

SLC29A1 (*ENT1*) polymorphisms and outcome of complete remission in acute myeloid leukemia

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Abstract

Purpose The solute carrier family 29 (equilibrative nucleoside transporter), member 1 (*SLC29A1*) is known to be involved in the transportation and resistance of the nucleoside analog cytosine arabinoside (AraC), one of the most effective drugs in the treatment of acute myeloid leukemia (AML).

Methods In vitro functional analysis in AML cells and genetic association study were performed.

Results Our functional analysis of *SLC29A1* on anticancer effects of AraC showed that cytotoxic effects of AraC in AML cell lines were decreased by the reduction of *SLC29A1* expression ($P < 0.05$). To investigate whether *SLC29A1* polymorphisms could affect the achievement of complete remission (CR) in AML, we genotyped a total of

six common single nucleotide polymorphisms on *SLC29A1* in 103 AML patients, including 17 successes and 86 failures in CR. As a result, rs3734703 in 3'-untranslated region was significantly associated with CR even after correction for multiple testing (Fisher's exact test, $P = 0.008$; $P_{\text{corr}} = 0.04$). A haplotype, ht3 (A–G–G–T–C–A; frequency = 0.294 in success group; frequency = 0.120 in failure group), also revealed a significant association with CR ($P = 0.01$; simulated $P_{\text{sim}} = 0.02$).

Conclusions Although further replication in larger subjects and further functional evaluations are required, our results suggest the contribution of *SLC29A1* to cytotoxic effects of AraC. In addition, genetic variations of *SLC29A1* could be a potential marker for the achievement of CR of cancers of white blood cells including AML.

Jeong-Hyun Kim and Chansu Lee have contributed equally to this work.

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Keywords Acute myeloid leukemia (AML) · AraC · Remission · *SLC29A1* · Single nucleotide polymorphism

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Introduction

Patients with acute myeloid leukemia (AML), a rare and heterogeneous cancer, are generally treated with cytosine arabinoside (1-beta-D-arabinofuranosylcytosine; cytarabine; AraC, which is one of the most widely used drugs in the treatment of AML) or AraC-combined chemotherapy [1, 2]. Generally, AML patients have shown a poor 5-year survival (in particular, 13.9 % for older patients over the age of 50 years) after the initial diagnosis [3]. In addition, the outcome of complete remission (CR) of AraC-based treatment in AML is still unsatisfactory, and clinical resistance to AraC and its adverse side effects have been observed [3–5]. However, the causes of resistance to AraC and eventual treatment failure in hematologic cancers such as AML remain unclear.

The solute carrier family 29 (equilibrative nucleoside transporter), member 1 (SLC29A1, or ENT1) plays a crucial role in the cellular uptake of anticancer nucleoside agents as well as physiologic nucleosides. For instance, a higher expression of SLC29A1 has been observed to be less sensitive to 5-fluorouracil (5-FU), a nucleoside analog used in the treatment of cancer, in pancreatic cancer, suggesting that the highly expressed SLC29A1 may interrupt the 5-FU function that blocks synthesis of pyrimidine thymidine [6]. In addition, a significant association of SLC29A1 expression with sensitivity to gemcitabine and its uptake into mantle cell lymphoma has been found [7]. These results suggest that SLC29A1 may play an important role in the sensitivity to anticancer drugs.

Although it is known that SLC29A1 transports about 80 % of AraC into leukemic cells [8, 9], the chemotherapeutic efficacy of AraC varies between patients [10]. Among the identified mechanisms of AraC resistance in leukemic cells, the altered SLC29A1 has been suggested to be a responsible factor for the resistance [11]. Many studies have also reported that functional abnormalities in SLC29A1 are associated with resistance and sensitivity to AraC in AML [4, 12, 13]. However, only limited studies have recently suggested potential contributions of *SLC29A1* genetic variants, rs3734703 and rs693955, to the clinical outcomes in AML patients [14, 15]. In addition, there are almost no studies which investigated the association between *SLC29A1* polymorphisms and remission in AML, except for one recent report [16].

Therefore, this study performed functional analysis of SLC29A1 on anticancer effects of AraC in AML cells and genetic association analysis of *SLC29A1* single nucleotide polymorphisms (SNPs) with the achievement of CR in AML patients during the induction and subsequent consolidation chemotherapies.

Methods

Cells and cell culture

The human acute myeloid leukemia cell lines, HEL and KG-1, were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in sodium pyruvate, vitamins, and L-glutamine contained RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) with 10 % heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 g/mL) (GIBCO, Grand Island, NY, USA). Cells were cultured and maintained in a humidified atmosphere of 5 % CO₂ and 95 % air at 37 °C in an incubator. All experiments were performed in the logarithmic growth phase of each cell line.

Reagents

Analog cytosine arabinoside was purchased from Sigma (St Louis, MO, USA). AraC was dissolved in saline solution and stored at 4 °C freezer as a stock solution. Cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan), which was stored at 4 °C freezer. All solutions were protected from light.

Cell proliferation assay

100 µL of cell suspension was seeded into 96-well culture plates at 1×10^4 cells per well and then incubated for 24 h. Reagents were added to each well in the plate, and cells were incubated for the indicated time period. Then, 10 µL of the CCK-8 solution was added to each well for another 4 h. The absorbance of each well was measured in a microplate reader (Becton–Dickinson Labware, Le Pont de Claix, France) at 450 nm. Means and standard deviations were generated from three independent experiments. Absorbance values were normalized to the values obtained from the control group to determine the value for % of survival. Values are the mean \pm SD.

Gene expression regulation using plasmid vector transfection

Plasmid vectors containing shRNA sequence for target genes were obtained from OriGene Technologies, Inc. (Rockville, MD, USA). The *SLC29A1* and targeted shRNA sequence were used. One set of shRNA constructs contains four different sequences of each shRNA expression vectors in the pGFP-V-RS plasmid. Plasmids were amplified with transformation method using DH5 α competent cell strain. In the transfection study, a uni-dose mixture of different

shRNA vectors for the same gene was used. The Amaxa[®] Nucleofector[®] Device and solution were used for electroporation method (Walkersville, MD, USA) using about 1×10^6 cells at one experiment according to the manufacturer's protocol.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from appropriate cells using TRIzol reagent (Gibco-BRL, Invitrogen, Carlsbad, CA, USA), and its concentration was measured by ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA). About 1 μ g RNA per sample was reversely transcribed with SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using random hexamers. The cDNA was synthesized by incubation at 45 °C for 1 h and then inactivated by heating at 95 °C for 5 min. Using cDNA, PCR was performed in a total volume of 20 μ L for 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 48 °C for 30 s, and 72 °C for 1 min, and finally 10 min at 72 °C. The *Beta-actin* was used as an internal control. PCR products were performed using electrophoresis in 1 % agarose gel and analyzed by ethidium bromide staining.

Study patients

A total of 103 AML patients (60 men and 43 women), with the median age of 50.4 years (range 16–76 years), were recruited from Seoul National University Hospital in Korea. Patients were divided into subtypes according to the French–American–British (FAB) classification: M0 ($n = 2$), M1 ($n = 14$), M2 ($n = 47$), M4 ($n = 33$), M5 ($n = 5$), and M7 ($n = 2$). This study excluded patients with acute promyelocytic leukemia (M3 FAB subtype). The criteria for cytogenetic clone and karyotype were based on the guidelines of the International System for Human Cytogenetic Nomenclature [17]. All 103 AML patients received standard induction chemotherapy (idarubicin 12 mg/m²/day intravenously for days 1–3 and AraC 200 mg/m²/day intravenously for days 1–7), with a modified dose of idarubicin to two-thirds of the total dose in patients older than 66 years of age. Two more cycles of high-dose AraC (3 g/m² per day intravenously twice a day on D1, 3, 5) were performed for consolidation therapies. In this study, the success of CR means the achievement of CR sustained during the follow-up period, whereas no success of CR means a failure of attaining CR after induction chemotherapy or relapse any time during the courses of the treatment. The study protocol was approved by the Institutional Review Board of Seoul National University Hospital, and all patients provided written informed consent.

Genotyping of SNPs and statistical analysis

A total of six common *SLC29A1* SNPs were selected based on minor allele frequency (MAF) > 0.05 and linkage disequilibrium (LD) using the genotype data of Asian populations (Chinese and Japanese) from the 1000 Genomes Project (<http://browser.1000genomes.org/index.html>). All SNPs were successfully genotyped in 103 study subjects using TaqMan assay based on the ABI prism 7900HT sequence detection system (Applied Biosystems, Carlsbad, CA, USA). Statistical analyses were performed using Fisher's exact test and the HaploStats program in R [18], adjusting for age and sex as covariates. To correct for multiple testing errors, we used the SNPSpD program (<http://gump.qimr.edu.au/general/daleN/SNPSpD/>).

Results

Functional analysis of *SLC29A1* on anticancer effects of AraC

To evaluate whether the decrease of *SLC29A1* expression affects anticancer effects of AraC or not, we transfected shRNA of *SLC29A1* into AML cell lines (HEL and KG-1) and the anticancer effects of AraC were examined. AraC showed cytotoxic effects on these cells, and HEL cells were more sensitive to the cytotoxic effect of AraC than KG-1 cells (Supplementary Fig. 1). Reduction of *SLC29A1* was detected by green fluorescence protein (GFP) that was contained in shRNA vectors and RT-PCR in cell lines (Fig. 1a, b). When expression of *SLC29A1* was inhibited by shRNA, cytotoxic effects of AraC in both of HEL and KG-1 cells were reduced by approximately 20 % ($P = 0.028$) and 12 % ($P = 0.017$), respectively, compared with parental cells (Fig. 1c).

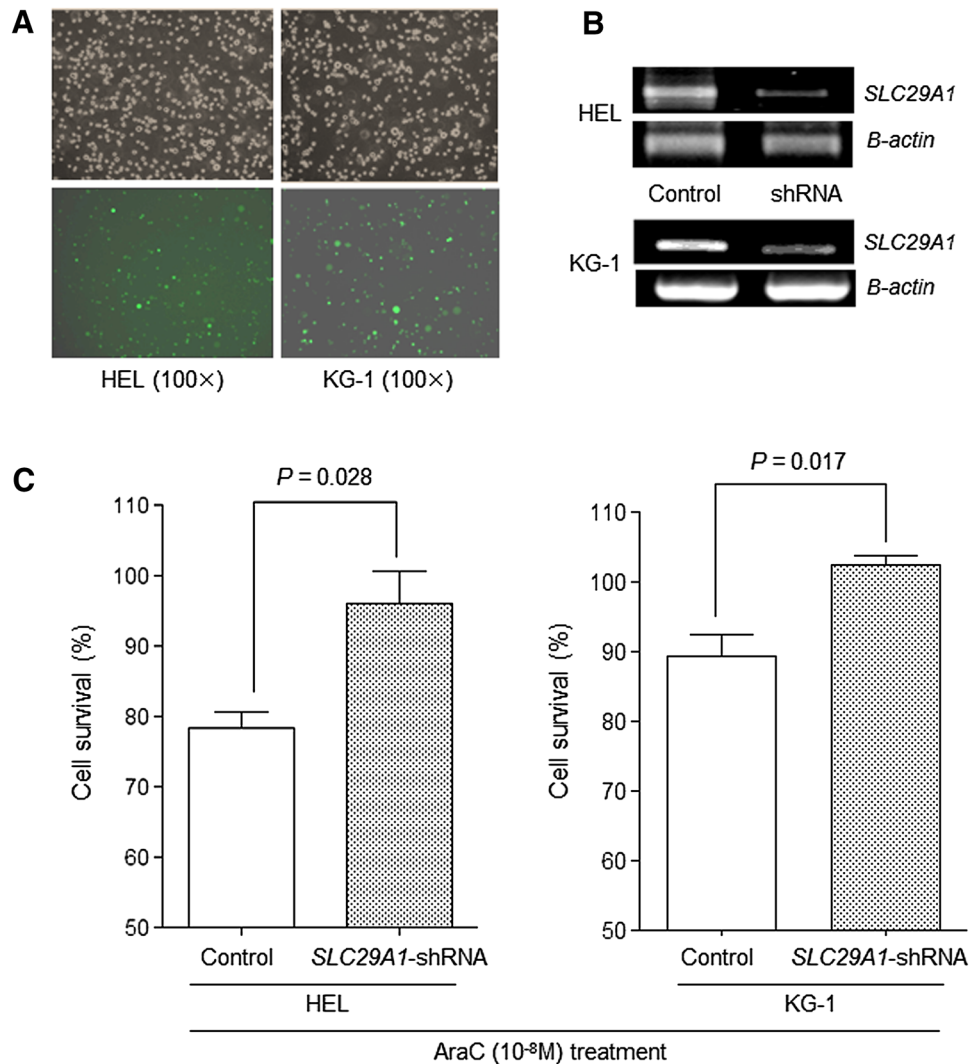
SNPs, LDs, and haplotypes of *SLC29A1*

To investigate genetic associations of *SLC29A1* with CR in AML, a total of six SNPs of *SLC29A1* on chromosome 6p21.1 were selected under the criteria in the Methods section and genotyped (Fig. 2a). Pair-wise comparisons revealed that six common *SLC29A1* SNPs showed a tight LD block in 103 Korean AML patients (17 successes and 86 failures in CR), and five major haplotypes with frequencies over 0.05 were inferred (Fig. 2b, c). For haplotype association analysis, only these common haplotypes were used.

Association analysis of *SLC29A1* SNPs and haplotypes with CR in AML

As a result, four SNPs were observed to have significances in the association with CR in AML under the Fisher's exact

Fig. 1 Effect of *SLC29A1* shRNA on the resistance against AraC in AML cells. **a** GFP expression in AML cells (HEL and KG-1) transfected GFP tagged expression vector. Cells are transfected with the 1 μ g of shRNA contained plasmid vector that targets against *SLC29A1*. After transfection (48 h), cells are treated with AraC at 10^{-8} M concentration. **b** Detection of shRNA-mediated downregulation of *SLC29A1* expression by RT-PCR in HEL and KG-1 cell lines. All RNA expression values are normalized with internal control of *Beta-actin* (*B-actin*). **c** Cell viability is determined by MTT assay method using CCK-8 cell proliferation kit. Data are expressed as mean \pm SE



test (minimum $P = 0.008$, Table 1). Among these statistically significant SNPs, rs3734703 in 3'-untranslated region (3'UTR) retained a significant signal even after correction for multiple testing ($P_{\text{corr}} = 0.04$), showing a higher MAF in remission success group (MAF = 0.324) than in failure group (MAF = 0.194). In addition, one haplotype (ht3, A–G–G–T–C–A), containing the minor "A" allele of the significant rs3734703, showed a significant association with CR in AML ($P = 0.01$; simulated $P_{\text{sim}} = 0.02$, Table 1), with about 2.5-fold higher rate of frequency in remission success group than in failure group.

In silico analyses for potential functions of rs3734703

To predict a potential function of the significantly associated rs3734703 in the 3'UTR, in silico analyses were performed. Intriguingly, in the UTRScan (<http://itbtools.ba.itb.cnr.it/utrscan>) as a program estimating the regulatory motifs in the untranslated regions of eukaryotic mRNAs,

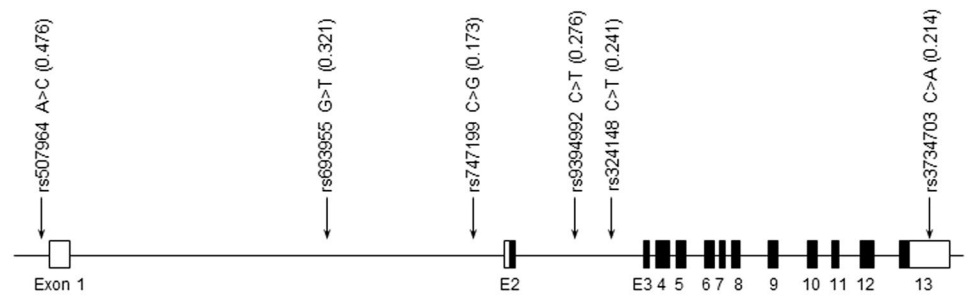
a sequence region including rs3734703 was predicted to be a potential regulatory element of upstream open reading frame (uORF), a motif involved in the interference with expression of its downstream primary ORF (Supplementary Table 1). In addition, using the expression quantitative trait (eQTL) browser (<http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/>), this rs3734703 was observed to act as a potential cis-acting variant for *SLC29A1* (*ENSG00000112759*) expression, with an eQTL score of 0.874 (Supplementary Fig. 2).

Discussion

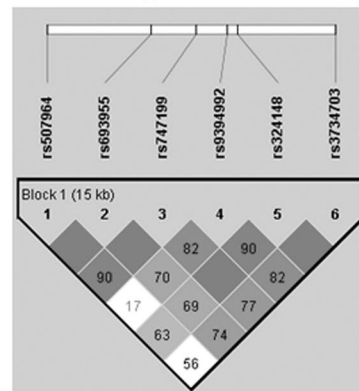
The cytotoxicity of AraC results from a combination of DNA polymerase inhibition and incorporation of AraC into DNA, in competition with deoxycytidine triphosphate [19]. AraC is considered as a primary antimetabolite treated for hematologic malignancies and is therefore used as one of the most effective drugs for the treatment of AML.

Fig. 2 Physical map, LD, and haplotypes of *SLC29A1*. **a** Schematic map of *SLC29A1* and its SNPs investigated in this study. *Black blocks* indicate coding exons; *white blocks* indicate 5'-untranslated region (UTR) and 3'-UTR. The MAF of SNP is indicated in the *bracket*. **b** LD plot of *SLC29A1*. *Number in block* represents the value of LD coefficient $|D'|$. **c** Haplotypes of *SLC29A1*. Only common haplotypes (frequency > 0.05) are shown and considered for further analyses

A Map of *SLC29A1* (solute carrier family 29, member 1) on chromosome 6p21.2-p21.1 (17 kb)



B LDs among *SLC29A1* SNPs



C Haplotypes in *SLC29A1*

Hap.	rs507964	rs693955	rs747199	rs9394992	rs324148	rs3734703	Freq.
ht1	C	G	C	C	C	C	0.321
ht2	A	T	C	C	T	C	0.193
ht3	A	G	G	T	C	A	0.149
ht4	A	T	C	C	C	C	0.111
ht5	C	G	C	T	C	C	0.076
Others	0.150

Table 1 Association of *SLC29A1* SNPs and haplotypes with complete remission in acute myeloid leukemia

SNP/haplotype	Allele	Position	MAF	Statistics			
				Complete remission success (n = 17)	Complete remission failure (n = 86)	P value (Fisher's exact test)	P_{corr}
rs507964	A > C	Promoter	0.471	0.465	0.03	NS	
rs693955	G > T	Intron 1	0.235	0.343	0.38	–	
rs747199	C > G	Intron 1	0.294	0.155	0.02	NS	
rs9394992	C > T	Intron 2	0.412	0.253	0.02	NS	
rs324148	C > T	Intron 2	0.176	0.256	0.30	–	
rs3734703	C > A	Exon 13 (3'UTR)	0.324	0.194	0.008	0.04	
				Frequency		P value*	P_{sim}
ht1 (C–G–C–C–C–C)				0.322	0.325	0.95	–
ht2 (A–T–C–C–T–C)				0.109	0.211	0.19	–
ht3 (A–G–G–T–C–A)				0.294	0.120	0.01	0.02
ht4 (A–T–C–C–C–C)				0.090	0.114	0.68	–
ht5 (C–G–C–T–C–C)				0.081	0.072	0.75	–

Bold values indicates the statistical significance of $p < 0.05$

P_{corr} value corrected for multiple testing (effective number of correction = 5.0016) using the SNPSpD program

Simulated P value (P_{sim}) is the re-calculated score statistics from a permuted reordering of the trait and covariates and the original ordering of the genotype matrix

MAF minor allele frequency, UTR untranslated region, NS not significant

* The HaploStats software is used to infer the haplotype frequencies and to perform the score test statistics

However, resistance to drugs, including AraC, is a major cause of chemotherapeutic failure among AML patients [20, 21]. Chromosomal abnormalities in pediatric AML have been reported to be related to AraC resistance [22]. In addition, candidate gene approaches have identified genetic variants associated with the resistance to AraC and its treatment outcome [23, 24]. These previous results suggest that abnormality of genes in the pharmacokinetic pathway of AraC may also be a potential cause of AraC resistance. Actually, based on our functional in vitro experiment in AML cells, reduced *SLC29A1* expression was observed to result in the resistant effect on AML cells. Although 7 + 3 (7 days of AraC; 3 days of idarubicin) is quite a standard regimen as an induction treatment for most subtypes of AML, the single most important agent in almost all AML subtypes except M3 AML is AraC throughout the courses of induction and consolidation treatment. Therefore, this study focused on AraC and related polymorphisms.

In our prior tests of karyotype and mutations for the study subjects, no associations with event-free survival and overall survival were observed. Therefore, to investigate other genetic effects on outcome in AML, this association study between genetic polymorphisms of candidate genes and CR in AML was performed. This study found the effect of *SLC29A1* genetic variants on the outcome of CR in AML patients. Considering that *SLC29A1*, as an equilibrative nucleoside transporter, is responsible for ~80 % AraC influx in leukemic cells [8, 9], our preliminary findings suggest that *SLC29A1* polymorphisms, including rs3734703 and the haplotype ht3, may be potential markers to identify patients more likely to have enhanced response to AraC-based chemotherapy in white blood cell cancers such as AML.

Recently, under the suggestion that SNP analysis could be an important tool for developing tailor-made treatments for AML patients who benefit by receiving consolidation of high-dose AraC therapy, *SLC29A1* rs693955 has been observed to be associated with a shorter time to relapse [14]. Another study has also shown that rs9394992 and rs324148 of *SLC29A1* might be potential prognostic factors to overall survival and disease-free survival in Chinese AML patients [16], supporting our result of the potential effect of *SLC29A1* genetic variants on CR in AML. However, MAFs of the two SNPs were opposite between Chinese and Korean patients. Considering different genetic distributions among ethnic population groups, further

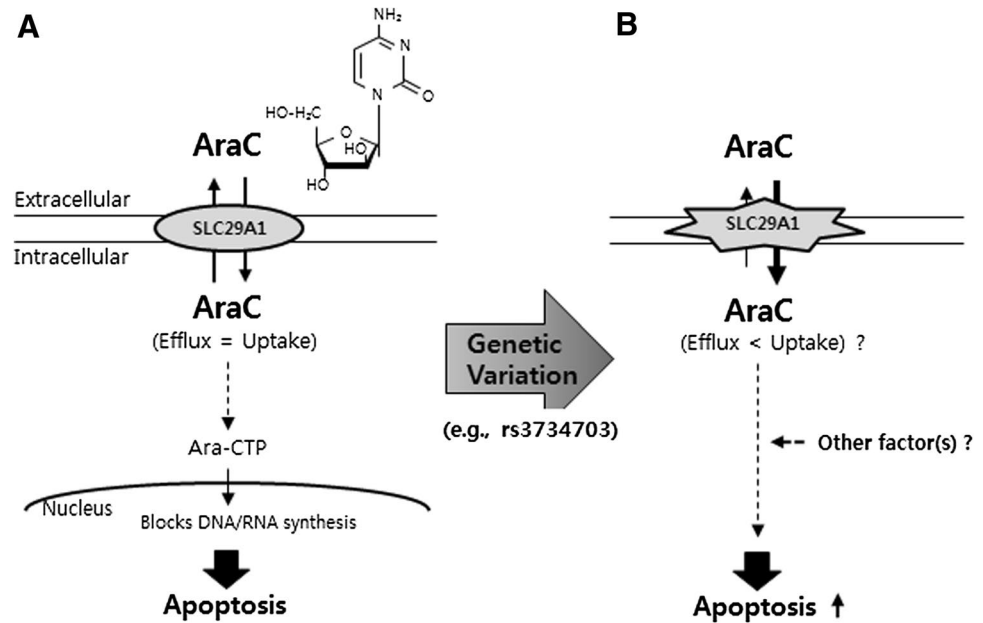
researches of *SLC29A1* SNPs may provide useful information to pharmacogenetics for drug responses in cancers of white blood cells including AML.

Functional abnormalities in human *SLC29A1* (such as loss-of-function mutations, downregulated gene expression and low promoter activity) have been implicated in the resistance and sensitivity to AraC as well as reduced cellular uptake of AraC in AML [4, 11–13, 25]. A recent genome-wide association study and its follow-up meta-analysis showed a significant association between intronic rs186556 of *SLC29A1* and AraC sensitivity in lymphoblastoid cell lines [26]. In addition, functional haplotypes in promoter regulatory region of *SLC29A1* have been observed to have potential roles in the gene expression and altered AraC chemosensitivity [27]. Furthermore, AML patients with *SLC29A1* deficiency were predicted to have a decreased survival and an increased rate of relapse [4]. These lines of evidence and our result suggest that *SLC29A1* variants and haplotypes may affect the attainment of CR and alter the uptake activity in AML patients. As presented in Fig. 3, a possible explanation is that genetic variations of *SLC29A1* (e.g., rs3734703) may lead to increased uptake activity of AraC, resulting in increased apoptosis and CR in AML.

In this study, high frequency for the major “C” allele (in other words, low MAF) of rs3734703 in 3′UTR showed a poor response to AraC-based therapy. Variants in the 3′UTR usually have a role in the expression of the gene by altering its stability and/or secondary structure [28]. Although further functional evaluations are required, it is a possible explanation that the genetic effect of rs3734703 on transcriptional regulations, such as stability and secondary structure of mRNAs, may lead to an expression change of the gene product and accompanying different outcome in CR in AML.

Among the haplotypes, only the ht3 (A–G–G–T–C–A) showed a significant association with CR in AML ($P = 0.01$) even after corrections for simulated statistics ($P_{\text{sim}} = 0.02$, Table 1). The ht3 was the unique haplotype containing the minor “A” allele of the significant rs3734703, with about 2.5-fold frequency rate in remission success group compared with failure group. Although there are several study limitations, such as no additional function studies and insufficient sample size, this ht3 haplotype of *SLC29A1* could be a potential genetic marker that is associated with CR in AML.

Fig. 3 Simplified schematic model for genetic association between *SLC29A1* and remission in AML. **a** In wild-type condition, a moderate exchange activity of *SLC29A1* is maintained. **b** Dysfunctional *SLC29A1* derived from genetic variation (e.g., rs3734703) can increase uptake activity of *SLC29A1*, which lead to apoptosis and increasing success in complete remission in AML



Conclusions

Although further replication and evaluation studies are needed, our results suggest that *SLC29A1* may have a role in the outcome of CR in AML, with providing potential information to identify patients more likely to achieve remission in AML.

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Compliance with ethical standards

Conflicts of interest There are no conflicts of interest.

Research involving human participants All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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