Original Article

The expression and clinical significance of murine double minute 2, lysosome-associated membrane protein 1, and P-glycoprotein in pediatric acute lymphoblastic leukemia

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Background: To analyze the expression and clinical significance of murine double minute 2 (*MDM2*), lysosome-associated membrane protein (*LAMP1*) and P-glycoprotein (*P-gp*) in children with acute lymphoblastic leukemia (ALL).

Methods: Thirty-three children with ALL who were admitted to our hospital between January 2017 and January 2018 were enrolled as the ALL group. The expression of *MDM2*, *LAMP1* and *P-gp* was compared between the two groups, as well as between ALL patients with different clinical characteristics. Logistic regression was used to analyze the risk factors that affect the prognosis and survival of ALL patients. Kaplan-Meier survival curves were used to analyze the correlations of *MDM2*, *LAMP1* and *P-gp* on the prognosis and survival of ALL patients.

Results: The expression levels of *MDM2*, *LAMP1* and *P-gp* in the ALL group were higher than those in the control group (P<0.05). The average survival time of the group with low expression of *MDM2* was (34.92±0.56) months, the average survival time of the group with high expression of *MDM2* was (31.32±0.42) months, and the difference was statistically significant (P<0.05). The average survival time of the group with low expression of *LAMP1* was (36.71±0.55) months, the average survival time of the group with high expression of *LAMP1* was (29.87±0.40) months, the difference was statistically significant (P<0.05). The average survival time of the group with low expression of *P-gp* was (36.29±0.41) months, the average survival time of the group with high expression of *P-gp* was (26.46±0.37) months, and the difference was statistically significant (P<0.05).

Conclusions: Abnormal expression levels of *MDM2*, *LAMP1* and *P-gp* protein are related to the occurrence and development of ALL, and are closely related to patient prognosis and survival. Therefore, MDM2, LAMP1and P-gp can serve as molecular markers for predicting the prognosis of children with ALL.

Keywords: Acute lymphocytic leukemia (ALL); murine double minute 2 (*MDM2*); lysosome-associated membrane protein (*LAMP1*); P-glycoprotein (*P-gp*); pathological parameters

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Introduction

Leukemias are a life-threatening disease that causes malignant disorders of the blood and bone marrow. In the adolescent and young adult population, the acute leukemias is most widespread, while chronic myeloid leukemias are infrequent (1). Acute lymphoblastic leukemia (ALL) is a common malignant tumor of the blood system arising from the hematopoietic precursors of lymphoid, including T-cell ALL (T-ALL) and B-cell ALL (B-ALL), and account for 20–25% of acute leukemia. T-ALL occupies 10–15% of pediatric and 25% of adult ALL cases, rooting in the malignant transformation of T cell progenitors (2). Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy and can seriously threaten the health of children (3). Chemotherapy is the main treatment option for children with this disease. However, due to side effects, drug resistance, and complications associated with chemotherapy, ALL can carry a poor prognosis for some patients, even resulting in death. Therefore, the identification of new therapeutic targets to improve the status and the prognosis of pediatric ALL patients is crucial (4). At present, discovery of targetable pathways that lead to drug resistance and recent breakthroughs in immunotherapy show great promise towards treating this aggressive disease in ALL (5).

Multidrug resistance (*MDR*) is the primary reason for the failure of leukemia treatment. A frequent cause of *MDR* is the overexpression of P-glycoprotein (*P-gp*), which is encoded by multidrug resistance gene 1 (*MDR1*) (6). Murine double minute 2 (*MDM2*) is an oncogene, the mutation and amplification of which have been found in numerous tumors. Lysosome-associated membrane proteins (*LAMPs*) is a type I penetrating protein that is mainly located in the outer membrane of lysosomes (7). *LAMP1*, which has the highest expressive in *LAMPs*, maintains the stability of the lysosomal membrane, and plays a part in the regulation of cell apoptosis, proliferation, and invasion during tumor progression (8). More than 50% mutations in human cancers along with the increase in expression of *MDM2*, has been found as one of the reason for cancer progression (9). The negative regulator of *P53*, *MDM2*, is frequently overexpressed in acute myeloid leukemia (AML) that retains wild-type T*P53* alleles. Targeting of *P53-MDM2* interaction to reactivate *P53* function is therefore an attractive therapeutic approach for AML (10). P-glycoprotein (*P-gp*) expression has been reported to be associated with chemoresistance and poor prognosis in various malignancies (11). *P-gp* expression in newly diagnosed childhood ALL is an independent prognostic parameter for dismal outcome. *P-gp* positivity at relapse tends towards an adverse clinical outcome compared to the *P-gp* negative relapsed population (12). Previous research has exhibited that Bcl11a transcript levels are significantly downregulated in B-cell acute lymphoblastic leukemia (B-ALL) patients with complete remission. The decrease expression characteristics of Bcl11a and *MDM2*, Pten may imply the complete remission of B-ALL patients (13). The decrease expression levels of *P53* and *MDM2* were seen in ALL patients compared with control group. In T cell subgroup of ALL the expression of *P14ARF* gene is more decreased among other subgroups (14). This study aimed to investigate the expression and clinical significance of *MDM2*, *LAMP1* and *P-gp* protein in pediatric ALL in order to provide ideas for clinical therapies. We present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/tp-20-307).

Methods

Study subjects

A total of 33 pediatric ALL patients who were admitted to Northwest Women and Children's Hospital between January 2017 and January 2018 were enrolled as the ALL group. The 33 patients included 16 males and 17 females, who ranged in age from 3–18 years old, with an average age of (9.39±3.14) years old. According to the French-American-British (FAB) classification system, there were 7 cases of Metastasis1 (M1), 10 cases of M2, 6 cases of M3, 4 cases of M4, and 6 cases of M5. The inclusion criteria were: (I) met the World Health Organization's diagnostic criteria for ALL (15); (II) newly treated; and (III) aged between 1 and 18 years. The exclusion criteria were: (I) unable to tolerate the test methods of this study; (II) with malignant tumors other than ALL; (III) unable to cooperate with the research due to severe mental illness or dementia; (IV) missing clinical data or withdrawal from the study. All patients and family members (Father or Mother) agreed to participate in this study and signed an informed consent form. The study was approved by the medical ethics committee of the Northwest Women and Children's Hospital. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

A second group, comprising 21 children with non-malignant hematological diseases (including 19 cases of immune thrombocytopenic purpura and 2 cases of infectious mononucleosis), was enrolled as a control group. This group included 10 males and 11 females, who ranged in age from 2–18 years old, with an average age of (9.41±3.11) years.

There were no differences in age, sex, or other baseline data between the study group and the control group (P>0.05).

Study methods

In all subjects, the expression of *MDM2* and *P-gp* was detected using immunohistochemical techniques, and the real-time fluorescent quantitative polymerase chain reaction (PCR) method was used to detect the expression of *LAMP1* in bone marrow specimens. The differences in the expression levels of *MDM2*, *LAMP1* and *P-gp* were compared between the ALL group and the control group. Further, the differences in the expression of *MDM2*, *LAMP1* and *P-gpin* ALL patients with different clinical characteristics were also analyzed. All patients with ALL were given two courses of standard chemotherapy. The patients were followed up for 2 years, and their survival after treatment was recorded. Logistic regression was used to analyze the risk factors that affected the prognosis and survival of the pediatric ALL patients. Kaplan-Meier survival curves were used to analyze the correlations of *MDM2*, *LAMP1* and *P-gp* on the prognosis and survival of pediatric ALL patients.

qRT-PCR

According to manufacturers' protocol, total RNA was isolated by employing the TRIzol reagent kit (Invitrogen, Beijing, China). The total RNA concentration was measured by adopting the Gene Quant ProRNA/DNA Calcula-tor (Amersham Pharmacia Biotec, UK). The PrimeScript RT reagent Kit (TakaRa, Dalian, China) was used to perform reverse transcription. The 2 SYBR Premix Ex Taq™ II (TakaRa, Dalian, China) was employed to assemble the reaction system of qRT-PCR. The reaction system is carried out in the Bio-Rad CFX-96 (Bio-Rad, CA, USA). GAPDH was used for normalizing. The qRT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression levels of mRNA.

Observation indicators

MDM2 and *P-gp* protein

Routine bone marrow puncture was performed for the ALL patients and control subjects. Bone marrow fluid (2 mL) was extracted and placed in a heparin anticoagulation bottle. Then, the bone marrow fluid was diluted with phosphate-buffered saline (PBS), and 3 mL of lymphocyte separation solution was injected into the upper layer of the centrifuge tube. The contents of the tube were then centrifuged (2,300 r/min). Subsequently, mononuclear cells were aspirated, washed with PBS, and the number of cells was adjusted to 1×10^6/L for inspection. If the test could not be performed immediately, the cells were suspended in RPMI-1640 culture medium containing 10% dimethyl sulfoxide (DMSO) +20% newborn calf serum and stored at –80 ℃.

The expression of *P-gp* was detected by first adding *P-gp* monoclonal antibody (UIC2, Immunotech) to mononuclear cells followed by incubation for 30 min in the dark, mononuclear cells was then washed twice with PBS, and then, the fluorescein isothiocyanate (FITC)-GAM IgG was added and incubated for 30 minutes in the dark, and washed once with PBS. The expression of *P-gp*in bone marrow mononuclear cells was examined; an expression rate of >20% was considered to represent high (+) expression, and an expression rate of ≤20% was considered to represent low (–) expression.

The expression of *MDM2* was detected using the 3,3'-diaminobenzidine (DAB) colorimetric method with an SABC kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) in bone marrow specimens according to the manufacturer's instructions. Lymphadenitis positive cases were used as positive controls. Positive *MDM2* protein expression was indicated by yellow-stained nuclei, with the intensity of staining graded as follows: no staining, no expression; light yellow, weak expression; yellow, positive expression; dark yellow or tan, strong positive expression. No expression and weak expression were regarded as low expression (–), and positive expression and strong positive expression were regarded as high expression (+).

P-gp

Routine bone marrow puncture was performed for the ALL patients and control subjects. Bone marrow fluid (2 mL) was extracted and placed in a heparin anticoagulation bottle. After that, 1 mL of normal saline was added to the bone marrow, and 2 mL of lymphocyte separation fluid was slowly injected into the upper layer for density gradient

Table 1 Comparison of the expression levels of *MDM2*, *LAMP1* and *P-gp* in the ALL group and the control group [n (%)]

Group	*MDM2*		*LAMP1*		*P-gp*	
	–	+	–	+	–	+
ALL group (n=33)	7 (21.21)	26 (78.79)	6 (18.18)	27 (81.82)	8 (24.24)	25 (75.76)
Control group (n=21)	15 (71.43)	6 (28.57)	16 (76.19)	5 (23.81)	14 (66.67)	7 (33.33)
t	13.404		17.887		9.567	
P	<0.001		<0.001		0.002	

MDM2, murine double minute 2; *LAMP*1, lysosome-associated membrane protein; *P-gp*, P-glycoprotein; ALL, acute lymphoblastic leukemia.

centrifugation (1,500 r/min). Then, the mononuclear cell layer was aspirated. The mononuclear cell was washed twice with physiological saline, and the supernatant was discarded. Finally, the cell concentration was adjusted to 1×10^6/L. The immunofluorescence labeling method was directly used to detect the expression of *P-gp* in the samples from the ALL patients. First, 50 μL of cell suspension was added into two 1.5 mL EP tubes, respectively, and then 10 μL of phycoerythrin (PE)-labeled *P-gp* monoclonal antibody (BD BIOSCIENCES Company, USA) was added to the measuring tube before incubation for 15 min in the dark at room temperature. Then, cell suspension was washed with physiological saline twice, the supernatant was discarded and 1 mL of physiological saline (0.9% Nacl) was added, which was then filtered through a 300-mesh screen. Finally, the supernatant was measured with a flow cytometer (Facscalibur, BD BIOSCIENCES Company, USA). *P-gp* ≥10% was considered to be high expression (+), and <10% was considered to be low expression (–).

Follow-up

After chemotherapy, the ALL patients were followed up for 2 years (until January 2020) or until death. The follow-up time of the patients ranged from 2–24 months, with an average follow-up time of (10.71±5.93) months. The main methods of follow-up were telephone calls and patient visits to the hospital for review.

Statistical analysis

The data in this study were statistically analyzed using SPSS18.0 software (SPSS Inc., Chicago, IL, USA). Count data were expressed as n (%), and analyzed using the F test. Measurement data were described as the mean ± standard deviation (± S), and analyzed using the χ^2 test. Logistic regression analysis was used to analyze the risk factors that affect the prognosis and survival of pediatric ALL patients, and Kaplan-Meier survival curves were used to analyze the effects of *MDM2*, *LAMP1* and *P-gp* on the prognosis and survival of pediatric ALL patients. Differences were considered statistically significant when P<0.05.

Results

The expression of MDM2, LAMP1 and P-gp

The expression rates of *MDM2*, *LAMP1* and *P-gp* in the ALL group were higher than those in the control group (P<0.05; *Table 1*).

The correlations of MDM2, LAMP1 and P-gp with various pathological parameters in children with ALL

No correlations were observed between the expression levels of *MDM2*, *LAMP1* and *P-gp* and age, sex, peripheral blood platelets, immunotype, brain white or white test, or infection during chemotherapy in ALL patients. However, the expression levels *MDM2*, *LAMP1* and *P-gp* were found to be related to newly diagnosed peripheral blood leukocytes, newly diagnosed peripheral blood hemoglobin, prednisone induction, and bone marrow examination at 35 days after chemotherapy induction (P<0.05), see *Table 2*.

Prognosis and survival of 33 pediatric ALL patients

After 2 years of follow-up, 13 pediatric ALL patients had died and 20 had survived, the survival rate was 60.61%.

Univariate and multivariate analysis of the prognosis and survival of pediatric ALL patients

Newly diagnosed peripheral blood leukocytes, newly

Table 2 The correlations between *MDM2*, *LAMP1* and *P-gp* and various pathological parameters in pediatric ALL patients

Pathological parameters	Cases (n=33)	*MDM2*				*LAMP1*				*P-gp*			
		+ (n=26)	− (n=7)	χ^2	P	+ (n=27)	− (n=6)	χ^2	P	+ (n=25)	− (n=8)	χ^2	P
Age (years)		9.31±1.14	9.35±1.32	0.080	0.937	9.46±1.23	9.41±1.29	0.089	0.929	9.59±1.24	9.51±1.36	0.141	0.889
Sex				0.113	0.737			0.007	0.935			0.010	0.922
Male	16	13	3			13	3			12	4		
Female	17	13	4			14	3			13	4		
Newly diagnosed peripheral white blood cells ($\times10^9$/L)				7.984	0.005			11.282	0.001			10.236	0.001
<50	13	7	6			7	6			6	7		
≥50	20	19	1			20	0			19	1		
Newly diagnosed peripheral blood platelets ($\times10^9$/L)				0.024	0.876			0.435	0.510			0.270	0.604
<20	15	12	3			13	2			12	3		
≥20	18	14	4			14	4			13	5		
Newly diagnosed peripheral blood hemoglobin (g/L)				9.351	0.002			6.991	0.008			11.933	0.001
<60	21	20	1			20	1			20	1		
≥60	12	6	6			7	5			5	7		
Immunophenotyping				0.113	0.737			0.007	0.935			0.008	0.930
B cell	17	13	4			14	3			12	5		
T cell	16	13	3			13	3			13	3		
Newly diagnosed brain white or white test				0.578	0.447			0.113	0.737			0.016	0.900
No	13	12	2			11	2			10	3		
Yes	20	15	5			16	4			15	5		
Prednisone induction				12.058	0.001			5.024	0.025			4.588	0.032
Sensitive	19	19	0			18	1			17	2		
Non-sensitive	14	7	7			9	5			8	6		
Bone marrow examination 35 days after induction (%)				4.160	0.041			6.902	0.009			5.475	0.019
<5	16	15	1			16	0			15	1		
≥5	17	11	6			11	6			10	7		

MDM2, murine double minute 2; *LAMP1*, lysosome-associated membrane protein; *P-gp*, P-glycoprotein; ALL, acute lymphoblastic leukemia.

Table 3 Univariate and multivariate analysis of the prognostic survival of pediatric ALL patients

Parameters	Univariate analysis			Multivariate analysis		
	OR	95% CI	P	OR	95% CI	P
Newly diagnosed peripheral white blood cells (<50×10^9/L *vs*. ≥50×10^9/L)	1.261	1.53–1.56	0.013	1.536	1.34–1.98	<0.001
Newly diagnosed peripheral blood hemoglobin (<60 *vs*. ≥60 g/L)	1.299	1.34–1.53	0.016	1.671	1.31–1.59	<0.001
Newly diagnosed peripheral blood platelets (<20×10^9/L *vs*. ≥20×10^9/L)	1.749	0.53–1.79	0.511	–	–	–
Immunophenotyping (B cell *vs*. T cell)	1.568	0.36–1.44	0.005	–	–	–
Prednisone induction (sensitive *vs*. non-sensitive)	1.478	1.16–1.67	0.025	1.631	1.12–1.89	<0.001
Bone marrow examination at 35 days after induction (<5% *vs*. ≥5%)	1.731	1.27–1.86	0.045	1.479	1.31–1.76	<0.001
MDM2 (high expression *vs.* low expression)	1.798	1.53–1.94	0.015	1.538	1.46–1.23	<0.001
LAMP1 (high expression *vs.* low expression)	1.735	1.43–1.70	0.046	1.496	1.11–1.80	<0.001
P-gp (high expression *vs.* low expression)	1.769	1.61–1.98	0.013	1.387	1.54–1.86	<0.001

MDM2, murine double minute 2; *LAMP1,* lysosome-associated membrane protein; *P-gp*, P-glycoprotein; ALL, acute lymphoblastic leukemia.

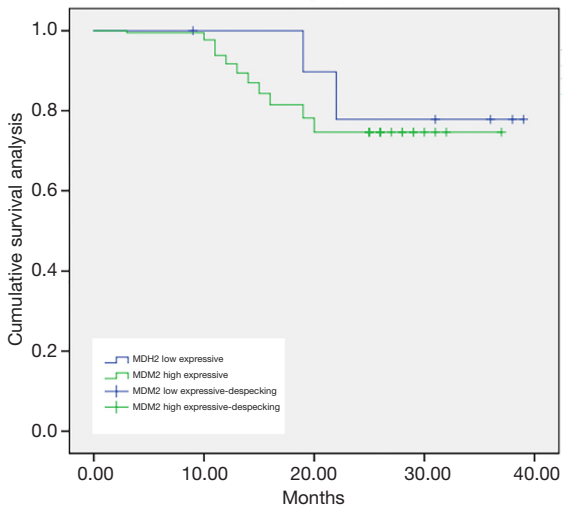

Figure 1 Comparison of the survival time of patients with different expression levels of *MDM2*. *MDM2*, murine double minute 2.

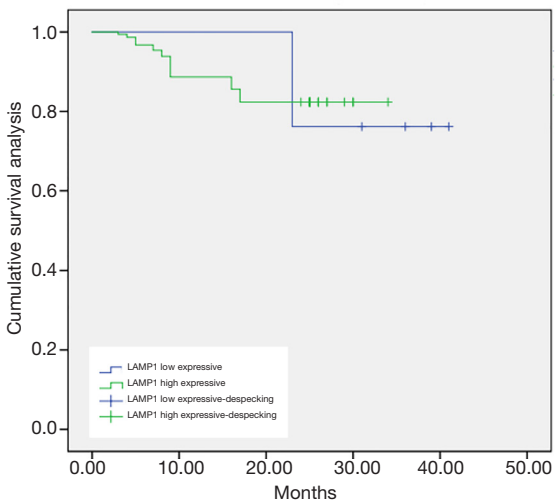

Figure 2 Comparison of the survival time of patients with different expression levels of *LAMP1*. *LAMP1*, lysosome-associated membrane protein.

diagnosed peripheral blood hemoglobin, prednisone induction, 35-day bone marrow examination, and high expression of *MDM2*, *LAMP1* and *P-gp* were found to be independent risk factors that affect the prognosis and survival of pediatric ALL patients (P<0.05; *Table 3*).

The effect of MDM2, LAMP1 and P-gp on the prognosis and survival of pediatric ALL patients

As shown in *Figures 1-3*, the Kaplan-Meier survival curves show significant differences under different *MDM2*, *LAMP1* and *P-gp* expression conditions.

The average survival time of the groups with low and high expression of *MDM2* was (34.92±0.56) months and (31.32±0.42) months, respectively. The difference was statistically significant (P<0.05).

The average survival time of the groups with low and high expression of *LAMP1* was (36.71±0.55) and (29.87±0.40) months, respectively. The difference was statistically significant (P<0.05).

The average survival time of the groups with low and

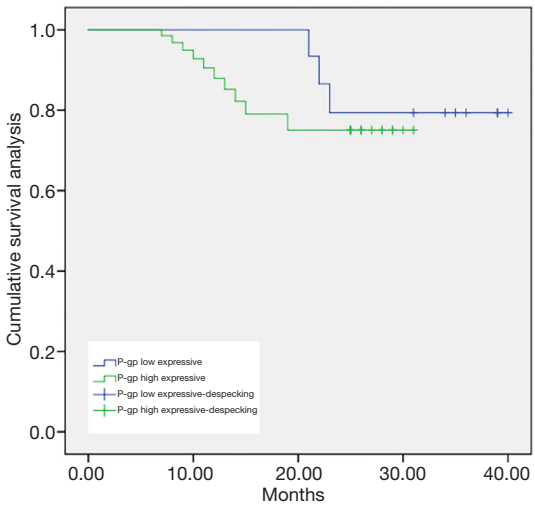

Figure 3 Comparison of the survival time of patients with different expression levels of *P-gp* protein. *P-gp*, P-glycoprotein.

high expression of *P-gp* was (36.29±0.41) months and (26.46±0.37) months, respectively. The difference was statistically significant (P<0.05).

Discussion

ALL is a malignant tumor caused by the abnormal proliferation of lymphocyte-derived B-line or T-line cells in the bone marrow. Abnormally proliferated primitive cells can inhibit normal hematopoietic function, even spreading to the meninges, lymphatic system, gonads, and liver (16,17). In recent years, various protein molecules have attracted widespread attention for their roles in tumor cell invasion and metastasis.

The *MDM2* oncogene is located on human chromosome 12q and encodes the *MDM2*, which is localized in the nucleus and has a short half-life (18). Studies have found that the *MDM2* gene is amplified in various cancers, including leukemia, soft tissue sarcoma, osteosarcoma, and some breast tumors, resulting in a high expression of the *MDM2* product (19). In this study, 78.79% of the 33 ALL patients had a high expression of *MDM2*, which was also much higher than the expression in non-ALL subjects. This result suggests that *MDM2* plays an important role in the pathogenesis of pediatric ALL.

Logistic regression analysis and Kaplan-Meier survival curves were used to explore the clinical significance of *MDM2* protein expression in pediatric ALL patients. The results showed that a high expression of *MDM2* is one of the independent risk factors that affect patient prognosis. The average survival time of patients with a high expression of *MDM2* was lower than that of patients with a low expression (P<0.05), which indicates that *MDM2* could potentially be used as a prognostic molecular biomarker for pediatric ALL patients. Further analysis have exhibited that *MDM2* may be closely related to the *P53* gene (20). Specifically, *P53* is an important tumor suppressor gene in the human body. *MDM2* overexpression can produce the effect of inactivating point mutations of the *P53* gene, and through the formation of *P53-P90* gene points, it affects the activation of *P53* transcription. Moreover, when *MDM2* is highly expressed, it can directly bind to the target DNA, produce an anti-*P53* effect and antagonize the effect of *P53*, promoting tumor invasion and development (16,21).

In recent years, it has been demonstrated that *LAMP* is a protein involved in a variety of cellular activities. The *LAMP* protein family are highly expressed in the plasma membrane and some metastatic tumor cells. Therefore, this study investigated the expression of *LAMP1* in pediatric ALL patients, in the hope of identifying new therapeutic targets (22). In this study, *LAMP1* was highly expressed in pediatric ALL patients and was significantly correlated with the levels of leukocytes and hemoglobin in the peripheral blood, which proved that *LAMP1* plays an important role in the occurrence and development of ALL.

LAMP1, as a lysosomal membrane stability protein, can also interact with *LAMP2*, E-selectin, and growth factor receptors to mediate the adhesion of tumor cells to the extracellular matrix and accelerate tumor cell migration (23). In this study, not only was a high expression of *LAMP1* found to be a risk factor affecting the prognosis of ALL patients, but the Kaplan-Meier survival curves differed significantly according to the levels of *MDM2*, *LAMP1* and *P-gp* expression. The average survival time of the group with negative low expression of *LAMP1* was longer than that of the high expression group (P<0.05), which further suggests that the high expression of *LAMP1* is not conducive to the remission of ALL patients and may even lead to a poor prognosis. Clinically, lysosomal targeted therapy may be used to overcome drug resistance in malignant tumors and improve the prognosis of patients.

MDR means that while anti-tumor cells are resistant to one anti-tumor drug, it is also resistant to other anti-tumor drugs with different structures and different targets (24). In recent years, with the innovation and development of medical technology, the rate of induction of remission in pediatric ALL has increased. However, some children still

cannot achieve complete remission, and even experience relapse of drug resistance, which eventually leads to death. Therefore, in China, the clinical treatment of *MDR* in pediatric ALL patients has attracted widespread attention. At present, *P-gp*-mediated overexpression of the *MDR1* gene is believed to be the principal mechanism underlying *MDR*. As an important efflux transporter in the ATP binding cassette transporter family, *P-gp* mediated efflux is also a major obstacle to drug delivery and cancer treatment (25). This study analyzed the expression of *P-gp* protein in bone marrow from the spinal cords of 33 children with ALL. The results showed that *P-gp* was the high expression, and the survival time of patients with *P-gp* high expression was much lower than that of patients with low expression, suggesting that *P-gp* could be used as a prognostic indicator in pediatric ALL.

Nevertheless, this study has some limitations. Firstly, the sample size is small, which may have led to sampling errors and makes the results unrepresentative. Secondly, follow-up was mainly conducted by telephone, and clinical verification was lacking. Therefore, it was impossible to determine whether the death of a patient was related to ALL. To further analyze the prognostic value of *MDM2*, *LAMP1* and *P-gp* expression in pediatric ALL patients, future studies with larger sample sizes and follow-ups with stronger authenticity should be carried out.

In conclusion, *MDM2*, *LAMP1* and *P-gp* are highly expressed in children with ALL. Abnormal expression levels of *MDM2*, *LAMP1* and *P-gp* are involved in the occurrence and development of pediatric ALL and are related to patient prognosis. Therefore, *MDM2*, *LAMP1* and *P-gp* can be used as molecular markers for predicting the prognosis of children with ALL and as potential therapeutic targets.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tp-20-307). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All patients and family members (Father or Mother) agreed to participate in this study and signed an informed consent form. The study was approved by the medical ethics committee of the Northwest Women and Children's Hospital. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

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