Nonisotopic Competitive RT-PCR Assay to Measure MDR1 Gene Expression

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We report an original application of competitive reverse transcription–polymerase chain reaction (RT-PCR) for the quantification of MDR1 mRNA in clinical specimens by simultaneous reverse transcription and PCR amplification of cellular RNA with decreasing amounts of an internal standard. The competitor RNA shares the same MDR1 primer sequences as the cellular mRNA, but yields a different-sized PCR product. This allows resolution of the amplified cDNA fragments after agarose gel electrophoresis and ethidium bromide staining. The concentration of MDR1 mRNA is derived from the ratio between the intensities of the bands corresponding to the amplified products. We have used this assay to measure MDR1 expression in breast carcinomas and assessed the precision, sensitivity, and accuracy of the method. Competitive RT-PCR is a simple, highly specific, nonradioactive procedure for the quantification of MDR1 mRNA and is particularly suitable for use in the clinical laboratory.

Indexing Terms: P-glycoprotein/cloning/electrophoresis, agarose/breast cancer/liver and cancer cell lines/polymerase chain reaction/RNA/mRNA

Drug resistance remains a problem in the chemotherapy of cancer. A particularly important type of tumor drug resistance, multidrug resistance (MDR), is manifested by cross-resistance to a number of structurally and functionally unrelated lipophilic drugs. Several mechanisms of drug resistance exist, but one important mechanism is through the overexpression of the MDR1 gene that encodes a 170-kDa integral membrane protein named P-glycoprotein (1-4). P-glycoprotein acts as a drug efflux pump that actively extrudes a wide variety of drugs out of the cell, thereby preventing them from accomplishing their intracellular action (5, 6). MDR1 gene expression is frequently observed in different human tumors, both untreated and treated with chemotherapeutic drugs, as well as in some normal tissues, where it most likely functions as a transport protein (7-10).

Several molecules such as immunosuppressors [cyclosporine (11), FK506, and rapamycin (12)], verapamil (13), ribozyme (14), and steroids (15) are known to reverse the MDR phenotype, and clinical trials are currently underway that are designed to inhibit P-glycoprotein function (16, 17).

The amount of MDR1 mRNA has been correlated with the degree of drug resistance (18), and even very low levels of MDR1 gene expression can confer a several-fold increase in drug resistance, which may be clinically significant (2, 10). Assay insensitivity, coupled with the often limited sample material available from tumors or its degradation, has often hampered the detection of what may be clinically significant amounts of MDR1 mRNA. In addition, reliable detection of MDR1 gene expression is further complicated by the existence of a homologous gene called MDR2 (1, 10), which has not been associated with resistance to chemotherapeutic drugs (19). To improve the ability to detect low amounts of MDR1 mRNA in clinical samples, we have developed a quantitative assay based on the competitive reverse transcription–polymerase chain reaction (RT-PCR) (20), which can be used with small RNA samples of varying quality.

At least four other groups have quantified MDR1 expression by PCR (21-24). Two of these assays (21, 22) use as internal controls housekeeping genes, making the assumption that expression of the control gene does not change during tumorigenesis. Unfortunately, mRNA concentrations of these genes do not always remain constant in the change from benign to metastatic growth (25). Another assay (23) includes no internal control and relies on the use of a high-performance thermal cycler. A more recent assay (24) is based on the technique originally described by Wang et al. (26) and includes a synthetic MDR1 RNA as an internal standard. It requires serial dilutions of the cDNA transcribed cRNA and sample mRNA, and uses 32P end-labeled primers for the PCR.

The PCR assay described herein quantitatively measures MDR1 mRNA with a synthetic “mimic” MDR1 cRNA added in serial molar dilutions to equal amounts of total RNA extracted from biological specimens. Following amplification, the amount of competitor cRNA yielding equal molar amounts of products gives the initial amount of target mRNA. The synthetic MDR1 RNA we created contains the same PCR primer sites as the cellular message, but gives after amplification a different-sized PCR product than the cellular MDR1 mRNA. The products are then resolved on an ethidium bromide–agarose gel and the data quantified by computer imaging of the gel.

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We have used this new quantitative PCR assay to measure MDR1 mRNA concentrations in eight patients with breast cancer as well as in cancer cell lines and normal human liver tissue. Results suggest that this nonradioactive assay will be useful for studying low levels of MDR1 expression in tumors and normal tissues of different origins.

**Materials and Methods**

**Cell Lines**

Eight transformed cell lines (KHOS-240, MCF-7, T-47D, 2R-75-1, A-431, HT 1080, HeLa, and Raji) were obtained from the American Type Culture Collection. The SW 613-S cell line derived from a colon carcinoma (27) was a gift from O. Brison, and the KB 3-1 and KB-A1 cells lines were provided by A. Pierré (Servier Lab., Courbevoie, France) (28, 29) with M.M. Gottesman’s authorization.

**Patients’ Samples**

The eight breast carcinomas were obtained by biopsy or tumorectomy from patients treated at “Institut Gustave-Roussy” (Villejuif, France). Four of the tumors had been treated by chemotherapy.

Human liver samples were obtained at the Paul Brousse Hospital (Villejuif, France), with the agreement of the local Ethics Committee, from adult kidney transplantation donors without known antecedents.

**RNA Preparation**

Total RNA from cell lines was extracted by using the guanidinium isothiocyanate procedure (30). Total cellular RNA from tumors was isolated by the guanidinium cesium chloride method (31).

**Construct of a Mimic MDR1 cRNA**

The following oligonucleotides (sense, +; antisense, −) were used as primers for cDNA synthesis and PCR amplification of two specific MDR1 fragments from total RNA extracted from a colon carcinoma cell line (SW 613-S).

- **A Sal 811** (−) 5′ CTAGTGAGCTTTGGCTCTGCCAATGCAATCAGGCTTATT 3′ HindIII
- **B Hind 320** (+) 5′ TACAAAGCTCAGGAAGATGACAGTGATGCTTATT 3′ BamHI
- **E Bam 586** (−) 5′ TCCGATTCTCTAGGCGAATTTTTCCTGGTTTATTAG 3′
- **F Bam 506** (−) 5′ CCGGATCCAGCTGAGCAGTCACAAAACGCTATCT 3′

The numbers used in the designation of the primers correspond to the 5′-end nucleotide of MDR1-specific sequence (underlined), taking +1 for the A of the initiation codon of human MDR1 cDNA (5). The recognition sequence of Sal I, HindIII, or BamHI restriction enzyme has been added at the 5′ end.

Two fragments, AE, and BF, were amplified from MDR1 cDNA: The 244-bp AE fragment was amplified from A Sal 811 (−) and E Bam 586 (−) oligonucleotides, and the 202-bp BF fragment was amplified from B Hind 320 (+) and F Bam 506 (−) oligonucleotides, as shown in Fig. 1. The PCR products were purified on DEAE cellulose and subcloned into a Sal I/HindIII-digested pBS+ vector (Stratagene, La Jolla, CA). An BamHI site was created within the MDR1 insert.

Mimic MDR1 cRNA was synthesized from EcoRI-linearized pBS-MDR1 recombinant by T3 RNA polymerase according to the manufacturer’s recommended reaction conditions (Pharmacia, Uppsala, Sweden). Template DNA was degraded with DNase and the synthetic MDR1 cRNA was extracted by Trizol reagent (Gibco-BRL, Life Technologies, Gaithersburg, MD) followed by ethanol precipitation and spectrofluorometric quantification at 260 nm. In repeated experiments, we obtained 18 μg of the synthetic 494-nucleotide cRNA from 10 μg of recombinant pBS-MDR1 plasmid vector.

**Competitive RT-PCR**

**cDNA preparation.** Total cellular RNA (1 μg) in combination with 1:5 serial dilutions of mimic MDR1 cRNA from 610 to 0.19 amol was reverse-transcribed according to the following method: RNAs and oligonucleotide MDR1 C 695 (−) 5′ CAGACAGCGCTGACAGTCCGAGCAGGACT 3′ in a volume of 42 μL of water were heated at 65°C for 15 min, followed by a 4°C quick chill. dNTP (1 mmol/L each; Pharmacia), 60 U of RNasin (Promega, Coger, France), and 60 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) were added to 1 × PCR buffer (10 mmol/L Tris-HCl, pH 8.3; 50 mmol/L KCl; 1.5 mmol/L MgCl₂) in a final volume of 50 μL. The reaction mixture was incubated at 42°C for 30 min and kept on ice.
MDR1 amplification. One-fifth (10 μL) of the serial cDNAs (target + mimic) was then amplified for MDR1 in a prechilled 50-μL reaction mixture having a final concentration of 200 μmol/L for each dNTP, 25 pmol of each MDR1 amplification primer [MDR1 C 695(−) and MDR1 D 410(+) 5′ GCCCTGGCAGCTGGAAAGACAAAT-ACACAAAAAT], 0.5 U of Taq polymerase (Appligene, Illkirch, France), and 2.5 μL of deionized formamide in 1 × PCR buffer. The PCR was performed in a Crocodile II thermocycler (Appligene) with the following temperature settings: initial denaturation at 94°C for 4 min followed by 30 cycles at 92°C for 1.5 min, 60°C for 1.75 min, and 72°C for 2.5 min, followed by a final extension at 92°C for 1.5 min, 60°C for 1.75 min, and 72°C for 15 min. One-fifth (10 μL) of each PCR product was then size-fractionated through a 2% agarose gel. The amplified cellular fragment (target) was 286 bp and the mimic was 211 bp. A 79-bp internal deletion within the MDR1 sequence and a 4-bp insertion (the engineered BamHI site) accounted for the 75-bp difference between the mimic and the cellular PCR amplification products.

Image analysis. We measured the intensity of the two bands corresponding to cellular (target) and competitor (mimic) amplification products with The Imager system from Appligene, consisting of a charge-coupled device (CCD) video camera connected to a Macintosh computer. Image processing and analysis were performed with the NIH Image 1.43 program. Fluorescence was recorded and the areas of the peaks were measured. The ratio of the intensities of the two bands, with a correction factor of 0.74 accounting for the difference in size of mimic/target, provided the basis of quantification.

Results

Exponential PCR Amplification of MDR1 mRNA

To determine the exponential range of PCR amplification for MDR1 mRNA and synthetic mimic RNA, we coreverse-transcribed 1 μg of total cellular RNA isolated from the human colon carcinoma cell line SW 613-S and 24.4 amol of mimic RNA into the first-strand cDNAs. The cDNA products were then amplified for various numbers of cycles of PCR. PCR products were size-fractionated through a 2% agarose gel, stained with ethidium bromide, and quantified.

Figure 2 shows the results obtained from the exponential range experiment. Both the synthetic and the cellular MDR1 cDNAs were amplified in an exponential fashion from 26 to 35 cycles. The relative amounts of the two amplified products stayed identical all along the PCR amplification, even after 35 cycles, when the plateau phase was reached.

Quantification of MDR1 mRNA in Human Cancer Cell Lines and in Normal Human Liver

MDR1-specific cDNA sequences synthesized from oligonucleotide C were amplified with a pair of amplimers (D,C) derived from exons 6 and 7 of the MDR1 gene, respectively (32), to prevent amplification of DNA sequences that may be present in RNA preparations. The MDR1-specific amplimers were selected so that they would not amplify the closely related MDR2 cDNA sequence. We screen biological samples by using 30 cycles of PCR, i.e., during the exponential phase of amplification. We probed a constant amount of cellular RNA (1 μg) with decreasing amounts of synthetic MDR1 cRNA (from 610 to 0.19 amol). Two examples of gel resolution are shown in Fig. 3. The 0.19 amol mimic-generated band was visible only when the MDR1 mRNA concentration of the target cellular RNA was inferior to that value. The data were quantified by computer imaging of the gel as described in Materials and Methods. The areas of the peaks were measured and the target/mimic ratios were plotted on a logarithmic scale against the mimic amounts in each reaction mixture of PCR amplification. The amount of MDR1 mRNA in the sample was then calculated by extrapolating from the intersection of the curve, where the amounts of target and competitor are equal (ratio = 1). When necessary, after a first assay in the conditions described above, intermediary mimic dilutions were added within the fivefold serial dilutions. Fig. 4 gives the curves obtained with total RNA extracted from SW 613-S cells (panel A) and from the HL1 (Table 1) human liver sample (panel B).

Results obtained by analyzing 7 normal human liver samples and 11 human tumor cell lines are given in Table 1. MDR1 gene expression was detected in all
Fig. 3. Competitive RT-PCR experiments showing fivefold serial dilutions of the MDR1 mimic added to RT-PCR reactions containing constant amounts of total cellular RNA (1 μg); (A) colon carcinoma cell line SW 613-S; (B) normal human liver (HL1 in Table 1). The amount of target RNA can be determined by noting how much mimic is required to produce equal molar quantities of PCR products. After 30 cycles of amplification, one-fifth of the products were resolved on a 2% agarose gel and stained with ethidium bromide. Lanes 4–9: 610, 122, 24, 4.9, 0.98, and 0.19 amol of MDR1 mimic, respectively; lane 1: 1-ng size-marker VI (Boehringer Mannheim, Mannheim, Germany); lane 2: mock reaction; lane 3: pure mimic RNA (610 amol); lane 10: pure cellular RNA (1 μg). The positions of the 286 and 211-bp fragments are indicated.

liver samples, although this expression was relatively low (0.3–3 amol/μg of total RNA, i.e., 1.8–18 molecules/10 pg of total RNA). As expected, the KB-A1 cell line expressed a high concentration of MDR1 mRNA. This cell line was derived from KB-3-1 epidermoid carcinoma cells selected in 2 μmol/L adriamycin (28, 29). In addition, detectable amounts of MDR1 mRNA were expressed in the osteosarcoma KHOS-240 and colon carcinoma SW 613-S cell lines.

Assay Evaluation

**Linearity.** In the experiments reported above, a constant amount of cellular RNA (1 μg) was probed with decreasing amounts of synthetic MDR1 cRNA in the presence of specific primers designed for RT-PCR. The following assay was performed to evaluate the linearity between the amount of initial cellular MDR1 RNA and the PCR product obtained after 30 cycles of amplification. We probed a constant amount of MDR1 competitor (24.4 amol) with increasing amounts of total RNA from colon carcinoma cell line SW 613-S (from 0.7 to 2.5 μg, i.e., 24.5–87.5 amol of MDR1 mRNA) and processed this as in the previous experiments. We observed a linear response (r = 0.98, Fig. 5). The wide range of linearity indicates that the assay can be used reliably with samples having a wide range of MDR1 mRNA concentrations.

**Accuracy.** To test the accuracy of this method, we mixed decreasing amounts of normal human liver RNA HL1 (9–1 μg, i.e., 18–2 amol of MDR1 mRNA) with increasing amounts of human colon carcinoma cell line SW 613-S-extracted RNA (1–9 μg, i.e., 35–315 amol of MDR1 RNA) to obtain a final concentration of 10 μg of RNA in 10 μL of water. Competitive RT-PCR was applied to 1:10 dilutions of the different mixes. The experimental values plotted against the expected ones (Fig. 6) showed the ability of competitive RT-PCR to

Fig. 4. Quantitative analysis of MDR1 gene expression in (A) human colon carcinoma cell line SW 613-S and (B) normal human liver tissue (HL1 in Table 1). Within the fivefold mimic serial dilutions, additional dilutions have been made (14.8 and 73.2 amol for SW 613-S, 1.96 and 3.9 amol for HL1). The gels were analyzed by computer imaging and the ratio of target to mimic was calculated. The data are plotted on a logarithmic scale vs the mimic amounts in the reaction tubes.
Table 1. Analysis of MDR1 mRNA expression in human cancer cell lines and normal human liver.

<table>
<thead>
<tr>
<th>MDR1*</th>
<th>Normal human liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL1</td>
<td>2</td>
</tr>
<tr>
<td>HL2</td>
<td>0.80</td>
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<tr>
<td>HL3</td>
<td>1.90</td>
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<td>2</td>
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<tr>
<td>HL5</td>
<td>3</td>
</tr>
<tr>
<td>HL6</td>
<td>0.30</td>
</tr>
<tr>
<td>HL8</td>
<td>0.85</td>
</tr>
</tbody>
</table>

* Amol/µg of total RNA (1 amol/µg corresponds to 6 molecules/10 pg of total RNA).

* Selected in adriamycin (2 µmol/L) from the drug-sensitive parental cell line KB-3-1.

discriminate between different levels of MDR1 gene expression.

We determined the sensitivity of the assay by diluting liver sample RNA HL1 (Table 1) in total RNA extracted from a HeLa cell line. In repeated experiments, as little as 0.08 amol of MDR1 mRNA could be detected per microgram of total RNA, i.e., 0.5 molecule/10 pg of total RNA.

Reproducibility. We calculated the CV of competitive RT-PCR by six replicate analyses of a sample (total RNA from colon carcinoma cell line SW 613-S, 35 amol/µg of total RNA). The values varied from 30 to 39.1 amol (30, 33.1, 34, 35, 38.8, 39.1) with a mean of 35 amol and a CV of 10%. These variations take into account variations associated with cDNA synthesis, PCR amplification, image scanning, and processing steps, since this assay was designed to process simultaneously target and mimic RNAs.

Quantification of MDR1 mRNA in Breast Carcinomas

Eight tumors were measured for MDR1 mRNA expression by quantitative RT-PCR. Quantitative PCR analysis of MDR1 mRNA was performed exactly as described for colon carcinoma cell line SW 613-S or human liver RNA. MDR1 expression in the tested samples is compiled in Table 2. In three of the four breast carcinomas treated by chemotherapy, MDR1 mRNA was expressed at concentrations much higher than in the nontreated tumors.

Table 2. Analysis of MDR1 mRNA expression in human breast carcinomas.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Chemotherapy</th>
<th>MDR1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
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</tr>
<tr>
<td>3</td>
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<td>4</td>
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<tr>
<td>6</td>
<td>+</td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>10.5</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>24.5</td>
</tr>
</tbody>
</table>

* Units as in Table 1.
Discussion

Using the approach originally described by Siebert and Larrick (20), we have developed a new assay for the quantification of MDR1 mRNA expression by PCR. The synthetic MDR1 RNA designed as an internal standard for this assay controls for the efficiency of both the reverse transcription reaction and the PCR. This synthetic RNA has the same primer sequences as the target mRNA, so that there are no differences in primer efficiencies. Further, the difference in size between mimic and target (75 nucleotides, taking into account the four additional nucleotides introduced with the BamHI cloning site) allows separation of the corresponding amplification products in an agarose gel, easily handled in clinical laboratories. The results of PCR-based measurements of MDR1 gene expression in various tumor-derived cell lines and normal tissues show a good correlation with studies carried out by traditional hybridization techniques (7–9). However, the PCR-based assay requires less tissue and ensures more reliability in detection of MDR1 gene expression in samples with a low level of drug resistance.

MDR1 expression in tumor cells is often heterogeneous and may lead to a very low signal when total RNA extracted from the tumor is analyzed. As a small increase in the levels of drug resistance may be sufficient for clinically refractory tumors, the need for high sensitivity in the analysis of such samples is obvious. The theoretical detection limit of our assay is more than sufficient to detect MDR1 expression in normal biological samples. In addition, false positives due to cross-reactivity with the closely related MDR2 gene are avoided by selecting MDR1-specific primer sequences.

Quantitative RT-PCR assays with radioactive detection have recently been reported in the field of endocrinology (33–35). The precision of the present nonradioactive assay is enhanced by the accurate measurement of the intensity of the bands corresponding to the PCR-amplified products, by using image analysis of fluorescence of ethidium bromide-stained DNA with a video camera and specific computer software. One can obtain a dose-dependent linear response by using a constant amount of cellular RNA and decreasing concentrations of competitor, and vice versa (fixed competitor and increasing cellular RNA). These results demonstrate that the two cDNA species are equivalent in relative efficiency of amplification.

We also evaluated the proposed method for accuracy, precision, and sensitivity. We evaluated the accuracy of our method for MDR1 gene expression by supplementing normal liver RNA in RNA extracted from a colon carcinoma cell line expressing MDR1. The results indicate that the present method of competitive RT-PCR precisely determines MDR1 gene expression over a wide range. The experiment in which we evaluated reproducibility, by assaying six replicates in the same batch analysis, established the good reproducibility of competitive RT-PCR as shown by the repeatability of the cellular/competitor ratio.

We have applied this method to the quantification of MDR1 gene expression in human breast cancer. Comparison of MDR1 mRNA amounts in samples from untreated patients showed an overexpression of MDR1 gene in the tumor of patients treated by chemotherapy, indicating that this assay may help define normal and drug-resistant levels of MDR1 expression in vivo.

In conclusion, competitive RT-PCR is a simple, reliable, accurate, and nonradioactive method for evaluating MDR1 gene expression in any clinical laboratory set up for molecular biology.

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