Overexpression of dihydrofolate reductase is a factor of poor survival in acute lymphoblastic leukemia

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Abstract. Dihydrofolate reductase (DHFR) has an important function in DNA synthesis and is a target of methotrexate, which is a crucial treatment option for acute lymphoblastic leukemia (ALL). However, the number of studies conducted to date on DHFR expression in childhood ALL is limited. The aim of the present study was to determine whether the expression of DHFR is associated with survival in childhood ALL. The expression of DHFR in 96 children with ALL and 100 control individuals was determined using reverse transcription-quantitative polymerase chain reaction. The results of the present study demonstrated that the expression of DHFR mRNA in children with ALL was significantly increased (P<0.001), compared with that in the control group. In addition, increased levels of DHFR mRNA were observed in patients with B-cell lineage, compared with patients with T-cell lineage ALL (P<0.05). The Kaplan-Meier estimator analysis revealed that children with ALL who exhibited increased levels of DHFR mRNA had a decreased overall survival time (P<0.05). It was observed that certain patient prognostic features (including age, sex, white blood cell count and high DHFR expression), are associated with poor survival (log-rank test, P<0.05). Therefore, the results of the present study indicated that DHFR upregulation is a factor for poor survival in ALL.

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer and the most frequent cancer in Hispanic children (1). In Mexico, it has been observed that childhood ALL has a relatively higher frequency amongst patients with leukemia, accounting for ~85% of childhood leukemia, whereas acute myeloid leukemia (AML) represents ~15% (2). Childhood ALL in Mexico has a mortality rate of 63.7 per million children, one of the highest rates reported globally (2).

Previously, methotrexate (MTX) has been used to target the folate metabolic pathway and is an important component for the treatment of cancer (3) including breast cancer (4), head and neck cancer (5), lung cancer (6) and osteosarcoma (7). Anti-folate therapies work against folate-dependent enzymes by inhibiting *de novo* pyrimidine and purine biosynthesis, resulting in cell death (8). At present, MTX is used in the chemotherapeutic treatment of patients with ALL. MTX functions as a binding inhibitor of dihydrofolate reductase (DHFR), an enzyme that catalyzes the reduction of DHF to tetrahydrofolate (an essential process for the biosynthesis of purines and thymidylate precursors), and therefore inhibits *de novo* DNA synthesis (9,10). The molecular mechanisms which underlie MTX resistance are attributed to decreased accumulation of MTX due to impaired transport and decreased retention of MTX, and an increase in DHFR expression (10-12).

Previous studies have demonstrated that increased levels of DHFR mRNA result in resistance to MTX therapy (13-15). However, there are a limited number of studies on the function of DHFR expression in patients with childhood acute leukemia. The purpose of the present study was to determine whether the expression of DHFR is associated with survival of patients with ALL and analyze whether the expression of DHFR may be used as a prognostic marker in acute leukemia.

Materials and methods

Patients. The present study was a hospital-based retrospective study that analyzed 96 children (60 males, 36 females; mean age, 7.78±4.97 years), with ALL validated by bone marrow aspirates and based on the French-American-British morphological criteria cytochemical staining properties and

immunophenotyping of blast cells (16,17), who were admitted to the Pediatric Oncology Service of the State Cancer Institute (Arturo Beltran Ortega, Acapulco, Guerrero, Mexico), between September 2005 and July 2015. The diagnosis of ALL was further subclassified as T-lineage ($CD3^+$, $CD7^+$plus $CD2^+$ or $CD5^+$ or both) or B-lineage ($CD22^+$, $CD19^+$, $CD20^+$, $CD79A^+$, $HLA\text{-}DR^+$ and $CD10^+$) according to the study by Gómez-Gómez *et al* (18). In addition, 100 healthy individuals were included in the present study as controls (53 males, 47 females; mean age, 10.21±5.53 years), all of whom had no familial history of cancer. All individuals (patients and controls) included in the study were aged between 1 and 17 years. The study was approved by the Ethics Committee of the State Cancer Institute (Arturo Beltran Ortega, Acapulco, Mexico).

Overall survival (OS) time was determined according to the study by Gómez-Gómez *et al* (18). Briefly, OS time was determined as the time between the day of registration into the study and the day of mortality (from any cause) or the day of last known contact. Patients with ALL were classified into one of the following two groups: Low-risk, aged between 1 and 10 years with <50,000 leucocytes/mm^3 at diagnosis; high risk, aged <1 and >10 years with >50,000 leucocytes/mm^3 at diagnosis.

Specimen collection and total RNA extraction. Bone marrow (patients) and blood (controls) samples from the 196 individuals were obtained and processed, according to the study by Gómez-Gómez *et al* (18). Different samples were used between patients and controls as bone marrow from healthy individuals was deemed ethically unacceptable. Total RNA was isolated from the bone marrow and/or blood samples using the TRIzol® method (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Quantification of mRNA using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA (1 μg) was converted to cDNA using the Superscript III First-Strand Synthesis System (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. In brief, a 20 ml cDNA reaction mix contained extracted total RNA (1 μg), 500 ng oligo (dT) (12-18) and dNTP mix (0.5 mM each). The mixture was heated at 65˚C for 5 min and incubated on ice for 3 min. 1x first-strand RT buffer, 5 mM DTT and 5 U of RNase Inhibitor (Invitrogen; Thermo Fisher Scientific, Inc.) were added into each tube. The tubes were incubated at 42˚C for 2 min, 200 U of SuperScript III (Invitrogen; Thermo Fisher Scientific, Inc.) was added and tubes were incubated at 42˚C for 50 min, and finally 70˚C for 15 min.

All PCR assays were carried out in triplicate in a 25 μl reaction volume, including the following: 5 μl cDNA template (500 ng), 12.5 μl SYBR-Green PCR Master Mix (SYBR Green PCR Reagents kit; Applied Biosystems; Thermo Fisher Scientific, Inc.), 0.5 μM of each oligonucleotide and ultrapure water. The following oligonucleotides were used: DHFR forward, 5'-TTCCTGAGAAGAATCGACCTTTAAA-3' and reverse, 5'-AAGGCATCATCTAGACTTCTGGAAA-3'; hypoxanthine-guanine phosphoribosyltransferase (HPRT) forward, 5'-AAGCTTGCTGGTGAAAAGG-3' and reverse, 5'-AAACATGATTCAAATCCCTGA-3'. The thermocycling conditions were as follows: 95˚C for 5 min followed by 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. All RT-qPCR reactions were performed in 96-well plates using the Applied Biosystems 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression levels of mRNA were determined according to the $2^{-\Delta\Delta Cq}$ method (19), using HPRT mRNA expression as a reference. The DHFR level in the ALL samples was defined as the relative value, compared with that of samples from the healthy individuals.

Detection of translocations. The detection of BCR-ABL, ETV6-RUNX1, AML1-ETO and CBFB-MYH11 translocations was realized by PCR and according to the protocol previously reported by Organista-Nava *et al* (20).

Statistical analysis. SPSS version 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) software were used to analyze the data obtained. The results are presented as the mean ± standard deviation or median (25-75% quartiles). To compare medians and frequencies between groups, χ^2 tests were used. The Mann-Whitney U test was used to compare differences in the DHFR mRNA levels between ALL patients and healthy individuals. OS time was determined using the Kaplan-Meier estimator method. Univariate logistic regression analysis was performed to define the risk of relapse, and multivariable logistic regression analysis was used to identify independent risk factors for ALL relapse. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of the patients and controls. The healthy individuals (controls) exhibited a mean age of 10.21±5.53 years and a normal leukocyte count (between 4 and $10x10^3$ leukocytes/mm^3; median 8,000 leukocytes/mm^3). The patients at diagnosis exhibited a mean age of 7.78±4.97 years, with a mean leukocyte count at diagnosis of 18,000 leukocytes/mm^3. Characteristics of the controls and patients are outlined in Table I. Of the patients included in the present study, 44.79% were classified as low-risk (aged between 1 and 10 years with <50,000 leucocytes/mm^3 at diagnosis) and the remaining 55.21% were classified as high-risk (aged <1 and >10 years with >50,000 leucocytes/mm^3 at diagnosis). The results of the present study demonstrated that B-lineage ALL was observed in 90.63% patients. Breakpoint cluster region (BCR)/Abelson murine leukemia viral oncogene homolog 1 (ABL) fusion gene was identified in 7.29% (7/96) patients with ALL. The ETS variant 6 (ETV6)-runt-related transcription factor 1 (RUNX1) rearrangement was revealed in only 0.01% (1/96) patients with ALL, and 60.42% (58/96) did not exhibit either the BCR-ABL or ETV6-RUNX1 gene rearrangement. 30 of the 96 patients were not considered for rearrangement analysis as analysis was not possible due to insufficient sample (Table I).

DHFR is significantly expressed patients with ALL. As presented in Table I and Fig. 1, the expression of DHFR was significantly increased in ALL, compared with that in

Table I. Characteristic and clinical data for patients with ALL compared with those of healthy individuals.

Variable	ALL, n=96	Healthy individuals, n=100	P-value
Mean age ± standard deviation, years	7.78±4.97	10.21±5.53	<0.001[a]
Leukocytes/mm^3	18,000 (4,700-42,875)[a]	8,000 (7,000-9,000)[a]	<0.001[a]
Sex			0.195
Female	36 (37.50)	47 (47.00)	
Male	60 (62.50)	53 (53.00)	
Status of participant			
Alive	34 (35.42)	100 (100.00)	-
Deceased	62 (64.58)	-	
Risk group			
Low	44 (45.83)	-	-
High	52 (54.17)	-	
Immunophenotype			
B-lineage	87 (90.63)	-	-
T-lineage	9 (9.37)	-	
Chromosomal translocation			
ETV6-RUNX1 [t(12;21)]	1 (1.04)	-	
BCR-ABL [t(9;22)]	7 (7.29)	-	
None	58 (60.42)	-	
Not determined	30 (31.25)	-	
DHFR level, median (25-75 percentiles)	9.38 (3.39-27.48)	1.07 (0.84-1.24)	<0.001[a]

Data are expressed as n (%), unless otherwise indicated. [a]P<0.05 vs. healthy individuals, obtained using the χ^2 test. ALL, acute lymphoblastic leukemia; low-risk group, aged between 1 and 10 years with <50,000 leukocytes/mm^3; high-risk group, aged <1 and >10 years with >50,000 leukocytes/mm^3; ETV6, ETS variant 6; RUNX1, Runt-related transcription factor 1; BCR, breakpoint cluster region; ABL, Abelson murine leukemia viral oncogene homolog 1.

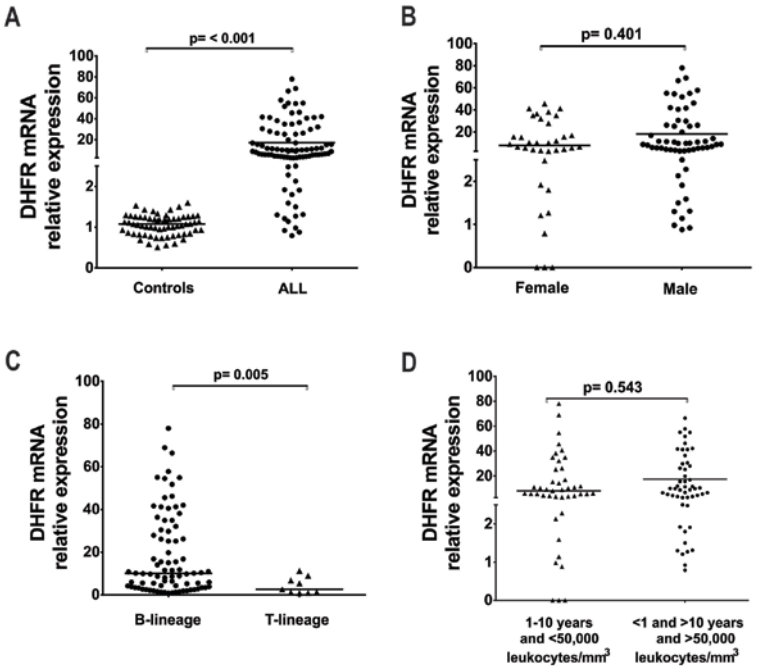

Figure 1. DHFR expression in childhood ALL compared with that in healthy control patients. (A) DHFR mRNA was identified to be significantly increased in patients with ALL compared with in the control patients [median (25-75 percentiles), 9.38 (3.39-27.48); P<0.001]. (B) The level of DHFR mRNA was not identified to be significantly different between male and female patients (P>0.05). (C) The expression level of DHFR in patient with B-lineage ALL was significantly increased, compared with that in patients with T-lineage ALL [10.10 (3.74-30.87); P=0.005]. (D) The expression levels of DHFR mRNA was not identified as significantly different between patients with ALL classified as low-risk (aged between 1 and 10 years with <50 m000 leucocytes/mm^3) and patients classified as high-risk (aged <1 and >10 years with >50,000 leucocytes/mm^3) (P>0.05). DHFR, dihydrofolate reductase; ALL, acute lymphoblastic leukemia.

Table II. Association between DHFR expression level, clinical features and the risk of relapse for patients with ALL.

Categories	Without relapse, n (%)	With relapse, n (%)	P-value	Univariate analysis			Multivariate analysis		
				OR	95% CI	P-value[a]	OR	95% CI	P-value
Sex			0.84	1.38	0.56-3.38	0.49	1.43	0.55-3.71	0.46
Female	12	24							
Male	16	44							
Risk group			0.04[a]	2.91	1.16-7.26	0.02[a]	3.20	1.23-8.29	0.02[a]
Low	18	26							
High	10	42							
DHFR level			0.04[a]	2.50	1.01-6.16	0.047[a]	2.81	1.09-7.24	0.03[a]
Downregulated	17	26							
Upregulated	11	42							

[a]P<0.05 vs. healthy individual, obtained using the χ^2 test. ALL, acute lymphoblastic leukemia; low-risk group, aged between 1 and 10 years with <50,000 leukocytes/mm^3; high-risk group, aged <1 and >10 years with >50,000 leukocytes/mm^3; OR, odds ratio; CI, confidence interval.

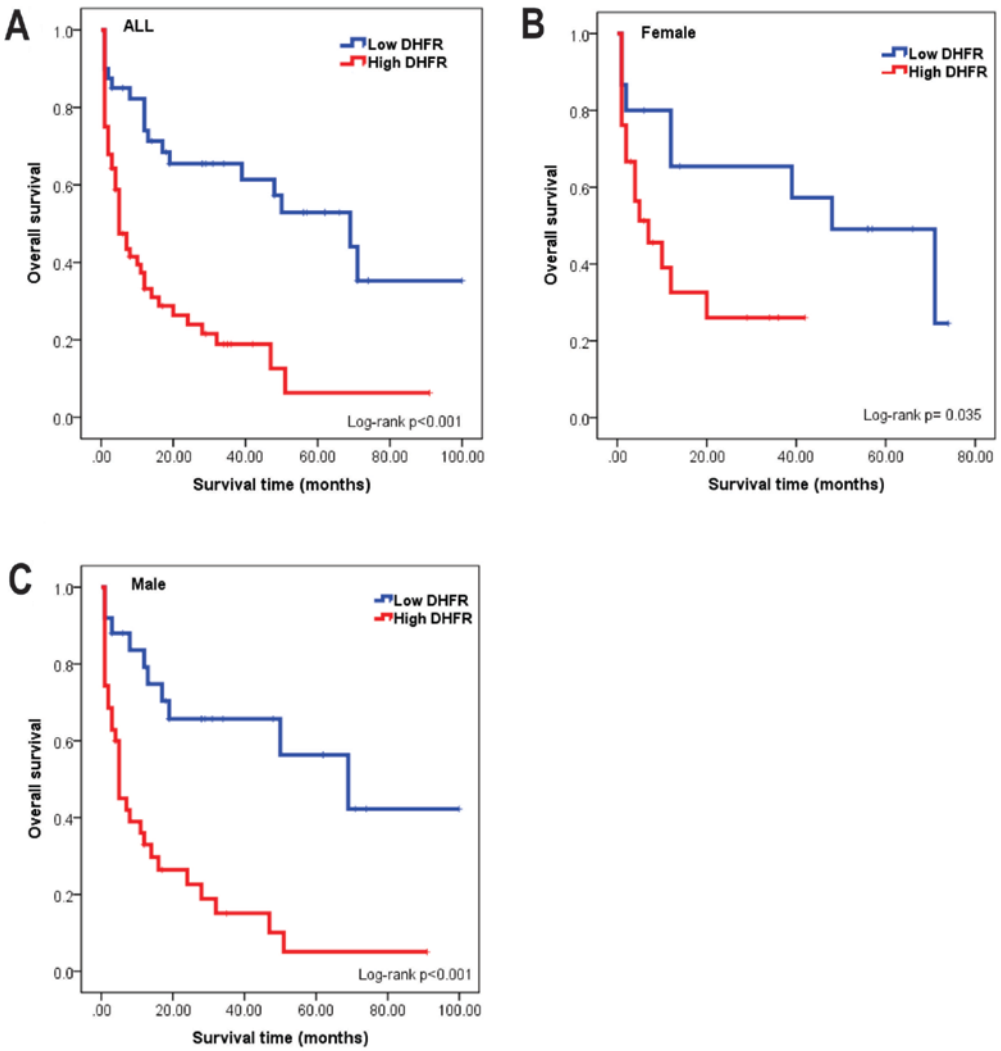

Figure 2. Association between sex, DHFR expression level and prognosis in patients with childhood ALL. (A) OS in patients with ALL exhibiting high or low DHFR expression levels. Patients who exhibited high levels of DHFR expression had a significantly decreased OS, compared with patients with low DHFR expression (P<0.001). (B) OS in female patients with high and low DHFR expression levels. Female patients with high levels of DHFR mRNA exhibited significantly decreased OS, compared with female patients with low DHFR levels (P=0.035). (C) OS in male patients with high and low DHFR expression levels. Male patients exhibiting a high level of DHFR mRNA had a significantly decreased OS, compared with male patients exhibiting low DHFR expression (P<0.001). DHFR, dihydrofolate reductase; ALL, acute lymphoblastic leukemia; OS, overall survival.

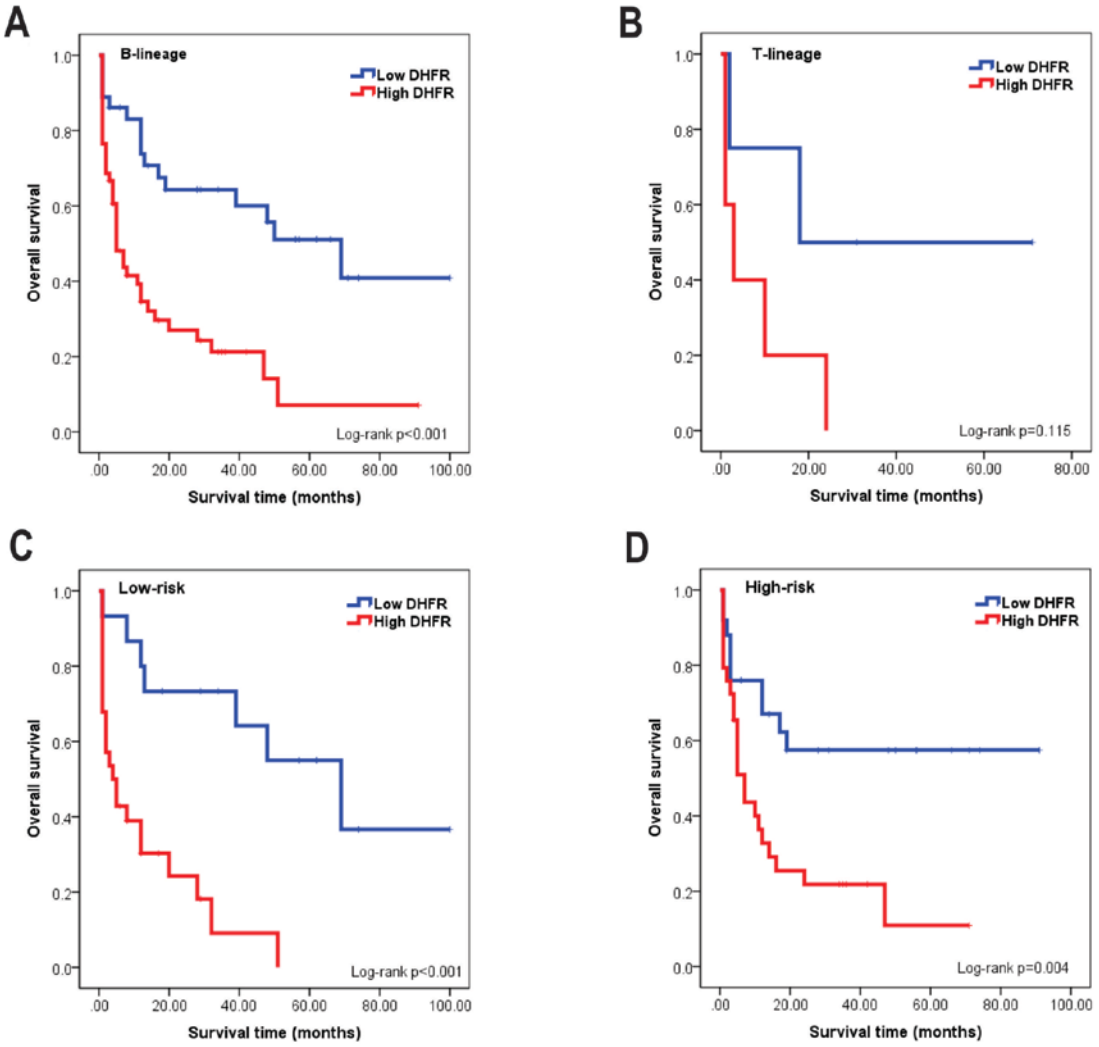

Figure 3. Association between cell lineage (B or T), risk-group, DHFR expression level and prognosis in patients with childhood ALL. (A) The OS was significantly decreased for patients with ALL with B-cell lineage and high DHFR expression, compared with patients with B-cell lineage ALL and low DHFR expression (P<0.001). (B) The OS was decreased for patients with ALL of the T-cell lineage and high DHFR expression, compared with patients with T-cell lineage ALL and low DHFR expression; however, this difference was not identified to be statistically significant (P>0.05). (C) In the low-risk group (aged between 1 and 10 years with <50,000 leucocytes/mm^3), the OS was significantly increased for patients with ALL and low DHFR expression, compared with that in patients with high DHFR expression (P<0.001). (D) In the high-risk group (aged <1 and >10 years with >50,000 leucocytes/mm^3), the OS was significantly decreased for patients with high DHFR expression, compared with those with low DHFR expression (P=0.004). DHFR, dihydrofolate reductase; ALL, acute lymphoblastic leukemia; OS, overall survival.

the controls (Fig. 1A; P<0.001). The median expression of DHFR in the control group was 1.07-fold (25-75 percentile, 0.84-1.24), whereas the median DHFR expression in the ALL group was 9.38 (3.39-27.48). No statistically significant difference was identified between the male and female patients (P=0.401; Fig. 1B). it was observed that DHFR was significantly increased in patients with B-lineage ALL, compared in with patients with T-lineage ALL (P=0.005); the mean was 18.63-fold (B-lineage) vs. 4.36-fold (T-lineage) (fold vs. control mean DHFR expression; Fig. 1C). In addition, no statistically significant difference between the low- and high-risk groups was observed (P=0.543; Fig. 1D).

Patients with ALL who exhibit increased DHFR expression have a higher risk of relapse during treatment. The associations between DHFR expression and the risk of relapse are presented in Table II. The ALL patients were divided into either the high or low DHFR expression groups, using as the median expression level of DHFR (9.38) as the threshold value. A total of 43/96 patients with ALL were classified in the low expression group and the remaining 53/96 were considered to exhibit high levels of DHFR expression. Patients in the high expression group exhibited a 2.81-fold [95% confidence interval (CI), 1.09-7.24; P=0.033] increased risk of relapse during treatment, compared with the low expression group (Table II).

Expression of DHFR and survival in ALL patients. The Kaplan-Meier estimator survival curves were performed to calculate the differences between the survival of ALL patients with high and low expression of DHFR. As presented in Fig. 2A, ALL patients in the high DHFR expression group exhibited poorer survival, compared with the low expression group. Similarly, in Fig. 2B and C, male and female patients with high levels of DHFR expression exhibited poorer survival, compared with patients with low DHFR expression (log-rank test, P=0.035 in females; P<0.0001 in males).

Patients with B-lineage ALL and high DHFR expression exhibited decreased survival, compared with patients with B-lineage ALL and low DHFR expression (Fig. 3A). Although a decrease in OS was observed in patients with T-lineage ALL and high DHFR expression, compared with patients with T-lineage ALL and low DHFR expression, no significant association was identified (log-rank test, P=0.115; Fig. 3B). In addition, patients with ALL classified as low- or high-risk, and with high DHFR expression, exhibited decreased survival, compared with patients with low DHFR expression (log-rank test; P<0.05; Fig. 3C and D).

Discussion

MTX is an important component in the chemotherapeutic treatment of several neoplasm types, including childhood ALL (15). The primary causes of MTX-resistance include alterations to its receptor (reduced folate carrier) and increased DHFR expression (9,21). MTX principally exhibits effects by inhibiting DHFR, which has an important function in folate metabolism (11). DHFR is involved in DNA biosynthesis and cell replication (9,22). If there is an increase in the expression of DHFR, DNA becomes more unstable, which may lead to abnormal cell proliferation (23). Therefore, it is important to identify novel biomarkers for patients with a high-risk of treatment failure (relapse), so the appropriate chemotherapeutic scheme may be selected and the survival time of these patients improved.

The overexpression of DHFR in certain cancer cell lines was identified to be a mechanism underlying resistance to MTX chemotherapy (9,21). However, limited information is available regarding the function of DHFR expression in patients with ALL. In the present study, the expression of DHFR was analyzed in samples from patients with ALL, which determined an association between clinical characteristics and patient survival. In addition, the results of the present study revealed that DHFR mRNA was expressed at a significantly increased level in patients with ALL, compared with in the controls (P<0.001), which was similar to the results of prior studies where high levels of DHFR were observed in acute leukemia (13,21).

To determine the clinical significance of DHFR expression in acute leukemia, a logistic regression analysis was performed to determine associations between the clinical characteristics of patients with ALL and the risk of relapse. The results of the present study demonstrated a significant association between the level of DHFR expression and the risk of relapse of leukemia [odds ratio (OR), 2.50; 95% CI, 1.01-6.16; P=0.047). Concordant with the results of Matheson *et al* (21), the results of the present study revealed that increased expression of DHFR in patients with ALL increased the risk of relapse. This suggests that DHFR expression levels may be important factor in ALL.

Similar to the results of previous studies (18,20,24), an association between age, sex, leukocyte count at diagnosis and prognosis was identified in the present study. The results of the present study revealed that patients in the high-risk group (aged <1 and >10 years with >50,000 leucocytes/mm^3 at diagnosis) exhibited a poor prognosis, compared with patients in the low-risk group (OR, 2.91; 95% CI, 1.16-7.26; P=0.02); these values have been established by the National Cancer Institute as the values to identify children with poor prognosis (25). Furthermore, DHFR expression was a poor independent prognostic factor (Table II).

The association between DHFR expression levels and the survival of patients with ALL was investigated in the present study. The results demonstrated that patients with high levels of DHFR expression exhibited decreased survival compared with patients with low DHFR expression levels (log-rank, P<0.05). Similar to the reports of Matherly *et al* (13), Matheson *et al* (21) demonstrated an association between the high expression of the DHFR and reduced survival in patients with ALL; this indicates that high levels of DHFR expression are an important prognostic factor in childhood ALL.

The results of the present study have supported the use of genetic factors to complement known biological or disease-based prognostic indicators in ALL. The demonstration of an increase in the expression of the DHFR in ALL is indicative of poor prognosis for patients with ALL.

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