYP2C19* mRNA (23). The control *MDR1* cDNA plasmid was generated from cDNA isolated from the P-gp-overexpressing cell line, MES-SA-DX5 (ATCC). The isolated *MDR1* cDNA was cloned into a linearized pCR3.1 TA vector (Invitrogen) containing cytomegalovirus (CMV) and T7 promoters capable of transcription. Restriction endonucleases, *Pst*I and *Eco*RI, were used to confirm correct orientation of the cDNA by electrophoretic analysis. After positive *MDR1* cDNA clones were identified, the clones were expressed in *E. coli* to generate a *MDR1* cDNA plasmid stock.

The *Bgl*II restriction enzyme was used to delete a 965-bp fragment from the interior of the *MDR1* mRNA (residues 258–1223; GenBank accession no. M14758), thereby generating the competitor plasmid designated as Δ*MDR1*. The digested products were separated on a 1% agarose gel, and the deleted Δ*MDR1* plasmid was extracted from the gel using the QIAquick gel extraction kit. The Δ*MDR1* plasmid was ligated using *T4* DNA ligase (Invitrogen) and transformed into Top10F′ competent cells (Invitrogen).

Since the pCR3.1 TA vector contained a T7 promoter capable of transcription, the control and internally deleted plasmids were used to synthesize *MDR1* and Δ*MDR1* RNA transcripts. After linearization, the DNA plasmids were transcribed according to Wang et al. (23). RNA quality and integrity were verified by electrophoresis on a 1% formaldehyde gel. The internally deleted Δ*MDR1* RNA product was 2.9 kb in length, while the positive control standard, *MDR1*, was 3.8 kb. The concentration of both RNA products was measured by spectrophotometric analysis at 260 nm, aliquoted, and stored at -80°C.

RT-PCR Amplification

The Titan One-Tube PCR System was used to amplify RNA samples in a GeneAmp® PCR system, model 9600 thermal cycler (Applied Biosystems, Foster City, CA, USA). The enzyme mixture included in the kit was comprised of Expand™ High Fidelity polymerase (Roche Applied Science) and AMV reverse transcriptase. Reactions were performed according to a method developed for competitive RT-PCR quantitation of CYP2C19 (0) with slight modifications. Each reaction mixture of 50 µL contained 50–200 ng total RNA and final $MgCl_2$ concentration of 2.5 mM. RT was performed at 45°C for 30 min, followed by a 2-min denaturation at 94°C and 10 PCR cycles consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 68°C for 3 min. An additional 35-cycle reaction followed, with denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 68°C for 3 min, adding 5 s to each cycle for elongation. The final extension step was a 68°C hold for 10 min. RT-PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide for detection of the cDNA

bands. The sample *MDR1* product (3.8 kb) and the internally deleted competitor, Δ*MDR1*, (2.9 kb) were well separated in the gel under these conditions.

Estimation of *MDR1* mRNA Levels

The method for estimation of *MDR1* levels was similar to that reported for quantitating message levels of *CYP2C19* (23). Total sample RNA (200 ng) was added to the initial RT-PCR with a known and fixed amount of Δ*MDR1* internal control (10^4 copies). Serial dilutions of total RNA (1:3), with fixed internal control, were made in each subsequent RT-PCR until there was no detectable *MDR1* signal. The reaction products were separated on a 1.5% agarose gel and stained with ethidium bromide. The amount of *MDR1* mRNA in each reaction was estimated employing the NIH image-processing program (18) on a digital image captured with a Kodak® DC120 camera (Eastman Kodak, Rochester, NY, USA). The NIH processing program accounts for the difference in band intensity that is due to the base pair lengths of the DNA products (3.8 vs. 2.9 kb). Estimations of *MDR1* mRNA levels were made by comparing to known amounts of Δ*MDR1* in each reaction. *MDR1* mRNA estimations were based on the average of duplicate or triplicate RT-PCR assays.

RESULTS

The first step in the development of a semi-quantitative method to detect full-length *MDR1* mRNA levels was the generation of an internally deleted competitor, Δ*MDR1*. The design of Δ*MDR1* provides for significant homology to *MDR1*; Δ*MDR1* differs from *MDR1* only by an internal deletion of 965 bp (Figure 1A). The deletion (residues 258–1223) allows for the simultaneous amplification of both *MDR1* and Δ*MDR1* in the same RT-PCR using identical forward and reverse primers. Since *MDR1* and Δ*MDR1* differ in length by a small amount, the efficiency of RT-PCR amplification is expected to be the same or very similar (5). The design of the 2.9-kb Δ*MDR1* internal control and the 3.8-kb full-length *MDR1* also allows for separation of the two bands on an agarose gel (Figure 1B, lanes 5–8).

Several rounds of optimization indicated that 42.1 fg Δ*MDR1* RNA (equivalent to 10^4 copies) appeared to be a sufficient amount of internal competitor in each RT-PCR. Therefore, for each quantitation reaction, 10^4 copies of Δ*MDR1* were used to produce a consistent and clearly detectable product. A typical titration of sample RNA to measure *MDR1* message levels is presented in Figure 1B. The amount of *MDR1* mRNA is estimated based on the dilution in which the band intensities of *MDR1* and Δ*MDR1* are equivalent.

Variation and confidence in *MDR1* quantitation by the long-range RT-PCR assay was evaluated over four days utilizing total cellular RNA from three cell lines with varying *MDR1* mRNA levels. The *MDR1* mRNA levels of these cells were estimated as follows [$\bar{x}$ ($\times 10^5$ copies/µg RNA) ± SD]: MES-SA-DX5=36.5 ± 5.91, HepG2=6.97 ± 1.44, and MB-228-3=2.59 ± 0.66. Based on these data, the CVs ranged from 16.2% to 25.5%. The assay was sensitive, with a lower limit of detection of 2000 copies *MDR1* mRNA.

With the semi-quantitative RT-PCR assay, we estimated *MDR1* levels in several cell lines and in lymphocytes of leukemia patients (Table 1). In the cell lines tested, we found that *MDR1*

Table 1. Full-Length *MDR1* mRNA in Cell Lines and Leukemia Patients

RNA Samples	*MDR1* mRNA Expression (10^5 Copies/µg RNA)
Cell Lines	
MES-SA-DX5	36.5
HT-209	7.15
HepG2	6.97
COS-7	5.24
Caco-2	5.08
FB3	5.02
Sk Hep-1	3.51
MB-228-3	2.59
Jurkat	1.45
Jurkat-Tat	0.53
Jar	0.41
HeLa	0.30
HT29	±[a]
U937	±[a]
Jeg	–[b]
LL2	–[b]
MES-SA	–[b]
Leukemia Patients	
Healthy donors (*n* = 3)	–[b]
W48	5.17
M92	4.64
D12	1.62

[a] *MDR1* expression was detected but levels were near the limit of detection (2000 copies) and therefore not quantifiable.
[b] *MDR1* expression not detectable.

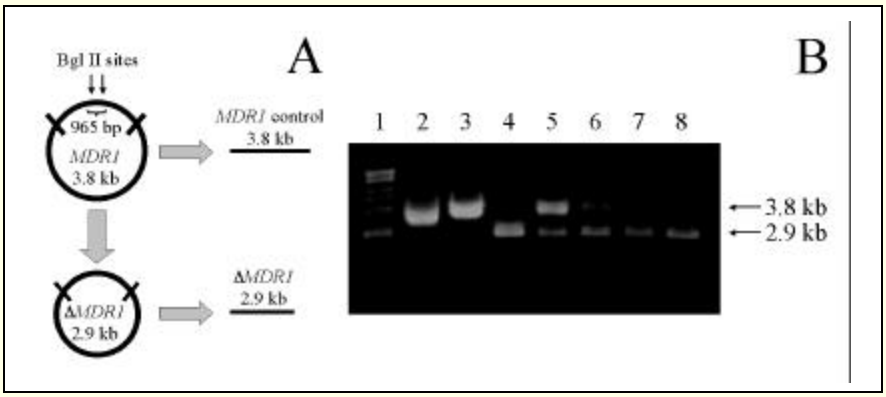

Figure 1. Development of a semi-quantitative RT-PCR technique to detect *MDR1* mRNA. (A) Schematic representation of full-length control and internally deleted vectors designed to transcribe *MDR1* and Δ*MDR1*. (B) Titration of *MDR1* mRNA using the internally deleted 2.9-kb Δ*MDR1* RNA as an internal control. Lane 1, DNA bp standard; lane 2, 10 ng *MDR1* mRNA transcribed from the positive control plasmid; lane 3, 100 ng total cellular RNA without the internal competitor; lane 4, 10^4 copies of Δ*MDR1* mRNA without cellular RNA; lanes 5–8, titration of total cellular RNA (serial 3-fold dilutions) with a fixed amount (10^4 copies) of Δ*MDR1* mRNA included as an internal standard comparison. Lane 5 was used to estimate the amount of *MDR1* mRNA since *MDR1* and Δ*MDR1* appear to have equivalent band intensity after adjusting for the difference in intensity due to the lengths of the products.

mRNA levels ranged from 0.3 to 36.5 × 10^5 copies/μg total RNA (a greater than 120-fold difference between cells). All three leukemia patients (M92, D12, and W48) had relatively high levels of *MDR1* mRNA, while the control healthy donors had undetectable levels.

DISCUSSION

RT-PCR approaches have been used to quantitate mRNA levels in a variety of applications, including HIV (16,24), hepatitis C virus (10,11), and drug-metabolizing enzymes (1,2,13,14,17,23). The use of a competitive synthetic RNA standard in these quantitative reactions is advantageous since the standard and native RNA share the same primer binding sites and can be amplified in the same RT-PCR to make estimates of native RNA levels.

RT-PCR techniques have also been used to measure *MDR1* mRNA levels by detecting short sequences of *MDR1* mRNA (9,12,25). Detection of full-length 3.8-kb *MDR1* mRNA has recently shown to be feasible by RT-PCR in a qualitative mode (7). With the successful synthesis of an internally deleted Δ*MDR1* RNA standard, we have now developed and characterized a semi-quantitative assay capable of quantitating full-length *MDR1* mRNA in a sensitive and reproducible manner. Since the detection of short RNA fragments identifies part, but not all, of the open reading frame, short RNA fragments may overestimate the amount of full-length mRNA necessary for protein expression. Because the primers used in this assay are located at the 5′ and 3′ ends of the *MDR1* mRNA, *MDR1* alleles containing point mutations or internal insertions or deletions would still be quantifiable. Only *MDR1* mutants with deletions at the 5′ or 3′ ends would not be detectable in this assay. Therefore, full-length *MDR1* mRNA estimates may provide a better estimate of P-gp expression. Whether *MDR1* mRNA levels may predict P-gp expression levels in cells and tissues remains to be evaluated.

The feasibility of using an internally deleted RNA competitor to detect and estimate *MDR1* mRNA levels has been demonstrated in these experiments. The assay allows for a semi-quantitative estimation of *MDR1* mRNA in different cell lines and in a preliminary sample of lymphocytes from leukemia patients. The *MDR1* mRNA levels varied over 100-fold between cell lines. In the leukemia patients, the data indicate that the transcription of *MDR1* mRNA is upregulated in lymphocytes isolated from leukemia patients compared to healthy

controls. Recently, Parasrampuria et al. (15) have shown that lymphocytes isolated from healthy individuals exclude rhodamine-123, a prototypic substrate of P-gp. These data indicate that normal lymphocytes express P-gp and may have low levels of *MDR1*. Thus far, in our small healthy human lymphocyte sample, we have not detected any *MDR1* expression. However, it may be possible to detect low *MDR1* expression in a larger sample size and with a larger quantity of total RNA used in each assay.

In summary, we have developed a semi-quantitate RT-PCR assay to measure full-length *MDR1* message levels. This assay requires only a small amount of total RNA to provide a reliable amplification and titration of *MDR1* mRNA. The semi-quantitative RT-PCR assay is applicable to studies of *MDR1* gene transcription in the regulation of P-gp expression in cells and tissues.

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