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Original Article

Differences in platelet proteins between acute lymphoblastic leukemia and healthy children

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Abstract: Background: Acute lymphoblastic leukemia (ALL) is the most common malignant cancer in the childhood. The researches about the changes of platelet function in ALL are still rare, and the specific mechanism is still unknown. Due to the limited detection technology, the functions of platelet in ALL are poorly understood. Purpose: To investigate the expression difference of platelet proteins between ALL and healthy children. Methods: Twenty-seven ALL children (ALL group), 25 ALL children who obtained complete remission after remission-induction therapy (ALL-CR1 group) and 27 healthy children (control group) were enrolled in this study. The surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technique was used to obtain and analyze the whole platelet protein spectrum of each group. Results: Compared with control group, ALL group had 9 protein peaks which had the stable expression differences ($P < 0.05$), the mass/charge ratio (m/z) were 2496.9, 4287.9, 7881.2, 4091.3, 3149.9, 2365.1, 9414.1, 5252.3 and 2280.7, respectively. There were 6 protein peaks with the stable expression differences between the ALL group and the ALL-CR1 group ($P < 0.05$), and the m/z were 2496.9, 4287.9, 9414.1, 7881.2, 3149.9 and 2280.7, respectively. The differentially expressed protein peaks were analyzed as endothelin-1, big endothelin-1, PF4, thrombin light chain, pituitary adenylate cyclase activating polypeptide 27, fibrinogen β chain and 3 unknown proteins. Conclusion: There are significant differences in platelet proteome between ALL children and healthy children, and the ALL children have the platelet function abnormalities, such as the obstacle of cell signal transduction, aggregation, and abnormalities of activation and coagulation.

Keywords: Acute lymphoblastic leukemia, platelets, SELDI-TOF-MS

Introduction

The acute lymphoblastic leukemia (ALL) is the most common malignant cancer in the childhood, accounting for about 25% of child cancers, and the 5-year survival rate of patients that received the protocol treatment is 76%-86% [1]. The bleeding is a common symptom and cause of ALL death. The leukemic cell infiltration into the vessel walls, decreased production of platelets, coagulation and anticoagulation dysfunction, as well as many other factors, can induce the severe bleeding, among which the most important reason is thrombocytopenia. The degree of thrombocytopenia is the determinant in the assessment of bleeding risk, but the clinics often find that the thrombocytopenia is not completely correlated with bleeding risk. Some patients exhibit the manifestation of only a small amount of bleeding, though the platelet count of is less than 10×10^9/L. In other patients, the platelet count is more than 50×10^9/L, but they still face with the symptoms of severe bleeding [1, 2]. Studies have found that, among all types of acute leukemia, when the platelet count decreases, there may be the platelet function abnormality [3, 4]. The possible causes of platelet count decrease in ALL may be as follows: (i) The abnormal progenitor cells and immature cells inside the bone marrow proliferate largely, which inhibits the normal hematopoietic function, induces the disorders of megakaryocyte generation and differentiation, thus reducing the normal platelet formation; (ii) During the suppression of bone marrow proliferation by partial chemotherapy drugs, the integrity of platelet structure will also be directly undermined, leading to the excessive releasing of the contents, i.e., α-particles and dense particles, into the peripheral blood, and accelerating the aggregation of platelets, thus making the plate-

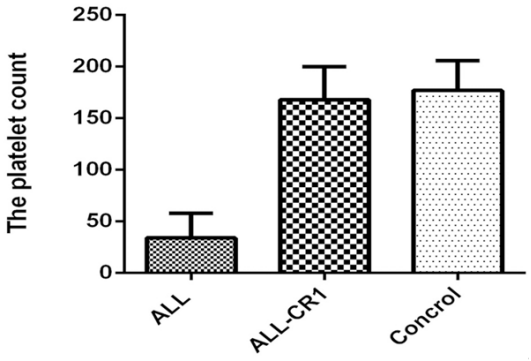

Figure 1. Comparison of platelet count among three groups.

let count inside the peripheral blood to continuously decrease; (iii) The repeated blood transfusion, severe infection, and a variety of inflammatory mediators can induce the immune response to accelerate the destruction and decline of platelets [5]. Currently, the researches about the changes of platelet function in ALL are still rare, and the specific mechanism is still unknown. Psaila *et al.* [6] have found that the expression of surface activation related receptor of platelet membrane in the acute myeloid leukemia (AML)/myelodysplastic syndromes (MDS) patients is decreased, and compared with ITP, the AML/MDS patients showed the low intrinsic activity and intrinsic reactivity of platelet. Sharma *et al*. (4) have found that the 3 main enzymes of platelet energy generation pathway show the functional abnormalities in the incidence and treatment of various types of leukemia. In the leukemia, the abnormalities of platelet metabolism and enzyme functions reflect that the origin of megakaryocyte is defective, which can also explain the absence of platelet functions in the leukemia. However, the platelet functions in ALL are not conclusive currently. Therefore, from the perspective of platelets, the further investigation towards the roles of platelet functions in the pathogenesis of ALL will have the great significance in elucidating its relative pathogenesis.

As a unique particle without the nuclear, the platelet cannot be studied through the conventional molecular and cellular biological techniques. The platelet proteomics has become an important tool to explore the biological information of platelets. In the last decade, the application of platelet proteomics technology has discovered a lot of new platelet receptors and signaling proteins [7, 8]. The platelet has a pivotal role in the pathogenesis and treatment processes of ALL. Along with the induction-remission role of chemotherapy drugs, the functions of bone marrow will gradually recover, and this will firstly be reflected in the changes of platelet. The parameters of platelet will exhibit the significant changes, thus it can be of an important value towards the evaluation of drug efficacy and disease prognosis. Due to the limited detection technology, the functions of platelet in ALL are poorly understood. This study applied the surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technique to detect the differential expressions of platelet proteins in the ALL children, aiming to study the changes, from the angle of platelet, of platelet functions during the pathogenesis and chemotherapy of ALL, and to investigate mechanisms of platelet dysfunction in ALL.

Subjects and methods

Subjects

Fifty-two patient children from the Department of Pediatric Hematology and 27 healthy children (control) from Department of Healthy Examination of Affiliated Hospital of Luzhou Medical College from Nov 2010 to Dec 2012 were enrolled in this study. The patients were divided into ALL group (27 cases) and ALL-CR1 group (25 cases, the patients obtained complete remission after remission-induction therapy). There was no significant difference in the age or gender among the 3 groups ($P > 0.05$). In ALL group, the patient met the basic diagnostic criteria of ALL by the morphological analysis of bone marrow cells and MICM classification, who had not received any related treatment and excluded the combination of other neoplastic diseases (9). There were 14 males and 13 females, with the age of 4.3±3.7 years. The specific sub-classification situations were as follows: (i) Morphological classification (morphology, M), including 15 cases of L1 type (55.6%), 10 cases of L2 type (37.0%) and 2 cases of L3 type (7.4%); (ii) Immunological classification (immunology, I), including 19 cases of B-lineage (70.3%), 5 cases of T-lineage (18.5%), 2 cases of T+B-lineage (7.4%), and 1 case of lymph-marrow-lineage (3.7%); (iii) Cytogenetic classification (cytogenetics, C), the chromosome analysis showed that 23 cases had the normal chromosome numbers (85.2%), 4 cases were abnormal (14.8%), 19 cases had the nor-

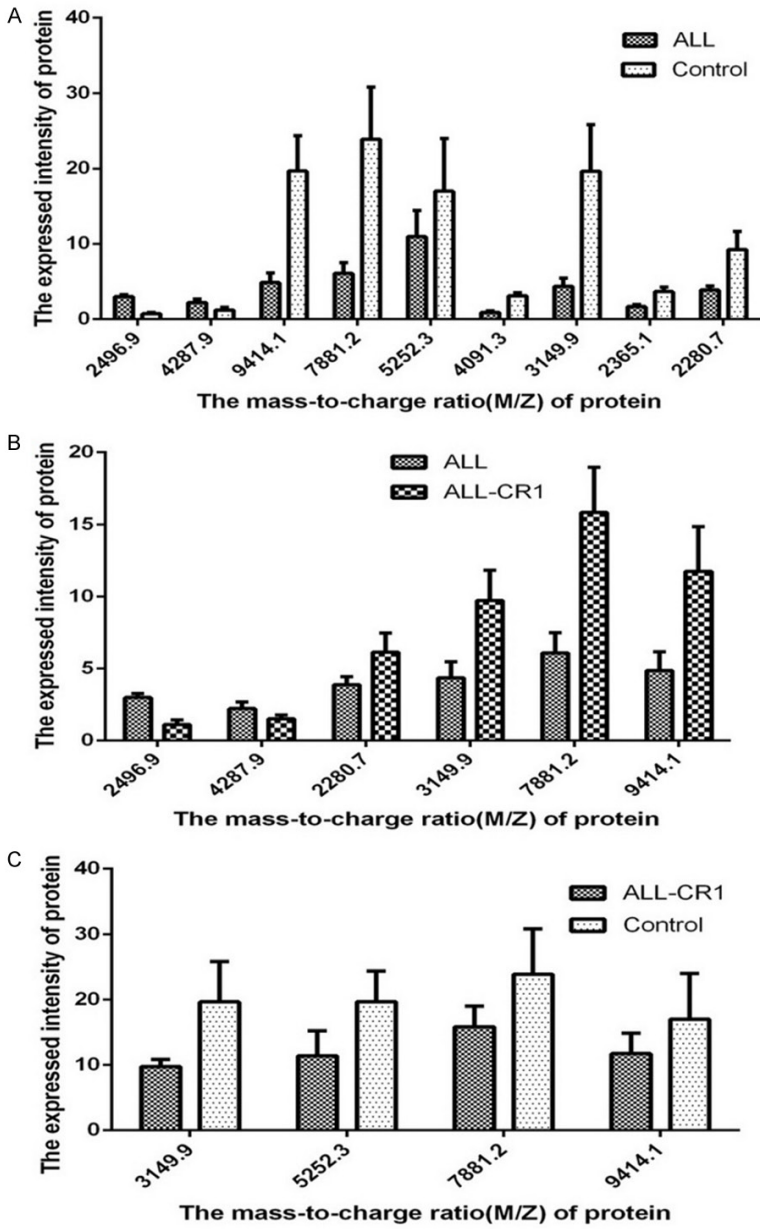

Figure 2. Comparison of protein peak intensity between ALL and control group (A), between ALL and ALL-CR1 group (B), and between ALL-CR1 and control group (C).

mal karyotypes (70.4%), while 8 cases had the abnormal karyotypes (29.6%); (iv) Molecular biology (molecular, M), 2 cases exhibited the positive of BCR-ABL fusion gene (7.4%), and 1 case exhibited the positive of MLL-AF4 fusion gene (3.7%); (v) Clinical risk classification, including 9 cases of high-risk type (33.3%), 8 cases of intermediate risk type (29.6%), and 10 cases of standard-risk type (37.0%). In ALL-CR1 group, the complete remission met the efficacy standards of acute leukemia (10). There were 12 males and 13 females, with age of 4.6±3.1 years. In control group, the children had normal indexes of peripheral blood examination. There were 15 males and 12 females, with age of 4.4±3.3 years. This study was approved by the ethics committee of Affiliated Hospital of Luzhou Medical College. Written informed consent was obtained from the family of all participants.

Separation of platelet samples and extraction of proteins

The negative-pressure blood lancet was used to extract 5 ml venous blood, which was performed with the anticoagulation in the vacuum blood sampling tube. 5 μL prostacyclin application solution was instantly added inside (with final concentration as 2.5 mM) to prevent the platelet activation. The mixture was then slightly mixed uniformly, and centrifuged at 800 rpm for 15 min. The upper layer plasma, which was about l/3 volume and rich of platelet, was then shifted into a new centrifuge tube. After adding 5 μl prostacyclin application solution, the plasma was centrifuged at 3000 rpm for 20 min, and then the upper plasma was discarded. 0.5 ml 7% ACD solution (citrate dextrose) (117 mM sodium citrate, 282 mM glucose and 78 mM citrate) was added inside to re-suspend the platelets, followed by centrifugation at 3000 rpm for 20 min. The supernatant was discarded, and the residual was then rinsed by Tyrodes-HEPES solution (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3, 1 mM EGTA), indomethaein (10 M) and ACD (7%). The platelets were then re-suspended to the concentration of 2×10^9/ml or 1×10^9/ml for the incubation at room temperature for 30 min.

Table 1. Comparison of the 9 significant protein mass peaks among three groups (mean ± SD)

M/z	ALL group	ALL-CR1 group	Control group
2496.9↑	2.99±0.27	1.12±0.30[a]	0.72±0.19[a]
4287.9↑	2.22±0.45	1.52±0.27[a]	1.22±0.38[a]
9414.1↓	4.86±1.30	11.75±3.10[a]	19.67±4.71[a,b]
7881.2↓	6.07±1.41	15.84±3.1[a]	23.89±6.91[a,b]
5252.3↓	10.95±3.47	13.36±4.68[a]	16.99±6.98[a,b]
4091.3↓	0.91±0.19	1.97±1.31[a]	3.10±0.43[a,b]
3149.9↓	4.36±1.11	9.73±2.10	19.65±6.18[a,b]
2365.1↓	1.65±0.27	2.56±0.53	3.67±0.57[a,b]
2280.7↓	3.88±0.55	6.15±1.31[a]	9.24±2.4[a,b]

Note: [a]P < 0.05 compared with ALL group; [b]P < 0.05 compared with ALL-CR1 group. ↑: high expression; ↓: low expression.

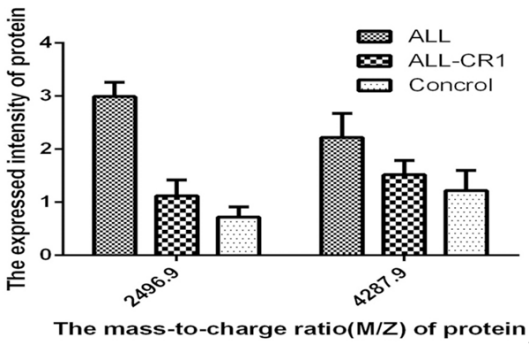

Figure 3. Comparison of 2 highly-expressed protein peaks among three groups.

After centrifugation at 15000 rpm, 2 μL/mL protease inhibitor was added, and the mixture was stored at -80°C. The isolated platelet concentration was detected by the blood cell automatic detector. The platelets were re-suspended in the lysis buffer, and the repeated frosting and thawing. The supernatant was collected for the protein content determination by the Bradford method, and the rest protein samples were stored at -80°C.

Chip pretreatment and detection of platelet protein spectrum

The SELDI-TOF-MS technique was used to obtain and analyze the whole platelet protein spectrum of each group. During the chip activation, 5 μL fused platelet lysate was added to the bottom of centrifuge tube, and 5 μL semi-saturated SPA was also added, followed by sufficient mixing and standing. 2 μL test sample was drawn and added to the well with the activated Au chip (Ciphergen Biosystems, USA). After drying, 1 μL semi-saturated SPA was added to each well for the detection. The protein Au chip was placed into the bioprocessor device, and 200 μL PBS was added to each well, followed by shocking for 3 times, 5 min for each time. The PBS was brushed off, and 200 μl HPLC water was added, followed by the quick dumping and patting-to-dry. The chips were then removed and dried at the room temperature, followed by the point-adding of 1 μl SPA, which was repeated after 10-min standing. The PBS II/C-type protein fingerprinting spectrometer (Ciphergen Biosystems, USA) was used to detect the platelet proteins which were bound to the protein chip surface. The range was optimized from 2000 to 20,000, and the highest molecular weight detected was set as 100,000, with the laser intensity of 215 and the detection sensitivity of 9. All-in-one protein standard molecule chips (Ciphergen Biosystems, USA) were used for correction with the mass deviation ≤ 0.1%. The Ciphergen Proteinchip software (Ciphergen Biosystems, USA) was used to automatically collect the original experimental data and store. The pre-treatment was performed for all the obtained platelet protein spectra.

Data analysis and protein screening identification

The Biomarker Wizard and Biomarker Pattern software were used to perform the data analysis, which used the analysis of variance to perform the preliminary statistical analysis towards the data obtained by the proteinchip software. The further analysis used the SPSS19.0 software to screen out the mass to charge ratios (m/z) of the platelet protein markers that had the statistical significance. The results were then input into the protein databases to get the corresponding proteins of these protein m/z peaks. The roles of screened differentially-expressed proteins in the platelet function were clarified, to the changes of protein expression were analyzed, for obtaining the molecular mechanism of platelet functional abnormality in ALL.

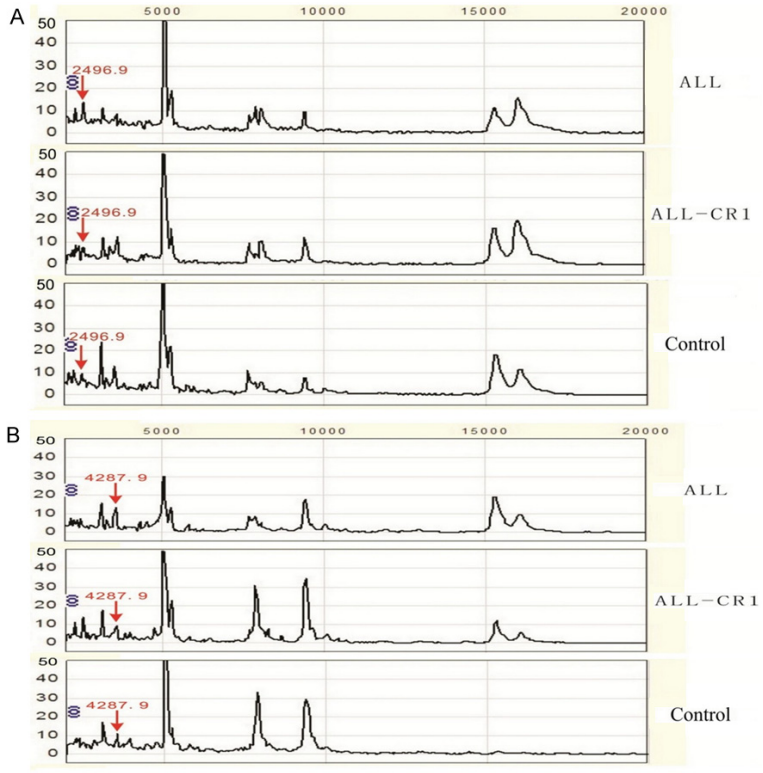

Figure 4. The platelet protein mass chromatograms of the protein peak with mass/charge ratio of 2496.9 (A) and 4287.9 (B) among three groups.

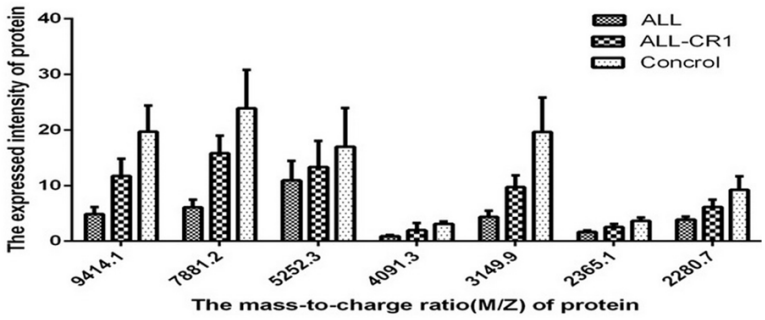

Figure 5. Comparison of 7 lowly expressed protein peaks among three groups.

Results

Changes of platelet count

The platelet counts of ALL group, ALL-CR1 group and control group were (34±24)×10^9/L, (168±32)×10^9/L and (177±29)×10^9/L, respectively. The ALL group exhibited the statistically significant differences with ALL-CR1 group and control group ($P < 0.05$), while the difference between the ALL-CR1 group and control group had no statistical significance ($P > 0.05$) (**Figure 1**).

Results of protein peaks detection

Compared with the control group, the ALL group had 176 protein peaks that were significantly different ($P < 0.05$), among which 25 protein peaks were highly expressed, and 151 were lowly expressed. When compared the variation coefficient (CV) of the aver age protein peaks between these 2 groups (CV = SD/Mean×100%), it could be seen that there were 9 proteins that could be stably expressed and had the small CV between the two groups, among which 2 were highly expressed, namely m/z as 2496.9 and 4287.9, and 7 were lowly expressed, namely m/z as 9414.1, 7881.2, 5252.3, 4091.3, 3149.9, 2365.1 and 2280.7 (**Figure 2A**).

Compared with the ALL-CR1 group, the ALL group had 112 significantly different protein peaks ($P < 0.05$), among which 15 were highly expressed and 97 were lowly expressed. There were 6 proteins stably expressed with the smallest CV between the two groups, among which 2 were highly expressed, with the m/z as 4287.9 and 2496.9, and 4 were lowly expressed, with the m/z as 9414.1, 7881.2, 3149.9 and 2280.7 (**Figure 2B**).

Compared with the control group, the ALL-CR1 group had 69 protein peaks that exhibited the significant differences ($P < 0.05$), and all were lowly expressed, 4 proteins were stably expressed and had the smallest CV between the two groups, with m/z as 9414.1, 7881.2, 5252.3 and 3149.9, respectively (**Figure 2C**).

The analysis towards the 9 protein peak between the ALL group and the control group revealed that, the 2 highly-expressed protein peaks also exhibited the significant differences during the comparison between the ALL group

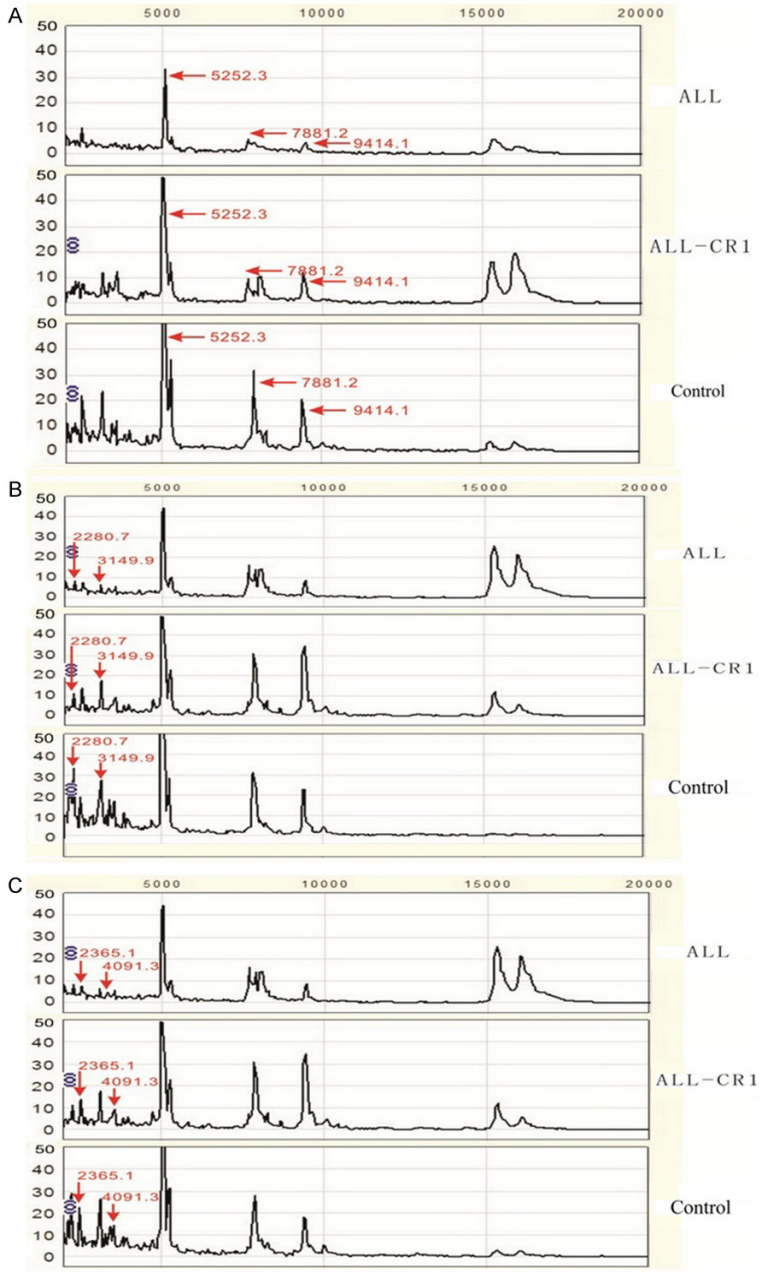

Figure 6. A: The platelet protein mass chromatograms of the protein peaks with mass/charge ratio of 5252.3, 7881.2 and 9414.1 among three groups; B: The platelet protein mass chromatograms of the protein peaks with mass/charge ratio of 2280.7 and 3149.9 among three groups; C: The platelet protein mass chromatograms of the protein peaks with mass/charge ratio of 2365.1 and 4091.3 among three groups.

and the ALL-CR1 group ($P < 0.05$), while the difference between the ALL-CR1 group and the control group was not statistically significant ($P > 0.05$) (**Table 1**; **Figures 3**, **4**). The 7 lowly expressed protein peaks were still lowly expressed in the ALL-CR1 group, and the difference was statistically significant when compared with the ALL group ($P < 0.05$). This indicated that, the functional improvement might exist, but there still existed the differences when compared with the control group ($P < 0.05$). The functions might not return to the normal (**Table 1**; **Figures 5** and **6**).

Results of protein identification

The m/z values of 9 differential proteins were detected between ALL group and control group, with 4 differential proteins between ALL group and ALL-CR1 group and 6 differential proteins between ALL-CR1 group and control group. They were input in the protein databases for the corresponding proteins towards these protein peaks. The differentially expressed protein peaks were predicted to be endothelin-1, big endothelin-1, PF4, thrombin light chain, pituitary adenylate cyclase activating polypeptide 27, fibrinogen β chain and 3 unknown proteins (**Table 2**).

Discussion

The platelet is the important part of blood, and generated from the megakaryocytes of bone marrow. Since the platelet has no nucleus and only a trace amount of mRNA, it could not be detected through the large quantities of cultivation or the conventional DNA recombinant technology, thus the research of platelets is a huge challenge towards the biologists. Due to the limited detection technology, the platelet research lags far behind the other nucleated cells [10]. The platelet proteome could be used to resolve the complex life processes of platelet proteome: base on the platelet, the specific proteins expressed by platelets could be identified, the platelet sig-

Table 2. Differences in protein database search results and related parameters of ALL patients

Number	M/z	Protein	PI	Protein functions
EDN1_HUMAN (P05305)	2496.9	Endothelin-1	4.54	Cytokine activity, endothelin receptor binding
EDN1_HUMAN (P05305)	4287.9	Big endothelin-1	5.31	Cytokine activity, endothelin receptor binding
FIBB_BOVIN (P02676)	2365.1	Fibrinogen β chain	4.37	Thrombospondin receptor activity
PF4V_HUMAN (P10720)	7881.2	Platelet factor 4	7.91	Platelet activation
THRB_HUMAN (P00734)	4091.3	Thrombin light chain	4.65	Blood coagulation, hemostasis
PACA_HUMAN (P18509)	3149.9	Pituitary adenylate cyclaseactivating polypeptide 27	9.69	Receptor signaling protein activity
	9414.9	Unknown		
	2280.7	Unknown		
	5252.3	Unknown		

Note: PI, isoelectric point.

nals or metabolic pathway might be analyzed, as well as the functional changes of platelet protein in the normal and pathological states could be analyzed, thus it could greatly improve our understanding towards the biological functions of platelet. The proteomics technology has identified more than 1100 kinds of platelet proteins, and many sub- proteomes have been distinguished, and proteomics data inside the comprehensive database has become the increasingly useful [11]. The identification of these proteins could help to elucidate the processes that involve the platelet, such as bleeding, coagulation disorders, and molecular mechanisms of transplantation treatment and regulation of blood vessel generation. As the important component of proteomics, the SELDI-TOF-MS technology could directly detect the proteomes of body fluid expression, such as blood, urine and cerebrospinal fluid, etc., and it has characteristics such as fastness, easiness, with less amount requirement and higher throughput, thus it has been widely used in the early diagnosis and monitoring of various diseases, including the hematologic diseases [12-15]. In the early stage, our group had applied the proteome into a variety of diseases and successfully detected the specific protein peaks [16-18], and we also applied the SELDI-TOF-MS technology towards the serum proteins detection of ALL children, and found that the serum proteomic of the ALL children had significantly different m/z (2770.43) when compared with the normal children, which might be the serum protein biomarker of ALL, so the SELDI-TOF-MS technology could provide a new method towards the monitoring of minimal residual disease after the complete remission of ALL [19].

ALL is the most common malignant hematological cancer disease in the childhood, accounting for 30% of malignant tumors which happen in the under-14-year-old children, and seriously threatening the lives of children. The bleeding is the one of most common clinical symptoms and death causes during the ALL incidence and chemotherapy [20]. A severe thrombocytopenia is an important factor that leads to the bleeding, and there are data showing that various types of acute leukemia occurred not only the decreasing of platelet counting, but also the abnormality of platelet functions. However, when the platelet counting is at a very low level, the detection of its functions would be limited, thus the platelet functions and bleeding risk would be difficult to be assessed when the platelets are in low levels [21]. There has been no report about the platelet proteomics in the ALL status. In this study, SELDI-TOF-MS technology was used to research the differences of platelet protein expressions before and after the ALL children's chemotherapy, as well as between the ALL children and the normal children, the results revealed that, there existed 176 differentially expressed proteins peaks between the ALL group and the control group, indicating that there were significant differences in the platelet proteome of peripheral blood between the ALL children and the normal children; and there were 112 significantly different protein peaks between the ALL group and the ALL-CR1 group. At the same time, there were 69 different protein peaks between the ALL-CR1 group and the control group, which were all lowly expressed, while there was no highly expressed protein peak, and the number of lowly expressed protein peaks that were statistically significant in the ALL-CR1 group was significantly reduced than the ALL group, indicating that the platelet functions of these ALL children might have certain improvements after the treatment, but there were still partial lowly expressed protein peaks, indicating that the

expressions of these proteins might not return to normal state yet.

Through this experiment, a total of 9 differentially expressed proteins between the ALL group and the control group were screened, among which 2 were highly expressed, and 7 were lowly expressed. Through the analysis towards these proteins, the 2 proteins significantly highly expressed were identified as endothelin-1 (ET-1), with the m/z as 4287.9, and the big endothelin-1, with the m/z as 2496.9. ET could not only exhibit the role of vasoconstriction, but also promote the mitosis, connecting with the growth, differentiation and metastasis of a variety of tumors; at the same time, it could induce the tumor angiogenesis [22, 23]. Wei *et al*. [24] found that when the AL children developed, the plasma ET was significantly higher than normal children. Wang *et al*. [25] found that when the AL children were accompanied with the bleeding, the ET levels were significantly higher than those without the bleeding, thus it revealed that ET might be involved in the processed of bleeding and coagulation. This study found that, ET existed in the platelet proteins of ALL children, with significantly increased level. The mechanism was not clear. The increased ET might be induced by the leukemia cells, and it would interact with the tumor cells, thus resulting in the abnormal clotting functions. The m/z of the 7 lowly expressed proteins were 7881.2, 4091.3, 3149.9, 2365.0, 2280.7, 9414.1, 5252.3, which were identified as PF4, thrombin light chain , pituitary adenylate cyclase-activating polypeptide 27, fibrinogen β chain and three unknown proteins. PF4 is a cytokine with small molecular weight, belonging to the CXC chemokine family, and plays an important role in regulating the inflammation and angiogenesis [26, 27]. Shi *et al*. [15] found by the proteomic analysis that the serum PF4 protein-corresponded peak in ALL was significantly reduced, which could be used as the potential protein biomarker to distinguish ALL, AML and the control group. This study found that the expression level of platelet PF-4 of the ALL group was low, though it was increased after the chemotherapy than the ALL group, it still existed the difference with the normal group. The low level of PF4 might be caused by the affected platelet activation functions. Kim *et al*. [28] found that in the AML-CR patients, the PF4 levels in the peripheral blood were significantly higher than the patients before the treatment and with the partial remission, the serum PF4 > 2.492 μg/ml might indicate that the platelet counting in the peripheral blood returned to > 100×10^9/L, and it could be used as a good indicator to evaluate the number restoring in the ALL complete remission period.

The thrombin light chain and fibrinogen β chain are involved in the normal coagulation process, and closely related to the platelets. These 2 proteins are both involved in the normal coagulation process. The experiment showed that the expressions of them were reduced, indicating that there was the abnormal coagulation in the pathogenesis of ALL, and this abnormality was presented not only by the bleeding, but also the thrombus formation. Giordano *et al*. [29] found that the laboratory indexes of newly diagnosed ALL children exhibited the increasing of thrombin generation and fibrin formation, inhibited fibrinolysis and activated endothelial functions and inflammations. But the specific roles of thrombin -like structure proteins within the platelet are still unclear, and their relationships with ALL still need the further exploration. The pituitary adenylate cyclase polypeptide 27 is a protease that exhibits the high activation role towards the pituitary adenylate cyclase. This protein may be related to the signal transduction pathway, but the exact mechanism is still unclear. This study found that in the ALL group, adenylate cyclase polypeptide 27 was lowly expressed, and might be associated with the disorder of platelet signal transduction pathway. This experiment also found 3 protein peaks that were significantly expressed, with the m/z as 2280.7, 9414.1 and 5252.3, respectively, while the corresponding proteins could not be found in the proteome database, these unknown proteins might be closely related to the platelet functions.

In this study, the platelet proteomics was used to study the platelet protein expressions in ALL, but the collected experimental cases were fewer, thus the results might not be comprehensive. In the future we should study the platelet proteins from various angles, and explore the changes of protein expressions at the different ALL periods. In addition, we should purify the proteins for the analysis and identification. This can help us to further clarify the mechanisms of diseases, providing a basis for

disease diagnosis, treatment monitoring and prognosis judgment.

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Disclosure of conflict of interest

None.

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