The impact of genetic polymorphisms on the development of drug-induced toxicities in acute lymphoblastic leukemia

PhD thesis

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Introduction

Nowadays, the constantly expanding toolkit of molecular genetics becomes increasingly widespread. Laboratory methods, online databases and software can contribute to the identification of the predisposing genetic factors in the background of diseases or conditions, which ultimately leads to personalized medicine.

Asparaginase, which eliminates asparagine from the circulation, has a crucial role in the treatment of pediatric acute lymphoblastic leukemia (ALL) patients. However, hipersensitivity reactions can occur against this bacterial enzyme, which is a great challenge. These reactions can manifest as anaphylaxis, which is directly life-threatening for patients. Also, the formation of neutralizing antibodies can lead to lower exposition to asparaginase resulting in suboptimal treatment response. For the time being, there is no tool available for identification of predisposed patients.

In my PhD work I investigated the genetic background of hypersensitivity to native *Escherichia coli* asparaginase applied as first-line treatment according to current protocols in patients with ALL. My aim was to identify patients with an increased risk to asparaginase hypersensitivity. In case of these patients, it can be suggested to use another asparaginase preparation with different immunogenic or antigenic properties as first-line treatment. My results may also contribute to a more accurate understanding of the mechanism of hypersensitivity reactions induced by other high molecular weight drug medications.

Pediatric acute lymphoblastic leukemia and its treatment

Leukemia is a malignant disease of blood-forming organs during which leukocytes proliferate unregulated and reduce the normal function of the bone marrow. Leukemic cells from the bone marrow can get into the circulation and infiltrate other organs i.e. nervous system, eyes etc. Symptoms are related to the reduction of normal blood-forming, the extensive expansion of malignant cells and the general toxic effects of malignancy. Both genetic and environmental factors can contribute to the development of leukemia.

In Hungary the number of new cases of pediatric leukemia ranges from 60 to 70 per year. ALL is the most frequent type of pediatric leukemia. Thanks to current treatment

methods based on constantly developing international protocols more than 85% of patients are cured.

The goal of the 2-3-year-long treatment is the complete eradication of the neoplastic cells which is both practically and theoretically possible. However, both the underlying disease and its treatment result in the suppression of the normal bone marrow function. Therefore, patients need close observation to provide them immediate care in case of an infection or any complication.

Several different but basically similar treatment protocols are applied worldwide. In Hungary, pediatric ALL patients are currently treated according to an international clinical study, ALL IC-BFM 2009. This treatment protocol can be divided into the following parts: induction, early intensification, consolidation, reinduction, late reintensification and maintenance therapy. Asparaginase is administered to patients during induction and reinduction. In addition, high risk patients receive asparaginase in their consolidation therapy as well.

Asparaginase hypersensitivity

Asparaginase is an enzyme with molecular weight of 130 kDa which hydrolyzes the circulating asparagine causing impairment of protein synthesis and consequently the apoptosis of lymphoblasts. While normal cells can replace asparagine by synthesis, lymphoblasts and certain tumor cells lack this ability and are dependent upon exogenic asparagines source.

Both clinical and subclinical hypersensitivities can occur against asparaginase. The most common manifestation of hypersensitivity reaction is urticaria. However, the signs and symptoms can range from local reactions of erythema, swelling or pain at the injection site to severe symptoms, including laryngeal edema, bronchospasm, hypotension and occasionally systemic anaphylaxis.

The previous BFM protocols and the current ALL IC-BFM protocol prescribe *E. coli* asparaginase as first-line treatment. Clinical hypersensitivity occurs in up to 45% of paediatric ALL patients, which necessitates the discontinuation of native *E. coli* asparaginase administration and subsequent switch to pegylated *E. coli* or *Erwinia chrysanthemi*-derived asparaginase. The reason for suspension of the treatment is most often hypersensitivity.

Several risk factors of asparaginase hypersensitivity have been described including different preparations, dosage, route of administration, readministration after a hiatus and concomitant chemotherapy. However, this type of adverse reaction is unpredictable and exhibits large interindividual differences.

The clinical hypersensitivity is associated with the formation of neutralizing antibodies. These antibodies can also form via so called silent inactivation, which has no clinical symptoms. This condition is characterized by very low or absent asparaginase activity following the administration of asparaginase. The unidentified subclinical hypersensitivity without proper intervention is associated with poor clinical outcome.

The genetic background of asparaginase hypersensitivity

In 2010 a genome wide association study (GWAS) has been carried out in the US to identify germline genetic variations contributing to the risk of asparaginase hypersensitivity in children with ALL. In this study variants of the *GRIA1* (Glutamate Receptor, Ionotropic, AMPA 1) gene located at 5q33 have been found associated with asparaginase hypersensitivity. Later, this finding has been confirmed by others.

The association of *HLA-DRB1*07:01* allele with an increased risk of asparaginase hypersensitivity was revealed in a candidate-gene study of Fernandez et al with 1870 pediatric ALL patients of European ancestry. In a subsequent GWAS of the same group on asparaginase hypersensitivity, a SNP linked to *HLA-DRB1*07:01* also acted as a risk allele in patients of diverse ancestry. In this study the rs6021191 variant in *NFATC2* was also associated with a higher risk of asparaginase hypersensitivity at the genome-wide significance threshold ($p=4.1 \times 10^{-8}$, OR=3.11). The minor allele frequency (MAF) of rs6021191 was only 0.001 among patients of European descent; therefore this result has more relevance in patients of non-European ancestry.

The role of *CYP3A4* polymorphisms in the survival of pediatric acute lymphoblastic patients

The most abundant CYP450 enzyme in the liver and the gut is the CYP3A4, one of the most important drug-metabolizing proteins in human. It has a role in the metabolisms of many drugs used in ALL therapy, for example, vincristine, cyclophosphamide, dexamethasone and doxorubicin. The heritability of the observed high enzyme variability of *CYP3A4* activity has been estimated at 90%, suggesting an important role of genetic variations in the gene and in the regulation of the gene expression. Interestingly, there are practically no publications about the role of *CYP3A4* polymorphisms in ALL pharmacogenomics. One possible explanation for this can be that the frequency of the functionally relevant variations are relatively low (maximum about 4–5%) and therefore the studies were underpowered in the usually small ALL populations.

Earlier, our group investigated the impact of *CYP3A4* variants on the survival rate of pediatric ALL patients. Beside the common SNPs (with more than 10% minor allele frequency in Caucasian populations) in the *CYP3A4* gene we also determined rarer functional polymorphisms in *CYP3A4* and in *CYP3A5* genes, which have overlapping substrate specificities and which SNPs in some studies significantly influenced the pharmacokinetics of certain drugs. We found that *CYP3A4* rs2246709 had significant influence on the survival of ALL patients which differed significantly by gender.

Objectives

1. Investigation of the association of polymorphisms in *GRIA1* and *GALNT10* genes (selected based on an earlier GWAS on asparaginase hypersensitivity) with asparaginase hypersensitivity in Hungarian pediatric acute lymphoblastic leukemia patients using competitive allele-specific PCR.

2. High resolution sequence-based typing of the *HLA-DRB1* and *HLA-DQB1* genes and investigation of the role of alleles and haplotypes in the development of asparaginase hypersensitivity.

3. Investigation of the impact of asparaginase hypersensitivity on the survival rate of pediatric acute lymphoblastic leukemia patients.

4. In the same population, investigation of the influence of *CYP3A4* rs2246709 on the survival of acute lymphoblastic leukemia patients with longer follow-up time.

Methods

Patients

For the candidate-gene association study samples and clinical data collection were carried out from 576 paediatric acute lymphoblastic leukaemia (ALL) patients who were treated between 1990 and 2012 in 9 Hungarian paediatric haematology centres according to four consecutive chemotherapy protocols from the Berlin—Frankfurt—Münster Study Group (ALL-BFM 90, 95, ALL IC-BFM 2002 and 2009). In this period of time two *E. coli* asparaginase, Kidrolase™ (Jazz Pharmaceuticals, Inc.) or Asparaginase medac™ (Kyowa-Hakko) were available as first-line asparaginase preparations.

Data collection was carried out retrospectively from the files of the patients.

The analysis of the *GRIA1* and *GALNT10* polymorphisms was carried out in a population of 505 pediatric ALL patients. For sequencing, DNA samples with suitable quality were available from 359 patients. The genotypic data regarding the *CYP3A4* rs2246709 polymorphism was available from 476 patients.

The National Cancer Institute Common Toxicity Criteria (CTC) system v3.0 was used to assess the grade of hypersensitivity.

Genotyping

A total of 20 SNPs in *GRIA1* and *GALNT10* genes were genotyped using KASPar-on-Demand prevalidated assays (LGC Genomics) on 7900HT Fast Real-Time PCR System (Applied Biosystems).

The high resolution (four-digit) sequence-based typing of *HLA-DRB1* and *HLA-DQB1* loci was carried out in a laboratory of Beijing Genomics Institute; in Hong Kong using an NGS-based genotyping method on Illumina MiSeq platform as previously described. Based on this, exon 2 was sequenced in *HLA-DRB1* and exons 2 and 3 were sequenced in *HLA-DQB1* genes.

Data regarding to *CYP3A4* rs2246709 polymorphism was available from the results of two different genotyping method (Sequenom iPLEX Gold MassARRAY and TaqMan® OpenArray®). Samples with discordant results were excluded and finally the analyses were performed with 476 patients.

Online databases and tools

The *HLA-DRB1* and *HLA-DQB1* genotypic data was used to search for three-gene *HLA-DRB1–HLA-DQA1–HLA-DQB1* haplotypes in the Allele Frequency Net Database — HLA Haplotype Frequency Search.

Amino acid sequences encoded by the *HLA-DRB1*, *HLA-DQA1* and *HLA-DQB1* exon 2 were inferred using the four-digit sequencing results of *HLA-DRB1* and *HLA-DQB1* as well as the inference results for *HLA-DQA1*. The Alignment Viewer of Database of Major Histocompatibility Complex (dbMHC) was used to assess the polymorphic amino acid positions in HLA-DRB1, HLA-DQB1 and HLA-DQA1 chains.

Statistics

Investigation of *GRIA1* and *GALNT10* polymorphisms

Multi-adjusted logistic regression was performed by using IBM SPSS Statistic software, version 20.0 to test for associations. Gender, ALL immunophenotype, age at diagnosis, risk group, *E. coli* asparaginase dosage during induction phase, BFM protocol and the polymorphisms in additive (11 vs. 12 vs. 22), dominant (11 vs. 12/22) and recessive (11/12 vs. 22) models were included in the analysis as categorical covariates (1, major allele; 2, minor allele). Odds ratios (ORs) and 95% confidence intervals (CIs) were obtained to estimate risks for each SNP to *E. coli* asparaginase hypersensitivity. The analyses were performed not only for the overall ALL cohort, but also for subgroups created by gender, ALL immunophenotype (pre-B vs. T-ALL), age at diagnosis (10 years vs. <10 years) and risk category (standard vs. medium vs. high risk). In order to deal with multiple comparisons the Benjamini-Hochberg false discovery rate (FDR) method with type I error rate of 5% ($p=6.76 \times 10^{-3}$) was applied as correction. Discrete-time survival analysis was used in R program to assess the impact of SNPs on the minimum number of doses at which *E. coli* asparaginase hypersensitivity developed.

HLA alleles and haplotypes

Multivariate logistic regression was used to test the associations of HLA class II alleles, *HLA-DRB1–HLA-DQA1–HLA-DQB1* haplotypes and polymorphic amino acid positions with *E. coli* asparaginase hypersensitivity. Gender, ALL immunophenotype (pre-B or T-ALL), age at diagnosis (≤10 or >10 years), risk group (standard or medium or high risk) and treatment protocol were included in the model as categorical covariates. Assuming an additive genetic model, odds ratios (ORs) and 95% confidence intervals (CIs) were obtained to estimate risks for each variable to asparaginase hypersensitivity. To account for multiple testing Bonferroni

correction was used. We also applied Bayesian network based Bayesian multilevel analysis of relevance (BN-BMLA) to extend our genetic association study by estimating *a posteriori* probabilities of strong relevance (posteriors)

Survival analysis

Kaplan-Meier method was applied to estimate the event-free and overall survival of ALL patients. The survival curves by asparaginase hypersensitivity and *CYP3A4* rs2246709 polymorphism were compared using Mantel-Cox test. Cox regression was performed using IBM SPSS Statistic software, version 20.0 to test for associations. Asparaginase hypersensitivity, the *CYP3A4* rs2246709, gender, ALL immunophenotype, age at diagnosis and risk group were included in the analysis as categorical covariates.

Results

A total of 20 single nucleotide polymorphisms in *GRIA1* and *GALNT10* genes were tested for associations in 505 patients.

Patients with *GRIA1* rs4958351 AA/AG genotype showed significantly reduced risk to asparaginase hypersensitivity compared to patients with GG genotype in the T-cell ALL subgroup (OR=0.05 (0.01–0.26); $p=4.70\times10^{-4}$), while no such association was found in pre-B-cell ALL.

In the medium risk group two SNPs of *GRIA1* (rs2055083 and rs707176) were associated significantly with the occurrence of asparaginase hypersensitivity (OR=0.21 (0.09–0.53); $p=8.48\times10^{-4}$ and OR=3.02 (1.36–6.73); $p=6.76\times10^{-3}$, respectively). Evaluating the genders separately in case of rs707176, however, the association was confined only to females.

The role of the *HLA-DRB1* and *HLA-DQB1* alleles and haplotypes in the Major Histocompatibility Complex II region was investigated in 359 pediatric acute lymphoblastic leukemia patients.

The next-generation-based high resolution typing of the HLA alleles was carried out in cooperation with the Beijing Genomics Institute. Based on genotypic data of the two *loci*, haplotype reconstruction was applied. In order to investigate the possible role of the HLA-DQ complex, the *HLA-DQA1* alleles were also inferred. Multivariate logistic regression analysis and a Bayesian network-based approach were used to identify relevant genetic risk factors of asparaginase hypersensitivity.

Patients with *HLA-DRB1*07:01* and *HLA-DQB1*02:02* alleles had significantly higher risk to develop asparaginase hypersensitivity compared to noncarriers ($p=4.56\text{x}10^{-5}$; OR=2.86 (1.73-4.75) and $p=1.85\text{x}10^{-4}$; OR=2.99 (1.68-5.31); n=359, respectively). After haplotype reconstruction the *HLA-DRB1*07:01-HLA-DQB1*02:02* haplotype was associated with an increased risk. After inferring the *HLA-DQA1* alleles the *HLA-DRB1*07:01–HLA-DQA1*02:01–HLA-DQB1*02:02* haplotype was associated with the highest risk of asparaginase hypersensitivity ($p=1.22\text{x}10^{-5}$; OR=5.00 (2.43-10.29); n=257).

Significantly fewer proportion of T-cell acute lymphoblastic leukemia patients carried the *HLA-DQB1*02:02* allele and the associated haplotype than did pre-B-cell acute lymphoblastic leukemia patients (6.5%; vs. 19.2%, respectively; p=0.047).

In our study of 476 patients, asparaginase hypersensitivity significantly influenced the survival rates of pediatric ALL patients (p=0.47). In the same population we identified that patients with *CYP3A4* rs2246709 GG genotype had significantly poorer survival.

Conclusions

1. Among the *GRIA1* and *GALNT10* genes, *GRIA1* polymorphisms showed significant associations with *E. co*li asparaginase hypersensitivity in our pediatric ALL population. We found that the effect of the rs4958351 A allele drastically differed from the previous findings in the T-ALL subgroup of patients. Another two polymorphisms, rs2055083 and rs707176 were associated with asparaginase hypersensitivity in certain subgroups. The identified *GRIA1* polymorphisms may influence the development of asparaginase hypersensitivity, but their effect may differ considerably by subgroups.

2. Patients with *HLA-DRB1*07:01* and *HLA-DQB1*02:02* alleles had significantly higher risk to develop asparaginase hypersensitivity compared to noncarriers. After haplotype reconstruction the *HLA-DRB1*07:01–HLA-DQA1*02:01–HLA-DQB1*02:02* haplotype was associated with an increased risk. In case of patients with the extended haplotype, it can be suggested to use another asparaginase preparation with different antigenic properties as first-line treatment.

3. The asparaginase hypersensitivity significantly influenced the event-free survival of ALL patients treated according the BFM protocols. Based on this, the monitoring of asparaginase level in patients is suggested.

4. Patients with *CYP3A4* rs2246709 GG genotype had significantly poorer survival compared to patients with AA genotype. This association was only significant in males. Further investigations are needed to clarify the function of this SNP.

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