

Establishment of a Doxorubicin-Resistant Subline from Acute Myeloid Leukemia Cell Line

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Abstract

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Introduction: In order to determine the multidrug resistance (MDR) phenotype due to P-glycoprotein expression in haematological malignancies including acute myeloblastic leukemia (AML), a well-characterized P-gp expressing cell line was required to validate and standardize flow cytometric assays and to calibrate instruments. Therefore, this resistant subline of K562 was established for the first time in Iran in order to study the MDR phenotype due to P-gp expression in some cancers.

Material and Methods: A resistant subline of K562 (KDI/20) to Doxorubicin from the same parental K562 was derived by stepwise increasing the concentration of Doxorubicin up to 20 ng/ml as a gold standard. For flow cytometric assessment of P-gp expression, 4E3 anti-P-gp was used. The resistant cell line was studied by rhodamine 123 for functional assay of P-gp. MDR1 gene expression was also confirmed using RT-PCR.

Results: P-glycoprotein was expressed in final concentration of 20 ng/ml of Doxorubicin on 70% of K562 cells after 120 passages. The Rhodamine 123 influx was 37%. The over-expression of MDR1 gene was observed in a 30-cycle PCR.

Conclusion: P-glycoprotein is expressed in human K562 cell line (K562) by continuous exposure to anticancer drug. P-glycoprotein expression is detected by several methods including flow cytometry and RT-PCR, and the number of PCR cycles is very important.

Keywords: P-glycoprotein, MDR-phenotype, K562 cell line.

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Introduction

Intrinsic natural and acquired drug resistance is one of the most important clinical problems in management of several cancers. This drug resistance, so called as multi-drug resistance (MDR), causes patient's resistance to several kinds of anticancer drugs with different structures and functions. One type of drug resistance results from decreased drug aggregation in the target cells because of the presence of an energy-dependent pump in them. This

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pump is a P-glycoprotein (P-gp) with a molecular weight of 170 kDa, which is encoded by two genes located on chromosome 7 in human being; they are called MDR1 and MDR2 (1, 2, 3).

P-gp is normally found in adrenal, kidney, small intestine, colon, liver, pancreas (4), CD34 positive bone marrow cells, T lymphocytes, and natural killer cells (5-6). Over-expression of P-gp coding gene is usually seen in different organ cancers including adrenal, colon, kidney, breast,

ovary, and thyroid, and in leukemias and lymphoma. P-gp expression in acute myeloblastic leukemia is reported to be 20-40% at the time of diagnosis (7). Therefore, the establishment of a simple and valid method for detection of P-gp expression in malignant cells of clinical samples is of great importance (8).

Although most laboratories can detect high levels of P-gp and MDR using techniques such as Immunocytochemistry, flow cytometry and RT-PCR, they have problems in detection of P-gp and MDR low levels (9). It is absolutely important to use a method that enables the detection of one to ten percent P-gp positive cells among the cell population and it is obvious that flow cytometry can detect low to moderate levels of P-gp expression (10). In order to determine MDR1/P-gp in multiparameter studies, we needed a low resistance P-gp expressing cell line as a gold standard to determine the protein cut-off (11, 12, 13).

As such a cell line was not available, we established a resistant cell line using human myeloblastic leukemic cell and low concentrations of Doxorubicin, for the first time in Iran, to use it as a gold standard for determination of the cut-off P-gp in hematologic malignancies, and so to provide necessary arrangements for complementary studies of elimination of drug resistance using gene therapy.

Material and Methods

Cell line and culture circumstances

Human acute myeloblastic leukemic cell (K562) was purchase from German DSMZ company and was cultured in RPMI-1640 medium (Gibco, UK) with 10% fetal bovine serum, 100 unit/ml penicillin, 100 µg streptomycin, and 20 mM HEPES buffer (Sigma, USA), and they were incubated at 37 °C with 5% CO₂ gas.

KD30 cell was received as a gift for positive control and was cultured in the same situation. Induction of P-gp expression and providing multidrug resistance (MDR) was achieved by continuous exposure of K562 cell to increasing concentrations of Doxorubicin (starting from 5 ng/ml and increasing up to 20 ng/ml) with 3 day intervals for cellular passage. We called this resistant cell as KDI/20 (K562 Doxorubicin Iran/20ng/ml).

P-gp determination with flow cytometry

We used monoclonal antibody against P-gp external epitope (4E3 clone made by Dako, Denmark). 5×10⁵ cells in 100 µl RPMI, 2% PBS-BSA (Sigma, USA) and 1µg anti-P-gp antibody were incubated at room temperature for one hour. After rinsing with 2% PBS-BSA twice, it was incubated with rabbit antibody against F(ab)₂ fragment conjugated with FITC at room temperature and darkness. The cells were fixed after rinsing twice with 1% paraformaldehyde and preserved at 4 °C and darkness until analysis. Monoclonal IgG2a mouse antibody (Dako, Denmark) was used as isotype control. These tests were repeated for at least five times.

P-gp activity determination by Rhodamine 123 (Rh 123)

2×10⁶ cells were incubated with 200 µl Rh 123 (Sigma, USA) in 1 ml PBS in 37°C for one hour. They were then rinsed with cold PBS twice and were incubated in one ml PBS in 37 °C for one hour. Subsequently, the cells were rinsed with cold PBS twice and were studied by flow cytometry promptly. K562 cells were used without any treatment for elimination of autofluorescence. These tests were repeated for at least five times.

P-gp activity determination by Daunorubicin (DNR)

2 µg DNR (Sigma, USA) in each ml was added to 10⁶ cells in one ml culture medium and the cells were incubated at 37 °C for 30 minutes. Then they were rinsed by cold PBS and preserved in darkness for flow cytometric analysis. These cells can be staged for one week with no change in the results. Untreated cell was used to eliminate the autofluorescence. The tests were repeated for at least five times.

Flow cytometric analysis

P-gp and Rh123 determination in FL1 (Fluorescence 1) channel and DNR examination in FL2 channel were performed by coulter EPICS XL flow cytometry (made by USA).

RNA extraction and RT-PCR method

1×10⁶ cells were rinsed in PBS twice and total RNA was extracted by Trizol solution (Sigma, USA) according the manufacturer's instructions. cDNA was synthesized by a kit (Fermantas, USA) using 1 µg RNA. PCR

reaction was performed in a total volume of 25 μ l using buffer 1x, 1.5 mM magnesium chloride, 15 pmol each primer, 200 mM dNTP, 1.5 unit Taq DNA Polymerase, and 2 μ l cDNA in Hybaid thermal cycler (France). MDR1 and β -2-microglobulin genes (as internal controls) were amplified in a tube. Thermal cycles included 4 minutes at 96 $^{\circ}$ C, 30 cycles of 96 $^{\circ}$ C for 50 seconds, 62 $^{\circ}$ C for 50 seconds, and 72 $^{\circ}$ C for 1.5 minutes, and a final 2 minutes at 72 $^{\circ}$ C as the terminal elongation.

The PCR product was undergone electrophoresis on a 2% agarose gel containing 0.05% ethidium bromide. The MDR1 gene product is a 1025 bp fragment, while the β -2-microglobulin gene product is a 135 bp one. MDR1 primers were designed using Oligo software, and β -2-microglobulin primers were obtained from literature.

MDR1 gene primer sequences

Forward: 5'-TTG GGG CTT TTA GTG TTG GA-3'
 Reverse: 5'-CTT TCT GTC TTG GGC TTG TGA-3'
 β -2- Microglobulin gene primer sequences
 Forward: 5'-CTC GGG CTA CTC TCT CTT TCT-3'
 Reverse: 5'-TGT CGG ATT GAT GAA ACC CAG-3'

Results

K562 resistant cell (KDI/20) was obtained by increasing concentrations of Doxorubicin from 5 ng/ml to 20 ng/ml, after 120 consequent passages with 3 day intervals in one year. 70% \pm 3.4% of the cells expressed P-gp (Figure 1).

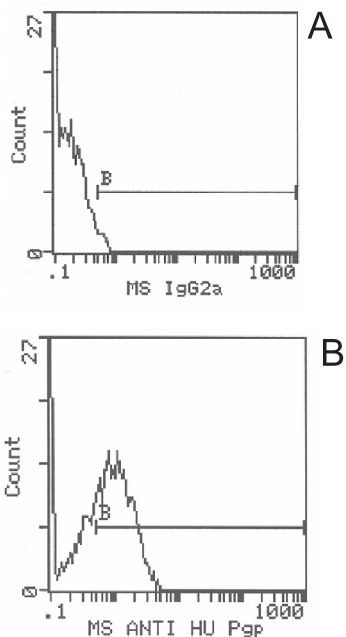


Fig. 1. (A) Isotype control; (B) Pgp positive cells (70%).

The results of P-gp activity determination by Rh123 after 120 cellular passages showed that 37% \pm 4.8% of the cells had Rhodamine influx and were positive for this stain. On the other hand, 76% of the cells were negative, in concordance with P-gp percent which was 70% positive. Resistant cells did not absorb Rhodamine stain and the cells showed P-gp activity, causing stain emission from the cells (Figure 2).

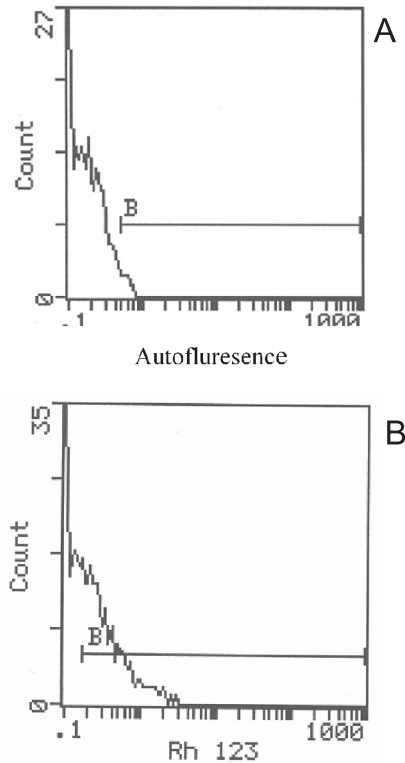


Fig. 2. (A) Cells without treatment; (B) Rh123 in Resistance Cells (37%).

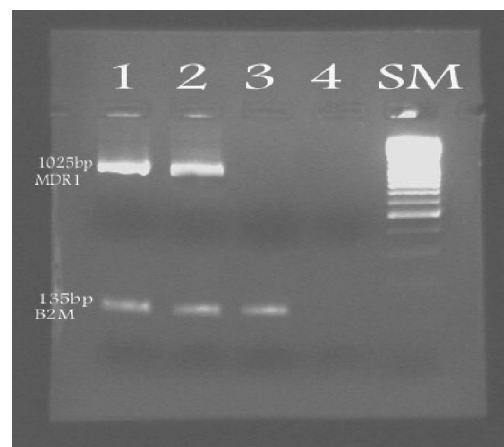


Fig. 3. (1) Positive Control; (2) KDI/20 cell; (3) Negative control; (4) No cDNA. SM; size marker.

Examination by RT-PCR molecular method showed the MDR1 gene over-expression compared to positive and negative control cells in a 30 cycle PCR (Figure 3).

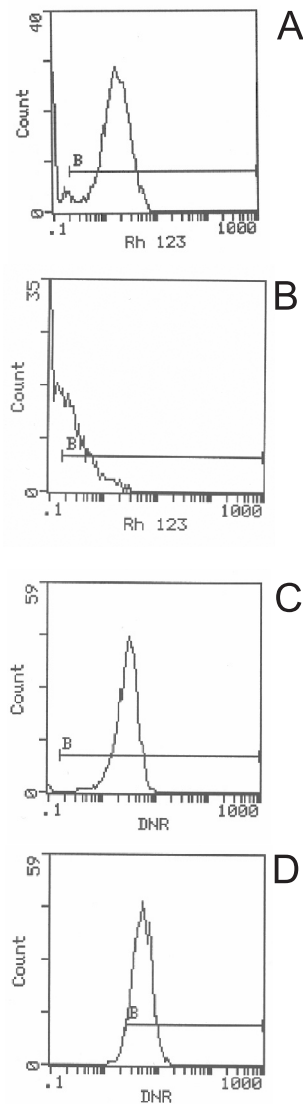


Fig. 4. (A) Rh 123 in parental K562 (98%); (B) Rh 123 in resistant cells (37%); (C) DNR in parental K562 (99%); (D) DNR in resistant cells (90%).

Discussion

Although the short-term and long-term outcomes in leukemic patients have been improved during the last decades because of combination chemotherapy, a considerable proportion of patients progress finally to leukemic relapse and die from complications of a decrease in cell number or a resistant disease. The 5 year survival in today lymphoblastic leukemic children is 75%, while it is 40%

in young adult patients with acute myeloblastic leukemia (14).

Treatment of the leukemic patients with relapse is difficult, as the leukemic clone is resistant to therapy and P-gp/MDR1 is a main cause of their cellular and eventually clinical drug resistance. Recently, MDR1 reversing medications including Quinidine, Cyclosporine A, Verapamil and antisense therapy have been used. Therefore, P-gp level detection by a sensitive method is necessary. Most laboratories are able to detect high levels of MDR1/P-gp, but they have some problems in detecting its low levels. As a result, we needed a P-gp expressing cell line as a gold standard for standardizing the methods and calibrating the instruments (10).

We established a cell line resistant to Doxorubicin. This process was not successful in passages of less than 50 and drug concentrations of less than 20ng/ml, in concordance with Slapak study who expressed P-gp in U937 cell line after 50 passages with a drug concentration of more than 5ng/ml (15). We obtained 44% cells positive for P-gp after 54 passage (data not shown here), which increased to 70% after 120 passage (Figure 1).

Another investigator successfully established a Doxorubicin resistant cell using 16 ng/ml of the drug (16). Furthermore, we approved P-gp over-expression by RT-PCR and the best results were obtained after a 30 cycles PCR, as in other studies (17, 18, 19, 20). Since several studies failed to show any direct relationship between P-gp presence and resistance to chemotherapy drugs, it is very important to perform P-gp activity determination in MDR examination (21, 22), a goal which can be achieved using Daunorubicin and Rh 123. Among these two fluorescent agents, we used Rh123 for this study, as required DNR dose is more than therapeutic doses and can result in changes in cell activity. On the other hand, DNR penetrates into the nucleus and its absorption may increase falsely. Furthermore, it can distinguish sensitive cells from poor resistant cells (23). However, Rh 123 does not have similar problems and enables distinguishing of sensitive from poor

resistant cells. In addition, there is a good relationship between P-gp positivity and Rh 123 (24). Our results approved this point, too (Figure 4).

Conclusion

Preliminary studies on resistant cell lines have shown that flow cytometry and RT-PCR are sensitive methods for detection of the cell surface P-gp and determination of MDR1 gene expression, respectively. However, the PCR cycle number is also important and Rh 123 is preferred over DNR for detection of drug resistance

phenotype.

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