# Proteomic Analysis on Multi-Drug Resistant Cells HL-60/DOX of Acute Myeloblastic Leukemia

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## Abstract

**Multi-drug resistance (MDR) is an important factor that causes treatment failure in acute leukemia. However, the full development mechanisms of MDR still awaits investigation. The purpose of this study is to investigate differentially expressed proteins in the multi-drug resistant acute myeloblastic leukemia (AML) cell line HL-60/DOX and the drug sensitive cell line HL-60, and to identify new potential multi-drug resistant related molecules with the proteomic approach. Two-dimensional gel electrophoresis (2-DE) maps of the proteins, extracted from two AML cell lines, HL-60/DOX and HL-60, were established respectively. The extracted proteins were digested by enzymes and identified with the matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The data of the peptide mass fingerprinting (PMF) was matched with databases of proteomics available on the Internet. Results showed that 16 proteins were identified to be differentially expressed between HL-60/DOX and HL-60 cells. They involved the protein disulfide isomerase precursor (PDI), the proteasomes α1 and other proteins which are related to drug resistance or cell metabolism, but their functional significances are required further investigation. Nevertheless, it is clear that this proteomic approach for studing the biology and development of MDR is a prerequisite in leukemia.**

**Key Words: leukemia , myeloblastic, multi-drug resistance, proteomics**

## Introduction

Resistance of tumors to chemotherapeutic agents is an important factor that limits the successful treatment of a wide range of malignancies (20). Although some multi-drug resistant related molecules have been found, such as the membrane transport-associated proteins, metabolism-related enzymes and modulating molecules of apoptosis, it is still difficult to explain all the mechanisms (1, 9, 12, 16, 20). High through put methods for gene expression analysis based on mRNA array or cDNA chips have facilitated functional research on those molecules. However, changes at RNA levels do not always correlate with that at protein levels due to the transcription and post-translational modifications contributing to alterations of proteins which are the executor of genetic information (14). Some reversing agents for multi-drug resistance (MDR), such as verapamil and cyclosporin A (CsA), have been used in combination with chemotherapy of leukemia. However, the effective dose of verapamil is somewhat larger than that of well - tolerant *in vivo* and is a major limited factor for clinical use because of its pronounced cardiovascular side-effects. Very high plasma concentrations of calcium channel antagonists are required to block the P-glycoprotein transport efficiently. The CsA seems to be a more effective

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MDR reversing agent than verapamil but the side-effects of CsA, such as dominant immunosuppression and toxicity to liver and kidney, restrict its clinical applications (17). So, further studies on mechanisms of multi-drug resistance would be helpful to identify new potential drug targets. Proteomic studies provide a new tool in this area (10, 21, 22); therefore, in the present study, we used some of the most popular and reliable proteomic methods, including the immobilized pH gradient (IPG) two-dimensional gel electrophoresis (2-DE), the peptide mass finger printing map, and bioinformatic analysis to study the differentially expressed proteins between the multi-drug resistant cells HL-60/DOX and drug sensitive cells HL-60 of acute myeloblastic leukemia (AML). Our purposes are to establish experiment model of leukemia proteomics, revealing the mechanisms of multi-drug resistance, and to identify new potential reversing agents of MDR in AML.

## Materials and Methods

*Materials*

N, N-methylenebisacrylamide (Bis), N, N, N', N'-tetramethylethylenediamine, urea, dithiothreitol (DDT), α-cyano-4-hydroxycinnamic acid, 3-[(3-cholaminopropyl)-dimethylamonio]-1-propanesulfonate (CHAPS), immobilize pH gradient Drystrip (IPG, pH 3~10, L=18), IPG buffer, IPG covering fluid, pharmalyte (pH 3~10), sodium dodecyl sulfate (SDS) and low molecular marker were products of Amersham-Pharmacia Biotech (APB, Uppsala, Sweden). Phenylmethyl-sulfonylfluoride (PMSF), ethylenedinitrilotetra acetic acid disodium salt (EDTA), pepstatin, leupeptin, benzamidine, and Coomassie Brilliant Blue G-250 were from Sigma (St. Louis, MO, USA); iodoacetamide, trifluoroacetic acid (TFA) and acetonitrile (ACN) were from Acros Organics (Morris Plains, NJ, USA). Sequencing grade trypsin was obtained from Boehringer Mannheim (Mannheim, Germany). Other analytical reagents were domestically produced.

*Cells Culture and Protein Extraction*

Acute myelocyte leukemia multi-drug resistant cells HL-60/DOX, induced by adriamycin from cells and whose MRP positively expressed (6), obtained from Department of Pharmacology, Hematology Institute, Chinese Academy of Medical Science, were maintained in RPMI 1640 containing 10% fetal bovine serum and 10 μg/ml adriamycin to logarithm growth phase. Acute myelocyte leukemia drug sensitive cells HL-60 were cultured in RPMI 1640 containing 10% fetal bovine serum. When the cells were exponentially growing, they were harvested by centrifugation and washed with PBS buffer, respectively. Then $1\times10^7$ cells were added up to 600 μl lysis buffer (9 M urea, 4% CHAPS, 0.8% pharmalyte, 1% DTT) which contain 35 μg/ml PMSF, 0.3 μg/ml EDTA, 0.7 μg/ml pepstatin and 0.5 μg/ml leupeptin. After three cycles of quick freezing and subsequent thawing in liquid nitrogen, 5 μg/ml RNAse and 20 μg/ml DNAse were added into the samples. Bathing in ice for 30 min, the samples were then centrifuged at 13,000 rpm for 20 min at 4°C. The protein extracts in the supernatant were stored at -86°C. The protein concentration was determined by a Bradford assay, using bovine serum albumin as a standard.

*Two-Dimensional Electrophoresis*

One mg of total proteins from HL-60/DOX cells and HL-60 cells were diluted to a total volume of 350 μl with a rehydration solution containing 8 M urea, 2% CHAPS, 20 mM DTT, 0.5% pharmalyte and a trace of bromophenol blue, respectively. After rehydration for 6 h, isoelectric focusing (IEF) was performed as follows: 30V for 6 h, 500V for 1 h, 1000V for 1 h and 8000V for 5 h. Isoelectric focusing separation was performed with 18-cm IPG strips (pH 3~10) and the IPGphor instrument (APB, Uppsala, Sweden). After IEF, the DryStrip covering fluid was removed and equilibrated for 15 min under gentle shaking in equilibration solution containing 50 mM Tris-HCl (pH8.8), 6 M urea, 30% glycerol, 2% SDS, 0.5% (w/v) DTT, and a trace of bromophenol blue. The strips were transferred to the second equilibration solution for 15 min with the same ingredients except the 2% (w/v) iodoacetamide, instead of DTT. In the second dimension, IEF strips were placed on the top of a continuous 13% SDS/PAGE gel, performed with a standard protocol at a constant current (20 mA per gel for 40 min and 30 mA per gel) using a PROTEAN II xi 2-D cell (BioRad, Richmond, CA, USA) until the bromophenol blue front reached the bottom of the gel. Next, gels were stained with Coomassie Brilliant Blue G-250 for 2 h, and then destained until the background was clear. ImageScaner (APB) scanned gels at 256 grayscale and 8 bits degree level. One of the HL-60 gels was selected as a reference gel. Other gels including those of the HL-60/DOX cells were matched with the reference gel. Quantification of spot abundance was carried out using ImageMaster 2D Elite 3.01 after removals of noise and background, calibrations of PI and Mr and volume normalization.

*Mass Spectrometry Analysis*

The differential protein spots were excised from gels, cut to 1-2 $mm^2$ slices with a blade, washed with 25 mM ammonium bicarbonate/50% acetonitrile, and

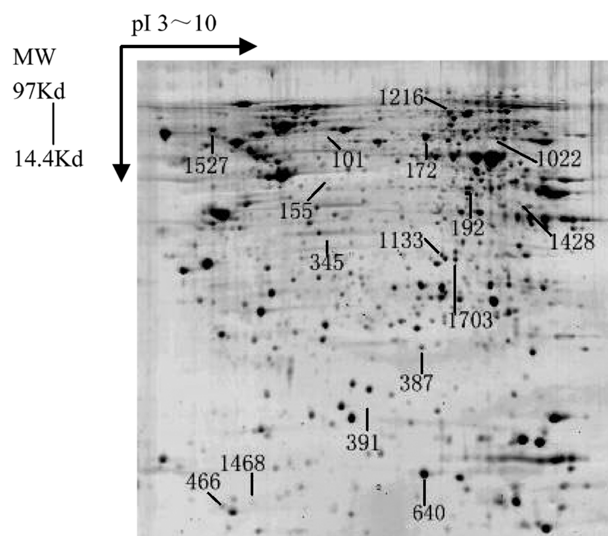


Fig. 1. Two-dimensional electrophoresis maps of proteins from HL-60/DOX cells

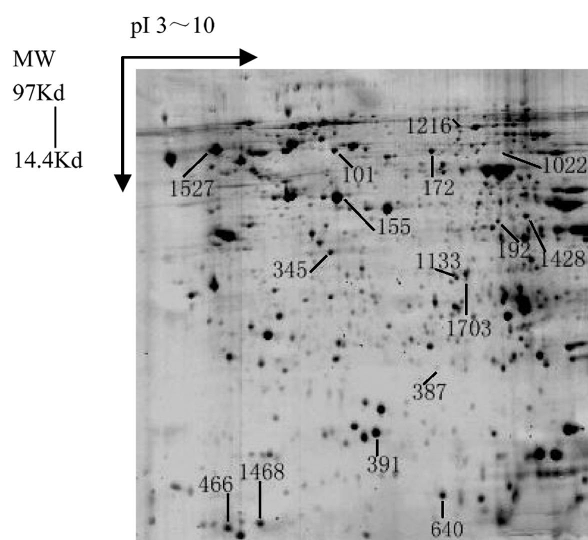


Fig. 2. Two-dimensional electrophoresis maps of proteins from HL-60 cells

dried in a vacuum concentrator. Tryptic peptides were first extracted using 5% TFA at 40°C for 1 h, then 2.5% TFA/50% acetonitrile at 30°C for 1 h. The extracted solutions were mixed in eppendorf tube, and dried with a vacuum concentrator. After the peptide mixture was solubilized with 0.5% TFA, peptide mass analysis of peptide mixture was performed by means of MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) analysis. One microlitter of peptide mixture was added onto the MALDI target, closely followed by adding 1 μl of α-cyano-4-hydroxycinnamic acid (10 mg/ml in 1:1 acetonitrile: 0.1% w/v trifluoroacetic acid) matrix solution. Mass spectra were externally calibrated with peptide standard from Bruker or internally calibrated with autodigest peaks of trypsin.

*Database Searching*

Database searching was performed by the Internet available program Mascot (http://www.matrixscience.com/). The search parameters were used as follows: S-carbamidomethyl-derivative (Cys-CAM) as cysteines, allowed maximum peptide mass error of 0.1-1 Da, more than four peptide mass hits required for protein match, up to 1-2 enzymatic missed cleavages, methionine in oxidized form, protein mass was restricted to range 30%, and species of origin restricted to humans or mammals.

## Results

*Two-Dimensional Electrophoresis Maps of Proteins Extracted from HL-60/DOX and HL-60*

Electrophoresis of the same samples extracted from HL-60/DOX or HL-60 were performed for three times in the same condition. Two-dimensional electrophoresis maps of proteins from HL-60/DOX and HL-60 are shown in Figures 1 and 2. The gels stained by Coomassie Brilliant Blue G-250 were analyzed using ImageMaster 2D Elite 3.01. 1162±79 spots in HL-60/DOX cells and 1102±52 spots in HL-60 cells were detected, respectively.

*Identification of Differentially Expressed Proteins by Peptide Mass Fingerprinting*

16 differential analysis of proteins labeled in Figures 1 and 2 were identified by MADLI-TOF. They include 6 spots upregulated and 8 spots downregulated, and protein spot 1022 uniquely expressed in HL-60/DOX, while protein spot 391 uniquely expressed in HL-60. These protein spots were excised from 2D gel. Low abundant proteins were excised from the same location of two to three gels, and then mixed. After in-gel digestion, 16 mass spectrums of proteins were acquired by peptide mass fingerprinting. The mass spectrum of spot 1527 is presented in Figure 3. Database search parameters used are listed in Table 1, and search result presents in Figure 4 using Mascot. It reveals that the score of spot 1527 is 118. Because protein scores greater than 60 are significant ($P < 0.05$), spot 1527 is identified as protein disulfide isomerase precursor (PDI). In addition, 16 differential proteins between HL-60/DOX and HL-60 were identified, as shown in Table 2.

## Discussion

Investigation on proteomics of multi-drug re-

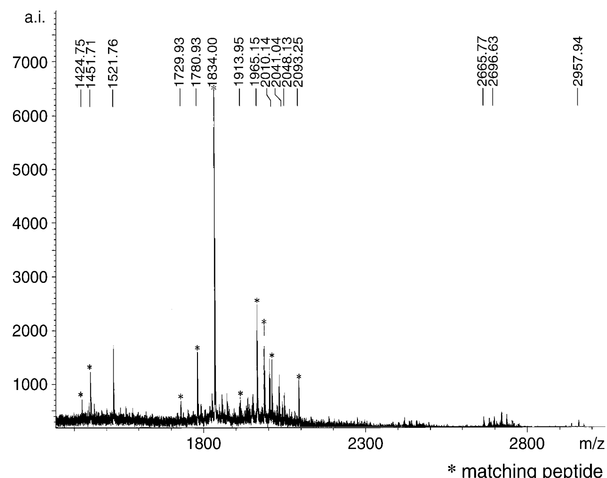


Fig. 3. Peptide mass fingerprint spectrum of protein spot 1527

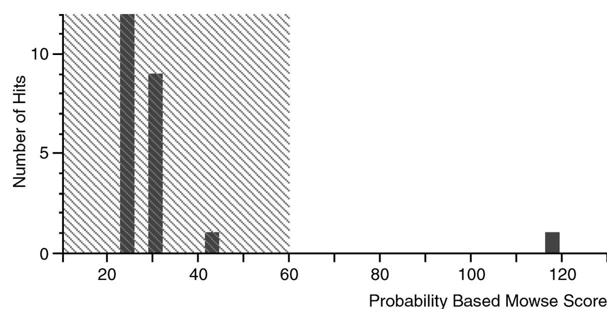


Fig. 4. Database searching results of PMF data from spot 1527

**Table 1. Database searching parameters of spot 527**

| | |
|---|---|
| Type of search | Peptide Mass Fingerprint |
| Enzyme | Trypsin |
| Variable modifications | Carbamidomethyl (C), Oxidation (M) |
| Mass values | Monoisotopic |
| Protein Mass | Unrestricted |
| Peptide Mass Tolerance | ±0.2 Da |
| Peptide Charge State | 1+ |
| Max Missed Cleavages | 1 |
| Number of queries | 15 |

sistance in acute myeloblastic leukemia has not been reported. So far in the present study, 16 differential proteins between multi-drug resistant AML cell line HL-60/DOX and sensitive cell line HL-60 were screened out with proteomic technology. Among those, following proteins are possibly related to tumor multi-drug resistance: [1] Protein disulfide isomerase precursor (PDI). It's a multifunctional cytoplasmic enzyme with additional chaperone activity and plays a role in the regulation of protein-bound SH groups. Expression levels of PDI are different between drug resistant and sensitive human liver cancer cells (24). Decrease of PDI can increase expression of surface thiol in B cell chronic lymphocytic leukemia and the expression of thiol leads to significant resistance of cells to the cytostatic drugs such as chlorambucil, vinblastine and cisplatin (8). [2] Proteasomes α1. Investigations indicated that the proteasome pathway may be involved in degradation of PML/RARα (4). Treatment of the retinoic acid (RA) resistant acute promyelocytic leukemia cell line NB4.007/6 with proteasome inhibitors lactacystin and LLnL partially restored the expression level of PML/RARα protein and resulted in a partial release of the RA-resistant phenotype. In addition, mammalian cells overexpressing Rpn11, regulatory particle of the proteasome, display moderate resistance to vinblastine, cisplatin and doxorubicin and exhibit a slower proliferation rate. These physiological events may promote tumor cells escaping from chemotherapeutic agents, and may serve as a marker for MDR-cells (23). [3] The purine nucleoside phosphorylase (PNP) is a major enzyme decomposing purine nucleoside and suppressing the effect of chemotherapeutic drugs that interfere in the synthesis of purine nucleoside (5). [4] The Cu, Zn-superoxide dismutase (Cu, Zn-SOD) plays a role in the antioxidant response by clearing up superoxide anion free radical. Change of redox state of K562/DOX cells, the doxorubicin- resistant human erythroleukemia K562 cells, can cause increase of Bcl-2 and suppression of P53 levels. The adaptive antioxidant in response to prooxidant effect of Dox promotes the development of cellular drug resistance (11). [5] The melallothionein-IA is a cysteine-rich metal binding protein. Human Melallothionein-IA induces multi-drug resistance of cells through enhancing the ability of DNA repair, decreasing the activity of topoisomerase, and inhibiting cell apoptosis etc(25). [6] An isoform of the non-neuronal cytoplasmic intermediate filament protein (IF) belongs to the intermediate filament networks in eukaryocyte, which attends message convection and whole adjustment of cells. Cytokeratin could be modified by mitoxantrone, a chemotherapeutic agent. The interaction of the cell damaging agent with cytokeratin may initiate signaling responses for cell survival and result in multi-drug resistance in tumor cells (3). We also found some proteins, closely related to modulation of cell metabolism. Functions of these proteins are not clear in MDR of tumor cells. These proteins include: [1] N-chimaerin, which is a GTPase-activating protein (GAP) and plays an important role in modulating signal switchover system (15). The GAP proteins have intrinsic GTPase and GDP/GTP switching

**Table 2. Identification of proteins between HL-60/DOX and HL-60 by Mascot in Swiss-port**

| Spot | Swissprot number | Protein name | Protein alteration (fold) # | Score | Sequence coverage (%) |
|---|---|---|---|---|---|
| 1527 | P07237 | Protein disulfide isomerase precursor | –14.80 | 118 | 27 |
| 1428 | P15786 | Proteasome α1 | –6.92 | 65 | 39 |
| 1703 | P00491 | Purine nucleoside phosphorylase | +16.70 | 120 | 44 |
| 1022 | P00491 | Purine nucleoside phosphorylase | only in HL-60/DOX | 79 | 31 |
| 466 | P00441 | Superoxide dismutase(Cu-Zn) | –9.34 | 75 | 58 |
| 391 | P04731 | Metallothionein | only in HL-60 | 82 | 100 |
| 1133 | P15882 | N-chimaerin | –2.96 | 82 | 23 |
| 101 | 9HIJ7 | WNT5B-precusor | –10.41 | 75 | 34 |
| 155 | P2288 | Non-neuronal cytoplasmic intermediate filament protein isoform | –19.64 | 75 | 100 |
| 387 | P23152 | Pre-mRNA splicing factor | +11.77 | 64 | 21 |
| 1216 | P31939 | Bifunctional purine biosynthesis protein | +6.41 | 61 | 25 |
| 345 | Q15181 | Inorganic pyrophosphatase | –13.53 | 100 | 37 |
| 1468 | Q9UPX8 | SH3 and multiple ankyrin repeat domains proteins 2 isoform | –14.29 | 60 | 34 |
| 192 | | No name in Swissprot database | +3.17 | | |
| 640 | | No name in Swissprot database | +3.01 | | |
| 172 | | No name in Swissprot database | +4.23 | | |

#HL-60/DOX *vs*. HL-60 + upregulaton – downregulation

activity. The signal switchover passage is opened when G-protein binds GTP and shut off when GTP is hydrolyzed into GDP by G-protein. [2] WNT5B-precursor, which belongs to the WNT family of secreted-type glycoproteins. It plays a major role in cell development, proliferation, migration and death. When aberrantly activated, the WNT signaling pathway leads to tumor formation (13). [3] The pre-mRNA splicing factor. Indolocarbazole, an inhibitor of topoisomerase, can block the assembly and splicing of spliceosome *in vitro* through inhibiting phosphorylation of pre-mRNA splicing factor, and thus modulate gene expression (19). [4] The bifunctional purine biosynthesis protein (PURP). Its specific structure provides us a framework for probing the catalytic mechanisms and designing specific inhibitors for clinical use in cancer chemotherapy (7). [5] Inorganic pyrophosphatase. The expression of inorganic pyrophosphatase was increased in lung adenocarcinomas cells, as compared with uninvolved lung cells by means of proteomic technology(2). [6] The ankyrin repeat domains proteins 2 (shank2). Shank2 is an ankyrin which firmly links many specific proteins in membrane and microfilament in the cellular skeleton. So it can maintain stable structure of cells and induce signal transduction (18).

Although 16 differential proteins in multi-drug resistant HL-60/DOX cells were identified with proteomic technology, the results may not reflect the entire information of differentially expressed proteins in MDR, due in part to the limitations of the separation flux and resolution of the two - dimensional gel electrophoresis. Moreover, functions of these proteins in MDR need to be further investigated to better understand the correlation between these proteins and multi-drug resistance of acute myeloblastic leukemia cells.

In summary, this is the first study on multi-drug resistance in acute myeloblastic leukemia using comparative proteomics. Sixteen differential proteins between HL-60/DOX and HL-60 were screened out with proteomics technology. These proteins involve the protein disulfide isomerase precursor (PDI), the proteasomes $\alpha$1 and other proteins which are related to drug resistance or cell metabolism. The findings will help us to further understand the possible mechanisms involved in MDR.

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