Cloning of adriamycin-resistant related (*arr*) gene in an adriamycin-resistant L1210 variant

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Abbreviations: *arr*, adriamycin resistant related; MDR, multidrug resistance; L1210AdR, adriamycin-resistant L1210 variant; DD-PCR, differential display polymerase chain reaction; RACE, rapid amplification of cDNA end; P-gp, permeability-glycoprotein

Abstract

A partial fragment of novel sequence (*arr*, adriamycin-resistant related) was previously identified using the differential display (DD)-PCR technique with adriamycin-resistant L1210 variant (L1210AdR), which shows a typical multidrug resistant (MDR) phenotypes. The present research shows the isolation of full length *arr* cDNA sequence. To clone the full length cDNA of *arr* gene, DD-PCR fragments were subjected to 5'- and 3'-Rapid Amplification of cDNA End (RACE) method. The cloned *arr* cDNA consisted of 770 bases and contained an open reading frame of 153 bases, encoding a protein of 51 amino acid with the molecular mass of 4 kDa by *in vitro* translation reactions. Northern blot analysis showed that a 770 bases transcript *arr* gene was overexpressed in adriamycin-resistant L1210 variant, but not in the parent suggesting that the *arr* gene may be involved in the adriamycin-resistant phenotypes.

Keywords: MDR, *arr*, DD-PCR, RACE

Introduction

The appearance of drug resistant cells is one of the major limiting factor in cancer chemotherapy. To overcome the drug-resistance, understanding of the mechanisms to obtain the resistance is necessary. Especially, some drug resistant cells selected for one drug *in vitro* show the cross resistance to other antitumor drugs different in their structures and mechanisms of action. The broad spectrum resistance to cancer chemotherapy has been

called multidrug resistance (MDR) (Kartner and Ling, 1989). Several researchers have investigated the mechanisms to drug resistance in cancer cells, including overexpression and amplification of Permeability-glycoprotein (P-gp) encoded by the *mdr1* gene (Gros *et al.*, 1986). P-gp is a transmembrane glycoprotein which acts as an ATP-dependent drug efflux pump and reduces the intracellular drug accumulation, thereby causing resistance to several antitumor drugs including adriamycin and vincristine (Gerlach *et al.*, 1986). In addition to P-gp, various mechanisms related to antitumor drug resistance have been extensively studied such as expression of multidrug resistant-associated protein (MRP) (Cole *et al.*, 1992), overproduction of detoxification factors such as glutathione, or its related enzyme, glutathione S-transferase (Schechter *et al.*, 1992). The decreased expression of a DNA topoisomerase II (Wolverton *et al.*, 1989) or increase of DNA topoisomerase I (Kotoh *et al.*, 1994) has been reported in antitumor drug resistant cells. The expression of sorcin (V19), a 22 kDa calcium binding protein has been shown to increase in some multidrug-resistant cell lines (Meyers *et al.*, 1987). These findings suggest that some proteins or genetic alterations are important in the development of antitumor drug resistance in cancer cells.

In the previous studies, we isolated and characterized antitumor drug resistant variants from mouse lymphoblastic leukemia L1210 cells; adriamycin-resistant (L1210AdR), vincristine-resistant (L1210VcR), and cisplatin-resistant (L1210Cis) variants (Kim *et al.*, 1991; Kim and Kim, 1994; Kim *et al.*, 1995; Kim *et al.*, 1996). Several differentially expressed fragments were identified between adriamycin-resistant L1210AdR and parent L1210 cells using the differential display polymerase chain reaction (DD-PCR) technique (Kim *et al.*, 1995). Northern blot analysis with DD-PCR fragment (190 bp) as a probe showed that a 0.7 kb transcript was identified to be overexpressed 10 times higher in L1210AdR cells than in the parent cells, leading us to designate this fragment as *arr* (adriamycin-resistant related) sequence (Kim *et al.*, 1995). There was no similar sequence to a partial sequence of *arr* gene through a homology search in GenBank DNA sequence database. In this paper, we report the cloning of the full length cDNA of *arr* gene.

Materials and Methods

Cell culture

Adriamycin-resistant mouse lymphoblastic leukemia L1210 variant was isolated as described previously

(Kim *et al.*, 1991; Kim and Kim, 1994; Kim *et al.*, 1995; Kim *et al.*, 1996). L1210 and the drug-resistant variants were cultured in Fisher's medium containing 10% heat-inactivated bovine serum (Hyclone) and 50 mg/ml of gentamycin. Adriamycin (10^{-5} M) was added to the medium to maintain the drug resistance phenotypes.

Total RNA and mRNA preparation

Total RNAs from the cells were isolated by acid guanidium thiocyanate/phenol/chloroform extraction methods (Chomczynski and Sacchi, 1987). The mRNAs were purified using a Poly(A) Tract mRNA purification kit (Promega) or oligo-dT cellulose column chromatography (Sambrook *et al.*, 1989).

Cloning of the *arr* gene by the Rapid Amplification of cDNA Ends (RACE) method.

The differentially expressed cDNA fragments were isolated from L1210AdR cells using DD-PCR as described previously (Kim *et al.*, 1995). To clone the full sequence, RACE was carried out using a Marathon cDNA amplification kit (Clontech) according to instructions supplied from the manufacturer with minor modifications. Based on the sequence of the partial DD-PCR fragment, the primers were designed as follows: *arrR* (5'-CAGAGGCA TAACTCATCAG-3') for 5'-RACE, *arrF* (5'-GTGTGCAA TGGTGGTTATAG-3') for 3'-RACE. First and second strands of cDNA were synthesized from the RNA extracted from the L1210AdR cells with random primers. The Marathon cDNA amplification kit contained primers for AP1 (5'-CCATCCTAATACGACTCACTATAGGGC-3'), AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3'), cDNA synthesis primers (5'-TTCTAGAATTCAGCGGCCGC(T)₃₀NN), and cDNA adaptor (5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'). The PCR was carried out in a mixture containing 4 ml adaptor-ligated cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.25 mM dNTP, 1 mM AP1 primer (for the adapter), 1 mM *arrF* primer (for the 3'-RACE) or *arrR* primer (for the 5'-RACE), and 2 U Taq DNA polymerase. The PCR cycles were initial denaturation for 1 min at 94°C; 30 cycles of 10 sec at 94°C, 15 sec at 60°C, and 30 sec at 72°C; and final extension for 5 min at 72°C. To identify the amplified DNA fragments, DNAs in the gel were transferred to a nylon membrane and hybridized with a ³²P-labeled *arrR* primer for detection of 5'-RACE fragments and with a ³²P-labeled *arrF* primer for detection of 3'-RACE fragments. Amplified DNA fragments were isolated from gel slices using a Qiagen gel extraction kit and subcloned into a pGEM-T vector (Promega), and sequenced by the dideoxychain-termination method using a Sequenase v2.0 sequencing kit (USB). To select colonies containing *arr*-specific DNA fragments amplified

by RACE, PCR was carried out using a forward primer (5'-GTTTTCCCAGTCACGACGTTGT-3'), a reverse primer (5'-TGGAATTGTGAGCGGATAAC-3') with vector and an *arrR* or *arrF* primer for 5'- or 3'-RACE, respectively.

In vitro transcription and translation of cloned *arr* sequence.

To determine the protein translated from the *arr*, *in vitro* transcription and translation were performed using pARR-R plasmid DNA as a template. The *arrR*1 fragments containing SP6 and T7 promoter were amplified from pARR-R1 plasmids. The amplified DNA fragments were separated on an agarose gel and purified using a Qiagen gel extraction kit. RNA from the amplified DNA fragments was transcribed using a Riboprobe *in vitro* transcription system (Promega) and the size and integrity of transcribed RNA was examined by an agarose gel electrophoresis. *In vitro* translation reaction to the RNA transcribed from *arrR* was performed in volumes of 20 ml of 1 mg RNA, 10 ml wheat-germ extract, 100 mM potassium acetate, 10 mCi ³⁵S-methionine, and 80 mM amino acid mixture without methionine at 25°C for 2 h. *In vitro* translated proteins were separated on a 15% SDS-polyacrylamide gel. The gel was fixed in 50% methanol/10% acetic acid, incubated in 1 M sodium salicylate (pH 6.0) for 30 min, and exposed to an X-ray film.

Northern and Southern blot analysis.

For Northern blot analysis, total RNA was isolated from indicated cells as described above, and equal amount of RNA (15 µg) was separated on 1.5% agarose gel containing 6.7% formaldehyde and MOPS buffer (20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0), and transferred to a Hybond N⁺ membrane (Amersham). The membrane was hybridized with [α -³²P]dCTP-labeled full size of *arr* at a 42°C in a solution containing 0.25 M NaCl, 1% SDS, 50% formamide, 2× Denhardt's solution, 100 mg/ml salmon sperm DNA, and 5% dextran sulfate. Blots were washed under high stringency (2×SSC/0.1% SDS at room temperature for 20 min followed by 0.5×SSC/0.1% SDS at 50°C 2 times for 30 min). For Southern blot analysis, genomic DNA was isolated from indicated cells and digested with *EcoRI*. The DNA digests were electrophoresed and transferred to a Hybond N⁺ membrane. Hybridization was carried out with the same conditions as used in Northern blotting.

Results and Discussion

Previously, adriamycin-resistant variant (L1210AdR) was selected and characterized from mouse lymphoblastic leukemia L1210 cells (Kim *et al.*, 1991; Kim and Kim, 1994; Kim *et al.*, 1995; Kim *et al.*, 1996). Using a DD-

PCR technique, a partial 190-bp PCR fragment, named *arr* (adriamycin resistant related) sequence, was isolated from L1210AdR (Kim *et al.*, 1995). Northern blot analysis revealed that 0.8 kb transcript could be observed in the total RNA fractions from L1210AdR cells, however, not detected in the poly(A)-RNA fractions using a probe with original 190-bp PCR fragment (data not shown). These findings imply that the transcript for *arr* gene does not contain the poly(A) tail. Thus, we tried to isolate the full-length sequence of *arr* gene on cDNA library synthesized

from L1210AdR-derived total RNA using the RACE method.

ArrR primers were synthesized on the basis of the original DD-PCR fragments. The cDNA was synthesized from L1210AdR-derived total RNA using oligo(dT) or random primers. 5'-RACE was performed and then amplified by a nested PCR with AP2 and *ArrR* primers. An each aliquots of PCR reaction was used as the template for the second amplification. Only the second step of amplification allowed the detection of fragments, showing 770 and 740 base pairs in length, by ethidium bromide staining after gel electrophoresis (Figure 1). Amplified DNA fragments were isolated from gel slices using a Qiagen gel extraction kit, and subcloned into a pGEM-T vector. Plasmids containing DNA inserts of the expected size were selected for sequencing analysis.

The complete *arr* sequence was obtained by matching the overlapping sequences between original DD-PCR fragments and each PCR product (Figure 2). Sequence analysis of the cloned DNA fragments demonstrated that 770 bp fragment contained the open reading frame of 156 base pairs encoding a polypeptide of 51 amino acids. The nucleotide sequence around the proposed initiation codon (ATG) is in agreement with the Kozak consensus sequence (Kozak, 1984). The deduced amino acid sequence of *arr* gene was not identical with any reported sequences in the data base and shared weak homology of less than 40%.

The *arr* gene was observed at about 770 bp in the total RNA fractions from L1210AdR cells, however, it was not detected in the poly(A)-RNA fractions using a probe with

Figure 1. Identification of DNA fragments by 5'-RACE. PCR products were purified and amplified on a cDNA library, prepared from L1210AdR-derived total RNA with random primers (lane 1) or oligo-dT primers (lane 2). A nested PCR was done with AP2 and *ArrR* primers. M indicates the 123 bp DNA ladder.

Figure 2. Nucleotide sequence and deduced amino acid sequence of *arr*. The nucleotide sequence data has been deposited in a GenBank with the accession number AF039839. The possible open reading frame is boxed, and the position of the stop codon is indicated by ***. The oligonucleotide sequences used in the primer for 5'-RACE (*arrR*) or 3'-RACE (*arrF*) is indicated by solid arrow or dashed arrow, respectively. The original 190 bp of DD-PCR fragment is underlined.

original DD-PCR fragment or cloned *arrR* (data not shown). In addition, a consensus polyadenylation signal (AAUAA) in *arr* sequence was not found. When the 3'-RACE from the 3'-end was performed it was found that the 204 and 860 bp long fragments were yielded (data not shown). However, these fragments failed to detect 0.8 kb transcript in L1210AdR cells by Northern blot analysis (data not shown). From these findings, it is suggested that *arr* transcript does not contain the poly(A) tail.

To define the protein translated from the *arr* sequence, *in vitro* transcription and translation were analyzed using pARR-R1 plasmid DNA containing T7 and SP6 promoter as a template. The *arrR* DNA was amplified from pARR-R1 plasmid with forward and reverse primers, and transcribed with T7 or SP6 RNA polymerase by using a Riboprobe *in vitro* transcription system. As shown in Figure 3A, antisense T7 and sense SP6 transcripts were observed as the same size. However, only the sense transcript with SP6 RNA polymerase was translated to a polypeptide with a molecular mass of 4 kDa in wheat germ extract (Figure 3B).

To determine the expression of the *arr* gene in the various drug resistant cells, Northern blot analysis was done. Equal amounts of total RNA isolated from L1210AdR variants (Kim *et al.*, 1991; Kim and Kim, 1994; Kim *et al.*, 1995; Kim *et al.*, 1996) was separated, blotted and

hybridized with ^{32} P-labeled *arrR*. As shown in Figure 4A, The *arr* gene was detected as a single RNA species (770 bp) and highly expressed in L1210AdR, but not in the parent L1210 cells. It was further confirmed by genomic Southern blot analysis. The Southern blotting pattern showed three distinct bands (0.7, 2.1 and 2.9 kb) were amplified in *Eco*RI fragments of L1210AdR as compared to those of parent L1210 cells (Figure 4B). These results suggested that the expression of *arr* gene appears to be closely associated with adriamycin and vincristine mediated drug resistance, however, it is unlikely to represent rearranged DNA amplification.

Permeability-glycoprotein (P-gp) encoded by the *mdr1* gene that functions as an ATP-dependent drug-efflux pump has been shown to confer resistance to several structurally unrelated drugs, including adriamycin, mitomycin, *Vinca* alkaloids, and other natural drugs (Fojo *et al.*, 1985; Shen *et al.*, 1986; Bradley *et al.*, 1988). Amplification of the *mdr1* gene has been observed in some solid tumors such as colorectal and renal-cell carcinomas (Fojo *et al.*, 1987; Goldstein *et al.*, 1989). In addition, the *mdr* gene was highly expressed in L1210VcR and L1210AdR cells, but not detected in the parent L1210 and L1210Cis cells (Kim *et al.*, 1991; Kim and Kim, 1994; Kim *et al.*, 1995; Kim *et al.*, 1996). Both L1210VcR and L1210AdR variants have exhibited typical multidrug resistant phenotypes; the cross resistance to plant alkaloids such as vinblastine and vincristine, etoposide, methotrexate, dactinomycin, daunorubicin and epirubicin (Kim *et al.*, 1991; Kim and Kim, 1994; Kim *et al.*, 1995; Kim *et al.*, 1996).

At present, it remains unclear whether the increased expression of *arr* gene is important in the development

Figure 3. *In vitro* transcription (A) and *in vitro* translation (B) of *arrR* sequence. A. The *arrR* DNA containing T7 and SP6 promoter was amplified from pARR-R1 with FP and RP primers and described with T7 or SP6 RNA polymerase using Riboprobe *in vitro* transcription system (Promega corp.). M, 123 ladder; 1, T7 transcripts of *arrR* containing antisense RNA sequence; 2, SP6 transcripts of *arrR* containing sense RNA sequence. B. The transcribed RNAs were translated with wheat germ extract and ^{35} S-methionine. The translated products were separated in 15% SDS-polyacrylamide gel. The gel was fixed, dried, and exposed to a X-ray film for 4 days. lane 1; The translated products from the T7 transcript, lane 1; The translated products from the SP6 transcript

Figure 4. Northern and Southern blot analyses. Fifteen mg of total RNA (A) or five mg of *Eco*RI-digested genomic DNA (B) isolated from indicated cells was separated and blotted onto the Hybond N⁺ membrane. Hybridization was carried out with the full-size of ^{32} P-labeled *arr*. L1210; parent L1210 cells, L1210AdR; adriamycin-resistant L1210 variant.

of antitumor drug resistance. However, it is probable that increased *arr* expression may be related to adriamycin-resistant phenotypes. Regarding the absence of *arr* transcript in the mRNA fraction, additional studies will be necessary to determine the significance of the expression of *arr* gene for the development of multidrug resistance.

In summary, we isolated a novel sequence, named as *arr*, in adriamycin-resistant L1210 variant by using DD-PCR and 5'-RACE methods. *arr* gene consists of 770 bases containing an open reading frame of 153 bases, which encodes a protein of 51 amino acid with the molecular mass of 4 kDa by *in vitro* translation reactions. The expression of *arr* is enhanced in adriamycin-resistant L1210 variant.

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