Pharmacogenetic Analysis of Liver Toxicity after Busulfan/Cyclophosphamide-based Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract. *The aim of this study was to evaluate the impact of genomic polymorphisms of methylene-tetrahydrofolate-reductase (MTHFR-C677T, MTHFR-A1298C) and various glutathione S-transferases (GSTP1-Ile105Val, GSTA1*a/b, GSTM1, GSTT1) on the occurrence of liver toxicity in patients receiving allogeneic hematopoietic stem cell transplantation (HSCT). Patients and Methods: Eighty-four adult patients were enrolled in this retrospective study. All patients were treated with busulfan/cyclophosphamide as a conditioning regimen and received cyclosporine and short-course MTX for GvHD prophylaxis. Genotyping was performed using PCR based restriction-fragment-length-polymorphism (RFLP) techniques. Results: Multivariate analysis identified the MTHFR-A1298C polymorphism as an independent predictor for maximum levels of bilirubin (p=0.0025) and duration of hyperbilirubinaemia (p=0.013). Furthermore, there was an association between this polymorphism and the occurrence of the sinusoidal obstruction syndrome (SOS) (p=0.048). No significant associations between the MTHFR-C677T or the various GST polymorphisms and liver toxicity were observed. Conclusion: The MTHFR-A1298C polymorphism might be associated with liver toxicity in patients receiving allogeneic HSCT.*

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Key Words: Allogeneic hematopoietic stem cell transplantation, glutathione S-tranferases, methylene-tetrahydrofolate-reductase, sinusoidal obstruction syndrome.

Allogeneic hematopoietic stem cell transplantation (HSCT) is still associated with a high incidence of therapy-related mortality (TRM) due to various complications such as graft-*versus*-host-disease (GvHD) and infectious diseases, and also toxic side-effects following high-dose chemotherapy. Liver toxicity including sinusoidal obstruction syndrome (SOS), also known as veno-occlusive disease (VOD), caused by the conditioning regimen, is very frequent and has an impact on therapy outcome. Interestingly, there is a remarkable interindividual variability in the observed occurence of liver toxicity in patients receiving identical treatment schedules. Pharmacogenetic approaches have potential to give an explanation for this observed interindividual variability.

Methylene-tetrahydrofolate-reductase (MTHFR) plays a central role in normal DNA synthesis and methylation, important steps in the repair of cell damages caused by drugs. MTHFR catalyses the reduction of 5,1,-methyleneTHF to 5-methylTHF, thereby providing one-carbon group for methionine synthesis, and regulating folate metabolism. Polymorphisms of MTHFR (C677T and A1298C) have been shown to alter enzyme activity (1). The C677T polymorphism has been shown to be associated with toxicity (such as mucositis) in HSCT (2, 3).

Additionally, many chemotherapeutics (such as busulfan and metabolites of cyclophosphamide) that have been linked to liver toxicity are metabolised by glutathione S-transferases (GSTs). GSTs catalyze the conjugation of busulfan, as well as the active metabolite of cyclophosphamide with glutathione, the main cellular antioxidant in the liver. Several tissue-specific and non-tissue-specific isoforms of GSTs are known and functional polymorphisms of these isoforms have been described. A null genotype polymorphism of one of these isoforms (GSTM1) could be linked to SOS in thalassemic children undergoing HSCT after busulfan/cyclophosphamide conditioning (4). An

A/G single nucleotide polymorphism (SNP) within the substrate-binding domain of GSTP1 decreases enzyme activity (5). The main GSTs present in the liver are GSTA1 and A2. Promoter polymorphisms of GSTA1 (GSTA1*a/b) have been shown to have a major impact on promoter activity (6).

The aim of our study was to evaluate the impact of MTHFR genomic polymorphisms and various GSTs on liver toxicity in patients receiving busulfan/cyclophoshamide-based allogeneic HSCT.

Patients and Methods

Eighty-four adult patients were enrolled in this retrospective study. DNA was isolated from leucocytes of stem cell recipients collected prior to HSCT using the QiaAmp kit (Qiagen, Valencia, CA, USA). The GSTP1-Ile105Val, GSTA1*a/b and MTHFR (C677T, A1298C) polymorphisms were detected by PCR-based restriction-fragment-length-polymorphism (RFLP) techniques and the null genotypes GSTM1 and GSTT1 were detected by multiplex-PCR as described previously (1, 6-9). For quality purposes a random 20% of samples was repeated and showed 100% concordance.

All patients were treated at the Bone Marrow Transplantation Unit of the University Hospital Hamburg, Germany, with standard busulfan (16mg/kg)/cyclophosphamide (2x60 mg/kg) as the conditioning regimen and received cyclosporine and short-course methotrexate (MTX) (day1:15 mg/m^2, days 3+6:10 mg/m^2) for GvHD prophylaxis. Busulfan was given orally at a fixed dose. Sixty seven patients (80%) additionally received anti-thymocyte globulin (ATG). Assessment of liver toxicity was based on the maximum bilirubin levels within 20 days after HSCT, the duration of hyperbilirubinaemia >2 mg/dl and the occurrence of SOS according to the Baltimore criteria. The bilirubin levels were measured daily right from the beginning of the conditioning regimen. In addition to the measurement of bilirubin levels, evaluation of SOS occurrence was performed by daily clinical examination including abdominal ultrasound (hepatomegaly, ≥5% weight gain, ascites). All patients were tested negative for previous hepatitis B or C infection. In addition, none of the patients had initial bilirubin levels >2.4 mg/dl.

Univariate and multivariate analyses were performed to assess associations between genotypes and toxicity parameters. Univariate analyses were performed for genotypes and adjustment factors (gender, age, ATG treatment, donor type, previous treatment with imatinib, hydroxyurea and/or interferon). Analyses with respect to the occurrence of SOS were done using the Chi-square test or Fisher's exact test. In the case of continuous toxicity outcomes the non-parametric Wilcoxon (2 groups) or Kruskall-Wallis tests (>2 groups) were used. Multivariate analysis was performed using the logistic-regression-model (SOS) and linear-regression-model (maximum bilirubin levels, duration of hyperbilirubinaemia) considering candidate factors (genotypes and adjustment factors) with *p*-values below 0.2 from the univariate analysis. All patients agreed to the genotype analysis in this study.

Results

Patient characteristics are given in Table I. The genotype distributions were as follows: MTHFR-677CC 33%, 677CT 50%, 677TT 17%; MTHFR-1298AA 46%, 1298AC 45%, 1298CC 9%; GSTP1-105Ile/Ile 55%, Ile/Val 34%, Val/Val 11%; GSTA1*a/a 45%, a/b 46%, b/b 9%, GSTM1-null 57%, GSTT1-null 18%. The distribution of genotypes among our study population was comparable to previous reports of other groups. There was no deviation from Hardy-Weinberg-Equilibrium. As expected, there was a highly significant linkage disequilibrium between the two MTHFR polymorphisms ($p<0.001$). None of the patients was homozygous for both variants (677TT + 1298CC) in our study population, which was consistent with previous reports.

Univariate analysis revealed a statistically significant association between the MTHFR-A1298C polymorphism and liver toxicity. The patients carrying the MTHFR-1298CC genotype demonstrated higher median maximum levels of bilirubin with 6.8 mg/dl than the MTHFR-1298AC and MTHFR-1298AA patients (3.0 mg/dl and 3.4 mg/dl, respectively) ($p=0.004$) (Figure 1). Furthermore, elevation of bilirubin levels >2 mg/dl lasted longer in the MTHFR-1298CC patients with a median of 14 days compared to 3 and 6 days in patients harbouring the MTHFR-1298AC and MTHFR-1298AA genotypes ($p=0.004$), respectively (Figure 1). There was a significantly higher incidence of SOS with 86% in patients that were homozygous for the C allele compared to 39% in patients harbouring at least one A allele (odds ratio 9.4; 95%CI 1.1;81.9) ($p=0.043$). Multivariate analysis confirmed the statistical independent impact of the MTHFR-A1298C polymorphism on the maximum elevation of bilirubin ($p=0.0025$), duration of hyperbilirubinaemia ($p=0.013$) and appearance of SOS ($p=0.048$). No significant associations between MTHFR-C677T, GSTP1-Ile105Val, GSTA1*a/b, GSTM1 and GSTT1 polymorphisms and liver toxicity were found in this study. Furthermore, none of the analyzed polymorphisms was linked to GvHD, overall survival, therapy-related-mortality or relapse.

Discussion

Our data suggested that the MTHFR-A1298C polymorphism might be associated with SOS in patients treated with busulfan/cyclophosphamide as a conditioning regimen followed by allogeneic HSCT. The MTHFR-A1298C polymorphism is located on exon 7 which codes for the C-terminal regulatory region. The observed effect of the A1298C polymorphism might be due to its location, since this region is known to regulate enzyme activity depending on adenosylmethionine levels. Our suggestion is that this polymorphism may lead to imbalances in MTHFR reactivity on cell damages.

A study by Mathews *et al.* also demonstrated an association between the MTHFR-A1298C polymorphism and liver complications in patients with acute promyelocytic leukemia treated with single agent As2O3 as induction, consolidation and maintenance chemotherapy (10). The

Table I. Patient characteristics.

Patient characteristics	n	(%)
Demographic information		
Age (years)		
Median	39.5	-
Range	16-59	-
Gender		
Male	49	58
Female	35	42
Disease		
CML	79	95
CML in 1st chronic phase	63	75
AML (M4)	2	2
MDS	2	2
Farber's Disease	1	1
Transplant related information		
Donor type		
Related	42	50
Unrelated	42	50
ATG treatment		
Yes	67	80
No	17	20
Previous imatinib treatment		
Yes	11	13
No	73	87
Previous interferon treatment		
Yes	31	37
No	53	63
Previous hydroxyurea treatment		
Yes	77	92
No	7	8
Survival status		
Alive	56	67
Dead	28	33
Relapse status (only CML patients)		
Relapse	5	6
No relapse	74	94
Acute GvHD		
Grade 0-2	68	81
Grade 3-4	16	19
Sinusoidal obstruction syndrome (SOS)		
Yes	36	43
No	48	57
Maximum bilirubin levels (mg/dl)		
Median	3.4	-
Range	0.7-25.4	-
Duration of hyperbilirubinaemia >2 mg/dl (days)		
Median	5	-
Range	0-32	-

CML: Chronic myelogenous leukaemia, AML: acute myeloid leukaemia, MDS: myelodysplastic syndrome, ATG: anti-thymocyte globulin, GvHD: graft-*versus*-host disease.

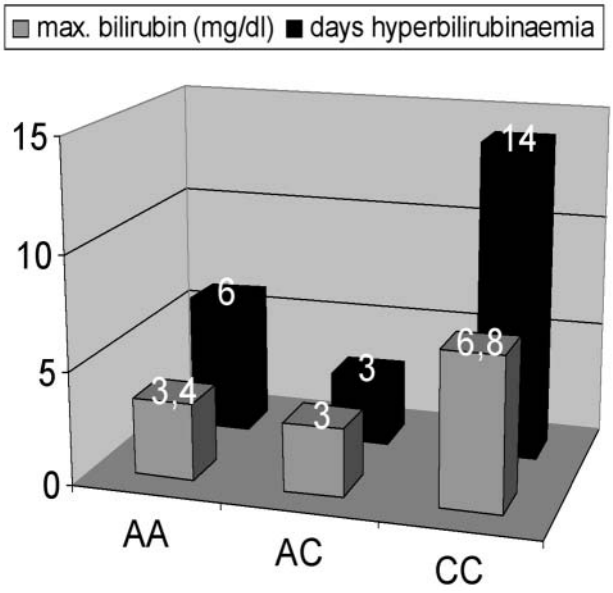

Figure 1. *Association between MTHFR A1298C and liver toxicity. Toxicities were assessed by median max. bilirubin levels (mg/dl) and median duration of hyperbilirubinaemia (days). Patients harbouring the less active MTHFR-1298CC genotype demonstrated higher median max. bilirubin levels (p=0.004) and prolonged hyperbilirubinaemia (p=0.004) compared to patients possessing at least one A allele.*

MTHFR-C677T polymorphism was not related to liver complications, which is in agreement with our results. The data suggest that there might be a general effect of the MTHFR-A1298C polymorphism on drug-induced cell toxicity in the liver. This effect may be a consequence of inadequate cell reactivity (lack of DNA-repair) to toxic influences. Such an imbalance could lead to a tendency for apoptosis of the injured cells and in consequence to increased toxicity. Interestingly, Nuckel *et al.* reported increased spontaneous apoptosis in B cells from chronic lymphocytic leukaemia (CLL) patients with MTHFR-1298CC genotypes (11). This might also imply a tendency for increased apoptosis in MTHFR-1298CC carrying normal cells (such as hepatocytes and hepatic sinusoidal endothelial cells) as a reaction to drug induced cell damage. This suggestion provides a possible explanation for the observed association of this genotype with SOS in this study since SOS is a consequence of toxic injury of hepatic sinusoidal endothelial cells. However, the exact molecular mechanism underlying this hypothesis remains unclear at this point and should be evaluated in the future.

The lack of a significant association between the MTHFR-C677T polymorphism and SOS or hyperbilirubinaemia in this study is in concordance with previous studies in allogeneic HSCT (3, 12). To date, the MTHFR-C677T polymorphism has been linked mainly to oral mucositis in allogeneic HSCT (2, 3). Mucositis was not analyzed in this study since documentation of mucositis

scores was not standardized during the evaluation period. Both MTHFR polymorphisms are linked to altered phenotypes and differing impacts of both MTHFR polymorphisms on clinical outcomes are common in the literature (13, 14).

While the GSTM1 null genotype has been identified as a risk factor for SOS after bone marrow transplantation in thalassemic children (4), it nevertheless, failed to be significantly associated with SOS in our study. One reason might be the fact that our patient population consisted only of adults which might imply a different impact of the GSTM1 null genotype with respect to age. In a study by Bredschneider *et al.* GSTA1 promoter polymorphisms (GSTA1*a/b) had no effect on GSTA1 protein expression or on GSTA1 function and the authors postulated that GSTA1 promoter polymorphisms were therefore unlikely to be associated with SOS (15). In fact, no association between GSTA1*a/b and SOS could be observed in our study.

To the best of our knowledge this is the first report linking the MTHFR-A1298C polymorphism to liver toxicity including the occurrence of SOS in allogeneic HSCT. Along with previous reports, our data provide evidence that pharmacogenetic approaches have potential for identifying patients who are at a higher risk of experiencing toxic side-effects in HSCT.

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Received June 5, 2007
Revised August 22, 2007
Accepted October 8, 2007