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# Pattern of *ABCC* Transporter Gene Expression in Pediatric Patients with Relapsed Acute Lymphoblastic Leukemia

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## Abstract

***Background:*** Abnormal expression of *ABCC* transporter genes has been associated with treatment failure in pediatric patients with acute lymphoblastic leukemia (ALL). The aim of this study was to evaluate the expression pattern of *ABCC1-6* and *ABCC10* genes in Iranian pediatric patients with ALL relapse and determine the potential predictive value of determining ALL relapse from *ABCC* expression.

***Methods:*** Patients with ALL were divided into two separate groups, either the case group with relapsed ALL or the control group in which ALL patients have been in progression-free survival for at least 3 years A total of thirty-nine participants (23 with relapsed ALL; 16 controls) were enrolled over 26 months. To determine the levels of *ABCC1-6* and *ABCC10* transporter gene expression RT-PCR was used. Cumulative doses of the chemotherapy drugs, VCR, DNR and L-ASP, were calculated for each patient.

***Results:*** Our findings showed elevated expression of *ABCC2-6* and decreased expression of *ABCC1* and *ABCC10* to be associated with an increased risk of ALL relapse. The mean-fold expression of *ABCC2* was significantly increased in the ALL relapse group. Additionally, the expression pattern of the *ABCC* transporter genes was associated with high doses of three chemotherapy drugs, VCR, DNR and L-ASP.

***Conclusions:*** Evaluating the expression pattern of *ABCC* transporter genes may be a potential biomarker for predicting the occurrence of ALL relapse in Iranian pediatric patients and improve cancer prognosis.

**Keywords:** ABC transporters, Childhood, Gene expression, Pattern, Recurrence.

## Introduction

The Iranian Cancer Registry has previously reported that in 2007, the incidence of pediatric cancer was 176 cases per 1 million children in Tehran, Iran (1). In patients with ALL, the most common factor involved in treatment failure is the occurrence of cancer relapse, often leading to a poor prognosis (3) The chemotherapy treatment is often rendered ineffective due to the development of multidrug

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resistance (MDR). This can be a result of abnormal ATP-binding cassette (ABC) transporter gene expression (4-6). The *ABCC* transporter subfamily of the ABC transporters is also known as multidrug resistance proteins (MRPs). These membrane proteins are able to render the chemotherapeutic agents ineffective due to their ability to transport anticancer agents across membranes and excrete substances out of the cell (7). Due to their ability to interfere with treatments, MRPs are an attractive target for improving the treatment of ALL and preventing the development of cancer cell resistance to chemotherapy (8). Important genes of this subfamily which relate to multidrug resistance in patients with malignancy are *ABCC1-6* and *ABCC10* (9).

Previous reports have examined the relationship between *ABCC* transporter gene expression and the prognosis of pediatric patients with ALL. Some research has found no significant relationship between the gene expression of *ABCC1* or *ABCC3* and ALL prognosis in pediatric patients (10-12). However, it has been found that one of the main functions of *ABCC1-6* is the transportation of chemotherapeutic agents, such as Adriamycin (ADR), Vincristine (VCR), Etoposide (VP16), 6-Mercaptopurine (6-MP), and Methotrexate (MTX) (13-16).

The aim of this study was to examine the gene expression profile of *ABCC1-6* and *ABCC10* in Iranian pediatric patients with ALL and determine if this expression pattern can predict ALL relapse. Additionally, we examined the relationship between chemotherapy dose and *ABCC* gene expression.

## Materials and methods

### *Ethics approval*

Permission to conduct this study and ethical approval was obtained from the Ethics Committee of the Shahid Beheshti University of Medical Science. The ethics approval code was IR.SBMU.RETECH.REC.1396.533.

### *Patient samples and demographic*

In this prospective case-control study, we evaluated the gene expression of *ABCC1-6* and *ABCC10* in pediatric ALL patients. The participants in this study were children referred to the MAHAL's Pediatric Cancer Treatment and Research Center in Tehran, Iran, with either a previous or current diagnosis of ALL. Patients included in this study were under the age of 15, had an immunophenotyping report of ALL, and had previously completed their chemotherapy treatment. The case group included patients who have had ALL relapse immediately following or during their chemotherapy regimen. The control group consisted of patients who successfully completed their chemotherapy treatment and had at least 3 years of progression-free survival.

Informed consent was obtained from each patient's parent prior to enrollment in the study. Consent was also given to collect 3 mL of peripheral blood (PB) in EDTA-treated tubes before beginning the chemotherapy cycle.

For each participant a questionnaire about their clinical, pathological, and demographic characteristics was completed by a physician. These characteristics included age at the time of diagnosis, sex, French-American-British (FAB) classification and immunophenotype of ALL, date chemotherapy regimen was initiated, date of first complete remission, and the total doses of each chemotherapy agent used during the treatment, prognostic factor and National Cancer Institute (NCI) risk group (17). In addition, the date, site, and number of recurrences were reported for the case group.

### *Cumulative dose of chemotherapy agents*

The chemotherapy regimen was based on the ALL-BFM (Berlin-Frankfurt-Munster) protocol 2009. Cumulative doses of the chemotherapy drugs administered during the induction phase were calculated. The cumulative dose of VCR was categorized as low (<20 mg/m$^2$), intermediate (20-40 mg/m$^2$), or high (>40 mg/m$^2$) (18, 19). According to the standard dose of Daunorubicin (DNR) (20), the cumulative dose was categorized as standard (<100 mg/m$^2$) or high (≥100 mg/m$^2$). The cumulative dose of L-Asparginase (L-ASP) was categorized as standard (<60,000 u/m$^2$) or high (≥60,000 u/m$^2$).

### *Total RNA isolation*

Each PB sample was transferred to the laboratory immediately after collection. White blood cells were separated by red blood cell lysis buffer and suspended in PBS (21). Total RNA was isolated by YTzol Pure RNA reagent according to the manufacturer's instructions (Cat No: YT9063, Yekta Tajhiz Azma, Iran). The concentration of total RNA was determined by Thermo Scientific NanoDrop (Thermo Fisher Scientific, USA), and the purity was examined via OD260/OD280 absorption. Total RNA samples were stored in -70 °C until cDNA synthesis.

### *cDNA synthesis*

The cDNA was prepared using standard total RNA. A cDNA synthesis kit (Cat No: YT4500, Yekta Tajhiz Azma, Iran) was used according to the manufacturer's instructions. The template RNA (based on 1 µg) was mixed with 1 µL random hexamer primer (184.84 µl) and finalized with DEPC treated water to the volume of 13.4 µL. After incubation at 70 °C for 5 minutes, each product was mixed with 4 µL 5× first-strand buffer (200 µl), 1 µL dNTP (50 µl), 0.5 µL RNasin (25 µl), and 1 µL MMLV (50 µl). The mixture was incubated for 1 hour at 37 °C and was terminated by heating at 70 °C for 5 minutes. The PCR products were stored at –70 °C prior to RT-PCR analysis (9).

### *Real-Time PCR*

The primers used for *ABCC1-6* and *ABCC10* genes are shown in Table 1 and designed as previously described (22) . The *GAPDH* gene was used to normalize the results through RT-PCR with the forward primer (5'–3'): GAAGGTGAAGGTCGGAGTC and reverse primer (5'–3'): GAAGATGGTGATGGGATTTC. All primers were obtained from Pishgam Biotech Company, Iran. Primers had been purified using the Macrogen Oligonucleotide Purification Cartridge™ purification method.

Prior to beginning RT-PCR, products of cDNA synthesis were diluted at a ratio of 1:3. The mixture for RT-PCR consisted of 0.5 µL forward primer (0.2 µM) + 0.5 µL reverse primer (0.2 µM) + cDNA template according to 1 µg/µL + 7.5 µL Syber Green QPCR master mix 2× (cat no: YT2551, Yekta Tajhiz Azma, Iran;) and nuclease-free water to achieve a final volume of 20 µL. The RT-PCR program was set up as follows: one cycle of pre-incubation (95 °C = 300 s), 38 cycles of 3-step amplification (95 °C = 60 s, 60 °C = 60 s, 72 °C = 30 s), and one cycle of melting (95 °C = 10 s, 65 °C = 60 s, 97 °C = 1 s). Quantifications were determined by Light Cycler 96 Real-Time PCR Cycler (Hoffmann-La Roche AG, Basel, Switzerland). For checking the fluorescent signals of specific bands, all RT-PCR products were subjected to 1.5% agarose gel electrophoresis.

**Table 1.** Forward and reverse primers of *ABCC1-6* and *ABCC10*

| Gene | 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 |
| ***ABCC10*** | GCGGGTTAAGCTTGTGACAGA | CCCACCCGCAGAACTTGA | XM_052745 | 1585–1646 |

### *Statistical analysis*

The raw data was analyzed by light cycler software. Relative-expression analysis was done by using REST 2009 software. Statistical calculations were performed using SPSS (version 22) software. The mean fold expression and ROC curve analysis was determined using GraphPad PRISM version 8.1.2 software. The relative changes in gene expression were analyzed by the Livak method (23). A P-value of ≤0.05 indicates statistical significance.

## Results

### *Patients*

A total of 39 pediatric patients with ALL were enrolled in this study over a period of 26 months. Table 2 shows the demographic data of our patient cohort. The case group included 23 patients, and the control group included 16 patients. In this study 18 children were from Tehran (the capital city of Iran) and others had been referred from different provinces of Iran.

Our cohort included a total of 22 boys and 17 girls. The mean age at diagnosis was 6.1 ± 4.1 years (range 7 months–14 years). The immunophenotypes of patients were determined as follows: Pre-B ALL (n=21); early Pre-B ALL (n=13); T-cell ALL (n=3); and Pro-B ALL (n=2). The BMA (Bone Marrow Aspiration) at the end of induction showed that 63% were in complete remission, 29.6% were hypocellular, and 7.4% were in partial remission.

The sites of relapse in the case group were as follows: central nervous system (CNS): 55%, bone marrow: 25%, testis: 13%, and one patient experienced relapse in the bone marrow and CNS. The median time to relapse from diagnosis was 23 months (range, 65 days–5 years).

Nine patients (8 from the case group and 1 from the control group) did not fall into a prognostic group. Additionally, eight participants in the case group did not have an NCI risk evaluation. Of the 31 patients who had NCI risk evaluations, 21 had low-risk ALL, nine had high-risk ALL, and one had very high–risk ALL.

**Table 2.** Patient characteristics and cumulative doses of chemotherapy agents

| | Sex (n) & Age | | | | Immunophenotypes type of ALL (n) | | | | BMA (%) at the end of induction | | | Prognostic group (n) | | NCI risk groups (n) | | | Vincristine dose (%) | | | Daunorubicin dose (%) | | L-asparginase dose (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | M/F | MA.DX (mean ± SD) | PB | EPB | TC | POB | CR | Hy | PR | FV | LFV | LR | HR | VHR | L | I | H | SD | H | SD | H |
| **Case group** | **17** | **6** | **2.8** | **5.1 ± 0.9** | **13** | **6** | **2** | **2** | **50** | **35.7** | **14.3** | **9** | **6** | **9** | **5** | **1** | **57.1** | **14.3** | **28.6** | **75** | **25** | **84.6** | **15.4** |
| **Control group** | **5** | **11** | **0.04** | **7.3 ± 1.0** | **8** | **7** | **1** | **-** | **76.9** | **23.1** | **-** | **6** | **1** | **12** | **4** | **-** | **13.3** | **26.7** | **60** | **90** | **10** | **15.4** | **73.3** |

Abbreviations: n, number; BMA, Bone Marrow Aspiration; NCI, National Cancer Institute; M, Male; F, Female; M/F, Male to Female; MA.DX, Mean Age at Diagnosis; PB, Pre-B; EPB, Early Pre-B; TC, T-Cell; POB, Pro-B; CR, Complete Remission; Hy, Hypocellular; PR, Partial Remission; FV, Favorable; LFV, Less Favorable; LR, Low Risk; HR, High Risk; VHR, Very High Risk; L, Low; I, Intermediate; H, High; SD, Standard

### *Patient Follow-up*

At the time of preparing this manuscript, nine patients from the case group had died of relapsed ALL. The median time for the follow-up was 4 years (ranging from 14 months–10 years) for all patients. The median time of follow-up for patients in the case group was 36 months, and for the control group was 61 months. The 3-year overall survival and 5-year overall survival, according to Kaplan-Meier analysis was 82.3% ± 0.06% and 75.4% ± 0.07%, respectively.

***Cumulative Doses of Chemotherapy Agents***

The maximum and mean (±SD) cumulative dose of VCR was 90.9 mg/m$^2$ 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 was 185 mg/m$^2$ and 67.3 ± 43.5 mg/m$^2$, respectively (standard dose: 81.8%, high dose: 18.2%). Results showed that the maximum and mean (±SD) cumulative dose of L-ASP were 92,500 u/m$^2$ and 43,894.26 ± 22,717.9 u/m$^2$, respectively (standard dose: 78.6%, high dose: 21.4%). Table 2 shows the dose categorizations of chemotherapy agents used during the induction phase for the two groups.

***Expression of ABCC1-6 and ABCC10 by RT-PCR***

Using RT-PCR, the reaction efficiency for *ABCC* transporter genes was 0.96, and 0.98 for *GAPDH*. Patients with ALL relapse had significantly (P-Value: 0.0161) higher expression of *ABCC2* in comparison to the patients with at least 3 years of progression free survival (figure 1). The expression level of *ABCC3*, *ABCC4*, *ABCC5* and *ABCC6* was higher in patients with relapse ALL, while the expression levels of *ABCC1* and *ABCC10* was lower in patients with ALL relapse (Fig. 1). Table 3 shows the mean fold expression in the both the control and case groups of our ALL patients.

**Table 3.** Mean fold of expressions in considered patients

| | MF±SEM | P-Value | MF±SEM | P-Value | MF±SEM | P-Value | MF±SEM | P-Value | MF±SEM | P-Value | MF±SEM | P-Value | MF±SEM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Case group** | **1.18 ± 0.4** | **0.3592** | **3.06 ± 0.63** | **0.0161** | **7.34 ± 3.93** | **0.8042** | **3.04 ± 1.01** | **0.1536** | **2.59 ± 0.54** | **0.2486** | **4 ± 1.4** | **0.1046** | **1.39 ± 0.56** |
| **Control group** | **1.63 ± 0.27** | | **1.09 ± 0.48** | | **6.11 ± 3.06** | | **1.52 ± 0.25** | | **1.84 ± 0.36** | | **1.71 ± 0.33** | | **1.45 ± 0.31** |

ROC Curve analysis for the expressed *ABCC* genes revealed that *ABCC1* and *ABCC2* had significantly increased sensitivity of expression in relapsed patients (Fig. 2 and Table 4).

**Table 4.** ROC analysis for *ABCC1* and *ABCC2*

| Area under the ROC curve | *ABCC1* | *ABCC2* |
|---|---|---|
| Area | 0.6808 | 0.7845 |
| Std. Error | 0.08592 | 0.07786 |
| 95% confidence interval | 0.5124-0.8492 | 0.6319-0.9371 |
| P-Value | 0.0377 | 0.0009 |

The one-way ANOVA test showed a significant relationship between high doses of administered chemotherapeutic agents (VCR, DNR, L-Asp) and types of expression (high expression of *ABCC2*-6 and low expression of *ABCC1*, *ABCC10*) in relapsed ALL patients (P-Value< 0.0001).

## Discussion

The abnormal expression of ABC transporters is a major factor leading to the development of multidrug resistant cancer cells. Their role in the regulation of chemotherapeutic agents across the plasma membrane can enable them to render chemotherapy treatments ineffective. This function has made ABC transporters an attractive target for improving cancer therapy and preventing the development of multidrug resistance (8).

Sensitivity to chemotherapeutic agents is regulated by the expression of ABC transporter genes, mainly the *ABCC* subfamily of transporters (6,9,24). *ABCC1-6* are responsible for transporting drug agents like ADR, VP-16, VCR, and 6-MP (25).

Studies examining the role of ABC transporter gene expression on the outcome of treatment response (e.g., poor prognosis, early relapse, reduced progression-free survival) have shown contracting findings.

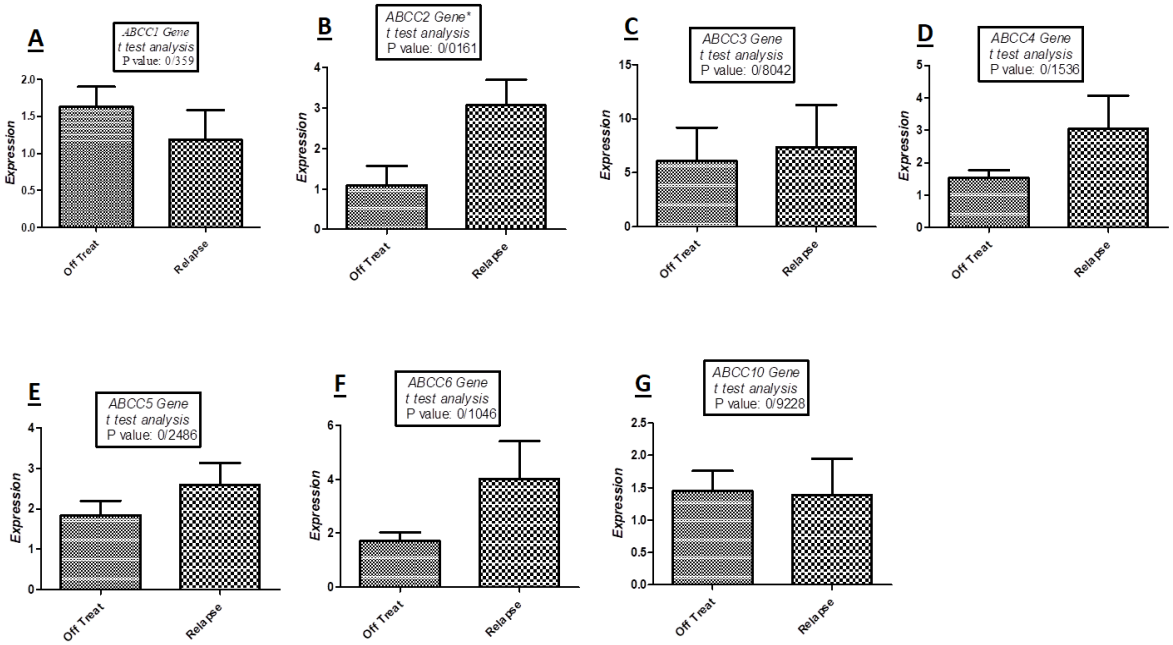


**Fig. 1.** Quantitative real-time PCR analysis with t-test for the mRNAs expression levels of *ABCC* transporter genes in two groups of relapse and off treatment (3 years progression free survival) patients:
A: low expression of *ABCC1* in relapse group in comparison to off treatment patients (P-Value: 0.359);
B: high expression of *ABCC2* in relapse group in comparison to off treatment patients (P-Value: 0.0161);
C: high expression of *ABCC3* in relapse group in comparison to off treatment patients (P-Value: 0.8042);
D: high expression of *ABCC4* in relapse group in comparison to off treatment patients (P-Value: 0.1536);
E: high expression of *ABCC5* in relapse group in comparison to off treatment patients (P-Value: 0.2486);
F: high expression of *ABCC6* in relapse group in comparison to off treatment patients (P-Value: 0.1046);
G: low expression of *ABCC10* in relapse group in comparison to off treatment patients (P-Value: 0.9228)

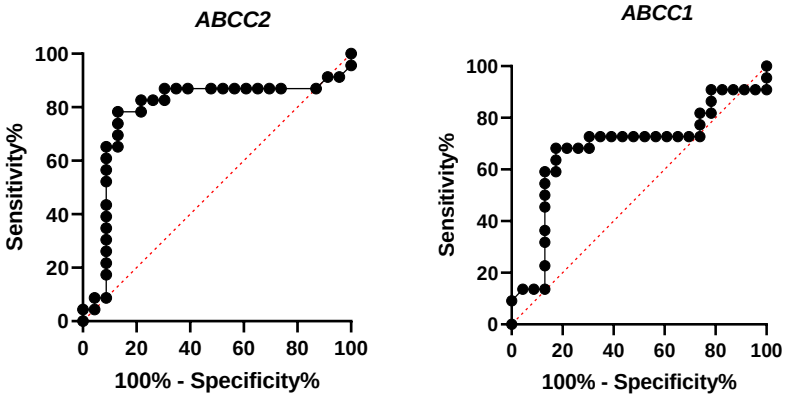


**Fig. 2.** ROC curves of *ABCC1* and *ABCC2* expression for evaluation of sensitivity and specificity in patients:
A: high sensitivity between low expression of *ABCC1* and occurrence of relapse in considered patients;
B: high sensitivity between high expression of *ABCC2* and occurrence of relapse in considered patients

Despite the improved survival of pediatric patients with ALL who receive modern treatment regimens, relapse remains to be the most common cause for treatment failure (26). Most studies have examined the effect of *MDR1* (P-gp) (27) and *MRP1* (*ABCC1*) in ALL (28).

A previous study evaluated the expression of *MDR1* and *MRP1* in 167 patients with ALL at different stages (new cases, complete remission, and relapses) by RT-PCR analysis. Their results showed that the level of *MRP1* expression was higher in patients with ALL relapse, and those individuals who expressed more *MRP1* did not experience complete remission. However, further research into the functional characteristics of *MRP1* through drug transporters is required to better understand the role of *MRP1* in ALL relapse (29).

A study by Gurbuxani et al. examined 32 adult and pediatric cases of untreated *de novo* ALL, observing a low correlation between *MDR1* and *MRP1* gene expression and a poor response to chemotherapy. Their results showed that there was no relation between gene expression and treatment outcomes or the occurrence of ALL relapse (30).

The prognostic value of *MDR1* and *ABCC1* in patients with acute leukemia is well known (31-34). A study of 34 pediatric patients with ALL revealed that high expression of *ABCC1* may affect complete remission and lead to decreased 2-year overall survival (35). Additional reports have demonstrated that there is no relationship between the expression of *ABCC1* or *MDR1* and poor treatment responses in adult or pediatric ALL patients (36). A separate controversial study of 140 pediatric patients with ALL showed that low expression of *ABCC1* and *ABCC3* is related to a high risk of death caused by treatment toxicity (37).

Plasschaert et al. examined 56 pediatric patients with newly diagnosed ALL to evaluate the effect of *ABCC1-6* expression on prognosis and treatment response. Their findings revealed that high expression of *ABCC1*, *ABCC3*, and *ABCC5* was related to poor prognosis. They suggested that characterizing the expression profile for the upregulated *ABCC* transporter genes may help identify early relapse in pediatric patients with ALL (24).

Few studies have explored the role of gene expression of ABC transporters in relation to the prognosis of Iranian pediatric patients with ALL. In a study by Mahjoubi et al. Iranian pediatric patients with relapsed ALL were observed to have high levels of *ABCC1* gene expression. (21).

In accordance with the Plasschaert et al. study, our cohort of Iranian children with relapsed ALL expressed higher levels of *ABCC2-6* than those who had finished their treatment regimens 3 years earlier without any disease recurrence. The expression of *ABCC1* and *ABCC10* was lower in patients with relapsed ALL than in those in the control group of our study. Our results also showed that high expression of *ABCC2-6* and low expression of *ABCC1* and *ABCC10* were significantly associated ($P < 0.05$) with high doses of the chemotherapeutic agents, VCR, DNR, and L-ASP. These results suggest that *ABCC* gene profiling should be done for pediatric patients with ALL to identify those who have a high probability of ALL recurrence.

The most common cause of treatment failure in children with ALL is relapse. Since the expression of *ABCC* transporter genes has been associated with the clinical outcomes of pediatric ALL patients, characterizing the expression pattern of *ABCC* genes could help predict the probability of treatment failure and improve patient prognosis. The results of this study show that characterizing the gene expression profile of *ABCC1-6* and *ABCC10* may offer a means of predicting the incidence ALL relapse in Iranian pediatric patients. Additionally, our study has revealed a relationship between high doses of chemotherapeutic agents (VCR, DNR, L-Asp) and *ABCC* transporter gene expression in ALL relapsed patients. As this study only recruited patients from a single center, a major drawback for this study was the small sample size. Future studies should examine patients across multiple different centers in order to better evaluate the role *ABCC* transporter gene expression has in ALL relapse and the role of high dose chemotherapeutic agents in *ABCC* gene expression.

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