Concentrative nucleoside transporter 3 as a prognostic indicator for favorable outcome of t(8;21)-positive acute myeloid leukemia patients after cytarabine-based chemotherapy

JU HAN SONG[1], KYUNG-MIN CHO[1], HYEOUNG-JOON KIM[2], YEO-KYEOUNG KIM[2], NAN YOUNG KIM[2], HEE-JE KIM[3], TAE-HYANG LEE[3], SEUNG YONG HWANG[4] and TAE SUNG KIM[1]

[1]Division of Life Sciences, School of Life Sciences and Biotechnology, Korea University, Seoul;
[2]Genome Research Center for Hematopoietic Diseases, Chonnam National University Hwasun Hospital, Hwasun;
[3]Catholic Blood and Marrow Transplantation Center, Seoul St. Mary's Hospital, The Catholic University of Korea, Seoul;
[4]Division of Molecular and Life Science and GenoCheck Co., Ltd., Hanyang University, Ansan, Republic of Korea

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Abstract. Although acute myeloid leukemia (AML) exhibits diverse responses to chemotherapy, patients harboring the t(8;21) translocation are part of a favorable risk group. However, the reason why this subgroup is more responsive to cytarabine-based therapy has not been elucidated. In the present study, we analyzed expression levels of cytarabine metabolism-related genes in patients diagnosed with AML with or without t(8;21) and investigated their correlation with clinical outcomes after cytarabine-based therapy. Among the 8 genes studied, expression of the concentrative nucleoside transporter 3 (*CNT3*) gene was significantly higher in t(8;21)-positive patients compared to the others in the test population and the validation cohort (P<0.001 in Mann-Whitney U test; P<0.002 in Pearson's correlation analysis). Additionally, in both multivariate and univariate analyses, t(8;21)-positive patients categorized in a higher *CNT3* expression tertile had longer disease-free survival [hazard ratio (HR), 0.117; 95% confidence interval (CI), 0.025-0.557; P=0.008] and overall survival (HR, 0.062; 95% CI, 0.007-0.521; P=0.010) compared to t(8;21)-positive patients in a lower *CNT3* expression tertile. Notably, these trends did not occur in t(8;21)-negative patients. Our results demonstrate that *CNT3* expression is associated with overall favorable outcomes and is predictive of clinical outcomes in AML patients with t(8;21). This suggests that *CNT3* expression can be used to optimize treatment strategies for AML patients.

Correspondence to: Dr Tae Sung Kim, Division of Life Sciences, School of Life Sciences and Biotechnology, Korea University, 5-ga, Anam-dong, Seongbuk-gu, Seoul 136-701, Republic of Korea
E-mail: tskim@korea.ac.kr

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Introduction

Acute myeloid leukemia (AML) is a highly heterogeneous hematologic malignancy that displays diverse responses to chemotherapy. Although a number of clinical factors affect treatment outcomes, the cytogenetic features of AML are generally accepted as strong predictors of therapeutic response (1). Pediatric and adult patients carrying the t(8;21) chromosomal translocation, which is one of the most frequent AML subtypes, are part of a favorable risk group (2). Although these patients are initially highly responsive to treatment, a number of patients relapse and fail to achieve long-term disease-free survival (DFS) (3-5). However, little is currently known concerning the functional mechanism by which t(8;21) is associated with both a higher remission rate in some patients, and occasionally with poor outcomes in others, except in cases that are characterized by other factors such as distinctive immunophenotypical changes and multidrug resistance genes (6-8).

Cytarabine is a nucleoside analog that is intensively used for the treatment of AML (9-11). Because of its hydrophilic nature, cytarabine can only enter cells via nucleoside transporters such as concentrative nucleoside transporters (CNT1/2/3) and equilibrative nucleoside transporters (ENT1/2) (12,13). Cytarabine must also be converted to its active form by specific enzymes such as deoxycytidine kinase (dCK) (14). Several groups have reported that low gene expression and protein activity of these factors confers resistance to nucleoside drugs of leukemia cells in *in vivo* and *in vitro* studies (15-18). Alternatively, activation of certain components of the metabolic machinery such as cytidine deaminase (CDA), ecto-5'-nucleotidase (CD73) and 5',3'-nucleotidase (NT5C), may cause resistance to cytarabine therapy (19-22). Microarray analysis of an *in vitro* gemcitabine-resistant model revealed upregulation of ribonucleotide reductase M subunits (RRM1/2) as part of the intracellular detoxification process (23). Nevertheless, the prognostic implications of changes in the expression of these genes have not been elucidated in specific subpopulations, such as in AML patients with t(8;21).

In the present study, we hypothesized that specific genes that are closely involved in the antitumor action of cytarabine may be associated with the clinical response to treatment of AML patients carrying t(8;21). Therefore, we attempted to identify a novel marker that was predictive of outcomes of patients carrying the t(8;21) abnormality and evaluated the prognostic impact of the candidate genes.

Materials and methods

Patient samples. Bone marrow (BM) mononuclear cells (MNCs) from adults newly diagnosed with AML and 4 healthy donors were collected at Chonnam National University Hwasun Hospital and Catholic University Seoul St. Mary's Hospital. Written informed consent for the cryopreservation and use of the samples for further research were obtained from the patients. The Institutional Review Board approved all research on the human subjects participating in the present study. The 54 individuals in the test study and the 44 individuals in the validation study had M2 subtype disease according to the French-American-British (FAB) classification criteria; their clinical information is summarized in Table I. In the test cohort, 16 patients had normal karyotypes, 25 patients had the cytogenetically t(8;21) abnormality, and 13 patients had diverse chromosomal abnormalities. In the validation cohort, 15 patients had normal karyotypes, 19 patients had t(8;21), and 10 patients had other abnormalities. Intensive remission induction therapy was executed by administration of the combination of 12 mg/m^2/day idarubicin for 3 days with 100 mg/m^2/day cytarabine for 7 days or N^4-behenoyl-1-*D*-arabinofuranosyl cytosine (BH-AC) at either 300 mg/m^2/day for patients younger than 40 years or 200 mg/m^2/day for patients older than 40 years. Four patients received cytarabine alone at a dose of 100 mg/m^2/day; one patient, daunorubicin plus cytarabine; one patient, cytarabine plus etoposide. Patients who failed to achieve complete remission (CR) after the first round of induction chemotherapy received re-induction chemotherapy with the same regimen. Patients achieving a CR received 3 courses of high-dose Ara-C (3 g/m^2 every 12 h/day on days 1, 3 and 5) or the combination of idarubicin and BH-AC for consolidation therapy. Thirty-three patients in the test cohort and 13 patients in the validation cohort received hematopoietic stem cell transplantation (HSCT).

RNA preparation and real-time polymerase chain reaction (real-time PCR). Total RNA was extracted from the BM-MNCs of patients using the Qiagen RNA isolation kit (Qiagen, Venlo, The Netherlands) and converted to cDNA with a reverse transcription kit (Invitrogen, Carlsbad, CA, USA). The cDNA was mixed with SYBR-Green PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA) and specific primers for 8 genes including *CD73*, *CDA*, *NT5C*, *dCK*, *CNT3*, *ENT1*, *RRM1* and *RRM2*. Amplification reactions were performed in triplicate using the ABI Prism 7900 Sequence Detection system (PE Applied Biosystems). *GAPDH* served as an experimental control. Primer sequences, PCR conditions, and data processing were previously described (24). In the validation study, the expression levels of the membrane transporters, *CNT1*, *CNT2*, *CNT3*, *ENT1* and *ENT2*, were evaluated by the method described above. However, we excluded the results for *CNT1* and *CNT2* from further analysis because they had low or undetectable signals that were not as reliable.

Table I. Patient samples.

Category	Test	Validation
Patients, n	54	44
Age, median years (range)	41 (20-72)	49 (17-73)
Gender, n (%)		
Male	28 (51.9)	26 (59.1)
Female	26 (48.1)	18 (40.9)
Blasts, mean % (range)	50 (2-92)	60 (20-91)
M2 (FAB), n	54	44
Cytogenetics, n		
t(8;21)	25	19
Normal	16	15
Others	13	10

Survival estimate and correlation analyses. The study population was separated based on the cytogenetic abnormalities of the patients. For the purpose of statistical analysis of the target gene, the patients were also subdivided into 3 groups (low, intermediate and high) according to candidate gene expression tertiles. DFS was defined as the time from first remission to relapse or death. Overall survival (OS) was defined as the time from diagnosis to the date of death or last follow-up. Kaplan-Meier estimates were used to construct survival curves. Statistical differences in the treatment outcomes were determined by the univariate analyses with log-rank test and Cox proportional hazards univariate model. In multivariate analysis, a backward stepwise method of Cox proportional hazards model was used to investigate the association of clinical variables and gene expression pattern with DFS and OS and to assess hazard ratio (HR) with 95% confidence interval (CI). Correlations between clinical and molecular features were obtained by Pearson's correlation statistics and represented as a correlation coefficient (r). All statistical analyses were performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA).

Results

Prolonged survival in patients carrying t(8;21). We collected BM-MNCs from 54 newly diagnosed patients with M2 subtype AML according to the FAB classification who received cytarabine-based chemotherapy. Although it is well established that AML patients with the t(8;21) abnormality respond better to chemotherapy than patients with normal cytogenetics or other cytogenetic abnormalities (1,2,25), we confirmed this in our study population. In a univariate analysis, Kaplan-Meier estimates showed longer survival rates in terms of DFS (P=0.028 in log-rank; P=0.037 in Cox model) and OS (P=0.029 in log-rank; P=0.034 in Cox model) in the t(8;21)-positive AML cohort compared to the t(8;21)-negative group (Fig. 1).

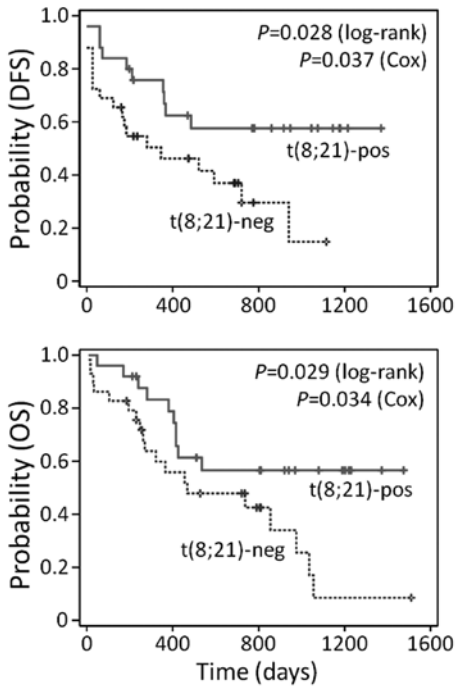
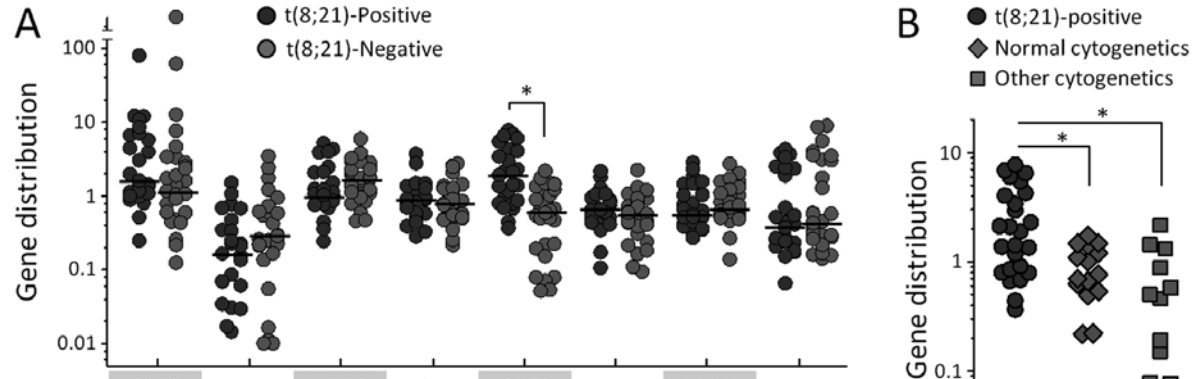


Figure 1. Survival estimate for AML patients who carry or lack the t(8;21) abnormality. Based on cytogenetic abnormalities, 54 patients with acute myeloid leukemia were classified into t(8;21)-positive (n=25) and t(8;21)-negative (n=29) groups. The curves for DFS and OS were generated using Kaplan-Meier analyses. P-values were obtained using log-rank test and univariate Cox regression analysis.

Elevated CNT3 expression in AML patients with t(8;21). To explore possible pathological reasons for the better response to cytarabine-based therapy in t(8;21)-positive patients, we examined the mRNA expression levels of 8 candidate genes in a total of 54 AML patients. These genes included *CD73*, *CDA*, *NT5C*, *dCK*, *CNT3*, *ENT1*, *RRM1* and *RRM2*, which are genes that are closely involved in metabolic processing of nucleoside analogs. Of the 8 genes, only *CNT3* mRNA levels were significantly different between the t(8;21)-positive and -negative patients in non-parametric statistical analysis with Mann-Whitney U test (P<0.001); no significant differences were observed for the remaining genes (Fig. 2A). Using the level of *CNT3* in the healthy donors as a reference, the median expression values were 1.89 (range, 0.36-7.67) for t(8;21)-positive patients and 0.63 (range, 0.05-2.16) for t(8;21)-negative patients. For the study of correlation between expression patterns of the candidate genes and cytogenetic characteristics of the patients, we conducted Pearson's correlation analysis. This analysis revealed that only the *CNT3* level had a definite correlation with the presence of cytogenetic abnormalities (r=0.520; P<0.001) (at bottom in Fig. 2A).

To further clarify the distinctive expression patterns of *CNT3* in patients, we divided the patients into 3 groups: t(8;21) (n=25), normal cytogenetics (n=16), and other cytogenetic abnormalities (n=13). The patients carrying t(8;21) still had higher *CNT3* expression compared to either of the other patient groups, even though the t(8;21)-negative population was divided into 2 subpopulations (Fig. 2B).

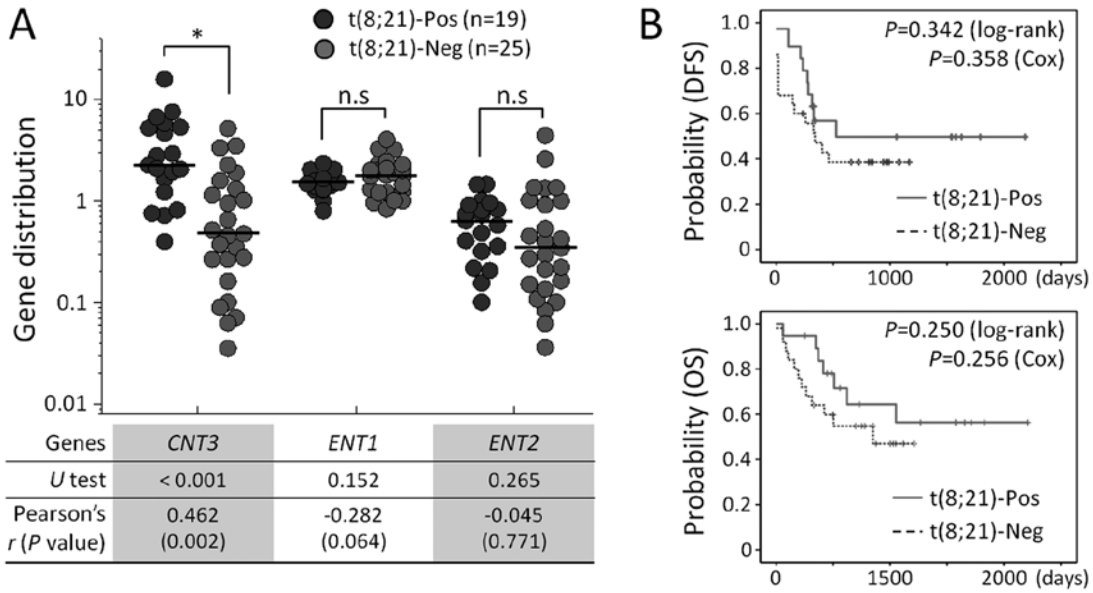
The t(8;21) abnormality-specific increase in *CNT3* expression was confirmed in a validation cohort of 44 patients diagnosed with M2 FAB subtype AML. To further examine the association between nucleoside transporter genes and cytogenetic abnormalities, we analyzed expression levels of solute carrier (SLC) family genes, including SLC28 (*CNT1*, *CNT2* and *CNT3*) and SLC29 (*ENT1* and *ENT2*). The levels of *CNT1* and *CNT2* mRNA were low or undetectable in most of the validation cohort and, therefore, results for these genes were excluded from the following analysis. As shown in Fig. 3A, we observed that *CNT3* expression was significantly elevated in patient samples that were t(8;21)-positive, compared to those that were t(8;21)-negative (P<0.001 in U test and P=0.002 in Pearson's correlation statistics). The median expression values were 2.23 (range, 0.39-15.76) for t(8;21)-positive patients and



Genes	*CD73*	*CDA*	*NT5C*	*dCK*	*CNT3*	*ENT1*	*RRM1*	*RRM2*
U test	0.099	0.114	0.136	0.683	< 0.001	0.215	0.445	0.544
Pearson's *r* (*P* value)	-0.121 (0.382)	-0.219 (0.112)	-0.106 (0.446)	0.009 (0.950)	0.520 (< 0.001)	0.149 (0.282)	-0.011 (0.938)	-0.132 (0.342)

CNT3	t(8;21)	Normal	Others
U test	–	0.003	0.001

Figure 2. Gene distribution in AML patients who carry or lack the t(8;21) abnormality. (A) Expression levels of 8 genes in 54 AML patients were normalized by comparison to the mean expression values in 4 healthy donors. Values are displayed separately according to the presence (n=25) or absence (n=29) of the t(8;21) abnormality. The solid lines denote the median levels in each population. (B) To further compare *CNT3* expression in detail, patients were subdivided into 3 groups: patients with normal cytogenetics, t(8;21)-positive patients, and patients with other cytogenetic abnormalities. The statistical differences were estimated by Mann-Whitney U tests. The association between gene expression pattern and cytogenetic status was determined using Pearson's correlation analysis. r, correlation coefficient.



Genes	*CNT3*	*ENT1*	*ENT2*
U test	< 0.001	0.152	0.265
Pearson's *r* (*P* value)	0.462 (0.002)	-0.282 (0.064)	-0.045 (0.771)

Figure 3. Distribution of nucleoside transporters in the validation population. (A) Expression levels of nucleoside membrane transporters were measured in 44 patients by real-time PCR. Values are presented according to the presence or absence of the t(8;21) abnormality. The solid lines denote the median levels of each gene in the indicated populations. The statistical differences were calculated by Mann-Whitney U tests. The association between the gene expression pattern and the cytogenetic status was determined using Pearson's correlation analysis. r, correlation coefficient. n.s, not significant. (B) The validation cohort was divided into a t(8;21)-positive group (n=19) and a t(8;21)-negative group (n=25). The curves for DFS and OS were generated using Kaplan-Meier analyses. P-values were obtained using log-rank tests and univariate Cox regression analysis.

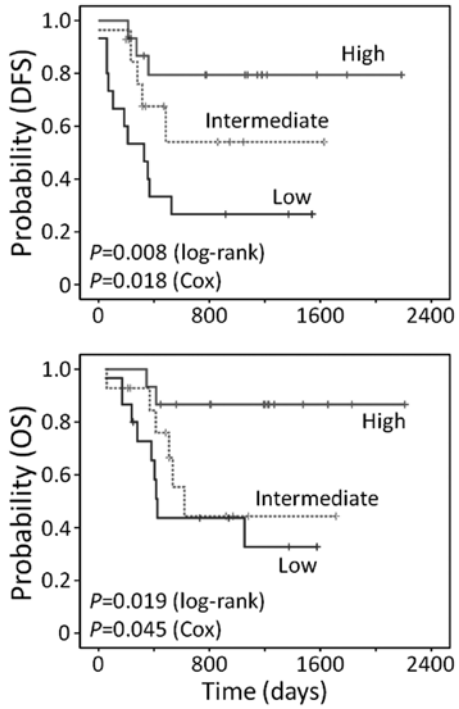


Figure 4. Prognostic value of *CNT3* in AML patients with t(8;21). The 44 t(8;21)-positive patients from the test cohort and the validation cohort were divided into tertiles based on the *CNT3* expression level: low (n=15; *CNT3* <1.34), intermediate (n=14; *CNT3* ≥1.34 and <3.37), and high (n=15; *CNT3* ≥3.37). The curves for DFS and OS were generated using Kaplan-Meier estimates. P-values were determined using log-rank tests and univariate Cox regression analysis.

0.47 (0.04-5.12) for t(8;21)-negative patients. Levels of *ENT1* and *ENT2* were not statistically different between the two groups. We also compared DFS and OS between t(8;21)-positive and -negative patient groups. Prolonged survival was observed in patients harboring t(8;21), although the results did not reach statistical significance for survival indices (P=0.342 for DFS and P=0.250 for OS in log-rank tests; Fig. 3B). The t(8;21)-specific higher expression of *CNT3* was consistent with the observation in Fig. 2 and implies a particular contribution of *CNT3* to favorable outcomes of these patients.

Prognostic impact of CNT3 expression on treatment outcomes. We hypothesized that expression of *CNT3* in patients with t(8;21) could enable more precise prediction of treatment outcomes. To examine the potential role of *CNT3* in predicting outcomes, 45 t(8;21)-positive patients from the test and validation cohorts were clustered into 3 groups (tertiles) based on their levels of *CNT3* expression (high, intermediate or low). First, the prognostic impact of *CNT3* was evaluated by univariate analysis. As shown in Fig. 4, patients with high *CNT3* expression had significantly longer survival compared to the other two patient groups (DFS, P=0.008; OS, P=0.019 in log-rank tests). In a Cox proportional hazards univariate model, HRs for the patients in the high *CNT3* tertile compared to the patients in the low tertile were 0.18 (95% CI, 0.05-0.63; P=0.008) for DFS and 0.14 (95% CI, 0.03-0.66; P=0.013) for OS. However, when the prognostic value of *CNT3* was investigated in a separate, restricted group of patients lacking the t(8;21), there were no significant differences in survival among the subpopulations with different *CNT3* expression levels (data not shown).

We conducted a multivariate analysis using the Cox proportional hazards model, which adjusted for the influence of the remission induction (RI) regimen, hematopoietic stem cell transplantation (HSCT), blast percentage, age, gender and *CNT3* mRNA; this model confirmed that high *CNT3* expression

Table II. Multivariate analysis of clinical outcomes in the t(8;21)-positive patients.

	Disease-free survival		Overall survival	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value
CNT3 expression				
CNT3 <1.34 (n=15)	1 (reference)		1 (reference)	
CNT3 ≥1.34 and <3.37 (n=14)	0.44 (0.14-1.38)	0.158	0.80 (0.24-2.64)	0.717
CNT3 ≥3.37 (n=15)	0.12 (0.03-0.56)	0.007	0.06 (0.01-0.52)	0.010
RI regimen				
Ida/AraC (n=24)	1 (reference)		1 (reference)	
Ida/BHAC (n=20)	1.11 (0.36-3.45)	0.860	2.20 (0.67-7.23)	0.196
HSCT				
Yes (n=24)	1 (reference)		1 (reference)	
No (n=20)	1.27 (0.45-3.59)	0.658	1.66 (0.51-5.39)	0.401
Blast (%)				
<50 (n=16)	1 (reference)		1 (reference)	
≥50 (n=27)	0.47 (0.14-1.56)	0.214	0.46 (0.12-1.72)	0.251
Age (years)				
≤60 (n=39)	1 (reference)		1 (reference)	
>60 (n=5)	0.54 (0.11-2.53)	0.431	0.69 (0.14-3.51)	0.654
Gender				
Male (n=26)	1 (reference)		1 (reference)	
Female (n=18)	1.37 (0.46-4.14)	0.573	1.80 (0.52-6.27)	0.355

HR, hazard ratio; CI, confidence interval; RI, remission induction; HSCT, hematopoietic stem cell transplantation.

was an independent risk factor associated with DFS (HR, 0.12; P=0.007) and OS (HR, 0.06; P=0.010) (Table II). Alternatively, clinical outcomes of patients with t(8;21)-negative AML were affected by the RI regimen and HSCT, but not by the *CNT3* expression status (data not shown). These findings suggest that *CNT3* expression levels can be used to stratify overall clinical outcomes after cytarabine-based standard chemotherapy in AML patients with t(8;21).

Discussion

We showed that cytogenetic abnormality-specific expression of *CNT3* in leukemic blasts from AML patients correlated with favorable responses to cytarabine-based chemotherapy and prolonged survival of patients with t(8;21). We also found that *CNT3* expression level was an independent predictor of the response to cytarabine-based chemotherapy in the t(8;21)-positive AML patients.

The t(8;21) translocation is one of the most frequent cytogenetic abnormalities, occurring in a large proportion (up to 15%) of AML patients. This type of AML has a high remission rate to chemotherapy, particularly when high-dose cytarabine is administered (2,9,26). Nevertheless, some patients with t(8;21) experience relapse and fail to maintain long-term survival (3-5). For this reason, most studies concerning the treatment response in AML with t(8;21) have been devoted to identification of useful markers that can be measured at diagnosis to predict poor outcomes. Representative clinically significant examples include the association of natural killer cell antigen CD56 and P-glycoprotein with inferior outcomes in t(8;21)-positive AML patients (6-8,27). However, neither of these factors is linked with favorable outcomes in patients carrying this aberration. Although a limited number of patients were examined in the present study, we were able to provide the first example of a gene related to cytarabine metabolism that can explain the varied responses of these patients to cytarabine therapy.

Nucleoside transporters including both influx and efflux pumps and drug metabolism-associated enzymes have been steadily identified as predictive markers of clinical outcomes and as potential therapeutic targets (24,28-31). In this study, we examined two types of specialized influx pumps of nucleoside analogs, ENTs and CNTs, as potential candidates for predicting patient outcomes. We chose these transporters because of previous reports indicating that they have a broad spectrum of substrate flux and are predominantly expressed in BM cells (32-34). *CNT3* expression significantly correlated with the presence of the t(8;21) aberration in both the test and validation data sets. Previous reports indicated that *ENT1* was clinically significant for cytarabine-mediated therapy; however, the present study found a slight but insignificant correlation between *ENT1* and the t(8;21) translocation in

correlation analysis and survival analysis in AML patients with t(8;21) (33,35,36). These results indicate that *CNT3*, but not *ENT1* or other functional genes, is a specific marker of cytogenetics in AML and is also a critical factor in the responsiveness of t(8;21)-positive patients to cytarabine.

Nucleoside transporters are required not only for nucleotide synthesis, but also for import of a variety of nucleoside-derived anticancer drugs. Specifically, CNT3 plays a role in the uptake of a broad range of nucleosides and their analogs, while CNT1 and CNT2 are responsible for specialized substrates (pyrimidine and purine nucleosides for CNT1 and CNT2, respectively) (34). Most studies that have only analyzed CNT3 have used *in vitro* assays to identify a critical role of CNT3 in the antitumor effect of nucleoside drugs and the resistance to these drugs (34,37,38). Alternatively, a study by Mackey *et al* (30) showed a negative correlation between CNT3 and clinical outcomes after fludarabine therapy in patients with chronic lymphocytic leukemia. Furthermore, distinct expression of *CNT3* has not been reported in AML with specific cytogenetics, even though there were several genome-wide approaches to dissect gene profiles in large numbers of patients (39-41). The present study is the first to demonstrate that *CNT3* levels can be used to stratify patients based on survival outcomes in, at least in part, t(8;21)-positive but not t(8;21)-negative AML patients. We questioned why the prognostic potential of *CNT3* preferentially applied to t(8;21)-positive AML patients. For the survival analysis, we divided t(8;21)-positive or -negative patients into subpopulations based on their *CNT3* expression level. The median values for *CNT3* expression were 2.07 (range, 0.36-15.76) for the t(8;21)-positive cohort and 0.56 (range, 0.04-5.12) for the t(8;21)-negative cohort. One potential explanation for this observation is that, at least in the t(8;21)-negative population, the small range of *CNT3* expression might be insufficient to allow stratification for outcome prediction. Although we did not determine whether *CNT3* transcript levels were consistent with protein expression levels in individual patients, and we did not ensure that the protein encoded by the *CNT3* gene was functional, it is believed that low mRNA levels are indicative of low CNT3 protein levels, which are insufficient for CNT3 to carry out its novel function.

In the CNT nucleoside transporter family, expression levels of C*NT1* and *CNT2* are known to be upregulated by extracellular stimuli including cell proliferation and activation (42-44). However, the mechanism of transcriptional regulation of *CNT3* remains largely unknown. Based on our novel finding that *CNT3* was upregulated in AML patients with t(8;21) but not in patients with other cytogenetic changes, we hypothesize that there may be a physiological interaction between the AML1 or AML1-ETO chimera proteins, which are derived from the t(8;21) translocation, and the promoter region of *CNT3*. Indeed, a virtual analysis for promoter prediction performed using transcription factor binding site search software identified 3 putative binding sites for AML1 at -3438 to -3433, -888 to -883, and +526 to +531 relative to the transcription start site of *CNT3*. Therefore, additional study is necessary to identify a role of these putative binding sites.

In summary, our results imply that higher *CNT3* expression in AML patients with t(8;21) contributes to the prolonged survival observed in this population. Our results also suggest that CNT3 can be used to predict clinical responses to cytarabine-based therapies in AML patients with t(8;21).

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