Clinical Study

Influence of Methylenetetrahydrofolate Reductase C677T, A1298C, and G80A Polymorphisms on the Survival of Pediatric Patients with Acute Lymphoblastic Leukemia

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1. Introduction

Leukemia is the most common childhood cancer. Recently the influence of polymorphisms in different genes is involved on the metabolism of chemotherapeutic agents and it has been studied especially in childhood acute lymphoblastic leukemia (ALL) [1]. Despite actual chemotherapy protocols cure almost 80% of pediatric patients with ALL, the majority of adult patients still die from this disease. Advances in cure rates in children could be attributable to identification of prognostic features together with the intensified chemotherapy and improved supportive therapy [2]. Genetic polymorphisms in patients with ALL can alter drug-metabolizing enzymes, transporters, and targets; therefore, they can influence both efficacy and toxicity of chemotherapeutic agents. Actually this type of genetic polymorphisms is not used in a specific treatment; however, they could be responsible for an altered sensitivity of leukemic cells to drugs [3]. The pharmacological pathway of MTX is useful to identify genes and polymorphisms that influence the response to chemotherapy for ALL. An important enzyme in the folate/methotrexate metabolism pathway is 5,10-methylenetetrahydrofolate reductase (MTHFR), which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in the folic acid cycle [3]. The MTHFR plays an important role in the folate metabolism and differences in its activity due to these two genic variants might modify the modulation of therapeutic response to...
antifolate chemotherapeutic agents. The frequencies of the C677T and A1298G allelic variants vary by ethnicity. In Europe, 8–20% of the Caucasian population is homozygous for the 677T allele and almost 40% is heterozygous [4]. The human reduced folate carrier (RFCh) is expressed in all cells and it is characterized as primarily responsible for the transport of folate and antifolate chemotherapeutic drugs, such as methotrexate (MTX), pemetrexed (Alimta), and raltitrexed (Tomudex) in mammalian cells, even when multiple other systems of assimilation are present [5]. However, the importance of physiology and pharmacology of G80A (RFCh) polymorphism remains unclear, although clinical and epidemiological findings have shown controversy [6]. In this study, we analyzed the association between the C677T, A1298C, and G80A polymorphisms and overall survival of Brazilian children with ALL submitted to treatment according to the Brazilian Group for Treatment of Lymphoblastic Leukemia in Childhood (GBTLI-99).

2. Materials and Methods

2.1. Patients and Samples. One hundred twenty-six children with acute lymphoblastic leukemia aged 0 ≤ 18 years were studied. They were enrolled in the Pediatric Hematology Oncology Center, Hospital Oswaldo Cruz (HUOC), Recife, Brazil, from 2003 to 2011. We evaluated 126 patients for C677T polymorphism and 118 patients for A1298C and G80A polymorphisms regarding to overall survival, and they presented clinical and laboratory diagnosis for acute leukemia using the GBTLI-99 treatment protocol [7]. The patient samples were collected from bone marrow puncture in the posterior iliac crest, according to the ethics committee of HUOC. The DNA samples were obtained by salting-out method (1988) [8].

2.2. Genotyping. The C677T polymorphism was genotyped by PCR-RFLP method as previously described [9]. The primers for MTHFR genotyping of the C677T polymorphism were (forward) 5′-TGA AGG AGA TGT CTG CGG GA-3′ and (reverse) 5′-AGG ACG GTG CGG TGA GAG TG-3′. The cycling: 1 cycle of 95°C/6 min, 40 cycles of 95°C/60 s, 62.5°C/90 s, and 72°C/60 s and 1 cycle of 72°C/7 min. Each PCR reaction of 24 μL contains the components: 2.5 μL Buffer (10x), 1 μL MgCl2 (50 mM), 1 U Taq polymerase (5 U/μL), 2 μL dNTP (200 μM), 1.5 μL primer (5 pmol/μL). The 677C → T base pair substitution creates a Hinf1 restriction site. The PCR product (198 bp) of C677T was digested for 48 hours at 37°C using Hinf1 and analyzed on agarose gel 3% with ethidium bromide (0.4 mg/mL) by electrophoresis. Digestion of PCR product with Hinf1 showed fragments of 175 bp and 23 bp for the TT genotype, 198 bp, 175 bp, and 23 bp for the CT genotype.

The A1298C polymorphism was genotyped by adapted allele specific PCR [10], were used (allele A) forward 5′-GGA GCT GAC CAG TGA AGA-3′ and reverse 5′-TGT GAC CAT TCC GGT TTG-3′; (allele C) forward 5′-CTT TGG GGA GCT GAA GGA-3′ and reverse 5′-AAG ACT TCA AAG ACA CTT G-3′. The cycling: 1 cycle of 94°C/2 min, 30 cycles of 95°C/30 s, 58°C/30 s, and 72°C/50 s and 1 cycle of 72°C/5 min. Each PCR, for 23 μL reaction contains the components: 2.3 μL Buffer (10x), 0.75 μL MgCl2 (50 mM), 1.5 U Taq polymerase (5 U/μL), 2 μL dNTP (200 μM), and 2 μL each primer (5 pmol/μL). The amplified products were analyzed using agarose gel 4% with ethidium bromide (0.4 mg/mL) by electrophoresis.

The G80A polymorphism was genotyped by PCR-RFLP using the primers and enzyme HhaI according to Chango et al. [11].

2.3. Evaluation of Toxicity and MTX Plasma Concentrations. The toxicity was assessed by toxicity scale for blood, liver, and kidney in accordance with the National Cancer Institute Common Toxicity Criteria (NCI-CTC), version 2.0. The MTX serum concentrations were evaluated in 24 hours and 48 hours during the maintenance phase, using the Methotrexate II kit (Abbott Laboratories) and the automatic analyzer of fluorescence—FLX TDX (Labclinics).

2.4. Statistical Analysis. The associations between categorical variables were performed using χ2 test. The overall survival analysis was performed using the patient followup according to the statistical models in conjunction with Kaplan Meier Log Rank (Mantel Cox) to assess the risk of death in 15.58 years (187 months) time. The serum MTX concentrations were analyzed by Kuskal Wallis test. All results with P value <0.05 were statistically significant. For this analysis we used the statistical programs BioEstat 5.0 and GraphPad Prism 5.0.

3. Results

The median age was 9 years; according to gender, the distribution of polymorphisms was similar, and except for the A1298C polymorphism, the G80A and C677T polymorphisms were in Hardy-Weinberg equilibrium. Regarding the rate (dead/alive), we observed that among the polymorphisms, the 677CC, 1298AC, and 80AA genotypes showed the higher death proportion (Table 1).

The C677T polymorphism showed a better overall survival for the 677T genotype than 677CC and 677CT genotypes in ALL, due to allele C. The survival of ALL patients with the 677TT genotype was about 98%, while for the 677CC genotype was 77%. The 677C allele favored the survival in 62%, while the 677T allele was 80% (Figure 1).

The A1298C polymorphism showed a better overall survival for the 1298CC genotype, showing a followup of 93%, while the AC genotype was 80% and AA was 85%, respectively. The survival of ALL patients with the A allele was about 68%, while that of patients with 1298C allele was 74% (data not shown).

Patients with 80GA genotype showed a better survival, while 80GG genotype patients showed worse survival up to 80 months and the survival analyses related to the allele were observed when the curves differ to 80 months (data not shown).

We analyzed the toxicity in only 18 patients. We observed that, according to NCI-CTC, the patients with 80AA genotype showed blood toxicity of grades 1 and 2, hepatic toxicity of grades 1 and 3, and no renal toxicity; patients with 80AG
Table 1: Characteristics of the patients to ALL according to genotypes of the C677T, A1298C, and G80A polymorphisms.

|                | n = 126 |          | n = 118 |          | n = 118 |
|----------------|---------|----------|---------|----------|---------|
|                | 677 CC (%) | 677 CT (%) | 677 TT (%) | 1298 AA (%) | 1298 AC (%) | 1298 CC (%) | 80 AA (%) | 80 GA (%) | 80GG (%) |
| Gender         |         |          |         |          |         |         |          |          |         |
| Male           | 32 (25.4) | 24 (19.0) | 5 (04.0) | 25 (21.2) | 17 (14.5) | 12 (10.1) | 21 (17.8) | 20 (17.0) | 13 (11.0) |
| Female         | 39 (31.0) | 22 (17.4) | 4 (03.2) | 25 (21.2) | 26 (22.0) | 13 (11.0) | 21 (17.8) | 31 (26.3) | 12 (10.1) |
| Age <9 years   | 37 (29.4) | 23 (18.2) | 4 (03.2) | 20 (17.0) | 27 (22.9) | 17 (14.5) | 19 (16.1) | 31 (26.3) | 14 (11.8) |
| ≥9 years       | 34 (27.0) | 23 (18.2) | 5 (04.0) | 30 (25.4) | 16 (13.5) | 8 (06.7)  | 23 (19.5) | 20 (17.0) | 11 (09.3) |
| Living         | 52 (41.3) | 34 (27.0) | 7 (05.5) | 40 (33.9) | 30 (25.4) | 19 (16.1) | 31 (26.3) | 42 (35.6) | 18 (15.3) |
| Dead           | 19 (15.1) | 12 (09.5) | 2 (01.6) | 10 (08.5) | 13 (11.0) | 6 (05.1)  | 11 (09.3) | 9 (07.6)  | 7 (05.9)  |
| Ratio (L/A)‡   | 0.36     | 0.35     | 0.28     | 0.25     | 0.43     | 0.31     | 0.35     | 0.21     | 0.39     |

†P value significant; L: living; D: dead.

Figure 1: Overall survival curve of ALL patients with genotype of the C677T polymorphism. (a) The survival of patients who carry the 677CC genotype is significantly lower than the survival of patients with the 677TT or 677CT genotypes and (b) the survival of patients who carry the variant 677C allele is significantly lower than the survival of patients with the 677T allele.

4. Discussion

In these last years it has been extensively debated the influence of C677T and A1298C MTHFR polymorphisms on hematological malignancies. Particularly, the roles of both polymorphisms on the susceptibility of the development of ALL have been broadly discussed [4]. Nowadays, it is very important to analyze the pathogenesis of the disease and, consequently, the risk stratification for a different genotypic profile population. De Jonge et al. [12] analyzed 245 dutch children and found that the T allele decreases the risk of leukemia in these patients according to the toxicity in course of chemotherapy with methotrexate (MTX), and, more recently, on the clinical response to chemotherapy [13], which can vary in populations, because leukemia is a multifactorial disease. In our country, the folic acid intake during pregnancy is impaired [14, 15], and this deficiency is...
patients with 677TT genotype had a better overall survival than the ALL, according to the treatment GBTLI-99 protocol. Although there was no statistical significance for the 677T allele serves as a toxicity predictor during the chemotherapy maintenance. These different results are probably attributable to several factors, such as the methotrexate/dose, treatment protocol, ethnic background, and number of patients analyzed [21, 24, 25].

In our protocol (GBTLI 99), the patients were treated with 2 g/m²/dose of methotrexate after 8 weeks of the induction phase, and it is the highest MTX dose given during the treatment for pediatric patients with ALL [8]. Kotnik et al. [28] evaluated different protocols (BFM95, BFM90, BFM86, BFM2002, BFM90NHL) of treatment to pediatric patients, which it was administered high doses of methotrexate (5 g/m²) and after analyzed the C677T and A1298C polymorphisms (also other genes SLCA9A1 and ABCB1) in comparison with the MTX toxicity on plasma. They observed only 26% of reduction in MTX clearance of patients with 677TT genotype, although the effect of magnitude on MTX clearance was not quantified for these studies [28], but it suggests an absorption of MTX by leukemic cells and, consequently, low toxicity in plasma levels. In addition to the A1298 > C polymorphism, they suggest it is also associated with lower risk of high-dose-MTX-associated leucopenia [28].

Unfortunately, it was not possible to examine all the genotyped patients relative to the G80A (RFCh) polymorphism. We show a small toxicity analysis in genotyped patients; however, the toxicity analysis (blood, kidney, and liver) suggests that the blood system has a toxicity degree more present among all the patients, presenting mainly leucopenia and thrombocytopenia. In the present study, we found that the 80AA genotype, although it had the lowest MTX plasma level up to 24 hours, showed a small reduction of MTX plasma concentrations in the period of 24 to 48 hours. Thus, we can suggest that patients carrying the 80AA genotype have a difficulty in metabolism of the chemotherapeutic to the transport the drug into the cell and, consequently, presentation of adverse effects. Chiusolo et al. [29] found in 54 patients with ALL a median age of 52 years (range 15–78 years); they found no influence of the G80A polymorphism on toxicity development and no correlation with MTX plasma levels (evaluated at 24 h and 48 h), but they point out a significant difference in overall survival rate according to genotypes; in fact, in the Kaplan–Meyer analysis, patients carrying the 80A variant had a better prognosis than the patients with the 80GG genotype, showing a better survival rate.

In our study, the patients with ALL-pediatric showed that the genotypic frequencies of the C677T polymorphism are similar to frequencies found in Egyptian, German and English populations, while the A1298C polymorphism showed frequency similar to Egyptian, French-Canadian, Italian, Japanese, and English populations [30].

Although there was no statistical significance for the A1298C polymorphism, the patients with mutant alleles (677T and 1298C) showed a better survival, suggesting that these polymorphisms could be involved in a prognostic good for leukemia.
Regarding to the G80A (RFCh) polymorphism, we show that up to 80 months of treatment, the patients with 80GG genotype had low survival. Chiusolo et al. [29] analyzed the overall survival of 49 patients, predominantly adult (15–78 years), noting a relation of worse survival in patients with 80GG genotype, but they did not identify the MTX toxicity related to genotypes.

In studies of population genetic, they show that the allele frequency varies considerably among different ethnic and geographical areas [4]. Moreover, our population is considered heterogeneous, originated from African, Caucasian, and Native American ancestral individuals. Compared to the studies of Thirumaran et al. [31] (174 Italian patients) and Thirumaran et al. [31] (460 German patients), our study found no association statistically significant for the A1298C polymorphism; this association is not only a difficulty just present in multietnic populations, but also is observed in German children [31] and Korean adults [32].

However, further studies should approach the treatment time and protocol, analyzing the risks of the polymorphisms and the folate route in different pediatric populations, because it would be important to conduct a meta-analysis study in order to get an appropriate treatment for the patients with polymorphisms unfavorable to the leukemia treatment.

Conflict of Interests
The authors declare no conflict of interests.

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