Int J Clin Exp Pathol 2017;10(2):1708-1718
www.ijcep.com /ISSN:1936-2625/IJCEP0044754

Original Article

Expression and clinical value of multidrug resistance-associated proteins (MRP) 1 to 6 in Chinese pediatric patients with B-precursor acute lymphoblastic leukemia

Jin Yang[1], Bao-Ling Qiu[1], Chen-Yan Zhou[1], Qi Zhou[1], Jian-Qin Li[1], Jian Pan[1], Wei-Ying Gu[2], Xiao-Fei Qi[3], Rui-Hua Chen[3], Yi-Na Niu[4], CS Chen[5], Shao-Yan Hu[1]

[1]Department of Hematology & Oncology, Children's Hospital of Soochow University, Suzhou, Jiangsu, China; [2]The First People's Hospital of Changzhou, Third Affiliated Hospital of Suzhou University, Changzhou, Jiangsu, China; [3]The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China; [4]Xinxiang Medical University, Xinxiang, Henan, China; [5]Loma Linda University, CA 92354, USA

Received November 21, 2016; Accepted November 27, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: In this study we detected the expression pattern of MRP1-6 in126 newly diagnosed B-precursor ALL (BP-ALL) children by real-time RT-PCR in China. We found that all 6 members of MRPs were expressed with a distinct pattern: MRP1 showed close relation with WBC counts, treatment response, and relapse; Higher expression of MRP2 in the TEL/AML1 positive group; MRP5 and MRP6 expressed lower in E2A/PBX1 positive group; High expression of MRP1, 5, 6 showed a close relation with poor response to the treatment; MRP1 and MRP6 expressed higher in relapse stage. Furthermore, each member expression alone didn't show any impact on the relapse-free survival in BP-ALL. However, when MRP1 was combined with other MRP members such as MRP5 or MRP6, the patient cohort could be stratified into 4 subgroups with relapse. Patients with high MRP1 and low MRP5 or 6 had the most favorable relapse-free survival. Our study illustrated a new pattern of MRPs related to relapse-free survival.

Keywords: Multidrug resistance-associated protein family members, acute lymphoblastic leukemia, B-precursor, relapse-free survival

Introduction

Acute lymphoblastic leukemia (ALL) is the most frequent malignancy in childhood. With the advancement of modern antileukemic agents and improved supportive care in contemporary clinical trials, the 5-year survival rate of childhood ALL has been incrementally improved in both developing and developed countries [1-4]. However, some patients still fail to respond to therapy and others relapse with resistant disease. Drug resistance remains a leading cause of death in pediatric ALL.

Several mechanisms of drug resistance were identified, of which ATP binding-cassette (ABC) transporters was mainly involved in chemosensitivity [5-11]. The superfamily of ABC transporters mainly consists of the multidrug resistance gene 1 (MDR1), breast cancer resistance protein (BCRP), and the family of multidrug resistance-associated proteins (MRPs). Currently, nine MRP genes have been identified, of which the MRP transporters (MRP1-6) are known to be involved in the effleux of chemotheraputic agents that are generally used in the treatment of ALL, including doxorubicin, vincristine, etoposide, 6-mercaptopurine, and methotrexate [12-15]. Although MRP family shares strong similarity on structure, function and substrate specificity, the reports on their clinical relevance in ALL are still controversial. The expression of MRP1 has been studied in larger groups of ALL patients and failed to show association with response to chemotherapy [9]. Higher levels of MRP3 were found in patients with a poor in vivo response to prednisone, but this could not be confirmed by an independent case-control study for prednisone response [16]. Relapsed patients showed a higher level

of expression of all MRP genes, except MRP4 which indicated that high MRPs expression correlate with an unfavorable prognosis independently of age [17].

In view of the strong overlapping functions of the members of MRPs, the aim of this study is to investigate the correlation of mRNA expression of all six relevant MRP genes (MRP1-6) with the clinical feature, cytogenetic abnormality, and clinical outcome in childhood ALL treated under Cooperative Study Protocol (CCLG-ALL 2008) in China.

Materials and methods

Patients

During the period of January 2012 to April. 2013, 126 patients newly diagnosed B-precursor ALL (BP-ALL) were enrolled to the study at the Children's Hospital of Soochow University. Follow-up time ended in May 2015. T-cell ALL and mature ALL were excluded from analysis because of their unique leukemia biology, associated risk characteristics, and treated under different protocols as well. Patients with BP-ALL were classified as standard-risk ALL (SR-ALL), intermediate-risk ALL (IR), and high-risk ALL (HR-ALL) and were treated according to the protocol from Chinese Children Leukemia Group (CCLG-2008) risk-stratified ALL regimens [18]. The study was reviewed and approved by the Institutional Review Board, and was conducted in accordance with the Declaration of Helsinki.

Analysis of minimal residual disease (MRD)

MRD was obtained during the study period from end-of-induction bone marrow specimens and was were analyzed with a six-laser FACS Calibur flow cytometer with CellQuest and CellQuestPro software (BD Biosciences, San Jose, CA) and were performed according to the established protocol [19-22]. MRD was analyzed either as continuous variable or as positive or negative (defined by using a threshold of 0.01% residual leukemia blasts) as established from previous large cohorts in pediatric ALL [23].

Definition

Relapse was defined as very early relapse (VER, less than 18 months from the first induction therapy), early relapse (ER, 18 months or more after first diagnosis and less than 6 months from stopping therapy), and late relapse (LR, 6 months or more after stopping therapy), respectively [24]. Complete remission (CR) was defined as <5% leukemic blasts in bone marrow, absence of blasts in peripheral blood, and absence of leukemic blasts in spinal fluid or other extramedullary sites. Central nervous system (CNS) disease at diagnosis was defined by a WBC count of greater than 5 cells/μL with identifiable blasts in the cerebral spinal fluid (CSF) or by a pathological mass detected by cranial computed tomography, with or without CSF pleocytosis. The presence of more than 1000 blasts/μL peripheral blood blasts on day 8 after prednisone treatment was defined as prednisone poor responder (PPR) [25].

Sample collection and RNA isolation and qRT-PCR

Mononuclear cells from bone marrow were isolated on Ficoll-Isopaque (Nycomed, Oslo, Norway) density gradient by centrifugation. The cells concentration at 2×10^6/ml was cryopreserved in RPMI 1640 supplemented with 10% FCS and 10% DMSO (Merck, Amsterdam, the Netherlands) and stored in liquid nitrogen. The median percentage of blasts in patient material was 80%±20% (mean ± SD). Total cellular RNA was isolated from ALL blasts using RNeasy Mini Kit including DNase digestion (Qiagen, Hilden, Germany). From some samples total cellular RNA was extracted using 1 mL of Trizol reagent (Life Technologies, Breda, Netherlands). The amount of RNA was measured by photometry. Subsequently, 1 microgram RNA was reverse transcribed in 20 microliter reverse transcriptase buffer containing 10 mmol/L DTT, 0.5 mmol/L each of dATP, dGTP, dCTP, and dTTP, 200 units of Moloney murine leukemia virus reverse transcriptase, 5 units of RNase inhibitor, and 10 ng/microliter random primers (MBI Fermentas, St. Leon-Rot, Germany).

Quantitative RT-PCR (qRT-PCR) was performed using the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers for the MRPs and b-actin and products were amplified based on the protocol previously described [26].

The expression of the MRP genes was standardized based on expression of β-actin gene.

Table 1. Initial patient data

	No.
Number of patients	126
Median age (mean ± SD), y (range)	5.8±3.6
Sex, male/female	62/64
Median WBC, 10^9/L (range)	26.8 (0.54-638)
Median percentage of leukemic cells, WBC (range)	26.8 (0.54-638)
Median HB, g/L (range)	79 (46-160)
Median PLT count, $\times10^9$/L (range)	71 (9-375)
Bone marrow blast, %	89 (41-98)
Peripheral Blood blast, %	72 (0-95)
TEL/AML1, yes/no	36/90
E2A/PBX1, yes/no	12/114
BCR/ABL, yes/no	4/122
MLL related	12/114
Response to prednisone, good†/poor‡	20/106
Relapse, VER/ER/no	8/4/114

†Less than 10^9 leukemic cells/L on day 8. ‡More than 10^9 leukemic cells/L on day 8.

Table 2. MRPs expression at different stage of BP-ALL

MRP expression*	Stage	Median**	Range	*p* value#
MRP1	Initial	5.82	2.56-10.98	<.001
	CR	1.99	0.55-3.76	
	Relapse	8.49	3.88-16.25	0.007
MRP2	Initial	2.99	1.05-5.23	>.05
	CR	1.17	0.25-3.25	
	Relapse	1.18	0.35-3.06	>.05
MRP3	Initial	0.52	0.19-2.01	<.001
	CR	2.09	0.69-4.02	
	Relapse	0.38	0.18-1.44	<0.001
MRP4	Initial	2.46	1.09-5.01	.039
	CR	1.64	0.54-3.91	
	Relapse	5.47	1.26-8.04	.029
MRP5	Initial	1.6	0.39-3.21	.002
	CR	0.91	0.28-2.01	
	Relapse	3.84	0.99-5.27	.002
MRP6	Initial	0.56	0.15-2.33	>.05
	CR	1.13	0.53-3.03	
	Relapse	2.00	0.90-4.03	>.05

NOTE: *The relative expression of MRPs was calculated by $2^{-\Delta\Delta Ct}$, namely, ΔCT (MRP1-6)=CT (MRP1-6)-CT (β-actin gene), ΔΔCt=ΔCT (MRP1-6) of patients -ΔCT (MRP1-6) of control. **The values are given as median (25th-75th percentile). #Compared with CR.

The expression of each gene in each sample was analyzed in duplicates. Meanwhile, samples from 20 cases without leukemia were used as control.

Statistical analysis

Because the levels of MRP expression were not evenly distributed, nonparametric methods were used. The Mann Whitney U test was done to compare MRP expression between two groups. For more than two groups, the Kruskal-Wallis test was employed. With the Spearman rank test, the correlation between MRP expression and other continuous variables was determined. Life-table estimates were employed to analyze double genes effects on relapse and Chi-square was run to compare subgroups' differences. Median values (the 50th percentile) were used as cutoffs for high versus low MRP expression. All *P* values are given for two-sided tests and $P<0.05$ was considered significant. Analyses were done using SPSS 16.0 for Windows software (SPSS, Chicago, IL).

Results

Patient characteristics

The clinical, cytogenetic findings and laboratorial features of 126 BP-ALL cases are shown in **Table 1**. Patients age range from 0.5 to 13.3 years with the median age of 5 years, and the predominant age group is in between age 1 and age 10 (n=100). The gender ratio is balanced (female:male; 62:64). WBC ranged from 1.6 to 395.6 ($\times10^9$/L) at median of 25.3 ($\times10^9$/L). The incidence of TEL/AML1 rearrangement is 36/126 (28.57%) which is consistent with reports in literature [27, 28]. E2A/PBX1 translocation occurs 9.5% (12/126) of the cohort which is similar to the literature reported by Italy [29] PPR patients accounted for 15.87% (20/126) of patients. MRD data were available in 109 cases during this study. Eight patients were VER, and 4 patients were ER, no LR at cut off data of data collection (May

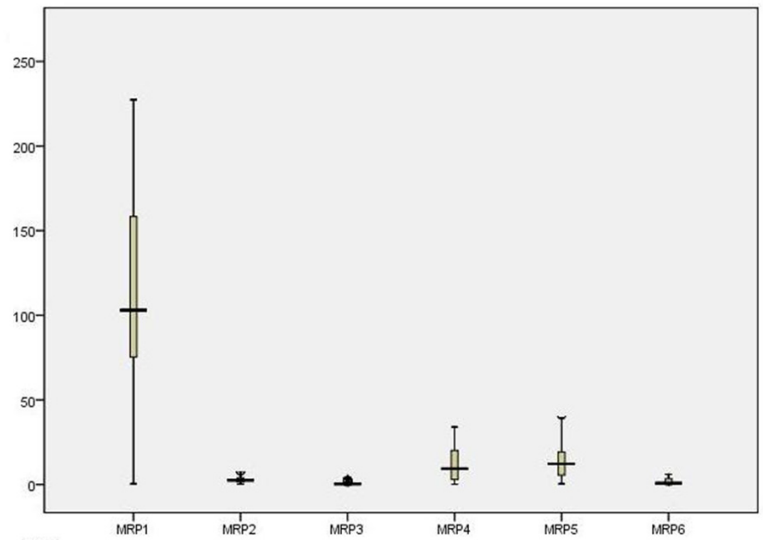

Figure 1. Relative expression of MRPs in initial patients with BP-ALL. The relative expression was calculated as following, the Ct value of each MRP divided by the Ct value of β-actin gene and amplified for 10000 times. The horizontal bar of the box represented median.

2015). No CNSL or testicular leukemia relapse was noted in this population.

MRP1-6 expression in BP-ALL during treatment course

Bone marrow specimen at diagnosis from 126 BP-ALL was analyzed for MRP1-6 using qRT-PCR. MRP1-6 expression can be detected in all cases. However, the expression levels of MRPs were significantly different as shown by median level (**Table 2** and **Figure 1**). Correlation analysis revealed that MRP5 had a statistic positive correlation with all other MRP members ($P<0.05$). MRP1 showed a relative poor correlation with other MRP members, except with MRP5. We further analyzed the expression levels of MRP1-6 in BP-ALL during treatment course, we found that the expression level of MRP1,3,4,5 had a significant difference at initial stage compared with complete remission, further increased after relapse. (**Table 2**), which indicated that MRPs expressing pattern has a close relation in predicting treatment response and outcome.

Correlation of MRP expression and clinical features in BP-ALL with univariate analysis

The number of cases is small and makes multivariate analysis unreliable. We analyzed MRPs in relation to initial WBC. MRP1 and MRP4 were statistically higher in the group of WBC above 50×10^9/L than that in WBC lower group (133.16 vs. 101.37, and 16.68 vs. 8.80, respectively) (**Table 3**). Analyzing the correlation of MRPs with known fusion genes as criteria for risk stratification of ALL, we found that MRPs correlates in different pattern. For example, in the group of patients with TEL/AML1 positive had a higher level of MRP2 and patients with E2A/PBX11 positive had a lower expression of MRP5 and MRP6 ($P<0.05$). Meanwhile, MRP6 in MLL related positive group expressed higher than negative group ($P=0.028$) (**Table 3**). In summary, we identify different member of MRPs showed different expression with clinical features of BP-ALL.

The correlation of MRPs with treatment response in patients with BP-ALL

MRP1 had a close correlation with treatment response. The PPR patients had a statistically higher expression of MRP1 ($P=0.01$) (**Table 3**). MRP1 expression also showed a positive correlation with MRD level on day 33 (122.65 versus 97.14, $P=0.045$), and 12 week post-chemotherapy (158.76 versus 87.97, $P<0.001$). MRP5 also had a higher expression in the group with positive MRD than negative MRD on day 33 and 12 week (**Table 3**). MRP4 and MRP6 expression showed statistically higher in the group with higher level of MRD on week 12 ($P<0.05$) (**Table 3**). However, MRP2 and MRP3 didn't show any statistic difference on the treatment response. Our results suggest that MRP1 is the most important parameter in predicting drug response of MRPs in BP-ALL patients. Pattern of MRPs expression can predict treatment response in BP-ALL patients. LR patients had lowest MRP1 expression, and HR patients had highest expression of MRP1 with the $P<0.001$ (**Table 2**). MRP6 expressed highest in the HR group and lowest in IR group ($P=0.012$). Meanwhile, relapse patients had a statistically higher expression of MRP1 and MRP6 at their initial stage (**Table 3**). However,

Table 3. The expression pattern of MRP family members in pediatric B-ALL patients

	n	MRP1	P	MRP2	P	MRP3	P	MRP4	P	MRP5	P	MRP6	p
WBC ($\times 10^9$/L)			.013*		.071		.07		.017		.944		.972
<50	86	101.37		2.89		0.27		8.80		12.76		1.06	
≥50	40	133.16		1.98		0.46		16.68		12.57		0.92	
TEL/AML1			.500		<.001*		.370		.672		.889		.061
Positive	36	111.05		3.28		0.28		11.00		12.61		0.90	
Negative	90	106.42		1.55		0.40		10.44		12.82		1.60	
E2A/PBX1			.310		.965		.147		.536		.021*		.001*
Positive	12	102.88		2.80		0.50		17.51		5.37		0.24	
Negative	114	89.72		2.60		0.28		10.48		13.25		1.24	
MLL			.118		.905		.357		.902		.364		.028*
Positive	4	209.95		2.61		0.35		9.35		22.21		10.23	
Negative	120	118.75		2.61		0.30		11.09		12.48		0.95	
BCR/ABL			.385		.050		.306		.182		.344		.969
Positive	12	131.89		1.01		0.43		16.21		16.60		0.81	
Negative	114	107.87		2.71		0.29		10.20		12.34		1.04	
Pred response			.010*		.181		.948		.475		.222		.635
Sensitive	106	104.47		2.78		0.30		10.41		13.10		1.10	
Nonsensitive	20	171.23		2.10		0.34		13.73		10.01		0.83	
$d33^{th}$ MRD			.045*		.078		.465		.854		.026*		.084
$<10^{-4}$	60	97.14		2.31		0.35		10.07		10.43		0.86	
$>10^{-4}$	49	122.65		2.91		0.25		11.09		14.86		1.41	
$W12^{th}$ MRD			<.001*		.210		.610		.030		.022*		.048*
$<10^{-4}$	60	87.97		1.96		0.37		10.12		11.06		1.13	
$>10^{-4}$	49	158.76		2.27		0.27		16.77		16.17		2.49	
Risk			<.001*		.052		.734		.479		.069		.012*
Low	42	85.58		2.41		0.35		11.54		11.25		1.13	
Intermediate	48	105.87		3.63		0.33		8.99		12.64		0.62	
High	36	156.36		2.05		0.25		11.57		15.38		1.83	
Relapse			.024*		.966		.082		.092		.17		.010*
No	114	83.75		2.55		0.29		10.48		12.48		0.91	
Yes	12	110.64		2.20		1.08		17.36		17.78		11.81	

Note: Huber's M-estimator was employed to compare the statistic difference. The weighting constant is 1.339. Other reason: Died from either chemotherapy related death or not to get remission. *: *p* value represented as the comparison between relapse group and no relapse that were still alive.

MRP1 or MRP6 alone didn't affect relapse-free survival with P>0.05 (**Figure 2A** and **2F**).

When we reclassified the patients combining MRP1 with MRP5, patients were classified into 4 subgroups, namely A ($MRP1^H/5^L$), B ($MRP1^H/5^H$), C ($MRP1^L/5^L$), and D ($MRP1^L/5^H$) (**Figure 3A**). The relapse rate was compared within these 4 subgroups with the method of life-table estimates, we found that subgroup A patients didn't develop relapse within follow-up term, and about 35% of subgroup D patients relapsed early, and group B and group C ranked the second and third relapse rate (P= 0.007) (**Figure 3C**). Same trend was observed when MRP1 and MRP6 was combined together (**Figure 3B** and **3D**). Our results indicated that MRP1 combining with other members may be useful in refining subtype of ALL for the highest risk of relapse.

Discussion

Our study indicates that MRP1-6 is commonly expressed in all BP-ALL though with significant variation and change diversely during the treatment course. Among all MRPs, MRP1 has the highest and MRP3, the lowest expression by Q-RT-PCR. Most members of MRPs except MRP2 and MRP3 increased at relapse stage. The population with $MRP1^H$ were characterized with PPR, high MRD level on day 33^{th} and 12

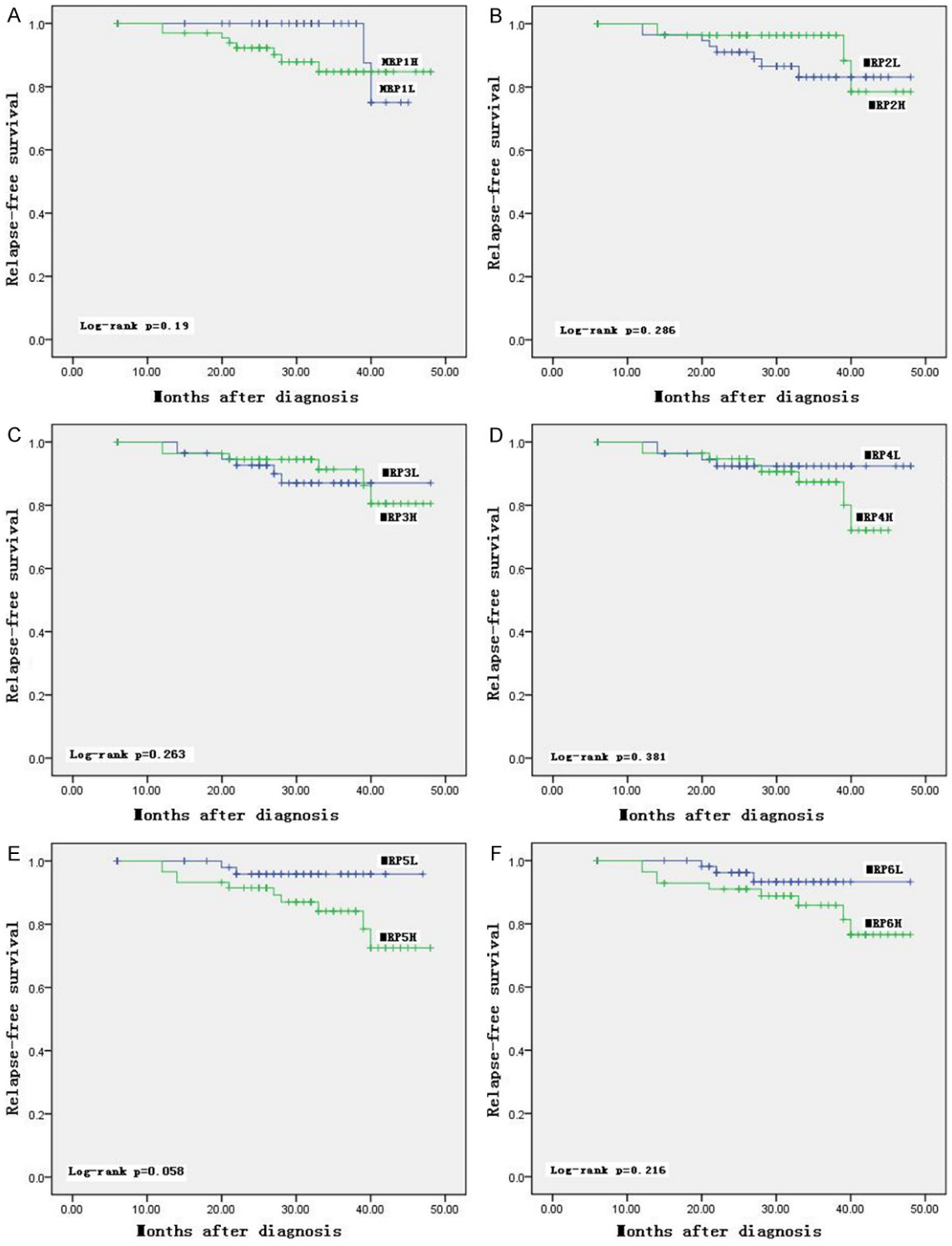

Figure 2. Relapse-free survival in children from the various MRPs subdivided into two groups of low (L) and high (H). A: MRP1; B: MRP2; C: MRP3; D: MRP4; E: MRP5; F: MRP6.

weeks and high WBC, age >10 year as well as. However, MRP1 expression alone is not significantly associated with relapse-free survival (**Figure 2A**) similar to other members of MRPs (**Figure 2B-F**). When the expression of MRP1 analyzed together with MRP6 (MRP1/6), sub-

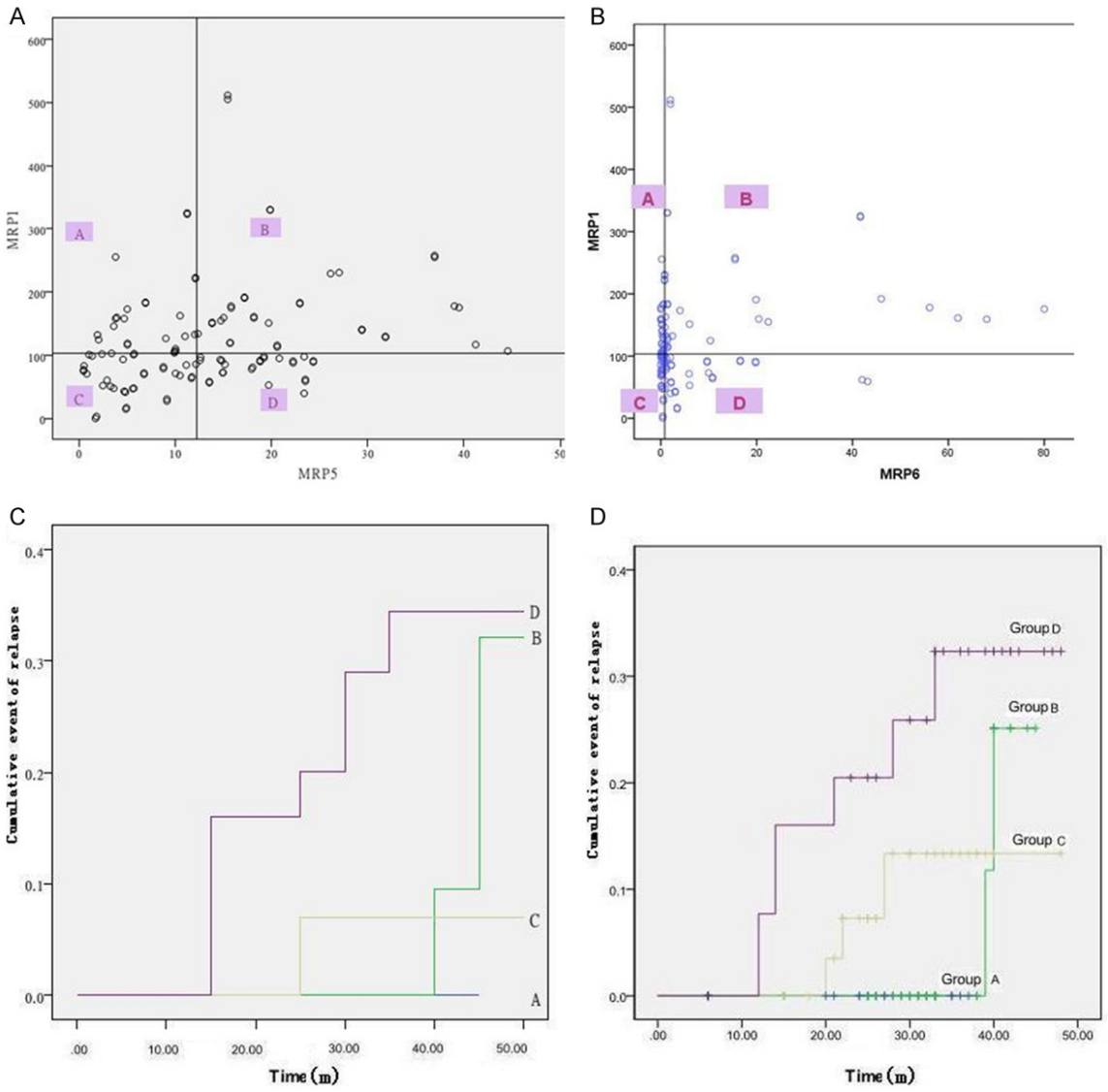

Figure 3. MRP1 and MRP5 (6) combinations were useful in refining subgroup of relapse. Patients subgroups were based on the 50th percentile of MRP1 and MRP5 (6). A. Four groups based on MRP1 and MRP5. B. Four groups based on MRP1 and MRP6. C. Relapse of four subgroups classified by MRP1 and MRP5. D. Relapse of four subgroups classified by MRP1 and MRP6.

groups with significant different outcome were identified. The rates of relapse from highest risk were patients with low MRP1 and high MRP6 (D group), and lowest in high MRP1 and low MRP6 (A group). To our best knowledge, such correlation between MRPs and clinical characteristics has not been reported in BP-ALL.

Anticancer drugs are highly subjected to MRPs effluex mechanism and render ineffectiveness of chemotherapeutic agents [30]. Elevated level of MRP1 is often found in malignant cells prior to drug treatment, just like our results. However, the impact of MRP1 on the outcome of ALL remains controversial. Some studies reported that increased MRP1 expression upon diagnosis had no impact on the event-free survival of children or adults [11, 31-33]. On the contrary, some authors declared that MRP1 expression influenced relapse-free survival [17, 34, 35]. Mahjoubi et al. reported overexpression of MRP1 occurred in Iranian pediatric leukemia patients at relapse [34] which was consistent with our results but they didn't find any relation between MRP1 mRNA levels and other

clinical characteristics, In our study, we not only found that MRP1 expression in relapse group was higher compared with CR group, but also found that high MRP1 expression is close correlated with PPR, high MRD level on day 33th and 12 weeks, high WBC, and age >10 year.

Recently, Rahgozar et al. [36] reported that MRP1 was positively related with the level of minimal residual disease (MRD) which was consistent with our findings. In our study we found that high expression of MRP1 had a positive correlation with poor response to therapy. Cortez et al. [37] reported that expression levels of MRP1 gene in patients classified as being at high risk was associated with higher rates of 5-year event-free survival (EFS) (P=0.01). Such findings were interpreted as low toxicity associated death rate in the high-risk patients with high expression of MRP1 gene [37]. Due to our follow-up cutoff duration is short, we focused on the relation of MRP1 expression upon diagnosis with relapse-free survival and found that MRP1 expression either high (>50% percentile) or low (<50% percentile) didn't exert influence on relapse-free survival (P=0.19) (**Figure 2A**). Our results are quite different from the report by Plasschaert et al. In 2005, they detected MRP1-6 expression in 56 pediatric patients, among them 15 cases were T-ALL, and only 39 cases were B-ALL. In our study, we enrolled 126 cases with B-ALL and treated under the protocol of CCLG-ALL-2008 which might partly explain the discrepancy.

Recently, MRP2 polymorphisms with methotrexate serum levels and its toxic effects in children with ALL was described [38, 39]. The expression level of MRP2 was rare reported in the literatures. Plasschaert reported that patients with higher MRP2 expression had a shorter term of relapse-free survival which was different from ours. The main reason might be our shorter follow-up duration though we detected a bigger size of patients (126 patients vs. 39 patients) [17]. In our findings we found that patients with TEL/AML1 showed a statistically higher expression of MRP2 than those negative patients (**Table 2**) which is first reported by our group. However, MRP2 expression didn't affect the relapse-free survival (P=0.286) (**Figure 2B**) Steinbach et al. [16] found that MRP3 expression was associated with a significantly worse prognosis (P=0.008). They detected the expressions of the MRP2, MRP3, MRP4, MRP5, and SMRP genes in B precursor ALL (n=76) and T-ALL (n=32) in 2003. They found that all 5 genes were expressed with a great variability which was consistent with our results. Meanwhile, they also found that MRP3 expression was associated with a significantly worse prognosis (P=0.008) independent of immunophenotype or sex [16]. In our study, the MRP3 gene was detected in 126 BP-ALL with lower expression at initial and relapse stage and higher expression after complete remission (CR). Further analysis also didn't show any correlation of MRP3 with clinical characteristics, cytogenetics, treatment response, and relapse.

Except the earlier reports by Plasschaert et al. group [17, 31], MRP4-6 expressions are rare reported. In our study, we found that relapse patients had higher expressions of MRP4-6 than CR group and showed significant correlation with high WBC, high MRD and gene rearrangements (**Tables 2** and **3**). However, we didn't find their impacts on relapse-free survival with univariate analysis (all *P* values >0.05) (**Figure 2**). Due to the complexity of MRPs, we attempt to reclassify patients based on the median expression of MRP1/5 or MRP1/6 into 4 subgroups and indicated that such classification is more useful in identifying patients with higher risk of relapse (**Figure 3**). Such classification may predict the outcome of pediatric BP-ALL in addition to conventional prognostic factors. However, more questions remain for the best use of such preliminary in future.

In conclusion, all MRPs were detectable in pediatric ALL and yet with distinct pattern. Prospective study in cooperated such markers are necessary to further define roles of MRPs in prognosis and treatment outcome.

Acknowledgements

The authors would like to thank the grants from Jiangsu Province's Clinical Medical Science and Technology Projects (Grant No. BL2013014), National Natural Science Foundation of China (No. 81100371 and No. 81370627), Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), and National clinical key subject construction project.

Disclosure of conflict of interest

None.

Address correspondence to: Shao-Yan Hu, Department of Hematology & Oncology, The Children's Hospital of Soochow University, 303 Jingde Road, Suzhou 215003, Jiangsu, China. Tel: 86-512-677-88409; Fax: 86-512-67786202; E-mail: hu_shaoyan@sina.com

References

[1] Conter V, Aricò M, Basso G, Biondi A, Barisone E, Messina C, Parasole R, De Rossi G, Locatelli F, Pession A, Santoro N, Micalizzi C, Citterio M, Rizzari C, Silvestri D, Rondelli R, Lo Nigro L, Ziino O, Testi AM, Masera G, Valsecchi MG; Associazione Italiana di Ematologia ed Oncologia Pediatrica. Long-term results of the Italian association of pediatric hematology and oncology (AIEOP) studies 82, 87, 88, 91 and 95 for childhood acute lymphoblastic leukemia. Leukemia 2010; 24: 255-264.

[2] Möricke A, Zimmermann M, Reiter A, Henze G, Schrauder A, Gadner H, Ludwig WD, Ritter J, Harbott J, Mann G, Klingebiel T, Zintl F, Niemeyer C, Kremens B, Niggli F, Niethammer D, Welte K, Stanulla M, Odenwald E, Riehm H, Schrappe M. Longterm results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leukemia 2010; 24: 265-284.

[3] Hunger SP, Lu X, Devidas M, Camitta BM, Gaynon PS, Winick NJ, Reaman GH, Carroll WL. Improved survival for children and adolescents with acute lymphoblastic leukemia from 1990-2005: a report from the Children's Oncology Group. J Clin Oncol 2012; 30: 1663-1669.

[4] Pui CH, Mullighan CG, Evans WE, Relling MV. Pediatric acute lymphoblastic leukemia: where are we going and how do we get there? Blood 2012; 120: 1165-1174.

[5] Pirker R, Wallner J, Geissler K, Linkesch W, Haas OA, Bettelheim P, Hopfner M, Scherrer R, Valent P, Havelec L, et al. MDR1 gene expression and treatment outcome in acute myeloid leukemia. J Natl Cancer Inst 1991; 83: 708-712.

[6] Marie JP. Drug resistance in hematologic malignancies. Curr Opin Oncol 2001; 13: 463-469.

[7] Sauerbrey A, Zintl F, Volm M. P-glycoprotein and glutathione S-transferase pi in childhood acute lymphoblastic leukaemia. Br J Cancer 1994; 70: 1144-1149.

[8] Kanerva J, Tiirikainen MI, Mäkipernaa A, Riikonen P, Möttönen M, Salmi TT, Krusius T, Saarinen-Pihkala UM. Initial P-glycoprotein expression in childhood acute lymphoblastic leukemia: no evidence of prognostic impact in follow-up. Pediatr Hematol Oncol 2001; 18: 27-36.

[9] den Boer ML, Pieters R, Kazemier KM, Rottier MM, Zwaan CM, Kaspers GJ, Janka-Schaub G, Henze G, Creutzig U, Scheper RJ, Veerman AJ. Relationship between major vault protein/lung resistance protein, multidrug resistance-associated protein, P-glycoprotein expression, and drug resistance in childhood leukemia. Blood 1998; 91: 2092-2098.

[10] Wuchter C, Leonid K, Ruppert V, Schrappe M, Büchner T, Schoch C, Haferlach T, Harbott J, Ratei R, Dörken B, Ludwig WD. Clinical significance of P-glycoprotein expression and function for response to induction chemotherapy, relapse rate and overall survival in acute leukemia. Haematologica 2000; 85: 711-721.

[11] Sauerbrey A, Voigt A, Wittig S, Häfer R, Zintl F. Messenger RNA analysis of the multidrug resistance related protein (MRP1) and the lung resistance protein (LRP) in de novo and relapsed childhood acute lymphoblastic leukemia. Leuk Lymphoma 2002; 43: 875-879.

[12] Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance associated proteins. J Natl Cancer Inst 2000; 92: 1295-1302.

[13] Kool M, van der Linden M, de Haas M, Baas F, Borst P. Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. Cancer Res 1999; 59: 175-182.

[14] Wielinga PR, Reid G, Challa EE, van der Heijden I, van Deemter L, de Haas M, Mol C, Kuil AJ, Groeneveld E, Schuetz JD, Brouwer C, De Abreu RA, Wijnholds J, Beijnen JH, Borst P. Thiopurine metabolism and identification of the thiopurine metabolites transported by MRP4 and MRP5 overexpressed in human embryonic kidney cells. Mol Pharmacol 2002; 62: 1321-1331.

[15] Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F, Borst P. MRP3, an organic anion transporter able to transport anticancer drugs. Proc Natl Acad Sci U S A 1999; 96: 6914-6919.

[16] Steinbach D, Wittig S, Cario G, Viehmann S, Mueller A, Gruhn B, Haefer R, Zintl F, Sauerbrey A. The multidrug resistance-associated protein 3 (MRP3) is associated with a poor outcome in childhood ALL and may account for the worse prognosis in male patients and T-cell immunophenotype. Blood 2003; 102: 4493-4498.

[17] Plasschaert SL, de Bont ES, Boezen M, vander Kolk DM, Daenen SM, Faber KN, Kamps WA, de Vries EG, Vellenga E. Expression of multidrug resistance-associated proteins predicts prognosis in childhood and adult acute lym-

phoblastic leukemia. Clin Cancer Res 2005; 11: 8661-8668.
[18] Lu J, Ashwani N, Zhang M, He H, Lu J, Wang Y, Zhao W, Cao L, Ji Z, He Y, Hunag Y, Chen R, Hu S. Children diagnosed as mixed-phenotype acute leukemia didn't benefit from the CCLG-2008 protocol, retrospective analysis from single center. Indian J Hematol Blood Transfus 2015; 31: 32-37.
[19] Weir EG, Cowan K, LeBeau P, Borowitz MJ. A limited antibody panel can distinguish B-precursor acute lymphoblastic leukemia from normal B precursors with four color flow cytometry: implications for residual disease detection. Leukemia 1999; 13: 558-567.
[20] Borowitz MJ, Pullen DJ, Winick N, Martin PL, Bowman WP, Camitta B. Comparison of diagnostic and relapse flow cytometry phenotypes in childhood acute lymphoblastic leukemia: implications for residual disease detection: a report from the children's oncology group. Cytometry B Clin Cytom 2005; 68: 18-24.
[21] Veltroni M, De Zen L, Sanzari MC, Maglia O, Dworzak MN, Ratei R, Biondi A, Basso G, Gaipa G; I-BFM-ALL-FCM-MRD-Study Group. Expression of CD58 in normal, regenerating and leukemic bone marrow B cells: implications for the detection of minimal residual disease in acute lymphocytic leukemia. Haematologica 2003; 88: 1245-1252.
[22] DiGiuseppe JA, Fuller SG, Borowitz MJ. Overexpression of CD49f in precursor B-cell acute lymphoblastic leukemia: potential usefulness in minimal residual disease detection. Cytometry B Clin Cytom 2009; 76: 150-155.
[23] Borowitz MJ, Pullen DJ, Shuster JJ, Viswanatha D, Montgomery K, Willman CL, Camitta B; Children's Oncology Group Study. Minimal residual disease detection in childhood precursor-B-cell acute lymphoblastic leukemia relation to other risk factors. A children's oncology group study. Leukemia 2003; 17: 1566-1572.
[24] Parker C, Waters R, Leighton C, Hancock J, Sutton R, Moorman AV, Ancliff P, Morgan M, Masurekar A, Goulden N, Green N, Révész T, Darbyshire P, Love S, Saha V. Effect of mitoxantrone on outcome of children with first relapse of acute lymphoblastic leukaemia (ALL R3): an open-label randomised trial. Lancet 2010; 376: 2009-2017.
[25] Dördelmann M, Reiter A, Borkhardt A, Ludwig WD, Götz N, Viehmann S, Gadner H, Riehm H, Schrappe M. Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. Blood 1999; 94: 1209-1217.
[26] Hu S, Chen R, Man X, Feng X, Cen J, Gu W, He H, Li J, Chai Y, Chen Z. The function and expression of IGFBP7 gene in childhood acute myeloid leukemia. Pediatr Hematol Oncol 2011; 28: 279-287.
[27] Loh ML, Goldwasser MA, Silverman LB, Poon WM, Vattikuti S, Cardoso A, Neuberg DS, Shannon KM, Sallan SE, Gilliland DG. Prospective analysis of TEL/AML1-positive patients treated on dana-farber cancer institute consortium protocol 95-01. Blood 2006; 107: 4508-4513.
[28] Shurtleff SA, Buijs A, Behm FG, Rubnitz JE, Raimondi SC, Hancock ML, Chan GC, Pui CH, Grosveld G, Downing JR. TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. Leukemia 1995; 9: 1985-1989.
[29] Chiaretti S, Vitale A, Cazzaniga G, Orlando SM, Silvestri D, Fazi P, Valsecchi MG, Elia L, Testi AM, Mancini F, Conter V, te Kronnie G, Ferrara F, Di Raimondo F, Tedeschi A, Fioritoni G, Fabbiano F, Meloni G, Specchia G, Pizzolo G, Mandelli F, Guarini A, Basso G, Biondi A, Foà R. Clinico-biological features of 5202 patients with acute lymphoblastic leukemia enrolled in the Italian AIEOP and GIMEMA protocols and stratified in age cohorts. Haematologica 2013; 98: 1702-1710.
[30] Deeley RG, Westlake C, Cole SP. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev 2006; 86: 849-899.
[31] Plasschaert SL, Vellenga E, de Bont ES, van der Kolk DM, Veerman AJ, Sluiter WJ, Daenen SM, de Vries EG, Kamps WA. High functional P-glycoprotein activity is more often present in T-cell acute lymphoblastic leukaemic cells in adults than in children. Leuk Lymphoma 2003; 44: 85-95.
[32] Kakihara T, Tanaka A, Watanabe A, Yamamoto K, Kanto K, Kataoka S, Ogawa A, Asami K, Uchiyama M. Expression of multidrug resistance-related genes does not contribute to risk factors in newly diagnosed childhood acute lymphoblastic leukemia. Pediatr Int 1999; 41: 641-647.
[33] Valera ET, Scrideli CA, Queiroz RG, Mori BM, Tone LG. Multiple drug resistance protein (MDR-1), multidrug resistance related protein (MRP) and lung resistance protein (LRP) gene expression in childhood acute lymphoblastic leukemia. Sao Paulo Med J 2004; 122: 166-171.
[34] Mahjoubi F, Akbari S. Multidrug resistance-associated protein 1 predicts relapse in Iranian childhood acute lymphoblastic leukemia. Asian Pac J Cancer Prev 2012; 13: 2285-2289.
[35] Huh HJ, Park CJ, Jang S, Seo EJ, Chi HS, Lee JH, Lee KH, Seo JJ, Moon HN, Ghim T. Prognostic significance of multidrug resistance gene 1 (MDR1), multidrug resistance-related protein (MRP) and lung resistance protein

(LRP) mRNA expression in acute leukemia. J Korean Med Sci 2006; 21: 253-258.
[36] Rahgozar S, Moafi A, Abedi M, Entezar-E-Ghaem M, Moshtaghian J, Ghaedi K, Esmaeili A, Montazeri F. mRNA expression profile of multidrug-resistant genes in acute lymphoblastic leukemia of children, a prognostic value for ABCA3 and ABCA2. Cancer Biol Ther 2014; 15: 35-41.
[37] Cortez MA, Scrideli CA, Yunes JA, Valera ET, Toledo SR, Pavoni-Ferreira PC, Lee ML, Petrilli AS, Brandalise SR, Tone LG. mRNA expression profile of multidrug resistance genes in childhood acute lymphoblastic leukemia. Low expression levels associated with a higher risk of toxic death. Pediatr Blood Cancer 2009; 53: 996-1004.
[38] Sharifi MJ, Bahoush G, Zaker F, Ansari S, Rafsanjani KA, Sharafi H. Association of -24CT, 1249GA, and 3972CT ABCC2 gene polymorphisms with methotrexate serum levels and toxic side effects in children with acute lymphoblastic leukemia. Pediatr Hematol Oncol 2014; 31: 169-177.
[39] Liu Y, Yin Y, Sheng Q, Lu X, Wang F, Lin Z, Tian H, Xu A, Zhang J. Association of ABCC2 -24C>T polymorphism with high-dose methotrexate plasma concentrations and toxicities in childhood acute lymphoblastic leukemia. PLoS One 2014; 9: e82681.