Identification and Validation of Serum Autoantibodies in Children with B-cell Acute Lymphoblastic Leukemia by Serological Proteome Analysis

Runhong Yu (yurunhong@163.com)
Henan Provincial People's Hospital https://orcid.org/0000-0002-1010-3543
Shiwei Yang
Henan Provincial People's Hospital
Yufeng Liu
Zhengzhou University First Affiliated Hospital
Zunmin Zhu
Henan Provincial People's Hospital

Research Article

Keywords: B-cell ALL,autoantibody,acute lymphoblastic leukemia,serological proteome analysis,children

Posted Date: September 7th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-867969/v1

Version of Record: A version of this preprint was published at Proteome Science on February 2nd, 2022.
See the published version at https://doi.org/10.1186/s12953-021-00184-w.

Abstract

Purpose: Study was by intention to screen serum autoantibodies that may contribute to the early detection of B-cell acute lymphoblastic leukemia (B-ALL) in children.

Patients and methods: The total protein from three pooled B-ALL cell lines(NALM-6, REH and BALL-1 cells) was separated using two-dimensional gel electrophoresis(2-DE), which was followed by Western blot by mixed serum from B-ALL patients (n=20) or healthy children(n=20).

We obtained and analyzed the images of 2-D gel and Western blot by PDQuest software,and then identify the spots of immune responses in B-ALL samples compared with those in control samples.The proteins from spots were identified using mass spectrometry (MS). The autoantibodies against α-enolase and voltage-dependent anion-selective channel protein 1(VDAC1) were further validated on the use of enzyme-linked immunosorbent assay(ELISA). The protein expression levels of the candidate antigens α-enolase and VDAC1 in B-ALL were thoroughly studied by immunohistochemical analysis.

Results: Six protein dots were identified with MS as Aconitase,apoptosis-inducing factor(AIF),dihydrolipoamide dehydrogenase(DLD), α-enolase,medium-chain acyl-CoA dehydrogenase(MCAD) and VDAC 1.The frequencies of autoantibodies against α-enolase and VDAC1 in children with B-ALL were 27% and 23%, respectively, which were significantly higher than those in normal controls(4% and 0). Immunohistochemical analysis showed the expression of α-enolase and VDAC1 was positive in 95% and 85% of B-ALL patients, respectively, but negative expression levels were showed in the control group.

Conclusion: This study incidates that α-enolase and VDAC1 may be the antigen associated with B-ALL .α-enolase and VDAC1 autoantibodies may develop into potential serological markers of B-ALL in children.Other proteins also need to be confirmed in a large number of serum samples.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common type of neoplasms in childhood(children aged 0–14 years),and its incidence peaks between the ages of 1 and 4 years[1,2]. 85% of pediatric ALL patients are of the B-cell lineage, and 15% are of the T-cell lineage[3].Currently, bone marrow aspiration is used for diagnosis. However, because of the substantial damage this approach causes, the majority of children exhibit significant psychological pressure regarding this examination. In recent years, with the administration of stronger chemotherapy, the prognosis of ALL has been greatly improved,while approximately 20% of patients with ALL still experience relapse, despite intensive chemotherapy; additionally, traditional chemotherapy drugs cause toxic side effects,which may lead to death[1,3].Therefore, there is an urgent need to find biomarkers which are noninvasive and specific for diagnosis and targeted therapy of pediatric B-ALL.

Mutated or aberrantly expressed proteins are produced in the process of tumorigenesis and progression of neoplasms,and these proteins are capable of eliciting an immunological reaction,which leads to the generation of autoantibodies[4].At present, vailable technologies couldn't detect tumor-associated antigens (TAAs) at low levels during the early stages of tumor growth; nevertheless, a large amount of autoantibodies which are existent may be detected for months or years before the clinical confirmation of premalignantcancer[5]. Autoantibodies have been suggested in the serum of patients with a wide variety of tumor and have shown possible for use as biomarkers for tumor diagnosis[6].A large number of studies have confirmed that autoantibodies were also involved in the development of tumors. For example, serum p53 antibody was detected in the serum of about 30% patients with colon cancer, lung cancer, breast cancer and hepatocarcinoma[7].The diagnostic specificity of serum pe53 antibody may be as high as 96%[7].

Therefore, we concentrated on screening autoantibodies as serum biomarkers of B-ALL using serological proteome analysis (SERPA) with combinations of 2-DE gel electrophoresis, immunoblotting and MS in this study.In recent decades, autoantibodies against TAAs were identified by SERPA in various diseases, containing hepatocellular carcinoma[8], colorectal cancer[9], cholangiocarcinomas[10],lung cancer[11],gallbladder carcinoma[12], prostate cancer[13], type 1 diabetes[14] and primary open angle glaucoma[15].However, as far as we know,there is no report on the screening of autoantibodies in children with B-ALL.The examination of serum autoantibodies could not only contribute to the diagnosis of B-ALL but also faciliate the development of targeted therapy. The main objective of this study was to screen new TAAs in B-ALL cell lines and confirm related autoantibodies in the serum from children with B-ALL by applying SERPA.

Material And Methods

Participants

The participants were recruited from the First Affiliated Hospital of Zhengzhou University. The study was performed according to the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the Department of Medicine of the First Affiliated Hospital of Zhengzhou University. Informed consent in writing was obtained from the parents or guardians before the initiation of this study. The primary ALL diagnosis was based on 2016 WHO classification. Age-matched healthy controls were recruited from hospital outpatient clinics. All participants were younger than 14 years of age. Basic clinical characteristics of patients and control subjects are described in Table 1.All serum and bone marrow samples were discarded after clinical use.

Table 1
Basic clinical characteristics of patients and control subjects

	Discovery stage		Validation stage	
	B-ALL	Healthy children	B-ALL	Healthy children
Number	20	20	30	25
Male	10	10	18	13
Female	10	10	12	12
Median age, years (range)	4(2 months-13 years)	6(1year-14 years)	4(2 months-13 years)	6(1year-14 years)

Cell culture and cell extracts

Three human B-ALL cell lines, namely, NALM-6, REH and BALL-1 cells, were obtained from DSMZ(Deutsche Sammlung von Mikroorganismen und Zellkulturen,Germany),the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and the Institutes of Biomedical Sciences of Fudan University,respectively.The B-ALL cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (BSA,Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml streptomycin and 100 U/ml penicillin.All the cells were incubated at 37°C in an atmosphere of 5% CO_2.The total proteins were extracted from the cell lines. The cells were harvested and washed 3 times with phosphate-buffered saline (PBS) at 800 × rpm for 10 min and incubated for 30 min at 4°C in SF cell lysis buffer (7 M urea, 40 mM Tris, 2 M thiourea, 2% CHAPS and 1% protease inhibitor cocktail(Roche, Germany)), followed by sonication at 20% amplitude (3×10 s). The cell lysates were then spun at 14000 × rpm for 40 min, and the supernatants were collected. The proteins from the cell lines were purified by the 2-D Clean up kit (GE Healthcare).The final protein concentration was quantified by Bradford assay. Proteins were extracted from all three B-ALL cell lines as described above. Finally, equal amounts of proteins were mixed for further experiments.

2D gel electrophoresis and Western blotting

Protein extracts (200 μg) from cultured cells were lysed in rehydration buffer(2 M thiourea, 7 M urea, 2% CHAPS, 0.5 % (v/v) immobilized pH gradient (IPG) buffer at pH 3–10,1.5 % (w/v) DTT and 0.002% bromophenol blue), which was used for passive rehydration of 7-cm, pH 3–10 nonlinear IPG strips(Bio-Rad, USA) by incubation at room temperature for 12 h in a strip holder; then, the samples were subjected to isoelectric focusing gel electrophoresis (the first-dimension gel).IEF was conducted at 50 μA/IPG strip, 250 V for 1.5 h, 1,000 V for 1 h, 4,000 V for 2 h, and 4000 V for 32,000 V-h. After focusing, the IPG strips were incubated in equilibration buffer(50 mM Tris-HCl buffer (pH 8.8),6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS) and 0.002% bromophenol blue) with 1% DTT for 15 min and then in equilibration buffer with 2.5% iodoacetamide for 15 min (Bio-Rad protocol).The treated gel strips were loaded onto a second-dimensional gel and subjected to 10 mA/gel. At this point, the gels were stained using Coomassie

blue dye method,by which proteins were visualized, or the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. After transfer, the PVDF membranes were blocked with blocking buffer (5.0% nonfat milk in Tris-buffered saline containing 0.1% Tween-20;TBST)and then incubated overnight at 4°C with diluted sera from B-ALL patients(n = 20) or normal controls(n = 20) as primary antibodies at a 1:200 dilution. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (GE, USA) as a secondary antibody at a 1:10000 dilution for 1.0 h at room temperature. The immunoreactive spots on the PVDF membranes were detected by an enhanced chemiluminescence kit (ECL Plus™ Western Blotting Detection kit, GE Healthcare, USA) according to the manufacturer's instructions. The images were acquired using the Luminescent Image Analyzer LAS-3000 v2.2.

In-gel digestion

The images of 2-D immunoblot were compared with the Coomassie Stained images ,by which the corresponding immunoreactive protein spots on the 2-D gel were identified with an ImageMaster 2D Elite 4.01 Software. The Coomassie-stained spots that were detected by the B-ALL serum but not by the healthy controlserum were manually excised. The excised gel fragments were destained with 200 µl of destainer, followed by the addition of 200 µl of 100% acetonitrile. After acetonitrile was cleared, the dried gel slices were digested with 0.01 µg/µl trypsin in 20 mM NH_4HCO_3 overnight at 37°C. Peptides were extracted with extraction buffer(5% formic acid in 50% acetonitrile) and incubated for 30 min. This procedure was repeated twice, and the extracted peptides were pooled and concentrated to complete dryness before MS analysis.

Mass spectrometry analysis

Each peptide sample was covered with 0.8 µL of 5mg/ml CHCA(α-cyano-4-hydroxycinnamic acid) solubilized in 50% acetonitrile and 0.1% trifluoroacetic acidand then spotted on a MALDI plate. The spotted samples were submitted for data acquisition on a 5800 MALDI-TOF/TOF mass spectrometer (AB SCIEX, CA). MS spectra were acquired from 700 to 3600 m/z for a total of 1000 laser shots. Laser intensity remained fixed for all the analyses. MS/MS analyses were performed using 2 kV collision energy with air as CID gas.Metastable ions were suppressed for a total of 1000 laser shots.Spectra analysis was performed manually.

Data analysis

All the MS/MS spectra were searched against the NCBI database using the Mascot server(version 2.3.2.0,Matrix Science, London, UK) search engine with scores of proteins above 71.The search parameters for the searches were as follows: NCBI database (created in December 2011) restricted to *Homo sapiens*;enzyme: trypsin;max missed cleavages: 1; peptide tolerance: 100 ppm; MS/MS tolerance:0.6 Da; variable modifications: oxidation (M). All the MS/MS spectra were manually verified.

ELISA for autoantibodies

The antigenic proteins α-enolase and VDAC1 were diluted to final concentrations of 1.0 μg/ml. α-enolase and VDAC1 were incubated in 96-well microplates in coating buffer at 4°C overnight,then free unbound sites were blocked with 1% BSA for 2 h at 37°C. The plates were incubated with human serum samples diluted at 1:100 with PBS for 1 h at 37°C, then they were washed and incubated with 100 μl of horseradish peroxidase(HRP)-conjugated goat anti-human IgG diluted at 1:5000 with PBS(Santa Cruz Biotechnology) for 30 min at 37°C. TMB substrate was added and incubated for 5 min at room temperature,and this reaction was terminated by adding the stop solution. The absorbance was measured by spectrophotometry at a wavelength of 450 nm.

Immunohistochemical analysis of α-enolase and VDAC1

We study the protein expression of α-enolase and VDAC1 in 20 children with B-ALL and 10 healthy controls(reviews in the convalescent phases of children with primary immune thrombocytopenia) by immunohistochemical analysis. A few drops of bone marrow(BM) were smeared directly onto slides,and BM slides were fixed with 4% paraformaldehyde fixative solution.

The blocked sections were incubated with primary antibodies against α-enolase (dilution 1: 200) and VDAC1 (dilution 1: 200) overnight at 4°C,and then they were incubated with secondary antibodies using polyperoxidase-conjugated anti-mouse/rabbit IgG (dilution 1:200) at room temperature for 30 min.The BM slides were incubated with freshly prepared 3,3'-diaminobenzidine working solution at room temperature for 5 min,counterstained with hematoxylin, and dehydrated. The distribution of leukemia cell staining was scored as 0(< 10% cell staining), 1 (10–40% cell staining),2 (40–70% cell staining) or 3 (≥ 70% cell staining).The staining intensity of leukemia cells scored as 0, 1, 2 and 3 indicated negative, light yellow, brownish yellow and brown staining in the cytoplasm and/or nucleus, respectively. Total scores of 0–1, 2, 3–4, and 5–6 indicated (-),(+),(+ +) and (+ + +), respectively. In this study, (+-+++) was classified as positive expression of α-enolase and VDAC1 proteins. Two experienced pathologists who blindly examined the specimens performed immunohistochemical analysis.

Statistical analysis

Statistical analyses were performed applying SPSS software version 20.0 (IBM Corp., NY, USA).The data were evaluated using the chi-square test, and comparisons between two groups were performed using an independent t-test, two-tailed. Significance was defined as a p value < 0.05.

Results

Identification of autoantibodies by SERPA

To identify serological autoantibodies against antigens from B-ALL cells, a mixture of the total proteins from three human B-ALL cells was separated by 2-DE and transferred onto PVDF membranes or visualized by Coomassie blue staining (Fig. 1A).Subsequently the PVDF membranes were incubated with pooled serum from 20 B-ALL patients or 20 matched normal controls. The antigenic protein profiles of each 2-D immunoblot were compared and matched to the original 2-DE. As a consequence,a total of 6

protein spots were significantly and specifically recognized by serum from B-ALL children, but no reactive spots were observed in the samples probed with serum from normal controls(Figs. 1B and 1C). Then, the proteins were identified by MS. Six autoantigens were identified, including aconitase, apoptosis-inducing factor(AIF), dihydrolipoamide dehydrogenase(DLD),α-enolase, medium-chain acyl-CoA dehydrogenase(MCAD),and VDAC1,and these antigens were subjected to further validation by ELISA.Information on the six identified proteins is presented in Table 2. Figure 2 shows that the peptide mass fingerprinting (PMF) map and MS/MS map of protein spots 4 and 6. The significance of proteins for further clinical verifications were performed based on literature searching.

Table 2
Summary of identified proteins from immunoreactive protein spots by MS analysis

Spot No.	Accession No.	Protein name	Theoretical pI/Mw	No.of Peptides matched	Score
1	gi|20072188	Aconitase	7.62/85564.57	30	257
2	gi|4323587	AIF	9.04/66900.61	15	116
3	gi|71042410	DLD	6.35/50147.55	15	122
4	gi|119339	Alpha-enolase	7.01/47168.96	17	253
5	gi|7542837	MCAD	8.57/47007.78	24	274
6	gi|130683	VDAC1	8.62/30772.60	11	170

Clinical validation by ELISA using recombinant proteins

The α-enolase and VDAC1 proteins were selected based on their important roles in tumor according to previous reports [16–19].To confirm the value of the autoantibodies for detecting B-ALL,we performed clinical verification for α-enolase and VDAC1 which were identified presenting immunoreactivity with sera from B-ALL patients. Clinical verification using individual serum samples from 30 patients with B-ALL and 25 healthy children showed the levels of autoantibodies against α-enolase and VDAC1 (Table 3).The results suggested increased levels of autoantibody against α-enolase and VDAC1 in B-ALL patients in comparison to controls. We demonstrated that autoantibody positivity against α-enolase and VDAC1 was observed in sera from 27% (8/30)and 7 of 30 (23%) children with B-ALL,which was significantly higher than that in healthy controls.

Table 3
Frequency of autoantibodies against α-enolase and VDAC1 in serum by ELISA

Group	n	Autoantibody positive(%)	
		ENO1	VDAC1
B-ALL patients	30	8 (27%)	7(23%)
Healthy controls	25	1(4%)	0(0/25)

Immunohistochemical detection of α-enolase and VDAC1

The expression of α-enolase and VDAC1 in bone marrow smears of 20 children with B-ALL and 10 healthy controls(reviews in the convalescent phases of children with primary immune thrombocytopenia) were performed by Immunohistochemical analysis. The expression of α-enolase and VDAC1 was positive in 95% and 85% of B-ALL patients, respectively, but the control group presented negative (Fig. 3).α-enolase and VDAC1 proteins were mainly expressed in the cytoplasm of cells.

Discussion

We usually observe that the prognosis of children with B-ALL with the same risk at diagnosis is significantly different in clinical practice. The prognosis of B-ALL is not only related to various factors, such as age, sex, and molecular genetic characteristics,but also closely associated with host immune function.Reports have demonstrated that the autoantibodies produce in the transition to precancer[20]. Different from other markers, autoantibodies emerged in the early process of oncogenesis, and they are shown in serum before TAAs can be detected[21];thus the examination of tumor autoantibodies using minimally invasive methods, has huge potentiality in early diagnosis, especially for asymptomatic patients.This can facilitate early detection and treatment, thereby protecting patients from early death.

Growing evidence has confirmed that there is a specific immune response in tumor patients,including patients with prostate cancer,hepatocellular carcinoma, breast cancer,lung cancer,pancreatic cancer and so on[22].In the earliest stage of leukemia, the number of leukemic cells in the peripheral blood is very small, and the patients have almost no clinical symptoms.It is difficult to detect leukemia-associated antigens in peripheral blood due to their low expression, but pathological changes could be detected by the immune system from the earliest stages.Immune system responds to them quickly by producing a large number of specific autoantibodies against antigens. The discovery of novel B-ALL antigens not only helps to explain the molecular mechanism underlying the occurrence and development of B-ALL in children but also can be used as a target for immunotherapy of B-ALL, and the corresponding autoantibodies can be useful as serum biomarkers for early diagnosis, disease monitoring and assessment of B-ALL prognosis.

SERPA has been widely applied as a hopeful means for screening and identifying all components of immunoreactive proteins on the basis of 2-DE,immunoblotting, and MS[23]. Autoantigens were confirmed

by SERPA in a variety of illnesses, containing gastric cancer[24],colorectal cancer[9],lung cancer[11],gallbladdercarcinoma[12], prostate cancer[13] and primary open angle glaucoma[15] in the past decade.In this study, mixed proteins from three B-ALL cell lines were dissolved by 2-DE, followed by Western blot analysis using mixed serum samples from children with B-ALL and healthy controls. As a result, 6 protein spots that were significantly and specifically recognized by serum from B-ALL patients were identified.In our SERPA analysis, aconitase,AIF, DLD,α-enolase, MCAD, and VDAC1 showed differential immunological reactions between children with B-ALL and healthy controls. To the best of our knowledge, we first showed that six proteins, aconitase,AIF,DLD,α-enolase, MCAD, and VDAC1, could induce the production of autoantibodies in B-ALL children. Based on literature searching of the identified proteins in patients with B-ALL or other cancers, we found that among these six proteins, AIF[25],DLD[26], and α-enolase[27,28] can induce autoantibody production in other tumor patients, but there is no report about the autoantibody production induced by aconitase, VDAC1 and MCAD. Among the immunogenic proteins, α-enolase and VDAC1 (spot nos. 4 and 6) attracted our attention.In light of the above, we selected α-enolase and VDAC1 for clinical verification and analyzed the autoantibody levels in children with B-ALL by ELISA.We then verified the production of autoantibodies against α-enolase and VDAC1 in 30 children with B-ALL and 25 normal controls. In the validation stage, we proved that α-enolase and VDAC1 autoantibodies were new representative targets for distinguishing patients with B-ALL from normal controls. To the best of our knowledge,this study was the first report of the existence of autoantibodies against α-enolase and VDAC1 in serum from children with B-ALL by a proteomic approach,nevertheless, the α-enolase autoantibody was not all specific to B-ALL serum and were found in the serum of patients with other tumors,as well as α-enolase antibodies were found in some autoimmune diseases[29–31].The mechanism which α-enolase and VDAC1 induce immune response in B-ALL is unclear.

One of these proteins, α-enolase,found on the surface of cells,is involved in several key biological processes of cancer, including proliferation, migration and invasion[32,33].According to previous reports,α-enolase is overexpressed in multiple tumors, including lung cancer[16],hepatocellular carcinoma[34],pancreatic cancer[19],and gastric cancer patients[35], and has been proposed as a biomarker for early detection and prognosis[36].Several reports have mentioned α-enolase as a tumor-associated antigen that induces autoantibody production in malignant tumors[27,28].Autoantibodies against α-enolase have also been previously reported in acute leukemia[37],chronic myeloid leukemia[38], non-Hodgkin's lymphoma[39] and other solid tumors[36].A recent study demonstrated that α-enolase and Annexin A1 autoantibodies could enhance diagnostic performance in lung cancers by combining CEA and CA125[28].Another study suggested that α-enolase could accelerate metastasis of lung cancer cell through HGFR and WNTsignaling pathway and presented a new antibody targeting α-enolase in lung cancer[33]. In multiple myeloma, α-enolase expression negatively correlated with overall survival,and targeting α-enolase can enhance immunity and improve outcome[40].Cui et al. found that positivity of α-enolase autoantibodies was observed in serum from 18 of 21 (86%) patients with acute leukemia(AL) and 20 of 22(90%) healthy controls[37].However,we found that α-enolase autoantibodies were detected in serum from

8 of 30 (27%) children with ALL and 1 of 25(4%) healthy controls.The reasons for the differences described above may be related to subjects in the different age and leukemia groups.

Similarly, we also selected another protein, VDAC1, for verification. We demonstrated that positivity for autoantibodies against VDAC1 was observed in sera from 7 of 30 (23%) children with ALL, but no such activity was observed in 25 (0%) normal children. VDAC1,which mainly located in the outer membrane of mitochondria,can control cell growth,energy production and Ca^{2+} homeostasis[41,42]. Moreover, VDAC1 also regulates apoptosis by mediating the release of apoptotic proteins from mitochondria and interacting with antiapoptotic proteins[17,41].The observation that VDAC1 is overexpressed in a variety of tumors shows that VDAC1 may be essential for cancer cell survival[18,41,43].Accumulating studies have proved that abrogation of VDAC1 expression significantly inhibits tumor growth in cancers[18,42–46],suggesting that VDAC1 may be a novel therapeutic target[47–50]. Furthermore, targeted drugs acting on VDAC1 against tumor growth and proliferation is a promising strategy for the treatment of cancer[51].However, as far as I know, VDAC1 has never previously been reported as an autoantigen and may become a new target antigen. Antibodies against VDAC1 can also be found in the serum of B-ALL children, indicating that VDAC1 triggers autoimmunity,and leads to elevation of VDAC1 autoantibody. However, the exact mechanisms require further study.Our results proved that VDAC1 may be a promising antigen which could be immunogenic and autoantibodies against VDAC1 might serve as a potential biomarker for B-ALL.

Aconitase, one of the other four proteins, was reported that mitochondrial aconitase activity played an important role in the development of advanced metastatic prostate cancer[52].AIF overexpression was found in a variety of malignant tumors,including uveal melanoma[53], pancreatic cancer[54],chronic lymphocytic leukemia[55], and diffuse large B-cell lymphoma[56]. Otherwise,downregulation of AIF was reported in testicular germ cell tumors[57] and renal cell carcinoma[58]. The relationship between AIF expression and disease prognosis has been reported in different kinds of tumors[53,55–57].In addition,Li et al found that there were autoantibodies against AIF in the serum of hepatocellular carcinoma using SERPA[25].A variety of evidences have shown that DLD is a mitochondrial enzyme which produces ROS and may be used an anticancer drug[59]. Yoneyama K et al found that DLD autoantibody may be a possible diagnostic marker for ovarian and endometrial cancer[26,60].The last protein,MCAD was upregulated in glioblastoma compared to normal brain and could protect mitochondria from lipid peroxidation[61].

Conclusion

In summary, except α-enolase, the other five proteins exist in mitochondria and are closely related to mitochondrial function in our study.This study suggested that α-enolase and VDAC1 autoantibodies have promising biomarkers for children with B-ALL, and measuring serum autoantibodies against α-enolase and VDAC1 may show promise for clinical application in terms of diagnosis, immunological surveillance,treatment and prognosis of children with B-ALL. However,the sample size of the B-ALL

patients used in this study was small. We will expand the samples for higher confidence in the results in the future.

Declarations

ACKNOWLEDGMENT

Thanks are due to Yan YANG with excellent technical assistance. This research was supported by the Natural Science Foundation of Henan Province, China (No. 162300410265).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All the data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS STATEMENT

Sample collection and data analysis were performed by Runhong Yu and Shiwei Yang. Runhong Yu performed 2D gel electrophoresis and Western blotting. Shiwei Yang performed the MS and statistical analysis. Yufeng Liu and Zunmin Zhu verified the statistical methods. The first draft of the manuscript was written by Runhong Yu. All authors commented on previous versions of the manuscript and approved the final manuscript.

ETHICS STATEMENT

The study was performed according to the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the Department of Medicine of the First Affiliated Hospital of Zhengzhou University. Informed consent in writing was obtained from the parents or guardians before the initiation of this study. All serum and bone marrow samples were discarded after clinical use.

References

1. Malard F, Mohty M. Acute lymphoblastic leukaemia. Lancet. 2020. 395(10230): 1146–1162.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin. 2020. 70(1): 7–30.

3. Inaba H, Mullighan CG. Pediatric acute lymphoblastic leukemia. Haematologica. 2020. Online ahead of print(11).
4. Casiano CA, Mediavilla-Varela M, Tan EM. Tumor-associated antigen arrays for the serological diagnosis of cancer. Mol Cell Proteomics. 2006. 5(10): 1745-59.
5. Macdonald IK, Parsy-Kowalska CB, Chapman CJ. Autoantibodies: Opportunities for Early Cancer Detection. Trends Cancer. 2017. 3(3): 198–213.
6. Tan HT, Low J, Lim SG, Chung MC. Serum autoantibodies as biomarkers for early cancer detection. FEBS J. 2009. 276(23): 6880 – 904.
7. Soussi T p53 Antibodies in the sera of patients with various types of cancer: a review. *Cancer Res*. 2000;60(7):1777–1788.
8. Looi KS, Nakayasu ES, Diaz RA, Tan EM, Almeida IC, Zhang JY. Using proteomic approach to identify tumor-associated antigens as markers in hepatocellular carcinoma. J Proteome Res. 2008. 7(9): 4004-12.
9. Wang H, Zhang B, Li X, et al. Identification and Validation of Novel Serum Autoantibody Biomarkers for Early Detection of Colorectal Cancer and Advanced Adenoma. Front Oncol. 2020. 10: 1081.
10. Mustafa MZ, Nguyen VH, Le Naour F, et al. Autoantibody signatures defined by serological proteome analysis in sera from patients with cholangiocarcinoma. J Transl Med. 2016. 14: 17.
11. Dai L, Li J, Tsay JJ, et al. Identification of autoantibodies to ECH1 and HNRNPA2B1 as potential biomarkers in the early detection of lung cancer. Oncoimmunology. 2017. 6(5): e1310359.
12. Akhtar J, Priya R, Jain V, et al. Immunoproteomics approach revealed elevated autoantibody levels against ANXA1 in early stage gallbladder carcinoma. BMC Cancer. 2020. 20(1): 1175.
13. Dai L, Li J, Xing M, Sanchez TW, Casiano CA, Zhang JY. Using Serological Proteome Analysis to Identify Serum Anti-Nucleophosmin 1 Autoantibody as a Potential Biomarker in European-American and African-American Patients With Prostate Cancer. Prostate. 2016. 76(15): 1375-86.
14. Massa O, Alessio M, Russo L, et al. Serological Proteome Analysis (SERPA) as a tool for the identification of new candidate autoantigens in type 1 diabetes. J Proteomics. 2013. 82: 263 – 73.
15. Beutgen VM, Perumal N, Pfeiffer N, Grus FH. Autoantibody Biomarker Discovery in Primary Open Angle Glaucoma Using Serological Proteome Analysis (SERPA). Front Immunol. 2019. 10: 381.
16. Chang GC, Liu KJ, Hsieh CL, et al. Identification of alpha-enolase as an autoantigen in lung cancer: its overexpression is associated with clinical outcomes. Clin Cancer Res. 2006. 12(19): 5746-54.
17. Shoshan-Barmatz V, Mizrachi D. VDAC1: from structure to cancer therapy. Front Oncol. 2012. 2: 164.
18. Arif T, Krelin Y, Nakdimon I, et al. VDAC1 is a molecular target in glioblastoma, with its depletion leading to reprogrammed metabolism and reversed oncogenic properties. Neuro Oncol. 2017. 19(7): 951–964.
19. Principe M, Borgoni S, Cascione M, et al. Alpha-enolase (ENO1) controls alpha v/beta 3 integrin expression and regulates pancreatic cancer adhesion, invasion, and metastasis. J Hematol Oncol. 2017. 10(1): 16.

20. Poletaev A, Pukhalenko A, Kukushkin A, Sviridov P. Detection of Early Cancer: Genetics or Immunology? Serum Autoantibody Profiles as Markers of Malignancy. Anticancer Agents Med Chem. 2015. 15(10): 1260-3.
21. Qiu J, Choi G, Li L, et al. Occurrence of autoantibodies to annexin I, 14-3-3 theta and LAMR1 in prediagnostic lung cancer sera. J Clin Oncol. 2008. 26(31): 5060-6.
22. Zhu Q, Liu M, Dai L, et al. Using immunoproteomics to identify tumor-associated antigens (TAAs) as biomarkers in cancer immunodiagnosis. Autoimmun Rev. 2013. 12(12): 1123-8.
23. Fulton KM, Ananchenko A, Wolfraim L, Martin S, Twine SM. Classical Immunoproteomics: Serological Proteome Analysis (SERPA) for Antigen Identification. Methods Mol Biol. 2019. 2024: 59–78.
24. Qin J, Yang Q, Ye H, et al. Using Serological Proteome Analysis to Identify and Evaluate Anti-GRP78 Autoantibody as Biomarker in the Detection of Gastric Cancer. J Oncol. 2020. 2020: 9430737.
25. Li L, Chen SH, Yu CH, Li YM, Wang SQ. Identification of hepatocellular-carcinoma-associated antigens and autoantibodies by serological proteome analysis combined with protein microarray. J Proteome Res. 2008. 7(2): 611 – 20.
26. Yoneyama K, Kojima S, Kodani Y, et al. Proteomic identification of autoantibodies in sera from patients with ovarian cancer as possible diagnostic biomarkers. Anticancer Res. 2015. 35(2): 881-9.
27. Qin J, Wang S, Shi J, et al. Using recursive partitioning approach to select tumor-associated antigens in immunodiagnosis of gastric adenocarcinoma. Cancer Sci. 2019. 110(6): 1829–1841.
28. Zang R, Li Y, Jin R, et al. Enhancement of diagnostic performance in lung cancers by combining CEA and CA125 with autoantibodies detection. Oncoimmunology. 2019. 8(10): e1625689.
29. Ye Y, Kuhn C, Kösters M, et al. Anti α-enolase antibody is a novel autoimmune biomarker for unexplained recurrent miscarriages. EBioMedicine. 2019. 41: 610–622.
30. Lu Y, Qin J, Xiang Y, et al. Experimental evidence for alpha enolase as one potential autoantigen in the pathogenesis of both autoimmune thyroiditis and its related encephalopathy. Int Immunopharmacol. 2020. 85: 106563.
31. Wei P, Xing Y, Li B, Chen F, Hua H. Proteomics-based analysis indicating α-enolase as a potential biomarker in primary Sjögren's syndrome. Gland Surg. 2020. 9(6): 2054–2063.
32. Zhan P, Zhao S, Yan H, et al. α-enolase promotes tumorigenesis and metastasis via regulating AMPK/mTOR pathway in colorectal cancer. *Mol Carcinog*. 2017;56(5):1427–1437.
33. Li HJ, Ke FY, Lin CC, et al. ENO1 Promotes Lung Cancer Metastasis via HGFR and WNT Signaling-Driven Epithelial-to-Mesenchymal Transition. *Cancer Res*. 2021;81(15):4094–4109.
34. Zhu W, Li H, Yu Y, et al. Enolase-1 serves as a biomarker of diagnosis and prognosis in hepatocellular carcinoma patients. Cancer Manag Res. 2018. 10: 5735–5745.
35. Sun L, Lu T, Tian K, et al. Alpha-enolase promotes gastric cancer cell proliferation and metastasis via regulating AKT signaling pathway. Eur J Pharmacol. 2019. 845: 8–15.

36. Zhang L, Wang H, Dong X. Diagnostic value of α-enolase expression and serum α-enolase autoantibody levels in lung cancer. J Bras Pneumol. 2018. 44(1): 18–23.
37. Cui JW, Li WH, Wang J, et al. Proteomics-based identification of human acute leukemia antigens that induce humoral immune response. Mol Cell Proteomics. 2005. 4(11): 1718-24.
38. Zou L, Wu Y, Pei L, et al. Identification of leukemia-associated antigens in chronic myeloid leukemia by proteomic analysis. Leuk Res. 2005. 29(12): 1387-91.
39. Ueda K, Nakanishi T, Shimizu A, Takubo T, Matsuura N. Identification of L-plastin autoantibody in plasma of patients with non-Hodgkin's lymphoma using a proteomics-based analysis. Ann Clin Biochem. 2008. 45(Pt 1): 65 – 9.
40. Ray A, Song Y, Du T, Chauhan D, Anderson KC. Preclinical validation of Alpha-Enolase (ENO1) as a novel immunometabolic target in multiple myeloma. *Oncogene*. 2020;39(13):2786–2796.
41. Shoshan-Barmatz V, Ben-Hail D, Admoni L, Krelin Y, Tripathi SS. The mitochondrial voltage-dependent anion channel 1 in tumor cells. Biochim Biophys Acta. 2015. 1848(10 Pt B): 2547-75.
42. Shteinfer-Kuzmine A, Verma A, Arif T, Aizenberg O, Paul A, Shoshan-Barmaz V. Mitochondria and nucleus cross-talk: Signaling in metabolism, apoptosis, and differentiation, and function in cancer. IUBMB Life. 2021. 73(3): 492–510.
43. Arif T, Vasilkovsky L, Refaely Y, Konson A, Shoshan-Barmatz V. Silencing VDAC1 Expression by siRNA Inhibits Cancer Cell Proliferation and Tumor Growth In Vivo. Mol Ther Nucleic Acids. 2014. 3(4): e159.
44. Arif T, Paul A, Krelin Y, Shteinfer-Kuzmine A, Shoshan-Barmatz V. Mitochondrial VDAC1 Silencing Leads to Metabolic Rewiring and the Reprogramming of Tumour Cells into Advanced Differentiated States. Cancers (Basel). 2018. 10(12).
45. Liu X, He B, Xu T, et al. MiR-490-3p Functions As a Tumor Suppressor by Inhibiting Oncogene VDAC1 Expression in Colorectal Cancer. J Cancer. 2018. 9(7): 1218–1230.
46. Arif T, Stern O, Pittala S, Chalifa-Caspi V, Shoshan-Barmatz V Rewiring of Cancer Cell Metabolism by Mitochondrial VDAC1 Depletion Results in Time-Dependent Tumor Reprogramming: Glioblastoma as a Proof of Concept. Cells. 2019. 8(11).
47. Leanza L, Romio M, Becker KA, et al. Direct Pharmacological Targeting of a Mitochondrial Ion Channel Selectively Kills Tumor Cells In Vivo. Cancer Cell. 2017. 31(4): 516–531.e10.
48. Reina S De Pinto V. Anti-Cancer Compounds Targeted to VDAC: Potential and Perspectives. Curr Med Chem. 2017. 24(40): 4447–4469.
49. Shoshan-Barmatz V, Krelin Y, Shteinfer-Kuzmine A, Arif T. Voltage-Dependent Anion Channel 1 As an Emerging Drug Target for Novel Anti-Cancer Therapeutics. Front Oncol. 2017. 7: 154.
50. Magrì A, Reina S, De Pinto V VDAC1 as Pharmacological Target in Cancer and Neurodegeneration: Focus on Its Role in Apoptosis. Front Chem. 2018. 6: 108.
51. Shoshan-Barmatz V, Shteinfer-Kuzmine A, Verma A. VDAC1 at the Intersection of Cell Metabolism, Apoptosis, and Diseases. *Biomolecules*. 2020;10(11).

52. Sawant Dessai A, Dominguez MP, Chen UI, et al. Transcriptional Repression of SIRT3 Potentiates Mitochondrial Aconitase Activation to Drive Aggressive Prostate Cancer to the Bone. *Cancer Res*. 2021;81(1):50–63.
53. Krasnik V, Furdova A, Svetlosakova Z, et al. Prognostic value of apoptosis inducing factor in uveal melanoma. *Neoplasma*. 2017;64(2):262–268.
54. Scott AJ, Wilkinson AS, Wilkinson JC. Basal metabolic state governs AIF-dependent growth support in pancreatic cancer cells. *BMC Cancer*. 2016;16:286.
55. Admoni-Elisha L, Nakdimon I, Shteinfer A, et al. Novel Biomarker Proteins in Chronic Lymphocytic Leukemia: Impact on Diagnosis, Prognosis and Treatment. *PLoS One*. 2016;11(4):e0148500.
56. Troutaud D, Petit B, Bellanger C, et al. Prognostic significance of BAD and AIF apoptotic pathways in diffuse large B-cell lymphoma. *Clin Lymphoma Myeloma Leuk*. 2010;10(2):118–124.
57. Letkovska K, Babal P, Cierna Z, et al. Prognostic Value of Apoptosis-Inducing Factor (AIF) in Germ Cell Tumors. *Cancers (Basel)*. 2021;13(4).
58. Xu S, Wu H, Nie H, et al. AIF downregulation and its interaction with STK3 in renal cell carcinoma. *PLoS One*. 2014;9(7):e100824.
59. Dayan A, Fleminger G, Ashur-Fabian O. Targeting the Achilles' heel of cancer cells via integrin-mediated delivery of ROS-generating dihydrolipoamide dehydrogenase. *Oncogene*. 2019;38(25):5050–5061.
60. Yoneyama K, Shibata R, Igarashi A, et al. Proteomic identification of dihydrolipoamide dehydrogenase as a target of autoantibodies in patients with endometrial cancer. *Anticancer Res*. 2014;34(9):5021–5027.
61. Puca F, Yu F, Bartolacci C, et al. Medium-chain acyl CoA dehydrogenase protects mitochondria from lipid peroxidation in glioblastoma. *Cancer Discov*. 2021.

Figures

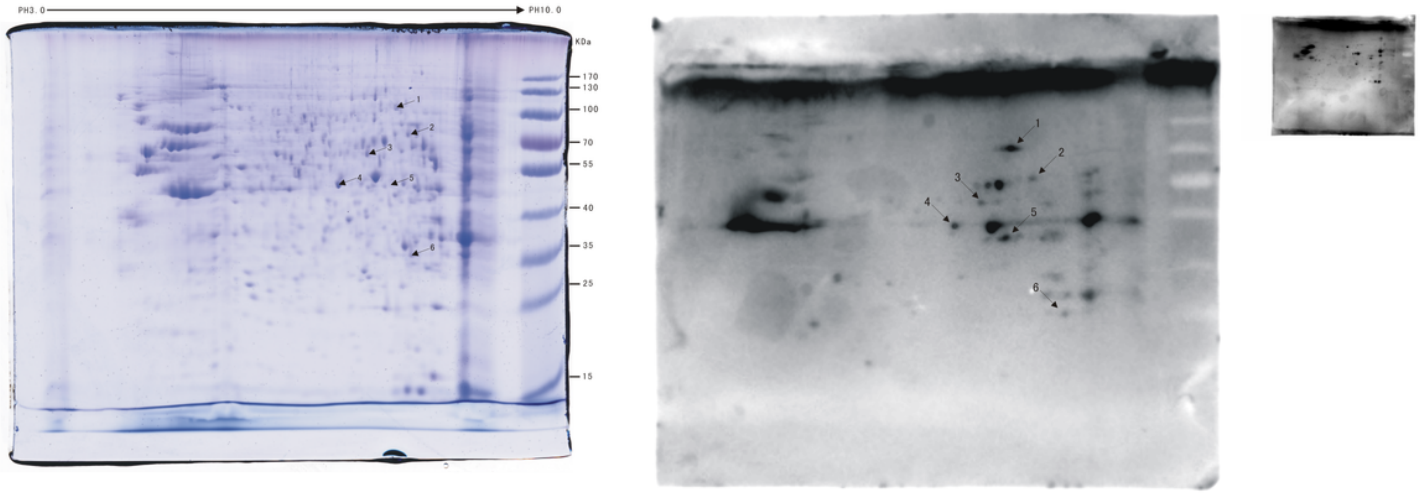

Figure 1

Analysis of the autoantibody response in B-ALL using 2-D immunoblotting. 2-D Immunoblotting images showed 6 different immunoreactive protein spots (marked with arrows) specifically recognized by serum from children with B-ALL. (A) Mixed proteins from 3 B-ALL cell were separated by 2-DE and visualized by Coomassie blue staining. (B) Mixed proteins were separated by 2-D PAGE and transferred to PVDF membranes. Then, the membranes were incubated with pooled serum from children with B-ALL. (C) PVDF membrane incubated with pooled serum from normal controls.

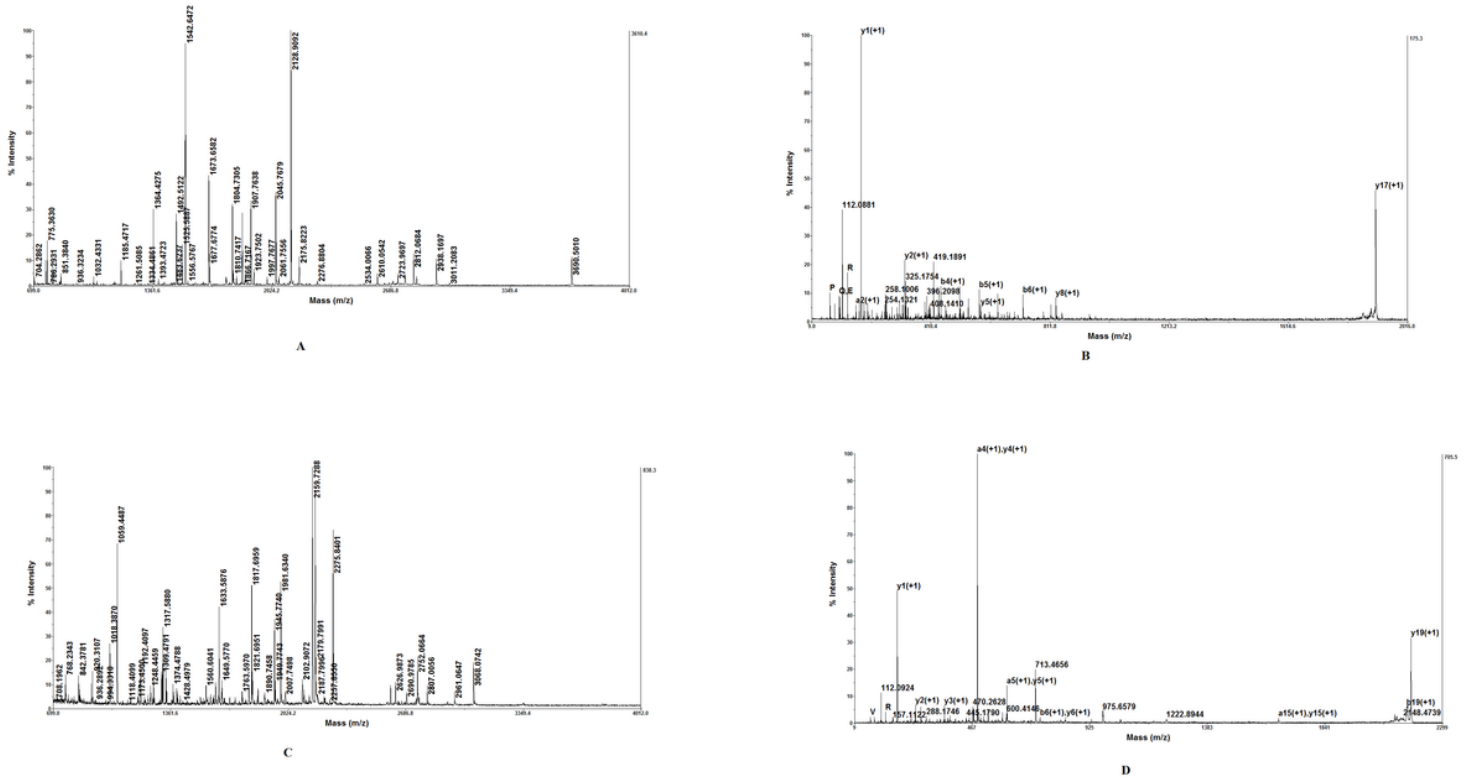

Figure 2

The peptide mass fingerprinting (PMF) map and MS/MS map of protein spots 4 and 6. (A) PMF map of protein spot 4.(B) MS/MS map of protein spot 4.(C) PMF map of protein spot 6. (D) MS/MS map of protein spot 6. The x-axis represents the mass charge ratio (m/z), the Y-axis represents the relative intensity, and the number beside the peak represents the mass number of a single isotope peak [M+H]+ of the peptide fragment.

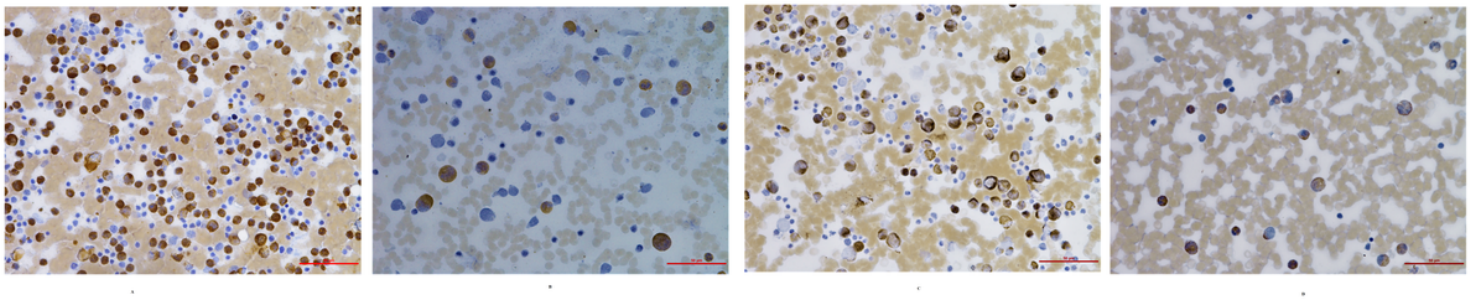

Figure 3

Immunohistochemical detection of α-enolase(A and B) and VDAC1(C and D) expression (400X. A and C: B-ALL group; B and D: Control group).