Characterizing Iranian Pediatric Patients With Relapsed Acute Lymphoblastic Leukemia Through Gene Expression Profiling of Common ATP Binding Cassette Transporters Subfamily C

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Background: The correlation between gene expression of ABCC transporters and recurrence as a treatment failure in pediatric patients with acute lymphoblastic leukemia (ALL) is an unsolved problem in scientific associations. The aim of this study was to evaluate the predictive value of *ABCC1-6* gene expression pattern for estimating recurrence in Iranian pediatric patients with ALL.

Materials and Methods: Iranian pediatric patients with approved ALL enrolled in this study as 2 groups of case (relapsed ALL) and control (treated individuals who lasted for >3 years following their final treatment). Real-time polymerase chain reaction was done with *GAPDH* for expressing *ABCC1-6* transporter genes. Cumulative doses of Vincristine, Daunorubicin, and L-Asparginase were checked for each patient. Gathered data analyzed with SPSS version 22 and REST 2009 software.

Results: Thirty-nine samples as 23 relapsed ALL and 16 controls enrolled. High expression of *ABCC2-6* and low expression of *ABCC1* were detected in pediatric patients with relapse. *ABCC3* and *ABCC4* had significant relation with high-risk patients of NCI group. Also, *ABCC4* and *ABCC6* had more expression with high doses of Daunorubicin and L-Asparginase.

Conclusions: Designed expression pattern have the predictive value for estimating of conferring relapse in Iranian pediatric patients with diagnosed ALL. The authors suggest of designing a multiple childhood malignancy center project to evaluate this pattern in a cohort study.

Key Words: ABC transporters, gene expression, pattern, relapse

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Relapse is still as the commonest treatment failure through chemotherapy of pediatric patients with acute lymphoblastic leukemia (ALL)[1] that can lead to poor prognosis and outcome in mentioned patients. Multidrug resistance (MDR) due to ATP binding cassettes (ABC) transporters' gene expression[2,3] specially subfamily C, which is known as multidrug resistance associate proteins (MRPs), is the main cause of relapse.[4] Literature review revealed controversial conclusions around the effect of ABCC transporters gene expression and prognosis of pediatric patients with ALL. Some studies showed that there is not any significant relation between gene expression of ABCC transporters and prognosis in pediatric patients with ALL.[5–7] According to various results from published studies, still the correlation between gene expression of ABCC subfamily and treatment failure or prognosis in pediatric patients with ALL is an unsolved problem.[8–11]

This project was planned because of rare reports with focus on the expression of ABCC transporters in Iranian pediatric patients with ALL. The aim of conducting this study was to design a gene expression profile pattern from *ABCC1-6* in Iranian pediatric patients with ALL. The most important question of this study was whether the considered profile could have the predictive value for estimating relapse in mentioned patients.

MATERIALS AND METHODS

Patients and Samples

Sampling of this study was done from patients who referred to 2 referral childhood's malignancy center in Tehran, Iran. The designed project was a case control study for evaluating gene expression of *ABCC1-6* by real-time polymerase chain reaction (RT-PCR) method. All of the enrolled patients were children younger than 15 years with ALL. The case group consisted of those mentioned patients who had relapse at the time of study and were managed with pediatric hematologists-oncologists for starting of administrating relapse protocol. The control group consisted of mentioned patients who successfully finalized their therapy and at the time of study 3 years were past for them without any treatment failure or relapse. After filling the informed consent with parents of patients, 3 mL of peripheral blood (PB) was obtained from children in the EDTA blood sample tubes.

Ethics Approval

This project was accepted in the ethical committee of Shahid Beheshti University of Medical Science with the ethical code of IR.SBMU.RETECH.REC.1396.533.

Chemotherapy Agents

Chemotherapy regimen of patients was based on ALL-BFM protocol 2009 (Berlin-Frankfurt-Munster). Cumulative

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doses of administered chemotherapy agents in induction phase were calculated. Cumulative dose of Vincristine (VCR) was categorized as low (< 20 mg/m^2), intermediate (20 to 40 mg/m^2), and high dose (> 40 mg/m^2).[12,13] According to the standard dose of Daunorubicin (DNR),[14] the cumulative dose categorized as standard (< 100 mg/m^2) and high (≥ 100 mg/m^2). Cumulative dose of L-Asparginase (LASP) categorized as standard (< 60,000 U/m^2) and high (≥ 60,000 U/m^2) doses.

Total RNA Isolation

After obtaining each sample, PB was transferred to the laboratory immediately. Then white blood cells separated from PB by Red Blood Cell Lysis Buffer and suspended in PBS.[15] Total RNA isolated by YTzol Pure RNA reagent according to the instruction manual (cat no: YT9063, Yekta Tajhiz Azma, Iran). The concentration of total RNA considered by Thermo Scientific NanoDrop (Thermo Fisher Scientific) and the purity checked by OD260/OD280 absorption. Total RNAs had been stored in −70°C until cDNA synthetizing.

cDNA Synthesize

cDNA was prepared using standard of total RNA. cDNA synthesis kit with cat no: YT4500, Yekta Tajhiz Azma, Iran was used. The template RNA (based on 1 μg) mixed with 1 μL random hexamer primer and finalized with DEPC-treated water to the volume of 13.4 μL. After incubation at 70°C for 5 minutes, each product mixed with 4 μL of 5× first strand buffer, 1 μL of dNTP, 0.5 μL of RNasin and 1 μL of MMLV. The mixture incubated for 1 hour at 37°C and terminated by heating at 70°C for 5 minutes. Finally PCR products of synthetizing were stored in −70°C for real-time PCR section.

Real-time PCR

Primers for *ABCC1-6* genes[16] were designed according to Table 1. *GAPDH* gene was used for the normalization of the results through RT-PCR with forward primer (5′-3′): GAAGGTGAAGGTCGGAGTC and reverse primer (5′-3′): GAAGATGGTGATGGGATTTC. All primers were obtained from Pishgam Biotech Company, Iran. Primers had been purified with MOPC purification method.

Before starting RT-PCR, products of cDNA synthesizes had been diluted as 1:3. For making mixture of RT-PCR, 0.5 μL of forward primer +0.5 μL of reverse primer + cDNA template according to 1 μg/μL + 7.5 μL of Syber Green QPCR master mix 2×(cat no: YT2551, Yekta Tajhiz Azma, Iran) added together and with nuclease free water the final reaction volume became 20 μL. The RT-PCR program had been set up as: 1 cycle of preincubation (95°C = 300 s); 38 cycles of 3-step amplification (95°C = 60 s, 60°C = 60 s, 72°C = 30 s), and 1 cycle of melting (95°C = 10 s, 65°C = 60 s, 97°C = 1 s). Quantifications had been done by LightCycler 96 Real-Time PCR Cycler (Hoffmann-La Roche AG, Basel, Switzerland). For checking the fluorescent signals of specific bands, all of the RT-PCR products were run by 1.5% agarose gel electrophoresis.

Statistical Analysis

Raw data were analyzed by light cycler software. Relative expression analysis were done by REST 2009 software. Statistical calculations were performed using SPSS version 22 software. *t* test was used for consideration differences between groups of case and control. *P*-value was defined as ≤ 0.05 for significant relations.

RESULTS

Patients

A total of 39 pediatric patients with ALL (23 case and 16 control) were enrolled in this study. Male to female ratio of the patients was 1:3 (male patients, 22; female patients, 17). The mean age at diagnosis was 6.1 ± 4.1 years (range, 7 mo to 14 y). Immune-phenotypes of patients were: Pre-B (21); early Pre-B (13); T-cell (3); Pro-B (2). Bone marrow aspiration at the end of induction showed that 63% had complete remission, 29.6% as hypo-cellular, and 7.4% as partial remission.

Sites of relapse for patients in the group of case were: CNS: 55%; bone marrow: 25%; testis: 13%, and 1 patient had relapse at bone marrow and CNS. The median time of relapse from diagnosis for those mentioned patients was 23 months (range, 65 d to 5 y).

Table 2 shows patients' characterizations according to case and control groups.

Chemotherapy Agents

The maximum and mean (± SD) cumulative dose of Vincristine were 90.9 and 38.5 ± 22 mg/m^2, respectively (34.5%, low dose; 20.7%, intermediate dose; and 44.8%, high dose). The maximum and mean (± SD) cumulative dose of DNR were 185 and 67.3 ± 43.5 mg/m^2 (standard dose, 81.8%; high dose, 18.2%). Results showed that the maximum and mean (± SD) cumulative dose of LASP were 92,500 and 43,894.26 ± 22,717.9 U/m^2 (standard dose, 78.6%; high dose, 21.4%). Table 2 shows cumulative doses of chemotherapy agents of induction phase based on the 2 considered groups.

Gene Expression of *ABCC1-6*

Patients with relapse had significantly (*P*-value <0.0161) more expression of *ABCC 2-6* and less expression of *ABCC1* in comparison with patients who were in control group. The mean fold of gene expressions in patients according to Livak test were: *ABCC1* (control: 1.63; case: 1.18), *ABCC2* (control: 1.09; case: 3.06), *ABCC3* (control: 6.11; case: 7.34), *ABCC4* (control: 1.52; case: 3.04), *ABCC5* (control: 1.83; case: 2.59), and *ABCC6* (control: 1.71; case: 4.00). Figure 1 shows relation between ABC transporters' genes in the 2 groups of case and control.

Patients at high-risk group of NCI showed significant relation with high expression of *ABCC3* (*P*-value: 0.004) and *ABCC4* (*P*-value: 0.03). High doses of DNR and LASP

TABLE 1. Forward and Reverse Primers of ABCC1-6

Genes	Forward Primer, 5′-3′	Reverse Primer, 5′-3′	Gene Bank Accession No	Position (bp)
ABCC1	GAAGGCCATCGGACTCTTCA	CAGCGCGGACACATGGT	L05628	3097-3166
ABCC2	TGCAGCCTCCATAACCATGAG	GATGCCTGCCATTGGACCTA	U63970	2728-2807
ABCC3	CACACGGATCTGACAGACAATGA	ACAGGGCACTCAGCTGTCTCA	AB_010887	2670-2745
ABCC4	AAGTGAACAACCTCCAGTTCCAG	GGCTCTCCAGAGCACCATCT	AF_071202	2026-2144
ABCC5	TGAAAGCCATTCGAGGAGTTG	CGGAAAAGCTCGTCATGCA	AF_146074	2979-3054
ABCC6	AGACACGGTTGACGTGGACAT	GCTGACCTCCAGGAGTCCAA	AF_168791	3156-3231

TABLE 2. Patients' Characteristics and Cumulative Doses of Chemotherapy Agents According to Case and Control Groups

			Immune-phenotypes (n)				NCI Risk Groups at the Time of DX			VCR (%)			DNR (%)		LASP (%)	
	M/F	Mean age at DX ± SD (y)	Pre-B	Early Pre-B	T-cell	Pro-B	LR (n)	HR (n)	VHR (n)	LD	ID	HD	SD	HD	SD	HD
Case (23)	17/6 (2.8)	5.1 ± 0.9	13	6	2	2	9	5	1	57.1	14.3	28.6	75	25	84.6	15.4
Control (16)	5/11 (0.4)	7.3 ± 1.0	8	7	1	—	12	4	—	13.3	26.7	60	90	10	73.3	26.7

DNR indicates Daunorubicin; DX, diagnosis; HD, high dose; HR, high risk; ID, intermediate dose; LASP, L-Asparginase; LD, low dose; LR, low risk; M/F, male/female; SD, standard dose; VCR, Vincristine; VHR, very high risk.

had significant relation with high expression of *ABCC4* (*P*-value: 0.01) and *ABCC6* (*P*-value: 0.05), respectively.

Patients' Follow-up

At the time of preparing this manuscript, 9 patients from case group died because of their relapse. The median time of follow-up for all of the patients was 4 years (range, 14 mo to 10 y). The median time of follow-up for patients in the case group was 36 months and for control group accounted as 61 months. The 3-year and 5-year overall survival according to Kaplan-Meier analysis were: 82.3% ± 0.06 and 75.4% ± 0.07, respectively.

DISCUSSION

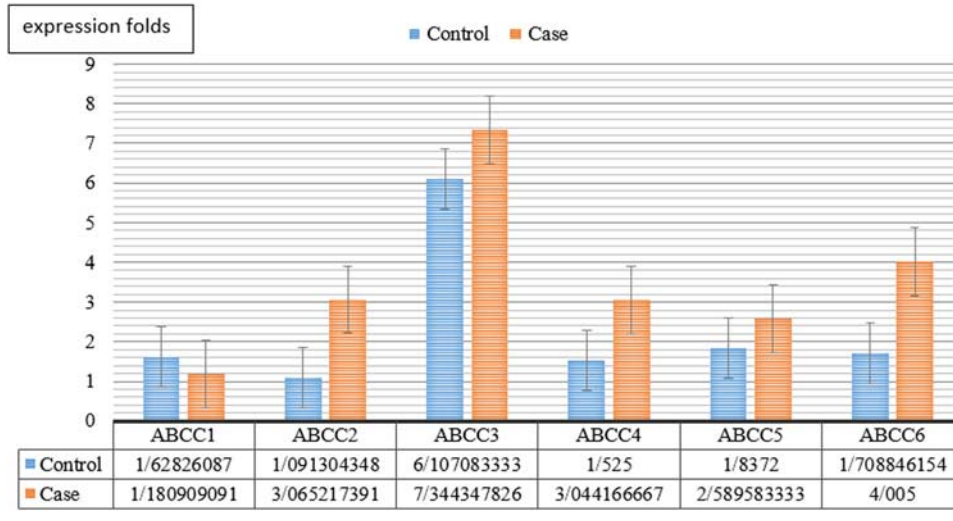
The main role of ATP Binding Cassette transporters is transportation of chemotherapeutic agents that make them as targets for drug therapies.[17] Sensitivity to chemotherapy agents are regulated by expression of ABC transporter genes mainly subfamily of C transporters.[18,19] As gene expression of ABCC transporters effect the quality of treatment so designing a pattern of expression could beneficially decrease treatment failures and improve prognosis. In Iran, adjusting updated chemotherapy protocols with universal decisions has specific complexity and even impossible.

There are obvious controversial conclusions according to variant studies about correlation of ABC transporters' gene expression and treatment response (poor prognosis, early relapse, and reduced relapse free survival). In spite of improving the survival rate of pediatric patients with ALL, still relapse is the main result of treatment failure.[20]

MRP1 was considered on 167 patients with ALL in different stages (new cases, complete remission, and relapses) by the RT-PCR method. Their results showed that *MRP1* was higher in relapse patients, and those individuals who expressed more *MRD1* had inability to achieve complete remission. Their suggestion was about further designed studies about functional characteristics of *MRP1* through drug transports.[21]

A study was designed on 32 cases with untreated do novo ALL showed low correlation between gene expression of *MDR1*, *MRP1*, and poor response of chemotherapy. Their results showed that there was not any relation between those genes' expression and treatment outcomes or even predicting for relapse in patients.[22]

Review articles demonstrated published reports that emphasized on the prognostic value of *MDR1* and *ABCC1* in patients with acute leukemia.[23–26] A study on 34 pediatric ALL patients revealed that high expression of *ABCC1* could affect complete remission and lead to decrease in the 2-year overall survival rate.[27] Also, other reports demonstrated that there is not any relation between gene expression of *ABCC1* or *MDR1* and poor treatment responses in considered patients with ALL.[28] Another controversial study was done on 140 pediatric patients



	ABCC1	ABCC2	ABCC3	ABCC4	ABCC5	ABCC6
Control	1/62826087	1/091304348	6/107083333	1/525	1/8372	1/708846154
Case	1/180909091	3/065217391	7/344347826	3/044166667	2/589583333	4/005

FIGURE 1. Gene expression of *ABCC1-6* in 2 groups of case and control. full color online

with ALL that showed low-level expression of *ABCC1* and *ABCC3* related with high risk of death caused by treatment toxicity.[29]

Systematic reviews around this topic revealed that there are rare published papers with the focus on gene expression of ABC transporters and prognosis in Iranian pediatric population with ALL. Mahjoubi and Akbari designed a study on 42 Iranian pediatric patients with ALL. In patients with relapsed, *ABCC1* showed high gene expression.[15]

In favor to that study, our data in Iranian pediatric patients with relapsed ALL had high expression of *ABCC 2-6* and low expression of *ABCC1* than those individuals who had finalized their therapies and lasted 3 years after their final treatment without any recurrence. Also, *ABCC3* and *ABCC4* had high expression in relapsed patients with high-risk NCI characterization. This result revealed that high-risk patients had more probability of higher expression of *ABCC3* and *ABCC4* than other groups of pediatric patients. High doses of DNR and LASP had related with high expression of *ABCC4* and *ABCC6*. Findings of this project for the first time as a population base are worthwhile according to the aim of the study. The most important limitation of this study was the number of enrolled patients as this project conducted with collaboration of single center.

CONCLUSIONS

Results of this study emphasize that high expression of ABCC2-6 and low expression of *ABCC1* had correlation with recurrence in pediatric patients with ALL. High doses of DNR and LASP led to high expression of *ABCC4* and *ABCC6* which are the risk factors of relapse in mentioned patients. Also due to NCI categorization, high risk group had more expression of *ABCC3* and *ABCC4* in relapsed pediatric patients with ALL.

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