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original research report

Effect of genetic alterations of cytarabine-metabolizing enzymes in childhood acute lymphoblastic leukemia

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BACKGROUND: Single nucleotide polymorphisms (SNPs) of deoxycytidine kinase (dCK) and cytidine deaminase (CDA) are known to alter their enzymatic activities, which affect the metabolism of cytarabine. Currently, treatment of childhood acute lymphoblastic leukemia (ALL) includes cytarabine, especially in high-risk patients. Therefore, we hypothesized that a genetic variation of *dCK* and *CDA* genes may influence the risk of cytarabine-related toxicities and early response to treatment.

PATIENTS AND METHODS: We included children diagnosed with ALL and lymphoblastic lymphoma (LL) stage III and IV. The patients received a modified St Jude Total Therapy Study XV protocol. Cytarabine was used during induction remission (low-dose cytarabine) and reinduction II (high-dose cytarabine) phases. Genotyping of *dCK* -360C>G and -201C>T and *CDA* 79A>C and 208G>A was performed. Minimal residual disease (MRD) at the end of the induction phase was measured using flow cytometry.

RESULTS: Ninety-four children with ALL (n=90) and LL (n=4) were analyzed. The median age at diagnosis was 5.8 years (range, 0.4-15 years). All four SNPs showed predominant wild type alleles. There was no *CDA*-208A allele in our population. Children with dCK-360G allele were at risk of mucositis after receiving low-dose cytarabine (OR=3.7; 95%CI, 1.2--11.3). Neither *dCK* nor *CDA* polymorphisms affected the MRD status at the end of induction phase.

CONCLUSION: The dCK-360G allele was found to increase the risk of mucositis after exposure to low-dose cytarabine in childhood ALL therapy.

Cytarabine (cytosine arabinoside, ara-C) is a deoxycytidine analogue that is a standard agent for treatment of acute myeloid leukemia (AML).[1] Cytarabine is intracellularly phosphorylated by deoxycytidine kinase (dCK) to 1-β-D-arabinofuranoxyl cytosine-5'-triphosphate (ara-CTP).[2] Its cytotoxicity results from a combination of DNA polymerase inhibition and incorporation of ara-CTP into DNA, in competition with deoxycytidine triphosphate (dCTP).[3,4] This incorporation causes chain termination resulting in blockage of DNA synthesis.[3] Cytidine deaminase (CDA) is an enzyme involved in the metabolism of cytarabine by converting cytarabine into an inactive product (ara-U).[4]

Nonsynonymous single nucleotide polymorphisms (SNPs) of the *dCK* and *CDA* genes have been reported.[5,7] The SNPs at the promoter of *dCK* gene (dCK-360C>G and -201C>T) affect mRNA expression whose dCK-360G/-201T expresses higher levels of *dCK* mRNA.[7] Shi et al demonstrated that AML patients with dCK-360CC/-201CC genotype have a significant inferior response to therapy as compared to the other genotypes.[7] The functional variations of CDA79A>C and 208G>A have been described previously.[6,8] The CDA79C and 208A alleles have reduced activity compared to wild type alleles in in vitro studies.[6,8]

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood. The current treatment of ALL includes cytarabine in several chemotherapeutic regimens, especially for high-risk patients.[9-12] We hypothesized that SNPs of *dCK* and *CDA* genes

Table 1. Primers, restriction enzymes, and PCR products of dCK and CDA polymorphisms.

Polymorphisms	Primers	PRC product (base pairs)	Restriction enzyme	PRC product after digestion (base pairs)
dCK -360 C>G	Forward 5′-CTG CAG GTG ACG CCC TCT-3′ Reverse 5′-GGG TGG CCA TTC CTT AGT CT-3′	469	EhE I	276, 100, 93 (CC) 376, 276, 100, 93 (CG) 376, 93 (GG)
dCK -201 C>T	Forward 5′-CTG CAG GTG ACG CCC TCT-3′ Reverse 5′-GGG TGG CCA TTC CTT AGT CT-3′	469	BsrB I	469(CC) 469, 259, 210 (CT) 259, 210 (TT)
CDA 208 G>A	Forward 5′-AAC ACA CGC AAC AGG AAG TG-3′ Reverse 5′-ATT GTT GCA ACC TGG CTT TC-3′	200	Rsr II	114, 86 (GG) 200, 114, 86 (GA) 200 (AA)

may have an effect on the risk of cytarabine-related toxicities in children with lymphoid malignancy.

PATIENTS AND METHODS

Children aged less than 15 years old diagnosed with ALL and lymphoblastic lymphoma (LL) stage III and IV at Ramathibodi Hospital between 2004 and 2008 were included in this study. The diagnosis of ALL was made from an analysis of lymphoblasts from the bone marrow. The investigations included morphology, immunophenotype, and cytogenetics. LL was diagnosed by pathological examination of tumor tissue.

Our institute treated children with ALL and advanced stage LL with a modified St Jude Total Therapy Study XV protocol.[12] The protocol divided ALL patients into low, standard, and high-risk groups according to age, initial white cell count, cytogenetics, and minimal residual disease (MRD) status. The components of therapy consisted of induction remission, consolidation, intensification (only high risk), reinduction I and II, and maintenance phases. Cytarabine was prescribed within remission induction and re-induction II phases. Cytarabine 75 mg/m^2 (low dose) was given subcutaneously on day 23-26 and day 29-32 of the remission-induction phase for all patients. The patients with ALL (standard and high risk) and LL (stage III and IV) received cytarabine 2 g/m^2 (high dose) infusion every 12 hours for 4 doses during the re-induction II phase. Common toxicities of cytarabine, including anemia, neutropenia, thrombocytopenia, mucositis, diarrhea, febrile neutropenia, and delay treatment (more than 2 weeks) were recorded and analyzed. The grade III-IV toxicities were used for statistical analysis of the association with dCK and CDA polymorphisms. This study protocol was approved by the Ethics Committee on Human Experimentation of Faculty of Medicine Ramathibodi Hospital, Mahidol University.

Genotyping of dCK *and* CDA *polymorphisms*

We collected 3 mL of peripheral blood of patients with complete remission for DNA extraction. The DNA was extracted by using a standard phenol chloroform method. Polymerase chain reaction (PCR) with restriction fragment length polymorphism (RFLP) technique was used for detection of dCK -360C>G and -201C>T polymorphisms. The reaction mix was composed of 1X buffer, 3 mM MgCl2, 0.48 mM dNTPs, 0.2 µM of each of the forward and reverse primers, DMSO, 0.06 U/µL of Taq and dH2O to the final volume 25 µL. The first round PCR reaction was performed using a pre-denaturation step of 5 min at 95°C followed by 20 cycles of 95°C for 1 min, 61°C for 1 min, and 72ºC for 1 min. This was followed by 20 cycles of 95°C for 1 min, 55ºC for 1 min, and 72ºC for 1 min and post extension of 72ºC for 10 min. The primers, restriction enzymes, and PCR products are shown in Table 1.

The CDA 208G>A polymorphism was identified by using PCR-RFLP technique. The reaction mix composed of 1X buffer, 3 mM MgCl2, 0.48 mM dNTPs, 0.2 µM of each of the forward and reverse primers, 0.02 U/µL of Taq and dH2O to the final volume 25 µL. The PCR reaction was performed using a pre-denaturation step of 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 60ºC for 1 min, and 72ºCfor 1 min and final extension of 72°C for 10 min. The primers, restriction enzyme, and PCR product are shown in Table 1.

The CDA 79A>C polymorphism was identified by ARMS-PCR technique. Each sample was tested in two conditions: (i) the condition for normal homozygous carried out with internal control and CDA 79 wild type primers, and (ii) the condition for mutant homozygous carried out with internal control and CDA 79 variant primers. The primers for internal control (β-actin) were forward 5'-CTAACACTGG CTCGTGTG ACAA G-3 and reverse 5'-GACTC GTCATACTCCTGCTTGC-3'. The primers for CDA 79 wild type were forward 5'-TTGCTC CCAGGAGGCC AAGA-3' and reverse 5'-CTTGCCACTGCCTGTGCCT-3'. The variant primers were forward 5'-ACCAACATGGCCC AGAAGCG-3' and reverse 5'-GTAGGGGC AGTAGGCTGACTG-3'. The reaction mix was composed of 1X buffer, 3 mM $MgCl_2$, 0.48 mM dNTPs, 0.48 μM of forward and reverse primers of internal control (β-actin), 0.12 μM (wild type) or 0.2 μM (variant) of each of the forward and reverse primer, 0.02 U/μl Taq and dH_2O to a final volume of 25 μL. The PCR reaction was performed at 94°C for 5 min followed by 35 cycles at 94°C for 30 sec, 68°C for 30 sec, and 72°C for 1 min and post-extension at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide.

Minimal residual disease measurement

Flow cytometry analysis was used for detection of MRD. Residual leukemic cells were characterized by expression of CD19, CD45, CD38/CD34, CD10/TdT and CD10/CD20 compared to normal B precursors. Briefly, the staining procedure was performed as follows: one million leukemic cells were incubated with the recommended amount of monoclonal antibodies-to-surface markers in dark surroundings at room temperature for 20 minutes. Then red blood cell lysis was done by 20-minute incubation with 2 mL FACS Lysing Reagents (Beckton Dickinson, San Jose, CA). The sample tubes that needed no further intracellular staining were washed twice and resuspended in 0.5 mL of phosphate buffer saline (PBS) with 0.5% bovine serum albumin and 0.02% NaN_3 and 0.5% paraformaldehyde before being kept in the dark at 4°C while waiting for acquisition. For intracellular staining, after surface staining and red blood cell lysis, leukemic cells were incubated with 0.5 mL FACS Permeabilizing Reagent (Beckton Dickinson, San Jose, CA) for 15 minutes and washed. The recommended amount of monoclonal antibody for intracellular markers was added and incubated for 10 minutes at room temperature. Cells were washed twice and resuspended in PBS (with 0.5% bovine serum albumin and 0.02% NaN_3) and 0.5% paraformaldehyde. Afterwards, all samples were analyzed by FACScan Flow Cytometer (Beckton Dickinson, San Jose, CA). One hundred thousand cells were acquired for each tube. List mode data files were analyzed using CellQuestPro Program (Beckton Dickinson, San Jose, CA). MRD was reported as the percentage of residual leukemic cells in all viable nucleated cells. MRD below 0.01% was reported as negative.

Table 2. Genotype frequencies of dCK and CDA polymorphisms.

Genotype	Cases (%) (n=94)	Controls (%) (n=100)
dCK -360 C>G		
CC	68 (72.3)	70 (70)
CG	25 (26.6)	26 (26)
GG	1 (1.1)	4 (4)
dCK -201 C>T		
CC	68 (72.3)	70 (70)
CT	25 (26.6)	26 (26)
TT	1 (1.1)	4 (4)
CDA 79 A>C		
AA	82 (87.2)	81 (81)
AC	12 (12.8)	18 (18)
CC	0	1 (1)
CDA 208 G>A		
GG	94 (100)	100 (100)
GA	0	0
AA	0	0

Statistical analysis

The SPSS 16 software package was used for the statistical calculation. Fishers exact and chi-square tests were used for estimation of the association between toxicity and genotype or alleles. The odds ratio (OR) with 95% confidence interval (CI) was determined.

RESULTS

Ninety-four patients (54 males and 40 females) who were diagnosed with ALL (n=90) and LL stage III (n=4) were included in this study. A median age at diagnosis was 5.8 years (range, 0.4-15 years). ALL subtypes were precursor B (n=80) and T cell (n=10) lineages.

Distribution of dCK *and* CDA *genotypes*

The genotype distribution of *dCK* and *CDA* polymor-

Table 3. Association between the genotype of dCK -360 C>G and cytarabine toxicity.

Toxicity	Low dose cytarabine (n=81)				High dose cytarabine (n=45)			
	CC n=59 n (%)	CG+GG n=22 n (%)	OR	95% CI	CC n=29 n (%)	CG=GG n=16 n (%)	OR	95% CI
Anemia	27 (45.8)	15 (68.2)	2.54	0.90-7.14	10 (34.5)	5 (31.3)	0.86	0.23-3.19
Neutropenia	31 (52.5)	15 (68.2)	1.94	0.69-5.44	13 (44.8)	7 (43.8)	0.96	0.28-3.27
Thrombocytopenia	12 (20.3)	2 (9.1)	0.39	0.08-1.91	20 (69)	10 (62.5)	0.75	0.21-2.70
Mucositis	3 (5.1)	6 (27.3)	7	1.57-31.15	2 (6.9)	1 (6.3)	0.9	0.08-10.77
Diarrhea	5 (8.5)	1 (4.5)	0.51	0.05-4.66	4 (13.8)	2 (12.5)	0.89	0.15-5.51
Febrile neutropenia	14 (23.7)	8 (36.4)	1.84	0.64-5.28	11 (37.9)	5 (31.3)	0.74	0.20-2.72
Delayed treatment	18 (30.5)	6 (27.3)	0.85	0.28-2.54	16 (55.2)	12 (75)	2.44	0.63-9.38

Table 4. Association between the genotype of CDA 79 A>C and cytarabine toxicity.

Toxicity	Low dose cytarabine (n=81)				High dose cytarabine (n=45)			
	AA n=71 n (%)	AC n=10 n (%)	OR	95% CI	AA n=40 n (%)	AC n=5 n (%)	OR	95% CI
Anemia	37 (52.1)	5 (50)	0.92	0.24-3.45	11 (27.5)	4 (80)	10.55	1.06-105.03
Neutropenia	38 (53.5)	8 (80)	3.47	0.69-17.52	17 (42.5)	3 (60)	2.03	0.31-13.51
Thrombocytopenia	13 (18.3)	1 (10)	0.5	0.06-4.26	26 (65)	4 (80)	2.15	0.22-21.18
Mucositis	9 (12.7)	0	-	-	2 (5)	1 (20)	4.75	0.35-64.74
Diarrhea	6 (8.5)	0	-	-	6 (15)	0	-	-
Febrile neutropenia	20 (28.2)	2 (20)	0.64	0.12-3.27	15 (37.5)	1 (20)	0.42	0.04-4.09
Delayed treatment	22 (31)	2 (20)	0.56	0.11-2.84	24 (60)	4 (80)	2.67	0.27-26.09

phisms in cases and controls were as shown in Table 2. Wild type was the predominant genotypes of all SNPs. The dCK -360 C>G and -201 C>T displayed a linkage disequilibrium of each other. The variant allele of CDA 208 G>A was not detected in our population. The genotype frequencies of these four SNPs were not statistically different between cases and controls.

Genetic polymorphisms and cytarabine toxicities

We compared the frequency of common adverse complications occurring after receiving cytarabine (low dose and high dose) and genotypes of dCK-360 C>G and CDA 79A>C (Table 3 and 4). After exposure to low-dose cytarabine, patients with dCK-360 CG or GG genotype had a significantly higher risk of mucositis compared to the ones with CC genotype (P=.01) (Table 3). The patients with the G allele had an increased risk of mucositis with an OR of 3.7 (95%CI, 1.2-11.3). In contrast, there was no association between dCK-360 C>G genetic variation and the side effects after high dose cytarabine. There was no association between CDA 79 A>C genotype and risk of side effects after either low- or high-dose cytarabine administration (Table 4).

Genetic polymorphism and MRD

In the 77 ALL children with available MRD data at the end of induction therapy, neither dCK -360 C>G nor CDA 79 A>C polymorphism had any significant effect on the MRD status (Table 5).

DISCUSSION

In this study, two major enzymes involved in cytarabine metabolism were selected and genotyped. We demonstrated that the frequencies of genetic polymorphisms were not similar with previous reports. Our population had the dCK-360 G allele frequency of 17%, which was similar to Chinese subjects (16%) but higher than in whites (2%).[5,7] The difference of allele frequencies be-

tween ethnicities was also seen in CDA 79 A>C polymorphism. The Thai population had a CDA 79 C allele frequency of 10%, whereas the Japanese and white subjects showed allele frequencies of 20% and 33%, respectively.[6,13] These data emphasize the importance of studying genetic variation individually for each ethnicity. One cannot use others' data for their research due to discordant genetic backgrounds.

We analyzed the association between the common side effects of cytarabine and the genetic polymorphisms of its metabolizing enzymes. We showed that children with dCK-360 G variant allele had a significantly greater risk of mucositis than the ones with wild type allele after receiving low dose cytarabine. Mucositis is one of the common side effects of standard-dose cytarabine.[4] The dCK -360 G allele was associated with higher dCK gene expression; therefore, it may increase the active metabolite of cytarabine in both cancer and normal cells.[7] The effect of the dCK -360 G allele on the incidence of mucositis was not found in patients after high dose cytarabine exposure. The CDA 79 A>C polymorphism showed no effect on the side effects of cytarabine in our population. In contrast, studies of AML patients revealed that CDA polymorphisms were associated with the treatment-related complications of cytarabine. Bhatla et al reported that AML children with CDA 79 CC genotype were at increased risk of mucositis after post-induction chemotherapy.[14] Adult AML patients with CDA 79 AA genotype had a lower incidence of liver toxicity after receiving cytarabine-based chemotherapy.[15] However, both studies did not perform genotyping of dCK polymorphism. The effect of CDA polymorphism on the side effects of cytarabine presents in AML studies may be due to the differences in the dosage and other concomitant drugs.

The MRD status at the end of induction chemotherapy has proved to be a significant prognostic factor determining the risk of relapse in childhood ALL studies.[16-18] Therefore, the dCK-360 C>G and CDA79 A>C alter enzymatic function, and might affect the treatment response of cytarabine. However, our data did not demonstrate any difference in MRD status between patients with wild type and variant. Because combination chemotherapy was used in the induction phase, the genetic effect of the cytarabine metabolic enzymes might be obscured. The treatment of ALL is composed of multiple chemotherapeutic agents in each phase, which may be a limitation of a study attempting to demonstrate the effect of a genetic polymorphism affecting metabolism of a single drug on the therapeutic outcome. In conclusion, our study has demonstrated that the dCK-360 G allele significantly increased the risk of mucositis after treatment with cytarabine. This finding should encourage clinicians to consider the role of genetic variation on the side effects of chemotherapy. To minimize the severe toxicities of chemotherapy, further studies on the polymorphisms of involved metabolic enzymes in childhood ALL are warranted.

Table 5. Association between genetic polymorphisms and MRD at end of induction therapy.

Genotype	MRD status		*P*
	Positive (n)	Negative (n)	
dCK -360 C>G			
CC	9	47	0.71
CG+GG	2	19	
CDA 79 A>C			
AA	11	57	0.34
AC	0	9	

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Author Contributions

Banklaui C: designed syudy, performed experiements, and wrote manuscript, Jindadamrongwech S: designed study and reviewed manuscript, Sawangpanich R: performed experiments and reviewed manuscript, Apibal S: designed study and reviewed manuscript, Hongeng S: designed study and reviewed manuscript, Paisooksantivatana K: performed experiment and reviewed manuscript, Pakakasama S: designed study and wrote manuscript.

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