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Analysis of drug resistance-associated proteins expressions of patients with the recurrent of acute leukemia via protein microarray technology

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Abstract. – INTRODUCTION: To detect the expressions of drug-resistance related proteins in bone marrow mononuclear cells of acute leukemia (AL) patients using protein microarray and to analyze the clinical value of protein microarray in predicting prognosis of AL patients.

PATIENTS AND METHODS: A total of 48 AL patients received chemotherapy were divided into four groups: recurrent acute myeloid leukemia group (R-AML; n=15); AML continue remission group (AML-CR; n = 13); recurrent acute lymphocytic leukemia group (R-ALL; n=13); and ALL-CR group (n=7). Fifteen age-matched patients with non-hematologic disease were used as controls.

RESULTS: Expression levels of P-gp, LRP/MVP, BCL-2, GST-π, PCNA, CXCR4 were increased significantly in both AML-R and ALL-R groups ($p < 0.05$). Besides, LFA-1 and TRAIL-R were also up-expressed significantly in ALL-R group ($p < 0.05$). In addition, the levels of P-gp, GST-π expressed in AML-R group were higher than those in AML-CR group ($p < 0.05$) and P-gp, LRP/MVP, GST-π, LFA-1 and CXCR4 in ALL-R were expressed higher than those in ALL-CR group ($p < 0.05$).

CONCLUSIONS: The recurrent of AL were related closely to the over expression of drug resistance-related proteins. Protein microarray can be used in the prediction of AL recurrence and would be beneficial in guiding individual therapy and patient prognosis.

Key Words:
Acute leukemia, Prognosis, Recurrent, Protein microarray, Multidrug resistance.

Introduction

Acute leukemia (AL) is one kind of malignant tumors of hematopoietic system. It is the most common cause of death due to cancer among children and young people[1,2]. Nowadays, chemotherapy is the main treatment of AL[3]. Most of the patient get relief from the chemotherapy, but some of them become more serious or even to die[4-6]. Multidrug resistance (MDR) is one of the main causes. MDR refers to a condition when cancer cells developed resistance to a drug and they begin to show cross resistance to several other chemotherapy drugs with distinct structures and mechanisms[5,7]. The mechanism of MDR is very complex, involving the up-regulated expression of MDR-related proteins, abnormal produced apoptosis and drug-metabolizing enzymes and so on[8-10]. Thus, it would not be accurate enough to guide individual therapy or evaluate the chemotherapy prognosis of AL patients, if only considering one single MDR-related protein.

Protein microarray, as a kind of microarray technology, has become a vital tool for large-scale and high-throughput miniaturized protein analysis with higher sensitivity and specificity[11-13]. At present, it is applied to analyze the interactions of protein to protein, nucleic-acid, lipid and small-molecule, as well as enzyme to substrate and antibody to antigen. Most of all, protein microarray can detect thousands of addressable elements at the same time[12].

In this study, the quantitative technology of protein microarray was utilized to detect 11 kinds of drug-resistance related proteins in bone marrow mononuclear cells of AL patients. The proteins were P-glycoprotein (P-gp), lung resistance-related protein/major vault protein (LRP/MVP), Bcl-2, topoisomerase IIA (Topo IIA), topoisomerase B (Topo IIB), glutathione S-transferase π (GST-π), vascular endothelial growth factor (VEGF), chemokine receptor 4 (CXCR4), proliferating cell nuclear antigen (PCNA), lymphocyte function-associated antigen-1 (LFA-1) and TNF-related apoptosis inducing lig-

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and-receptor (TRAIL-R). The main aim of this study was to analyze the relationship of drug resistance-associated proteins expressions with the recurrent of AL via protein microarray technology and to predict the prognosis after chemotherapy of the AL patients.

Patients and Methods

The Patients, Chemotherapy and Bone Marrow Collection

Forty-eight AL patients hospitalized in Xinqiao Hospital (the Second Affiliated Hospital of the Third Military Medical University, Chongqing, China) from January 2008 to December 2009 were chosen for the research. There were 31 males, 17 females, and the median age were 30 years old. According to the FAB diagnosis standard[14], the patients were classified in details (Table I). At the same time, 15 age-matched patients (male 8 cases, female 7 cases, median age 34 years old) were chosen as control group, including 7 cases of idiopathic thrombocytopenic purpura, 5 cases of iron-deficiency anemia and 3 cases of healthy donor. All people of the research were told possible complications of the check and chemotherapy, and signed informed consent. This study was approved by the Chongqing Xinqiao Hospital Ethics Committee.

AML patients except the M3 were treated with DA, MA (D: daunorubicin; M: mitoxantrone; A: cytarabine) as the main chemotherapy. M3 patients were adopted all-trans- retinoic acid, arsenic trioxide as the double induction therapy. The main chemotherapy drugs for ALL patients were vincristine, cyclophosphamide, daunorubicin, L-asparaginase and prednisone. The treatments of the patients were followed up for at least nine months. Whether the AL patients got complete remission (CR) or not were detected according to the diagnosis standard of blood diseases and curative standard[15].

The bone marrow specimens of AL patients were collected after the tracked treatment. The control group's bone marrow specimens were gathered at any time. All the bone marrow were put into heparin anticoagulation tube and stored in –70°C preparing for next experiment.

The Preparation of Mononuclear Cells Lysate

Physiological saline (2 ml) was added to the bone marrow (2 ml) in heparin anticoagulation tube, and then the mix was blended slowly into a centrifugal tube which had 4 ml lymphocyte separation fluid downside. The mixture and the lymphocyte separation fluid should be kept separately and centrifuged at the speed of 1800 r/min for 20 min. The mononuclear cells between the supernatant and precipitate were absorbed and washed twice with phospate buffered saline (PBS). Cold protein extraction reagents protease (with 1/200 protein inhibitors) was added to the mononuclear cells, according to the proportion of 10^7 cells with 1 ml protein extraction reagents. Followed the gently shaking 1 hour in ice bath, the mixture was centrifuged at the speed of 14000 r/min (15 min). The supernatant was the protein samples and should be measured to ensure the protein concentration under 500 μg/ml.

Protein Microarray Slides Preparation

The preparation of protein microarrays has been previously described in detail. People cell factors antibody microarrays (RayBio®, Noreross, GA, USA) slides were dealt with sulfuric acid and alkali respectively and cleaned with deionized water. Then they were dried and immerged into 5% amino silane alcohol solution for 30 min. After the process, the slides were cleaned, dried, and immersed into PBS (0.2 mmol/L, pH8.0) with 2.5% glutaraldehyde for 60 min. At the end, the slides were cleaned and dried again.

Table I. Classification and chemotherapy results of AL patients.

	AML							ALL			
Classification	M1	M2	M3	M4	M5	M6	Total	L1	L2	L3	Total
Total	5	10	2	5	5	1	28	6	6	8	20
CR	3	4	2	3	1	0	13	2	3	2	7
Recurrent	2	6	0	2	4	1	15	4	3	6	13

CR: complete remission.

The antibodies (purchased from Lab Vision Corporation, Fremonnt, CA, USA) of the 11 chosen proteins, which were diluted into 0.1 mg/ml with PBS, were added to the prepared slides by Omnigrid Accent Spotter with humidity of 58%-60% at 25°C. The space between each two antibodies was 350 μm. Each antibody was attached 10 points, so that the mean of the data would be more accurate. Afterwards, the slides were put in a quiet place for at least 12 h to ensure that antibodies were connected with the slides completely, and then were conserved in 4°C.

Protein Microarray

500 μl protein samples were added to prepared slides hole. The protein microarray operating procedures were according to the instructions of people cell factors antibody microarrays kit (RayBio®). Finally, the microarrays were scanned by laser scanner (Genpix4000B, Cambridge, UK) and the outcomes were saved into picture file of TIFF format.

Statistical Analysis

The green brightness of each point of picture was changed into digital data by scanalyze software (Figure 1), and the ratio of each sample hole was analyzed according to the following calculation formula.

Ratio (%) = (the brightness of the determination hole-the brightness of the blank hole)/the brightness of the inner basis hole-the brightness of the blank hole.

The data were expressed as mean±standard deviation (SD, n=10). The level of significance was statistically detected with analysis of variance (ANOVA, SPSS.13.0, SPSS Inc., Chicago, IL, USA). If $p < 0.05$, the difference has statistically significance.

Results

The Result of AL Chemotherapy

According the clinic diagnosis standard of blood diseases and curative standard[15], there were 13 AML and 7ALL patients achieved complete remission after the chemotherapy while the other patients became to be recurrent cases. The detailed information was shown in Table I.

Determination of Cutoff Value

The bone marrow samples of control group were detected according to methods described before, and the result was showed in Table II. The Cutoff value of leukemia patient bone marrow samples was calculated as the mean value plus 3 SD.

Protein Microarray Result of AL Patients

According to fluorescence intensity, microarray results of AL patients were analyzed and shown in Table III. Compared with the control group, the amount of P-gp, LRP/MVP, Bcl-2, GST-π, PCNA and CXCR4 in AML-R were over expressed significantly ($p < 0.05$), while protein expression of P-gp, LRP/MVP, Bcl-2, GST-π, PCNA, CXCR4, LFA-1 and TRAIL-R in ALL-R group were higher than those in the control group ($p < 0.05$). P-gp, GST-π protein expression of AML-R group was higher than that of AML-CR group ($p < 0.05$). In comparison to ALL-CR group, P-gp, LRP/MVP, GST-π, LFA-1, CXCR4 protein expressed higher in ALL-R group ($p < 0.05$). In addition, ALL-R group expressed larger amount of LFA-1, CXCR4 than those in AML-R group ($p < 0.05$).

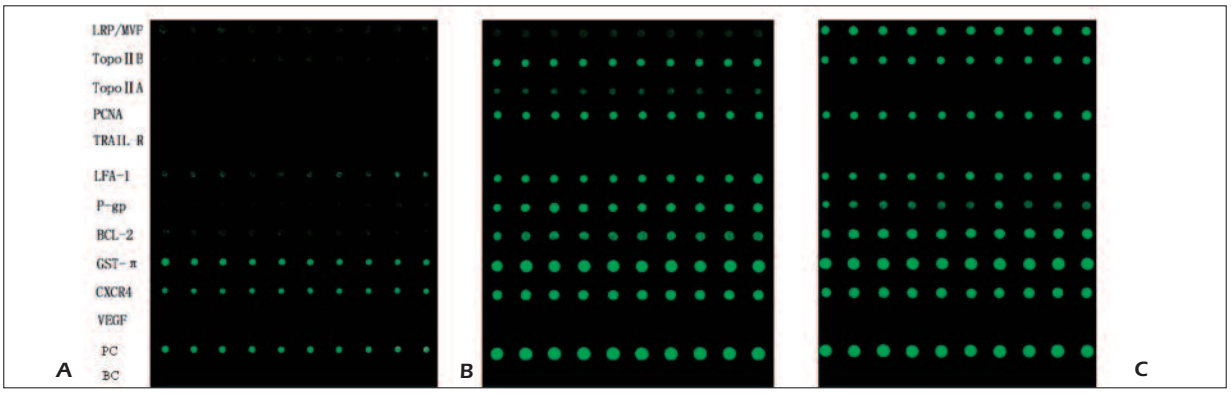

Figure 1. Assay figure of protein microarray.

Table II. The results of normal control group and the determination of Cutoff value.

	P-gp	LRP/ MVP	BCL-2	GST-π	Topo II A	Topo II B	VEGF	PCNA	LFA-1	CXCR4	TRAIL-R
Mean	0.0113	0.0027	0.0024	0.0211	0.0022	0.0048	0.0019	0.0009	0.0123	0.0278	0.0003
SD	0.0064	0.0020	0.0021	0.0046	0.0001	0.0008	0.0007	0.0005	0.0052	0.0075	0.0001
Cutoff	0.0305	0.0087	0.0087	0.0349	0.0025	0.0072	0.0040	0.0024	0.0279	0.0503	0.0006

SD: standard deviation.

Prognosis Analysis with Drug-Resistance Proteins

From the results of Table IV, there were 16 AL patients who got one protein index higher than the Cutoff value, 21 patients have two protein expressed higher and 6 patients have 3 or more protein indexes more than the Cutoff value.

Besides, protein expression of P-gp in 22 cases had increased, of which 16 cases were recurrent patients. In R-AML group (15 patients), 4 cases had the P-gp and LRP/MRP increased at the same time, 3 cases had P-gp and GST-π increased, while there were another 2 patients whose P-gp and VEGF were both higher than the Cutoff value. Among 13 cases of R-ALL patients, 2 cases had the P-gp and LRP/MVP increased, 3 cases the P-gp and GST-π were higher than Cutoff value, 2 cases the P-gp and CXCR4 were over expressed.

Discussion

AML is the original remit or secondary to some hematological disease or a history of chemotherapy and radiotherapy. With the understanding of cytogenetic and molecular basis for AML, many prognostic factors had been defined, such as age, amount of white blood cell (WBC) and the biological characteristics of subtype[16,17]. ALL, one of the most common childhood malignancy for which large-scale therapeutic trials were conducted, has served as a paradigm for cancer research for over four decades[18]. While after ALL is confirmed, the immune phenotype results should be checked to clear whether it is B-ALL or T-ALL[19], following the cytogenetic and molecular diagnosis, the age and WBC numbers are considered to ensure the patient's condition. Meanwhile, corrective evaluation of the patients' prognosis and dynamic monitoring of the patients can provide the useful method and improve the cure rate of AL[20].

In the clinical treatment, MDR is one of the main reasons for the failure of AL chemotherapy. Studies have indicated that resistance proteins' expression can reflect the state of leukemia disease and the prognosis effects[21]. For example, Shanghai Leukemia Cooperative Group have concluded that P-gp is an independent prognostic indicators. When P-gp has a positive expression, there would be a poor prognosis of the AL patients[22]. Actually, drug resistance of AL patients was not caused by only one resistance protein, it refers to P-gp, LRP/MVP, GST-π, VEGF and the others. So it is with a higher value to study the combined expression of multidrug related proteins in AL patients.

P-gp is a membrane glycoprotein which plays an important role in the development of MDR. Its relative molecular weight is 170,000 and belongs to the ABC transporter family. Consuming ATP molecule energy, P-gp will drive the chemotherapy drugs to the extracellular environment, making the intracellular drug maintains at a lower concentration continuously, so that cytotoxicity of the target cell is reduced or completely lost and drug resistance was generated. Studies have shown that P-gp is the molecular basis of MDR, and the degree of MDR is proportional to gene expression level of P-gp[23,24].

Lung Resistance-Related Protein (LRP) is a recently discovered multidrug resistance protein, as its amino acid sequence was 57-88% similar to major vault protein (MVP). LRP widely distributed in cell cytoplasm and organelles. It can combine with chemotherapy drugs, transport them along the route from perinuclear, cytoplasm to extracellular environment[25,26]. The mechanism of LRP/MVP creating MDR is different from P-gp, but they all transport the intracellular drugs to the outside of cell.

Glutathione-S-transferase (GST) is a versatile drug-metabolizing enzyme, which can be divided into α, μ, θ, π, and membrane-bound particle

Table III. Results of 11 proteins expressed in AL patients ($\bar{x} \pm SD$).

	P-gp	LRP/MVP	BCL-2	GST-π	Topo II A	Topo II B	VEGF	PCNA	LFA-1	CXCR4	TRAIL-R
AML-CR (n=13)	0.0231 ± 0.0125	0.0035 ± 0.0017	0.0033 ± 0.0019	0.0454 ± 0.0132	0.0003 ± 0.0001	0.0033 ± 0.0006	0.0011 ± 0.0005	0.0014 ± 0.0011	0.0005 ± 0.0002	0.0355 ± 0.0183	0.0004 ± 0.0001
AML-R(n=15)	0.0748 ± 0.0264$^{*,\Delta}$	0.0066 ± 0.0044*	0.0047 ±0.0025*	0.1278 ± 0.0859$^{*,\Delta}$	0.0017 ± 0.0001	0.0029 ± 0.0017	0.0015 ± 0.0009	0.0314 ± 0.0311*	0.0006 ± 0.0002	0.0629 ± 0.0145*	0.0005 ± 0.0002
ALL-CR (n=7)	0.0198 ±0 .0096	0.0114 ± 0.0047	0.0041 ± 0.0016	0.0358 ± 0.0065	0.0004 ± 0.0002	0.0025 ± 0.0009	0.0021 ± 0.0014	0.0012 ± 0.0007	0.0126 ± 0.0077	0.0317 ± 0.0097	0.0006 ± 0.0003
ALL-R (n=13)	0.05911 ± 0.0182$^{*,\Diamond}$	0.0731 ± 0.0354$^{*,\Diamond}$	0.0066 ± 0.0083*	0.0741 ± 0.0652*	0.0016 ± 0.0012	0.0041 ± 0.0027	0.0017 ± 0.0018	0.0340 ± 0.0241*	0.0726 ± 0.0054$^{*,\Delta,\#}$	0.4848 ± 0.2235$^{*,\Delta,\#}$	0.0011 ± 0.0009*
Control (n=15)	0.0113 ± 0.0064	0.0027 ± 0.0020	0.0024 ± 0.0021	0.0211 ± 0.0046	0.0022 ± 0.0001	0.0048 ± 0.0008	0.0019 ± 0.0007	0.0009 ± 0.0005	0.0123 ± 0.0052	0.0278 ± 0.0075	0.0003 ± 0.0001

Difference was considered significant when p value was < 0.05. *Means $p < 0.05$ when compared with control group; $^{\Delta}$Means $p < 0.05$ when R-AML group compared with AML-CR group; $^{\Diamond}$Means $p < 0.05$ when R-ALL group compared with ALL-CR group; $^{\#}$Means $p < 0.05$ when R-ALL group compared with R-AML group.

Table IV. Proteins expression changes compared with Cutoff.

	0 Peh-C	1 Peh-C	2 Peh-C	3 Peh-C	Total
Recurrent	0	5	17	6	28
CR	5	11	4	0	20
Total	5	16	21	6	48

CR: complete remission; Peh-C: Protein expressed higher than the Cutoff value.

types. GST-π is closely related to MDR. It is able to catalyze the reduced glutathione (GSH) combined with the electron affinity substrate (including most of the chemotherapy drugs) to form a more water-soluble product, which is easier to discharge from the bile or kidney[27,28].

Induction of cell apoptosis is a common pathway of many chemotherapy drugs to kill tumor cells.

Bcl-2 is a drug target of leukemia. It can induce MDR together with other MDR factors[29,30]. Topoisomerase II (Topo II) is an essential ribozyme to the survival of eukaryotic cells which is the main target for a variety of anti-tumor chemotherapy drugs. Changes in the quality and quantity of Topo II are related the yield of MDR[31,32]. VEGF as is of the key factors of tumor angiogenesis, and its expression of tumor cells is related to multidrug resistance[33,34].

Proliferating cell nuclear antigen (PCNA) is a new indicator to detect cell proliferation activity in recent years. In theory, high proliferative activity of leukemia cells is sensitive to chemotherapeutic drugs, thus high rate to relieve the AL patients. However, due to the its malignant proliferation potential, it is easy to relapse[35].

CXCR4 and LFA-1 are specific receptors. CXCR4 plays an important role in invasion, migration[36], while LFA-1 combines with specific receptors on the tumor cell membrane to activate the death pathway, and induce apoptosis of tumor cells[37]. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is regarded as a potential anticancer agent, but reports indicated that many cancer cells are resistant to the apoptosis induced by TRAIL. TRAIL is related to the recurrent of AL patients[38].

From the result of Table III, the experiment prompted the recurrent of AML might be related with unusual expression of P-gp, LRP/MRP, GST-π, Bcl-2and PCNA, while ALL recurrence was closely related with higher expression of P-gp, LRP/MRP, GST-π, PCNA, LFA-1 and CXCR4. The results were same with the former researches.

In this research, among the 18 patients with P-gp expression over Cutoff patients, 72.7% (16/22) people became into recurrent. This finding suggests that expression level of P-gp might be a predictive factor for recurrence of AL. It also proved P-gp might be a key factor related to the MDR.

Besides, AL patients with 2 proteins expression higher than the cutoff value would have 81% (17/21) to become recurrent cases. AL patients with more than 3 proteins expression higher than the cutoff value would recurrent at 100% rate. Above all, with the increasing number of the examination proteins, the predication results are more reliable.

Among the proteins selected in this study, Topo II showed a decreasing expression and needed further studies to define the cutoff value for the judgement of the test result. More rational research programs and more sensitive test index should be designed to confirm the accurate relationship of drug-resistance associated proteins and the recurrence of acute leukemia.

Conclusions

AL recurrence can be predicated by utilizing protein microarray. The predicted accuracy for recurrence of AL would be increased with the number of MDR related proteins whose expression value higher than the cutoff value. At this case, the patients should be treated with a more reasonable and positive chemotherapy method.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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