Expression Levels of Human Equilibrative Nucleoside Transporter 1 and Deoxycytidine Kinase Enzyme as Prognostic Factors in Patients with Acute Myeloid Leukemia Treated with Cytarabine

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Key Words

Pharmacogenetics · Cytarabine response · Nucleoside transporters · Acute myeloid leukemia

Abstract

Background: Cytarabine (Ara-C) is the primary drug in different treatment schemas for acute myeloid leukemia (AML) and requires the human equilibrative nucleoside transporter (*hENT1*) to enter cells. The deoxycytidine kinase (dCK) enzyme limits its activation rate. Therefore, decreased expression levels of these genes may influence the response rate to this drug. **Methods:** AML patients without previous treatment were enrolled. The expression of *hENT1* and *dCK* genes was analyzed using RT-PCR. Clinical parameters were registered. All patients received Ara-C + doxorubicin as an induction regimen (7 + 3 schema). Descriptive statistics were used to analyze data. Uni- and multivariate analyses were performed to determine factors that influenced response and survival. **Results:** Twenty-eight patients were included from January 2011 until December 2012. Median age was 36.5 years. All patients had an adequate performance status (43%

with ECOG 1 and 57% with ECOG 2). Cytogenetic risk was considered unfavorable in 54% of the patients. Complete response was achieved in 53.8%. Cox regression analysis showed that a higher *hENT1* expression level was the only factor that influenced response and survival. **Conclusions:** These results highly suggest that the pharmacogenetic analyses of Ara-C influx may be decisive in AML patients.

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Introduction

Cytarabine (Ara-C) is a cytosine analog that is currently indicated for the treatment of acute myeloid leukemia (AML) [1]. Ara-C is a prodrug that requires cellular influx primarily through the human equilibrative nucleoside transporter (*hENT1*) and active phosphorylation within the cell by the deoxycytidine kinase (dCK) enzyme to produce a monophosphorylated product (dCMP), which is bi- and triphosphorylated to act as an antineoplastic. The cytidine deaminase enzyme catalyzes this prodrug. The antineoplastic activity of Ara-C occurs via

two independent mechanisms: triphosphorylated metabolite (dCTP) inhibits DNA polymerase and then acts as an antimetabolite in DNA synthesis [2, 3].

Cell lines with lower dCK activity are resistant to nucleoside analogs and cells with higher activity are associated with higher sensitivity to these agents [4, 5]. Similarly, a lineal correlation between mRNA expression levels and enzymatic activity has been demonstrated [6], and decreased *dCK* expression also lowers activity in Ara-C-resistant cell lines [7]. The expression of *hENT1* is also correlated with intracellular Ara-C concentrations and clinical response [4, 5, 8]. The mechanism of primary Ara-C resistance is not completely defined, but inadequate cellular influx, either secondary to the downregulation of *hENT1* expression, reduced dCK activity or the increased activity of the enzymes responsible for Ara-C metabolism, such as 5'-nucleotidase (NT5C2) and cytidine deaminase, may play a role. Therefore, we evaluated the expression levels of the *hENT1* and *dCK* genes and correlated their expression with response rates and survival in patients with de novo AML.

Patients and Methods

Patient Samples

A prospective, pilot, open trial was designed. De novo AML patients, ≥ 15 years of age, with adequate renal and liver function, who were candidates for treatment with Ara-C (100 mg/m²/dose every 12 h for 7 days) + doxorubicin (60 mg/m²/day on days 1–3) in a 7 + 3 schema were included. Patients with a poor performance status (ECOG >3) were excluded. Peripheral blood (15 ml) was collected from the untreated patients. Additionally, blood samples from healthy volunteers were collected to compare expression levels of the *hENT1* and *dCK* genes. RNA was isolated using standard methods, and the *hENT1* and *dCK* genes were amplified as follows. RNA was extracted using the RNA kit PCR Core Gene Amp[®] (manufactured for Applied Biosystems by Roche Molecular Systems, Inc., Branchburg, N.J., USA) following the provider's recommendations. The local IRB approved this protocol, which was registered with www.clinicaltrials.gov (Identifier No. NCT01307241). All patients provided signed informed consent.

Real-Time Quantitative PCR

Each sample was tested in triplicate using quantitative PCR, and mRNA ratios relative to the *18S* housekeeping gene were calculated to standardize gene expression levels. The *18S*, *hENT1* and *dCK* genes were amplified using the following oligonucleotide primers: *18S*: forward: 5'-TCGGAAGTGGCCATGATT-3', reverse: 5'-CGAACCTCCGACTTTCGTTCT-3'; *hENT1*: forward: 5'-GCAAAGGAGAGGAGCCAAGA-3', reverse: 5'-CCCAAC-CAGTCAAAGATATTG-3'; *dCK*: forward: 5'-CGATCTGTG-TATAGTGACAG-3', reverse: 5'-GTTGGTTTTTCAGTGTCTA-TG-3'. The SYBR Green reaction was performed using the Maxima SYBR Green/ROX qPCR Master Mix (2 $\times$) reagents kit

Table 1. Baseline clinical characteristics

Gender	
Male	11 (39.3)
Female	17 (60.7)
Median age, years (range)	36.5 (17–75)
Performance status	
ECOG 0–1	12 (43)
ECOG 2	16 (57)
Cytogenetic risk	
Favorable	7 (25)
Intermediate	5 (18)
Unfavorable	15 (54)
Not evaluable	1 (3)
Values are expressed as n (%), unless otherwise indicated.	

(Thermo Scientific, Waltham, Mass., USA) following the manufacturer's recommendations. A reaction optimization was performed for each gene-specific pair of primers before real-time reverse-transcription PCR (RT-qPCR) was performed to confirm the specificity of the amplification signal. The following PCR conditions were used: 1 cycle of 10 min at 95°C and 40 cycles of 30 s at 95°C, 30 s at 59°C and 30 s at 72°C. Changes in fluorescence were recorded as the temperature increased from 65 to 95°C at a rate of 0.2°C/s to obtain a DNA melting curve.

Data Analysis Using the $2^{-\Delta\Delta CT}$ Method

Real-time PCR was performed for selected genes on the corresponding cDNA that was synthesized from each sample. Data were analyzed using the equation described by Livak and Schmittgen [9] as follows: amount of target = $2^{-\Delta\Delta CT}$. Validation of the method was performed as reported previously [10]. Data are presented as the mean $\pm$ SD. Statistical evaluation of significant differences was performed using Student's t test. p values <0.05 were considered statistically significant. This protocol was approved by the Institutional Ethics Committee and all patients signed informed consent before being included.

Descriptive statistics were used for clinical variables. Expression levels were correlated with response rate and survival. Overall survival (OS) was calculated using the Kaplan-Meier method. Cox regression analyses determined the factors that influenced response and OS.

Results

Twenty-eight patients were included from January 2011 until December 2012. Their median age was 36.5 years (17–75 years) and 60.7% were female. The M2 subtype, according to FAB classification, was the most frequent (39.3%). All patients had adequate performance status (43% with ECOG 1 and 66% with ECOG 2). Cytogenetic risk was assessed as unfavorable (in 72%), intermediate (in 18%) or favorable (in 25%) (table 1).

Fig. 1. Comparison of gene expression between the healthy and leukemic groups.

Gene Expression Levels

No blast selection was done to analyze gene expression but, in all patients, >75% of circulating cells were blasts. Initially, expression levels of *hENT1* and *dCK* were graded in comparison with the expression of *18S*. No correlation was found between *hENT1* or *dCK* expression levels and clinical baseline characteristics including cytogenetic risk, gender, age and morphological AML classification.

Expression levels of *hENT1* and *dCK* were compared with response rate, and arbitrarily 'normal levels' were defined as the median within responder patients. This cut-off level was compared with a nonresponder population. *hENT1* expression levels were considered overexpressed or normal in 8 cases (29%) and underexpressed in most cases ($n = 20$, 71%). In contrast, *dCK* expression levels were evaluated as underexpressed in 13 cases (46.4%) and considered normal or overexpressed in more cases (53.6%).

Thereafter, expression levels of both genes were compared with a second, new cut-off obtained from 50 healthy volunteers. Of interest, we found that for *hENT1* expression, the leukemic patients were within the range of the healthy volunteers but none could be considered as having underexpression of the *dCK* gene. This comparison is shown in figure 1.

Response

Five patients died during the aplasia period. Therefore, response rates were evaluated in 23 patients. Complete response was achieved in 65% of cases. Cox regression analysis determined that blasts percentage in bone marrow ($p = 0.01$) and *hENT1* and *dCK* expression levels ($p = 0.012$ and $p = 0.04$, respectively) influenced patient

Table 2. Cox regression analysis to evaluate factors influencing treatment response

Characteristics		Significance
Leukocytes		
Range	1,000–389,700	n.s.
<10,000/mm ³	64.7	
≥10,000/mm ³	35.3	
Hemoglobin, g/dl		
<8	23.5	n.s.
8–10	41.2	
>10	35.3	
Platelets, /mm ³		
<30,000	29.4	n.s.
30,000–50,000	29.4	
51,000–99,000	11.8	
≥100,000	11.8	
LDH value		
Normal	29.4	n.s.
Increased	70.6	
Albumin, g/dl		
<2.5	5.9	n.s.
2.5–3.5	58.8	
3.6–4.6	35.3	
Percentage of bone marrow blasts		
20–39	11.8	0.012
40–49	17.6	
>50	70.6	
<i>hENT1</i> expression levels		
Normal	40	0.012
Underexpressed	60	
<i>dCK</i> expression levels		
Normal	36	0.04
Underexpressed	64	

Values are expressed as percentages, unless otherwise indicated. LDH = Lactate dehydrogenase; n.s. = not significant.

Fig. 2. **a** OS in the whole group. **b** OS according to *hENT1* expression. **c** OS according to *dCK* expression.

response (table 2). Patients with normal levels of *hENT1* or *dCK* enzyme genes had a higher response rate and survival (table 3).

Survival

OS in the entire group was 58% at 1 year but decreased to 38% at 30 months (fig. 2). Gene expression levels were statistically significant because patients with normally expressed *hENT1* or *dCK* had a 50% longer median OS (table 3).

Table 3. Gene expression levels

Levels	n	Response rate, %	Median survival, months
<i>hENT1</i> normal	8	57	15.1*
<i>hENT1</i> underexpressed	20	50	10.5*
<i>dCK</i> normal	15	53.8	15.1
<i>dCK</i> underexpressed	13	61.5	8.1

* $p < 0.05$.

Discussion

Ara-C is included in different treatment schemas, and it remains the standard of care for AML patients. However, resistance to Ara-C incapacitates the therapeutic effort during treatment. Long-term results of standard treatment in adults with AML range from minimal to optimal. The health economic impact of induction chemotherapy using high-dose Ara-C compared to the standard dose was proposed by Fedele et al. [11]. High-dose Ara-C has a higher toxicity rate and higher costs for patients who require complete hospitalization until neutrophil recovery, and this schema is only adopted for young and fit patients [12]. Along the same lines, allotransplantation has been proposed for normal-karyotype AML patients who achieve a median survival of almost 18 months [13].

Nonbiological factors, including insurance status and county-level income, have also been associated with a poorer outcome [14], as in our series of patients in which we documented a 17.8% mortality rate during the aplasia period. Therefore, biomarkers related to predicted drug response are required in AML patients who are candidates for Ara-C treatment.

Ara-C is a prodrug that requires internalization into the cell via *hENT1*. Thereafter, the *dCK* enzyme produces a monophosphorylated product (dCMP), which is bi- and triphosphorylated to act as an antimetabolite. Resistance to Ara-C has been documented in AML cells with defective *dCK* or *hENT1* expression [12, 15, 16]. Our results clearly show that recently diagnosed, untreated AML patients with higher *hENT1* and *dCK* expression

levels have a higher response rate and a better OS. Notably, higher expression levels of the *hENT1* and *dCK* genes correlated with better input in patients who were treated with other pyrimidine nucleotide analogs for pancreatic, lung and biliary tract cancer [17–20]. In particular, a recently published meta-analysis [17] concluded that pancreatic cancer patients with high *hENT1* expression had longer disease-free survival with a hazard ratio of 2.62. These results are similar to in the study by Greenhalf et al. [18] in pancreatic patients who received adjuvant gemcitabine after resection.

The importance of *hENT1* for Ara-C influx to cells was demonstrated previously, and this transporter defines the scope of new drugs or chemical modifications of Ara-C, such as the addition of elaidic acid to create the elaidic acid ester derivative of Ara-C or elacytarabine, a modified drug that has cellular influx independent of *hENT1*. Clinical trials to evaluate the efficacy of this drug are ongoing. Preliminary results [3] in AML patients with persistent blasts after the first induction course showed an encouraging overall response rate of 41% and an acceptable safety profile.

Other metabolic steps are also implicated in chemotherapy-resistant leukemic cells including kinase activities, such as inactivation of the dCK enzyme [21, 22]. This modification was also documented in clinical conditions, including pancreatic, esophageal and biliary tract cancer [23, 24]. Therefore, other analogs as clofarabine were

evaluated in Ara-C-resistant leukemia [25]. Clofarabine primarily requires *hENT2*, and only a minor proportion of *hENT1*, to be transported into the cell, and it also requires phosphorylation by dCK and deoxyguanosine kinase. The sensitivity of Ara-C-resistant leukemia to clofarabine may be related to the affinity of this drug for other transporters and activation by other kinases [26–28].

Advances in cancer genomics have revealed a spectrum of somatic mutations that give rise to human AML, drawing our attention to its molecular evolution and clonal architecture [26–28]. Therefore, new target drugs were developed and evaluated in AML patients either alone or in combination with chemotherapy [28]. As an example, the addition of cladribine to regimens containing Ara-C and anthracyclines (idarubicine) allows not only a higher complete remission rate, including patients with unfavorable karyotype, but also has a more significant advantage in OS [29]. However, the cost of these new regimens may be unacceptable for patients with limited resources. Therefore, the optimization of standard treatments requires evaluation using a pharmacogenetic and pharmacoeconomic scope.

Disclosure Statement

The authors declare that they have no conflict of interest.

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