Role of ABC-transporter gene polymorphisms in childhood acute lymphoblastic leukaemia

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Ph.D. thesis

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Introduction

It was 50 years ago when first time some individual differences of drug effects and side-effects were identified as being hereditary. The exponential development of informatics and biotechnology experienced over the recent one or two decades made it possible to map the human genome and its variations. This vast set of newly available information stimulated an enormous acceleration of pharmacogenetic research.

My studies were focussed on the ABC-transporter (adenosine triphosphate binding cassette) gene family, on polymorphisms of *ABCB1* and *ABCG2* in particular. These transporters were originally identified in malignant cell lines showing cross-resistance to various chemotherapeutic agents (so called multidrug-resistance). Later they turned out to have important physiological role in detoxification pathways. They are expressed in all the organs of drug absorption and excretion, on the surface of haemopoietic stem cells as well as surrounding certain other organs that need special chemical protection (brain, gonads, placenta) and transport a wide spectrum of xenobiotics outwards, e.g. carcinogens and cytostatic drugs. Their relevance is well demonstrated by animal experiments: in *Mdr1a* (an *ABCB1*-homologous gene) knock-out mice the same oral dose of digoxin causes 1.9 times higher plasma-concentration and 35 times higher cerebrospinal fluid concentration of digoxin than in control animals. The substrates of these human genes include daunorubicin, doxorubicin, etoposide, methotrexate (both transporters), dexamethasone, vinblastine, vincristine (*ABCB1* only) mentioning only those used in chemotherapy protocols of cohorts analysed in this essay.

The most examined three frequent polymorphisms of *ABCB1* are 1236C>T (synonymous; rs1045642), 2677G>T,A (Ala893Ser,Thr; rs2032582) and 3435T>C (synonymous; rs1128503). They are in strong linkage. Numerous theories were suggested to explain the mechanism of these transporters, but lately two in vitro studies proved that only the 3435T>C polymorphism is functional: 1) the mRNA (messenger

ribonucleic acid) of the T-allele is less stable than that of the 3435C variant, and 2) most probably because a rare codon causes delay in translation and this alters protein folding, the transporter synthesized from the T-allele has modified affinity to inhibitors and altered transport of certain substrates in the presence of these inhibitors.

Polymorphisms of *ABCG2* are less frequent. Regarding *ABCG2* 421C>A (Gln141Lys, rs2231142, variant allele frequency approximately 10% in Caucasians), the protein coded by the A-allele is instable, is mostly degraded in proteosomes after synthesis. The even more rare 34G>A (Val12Met, rs2231137) polymorphism modifies mRNA-expression presumably because of association with a splice-variant.

Acute lymphoblastic leukaemia (ALL) is the most frequent childhood malignancy, adding up to almost one quarter of all the malignant diseases in this age group according to Hungarian epidemiology data. Patients are treated with risk-group adjusted, complex therapeutic protocols which are being continuously developed further. Recently around 80% overall (long term) survival rate has been achieved. This patient group is a frequent target of pharmacogenetic studies.

The planning and administrative preparations of our studies were done in 2002-2003, at the time when the first online large scale bioinformatic databases were emerging. The enormous relevance of the continuously appearing new tools was easy to recognize but, at that time, there were hardly any data available on the functionality or frequency of identified gene polymorphisms. Therefore our aim was to establish a DNA and databank that can be used later for a large number of studies in a flexible manner, and to start the examination of at least a few, seemingly functional polymorphisms that are candidates for chemotherapy individualization in the future.

Objectives

1) Establishing a large scale DNA and data-bank in paediatric oncology for a wide range of future pharmacogenetic studies:

- To collect DNA-samples and data relevant in pharmacogenetics from the childhood ALL population treated according to the ALL BFM (Berlin-Frankfurt-Münster) 90 and 95 protocols in Hungary;
- To collect DNA-samples and data relevant in pharmacogenetics from osteosarcoma patients treated at the 2nd Dept. of Paediatrics, Semmelweis University and at the National Medical Center, Budapest, according to the COSS (Cooperative Osteosarcoma Study Group) 86 and 96 chemotherapy protocols;
- To collect DNA-samples and data on etoposide pharmacokinetics and toxicity of young adults with testicular tumours (National Institute of Oncology, Budapest).
- To collect DNA from a control group to represent the healthy Hungarian population;

2) Examination of the clinical relevance of *ABCB1* 3435T>C, 2677G>T,A and 1236C>T as well as *ABCG2* 34G>A and 421C>A polymorphisms (suggested to be functional in publications of other groups) in childhood ALL:

- Do *ABCB1* and *ABCG2* variants have an impact on the immuno-suppressive side-effect of ALL-chemotherapy that is severe leukocytopenia and infections;
- Are the above gene polymorphisms associated with the acute toxic encephalopathy caused by chemotherapy;
- Whether carrying the above polymorphisms or the related haplo-types alter the risk for developing ALL or the efficacy of chemotherapy?

Methods

We received the list and basic clinical data of ALL children from The Hungarian Paediatric Tumour Register in order to plan our studies. We contacted every Hungarian paediatric oncology centre that were operating in 2003. We asked them to collect blood samples from patients arriving for follow up and to allow us to collect clinical data. Later, we contacted missing patients and their relatives by post, asked their consent so that we can use various samples preserved in clinical laboratories. Peripheral blood was taken from osteosarcoma patients in a retrospective manner, from testicular tumour patients in a prospective study, and at the National Blood Bank from healthy blood donors (we used no other specimen types from these latter cohorts).

The full documentation of each patient was reviewed and data of the course of disease, therapy and complications were entered in a Microsoft Excel table previously prepared for this purpose. Symptoms were graded according to the Common Terminology Criteria for Adverse Events 3.0 of the National Cancer Institute, USA.

For DNA extraction, QIAamp DNA Blood Maxi and Midi kits (Qiagen) were used in case of peripheral blood samples, while mononuclear cell suspensions, peripheral and bone marrow smears were processed with High Pure PCR Template Preparation Kit (Roche). Neonatal Guthrie blood spots were treated with Chelex-100 reagent to obtain DNA.

The *ABCB1* 3435T>C, 2677G>T,A and 1236C>T genotypes were determined with multiplex minisequencing. *ABCG2* 34G>A and 421C>A polymorphisms were tested with LightCycler PCR and melting point analysis.

Data of the samples, the clinical datasheets, survival reports from the Tumour Register and our genotype result sheets were joint together with Microsoft Access software, these can therefore be filtered and their data combined and extracted in a very flexible way.

When studying immunosuppression and encephalopathy, genotype

data were handled as dichotomous variables: homozygotes for the more frequent allele versus those harbouring at least one variant allele. In all analyses, 95% confidence intervals were calculated. When testing association of genotypes with numeric variables not of normal distribution, Mann-Whitney U-test was used. Categorical variables were analysed with uni- and multivariate logistic regression procedure. For comparing survival, Cox-regression was chosen. Analyses were performed using SPSS 13.0, Statistica 7.0, Medcalc 5.0 and Haploview 4.1 softwares.

The following factors were included in multivariate analyses. 1.) Immunosuppression: treating hospital, chemotherapy protocol, anthracycline dose, gender, age. 2.) Encephalopathy: treating hospital, chemotherapy protocol, anthracycline dose, dose intensity (full time till protocol completed), gender, age. 3.) ALL-susceptibility: age, gender; 4) Survival analysis: age, gender, immunophenotype of blasts, chemotherapy protocol (ALL BFM 90 or 95), risk group.

Results

The total DNA bank consisted of samples from 626 ALL, 93 osteosarcoma, 70 testicular tumour patients and 193 blood donors. Most were extracted from peripheral blood, however, in case of ALL patients, 62 samples were originated from mononuclear cell suspension, 13 from Guthrie spots and 213 from bone marrow or peripheral blood films. These are overlapping numbers, since multiple DNA-samples were prepared in many cases due to difficulties of genotyping specimens extracted from Guthrie spots or smears.

We selected a group of 445 ALL patients as our study cohort: those treated according to the ALL BFM 90 or 95 protocols, whose samples were successfully genotyped and were not excluded from analyses because of co-morbidities or other circumstances. We restricted the data-collection to these ALL patients. This group includes 68.1% of the total similar Hungarian patient population and is among the ten largest similar childhood ALL DNA-banks in the world. The only bias in

sampling was related to retrospective collection, patients who died during the intravenous chemotherapy phase are underrepresented. The thesis is based on publications written before the sample and data collection would have been finished. Therefore these articles describe observations made on smaller numbers of patients than the final study population, see details below. There are ongoing analyses on the full study population. The evaluation of data obtained from osteosarcoma and testicular tumour patients is also in progress, these results are not included in the thesis.

Chemotherapy induced immunosuppression was studied on 138 patients treated according to the standard and medium risk arms of ALL BFM 95 study (56% of all similar Hungarian children). The analysis was based on the total number of days on which patients received intravenous antimicrobial therapy throughout the 6-8-month-long intensive chemotherapy phase. We found that patients of *ABCB1* 3435TT genotype needed more antimicrobial treatment than the 3435CC/CT group (OR=2.3 CI95% 1.1-5.5 in univariate and OR=2.1 CI95% 0.9-4.8 in multivariate analysis). The difference was more pronounced when the group needing excessive anti-infective therapy was examined (the cohort in the upper quadrant of time-span of antimicrobial courses; OR=2.9 CI95% 1.3-6.4 and OR=2.5 1.1-5.6 in uni- and multivariate analyses, respectively). Only a similar tendency without statistical significance was observed when the most frequent linked haplotype was analysed (1236T-2677T-3435T homozygotes). There was no association between the *ABCB1* genotype and the occurrence of severe leukocytopenia during the reinduction phase. This phenomenon may be related to the fact that the *ABCB1*-szubstrate drugs of this part of the therapy have opposite effect on leukocyte count. While vincristine and doxorubicin are myelotoxic, the dexamethasone mobilizes leukocytes from their pools this way increasing the peripheral leukocyte count. All three, however, have immunosuppressive effect.

No association was found between *ABCG2* 421C>A genotype and the above clinical parameters.

Published data from other centres examining these polymorphisms, asking similar clinical questions are based on different chemotherapy protocols and therefore the results are not directly comparable.

We examined the occurrence of acute toxic encephalopathy episodes during the whole intravenous chemotherapy phase of ALL BFM 90 and 95 protocols, only the low/standard and medium risk arms, in 275 patients (50% of the total Hungarian cohort). Patients with ABCB1 3435TT genotype suffered significantly more acute toxic encephalopathy episodes than 3435CC+CT children (OR=2.6; CI95% 1.0–7.1 in univariate and OR=3.5; CI95% 1.2–10.7 in multivariate analysis). The statistical significance was lost when the same calculations were performed with the most frequent linked haplotype (1236T-2677T-3435T homozygotes versus all other genotypes; OR=1.7; CI95% 0.6–5.1 and OR=2.2; CI95% 0.7–7.2, respectively). In case of *ABCG2,* we observed a tendency: those harbouring the variant 421A allele suffered more encephalopathy episodes than those homozygotes for the wild allele (OR=2.1 CI95% 0.7–5.8 and OR=2.0 CI95% 0.6–6.1).

Multiplicative synergistic interaction was identified between the *ABCB1* 3435T>C and the *ABCG2* 421C>A variations regarding this complication (p=0.036). Patients harbouring *ABCB1* 3435TT genotype as well as carrying *ABCG2* 421A allele developed encephalopathy in 27.8%, while the frequency of this complication was 2.4-5.9% of those with any one of the two or neither of these genetic features.

There were 124 cerebrospinal fluid methotrexate concentration values available from 40 ALL patients from an earlier research study. Samples had been taken at the end of the 24-hour-long intravenous methotrexate infusion, repeatedly in subsequent courses of therapy. These concentrations did not differ in genotype groups.

According to our results, there is a small group of patients (about 7%) with altered function of both transporters at the blood-brain-barrier

who are extremely sensitive to the central nervous system (CNS) toxicity of chemotherapy. Further research is needed to identify the drug or drug-combination responsible for this phenomenon.

In a study published by another group there was no association found between the *ABCB1* polymorphisms and the central nervous system complications, however those patients received dissimilar chemotherapy and the applied clinical definitions were also different. There is no publication on the role of *ABCG2* or the gene interaction besides ours.

Regarding ALL-susceptibility, we compared allele-frequencies of the above polymorphisms in 396 patients diagnosed between 1990 and 2003 (51% of all those registered in Hungary) and 189 healthy blood donors. When examined one by one, allele frequencies did not differ in the two populations. Some rare *ABCB1* haplotypes and genotype combinations showed differences, however, statistical significance is lost if rules of multiple comparison are applied. To compare our result with the literature, two smaller studies found the *ABCB1* 3435TT genotype more frequent in ALL, and one large study's results agree with ours.

Our unpublished results on survival are based on a sample biased at early mortality. The *ABCB1* 3435T>C polymorphism was associated with altered event free survival (p=0.048 and p=0.039 in uni- and multivariate analyses, respectively) as well as overall survival (p=0.057 and p=0.045, respectively). Children with 3435CC genotype have poorer survival compared to the TT and CT groups, the latter two being similar to each other. These results were calculated based on 427 patients treated with ALL BFM 90/95 protocols (all risk groups included, 67% of all Hungarian patients), with a median follow up of 9.0 years. This observation agrees with the results of two other, similarly BFM-protocol-based studies in other European centres. In our cohort, we didn't find association between the survival and the *ABCB1*-haplotype (1236T-2677T-3435T homozygotes versus all others) or the *ABCG2* 421C>A and 34G>A polymorphisms.

Conclusion

The established childhood ALL DNA- and databank is large and relevant in international comparison, suitable for studying the clinical implications of gene polymorphisms.

ALL children with *ABCB1* 3435TT genotype are prone to increased infectious complications during the intensive phase of BFM chemotherapy protocols, and this is observed beside unchanged rate of leukocytopenia. Those with 3435TT genotype are also at higher risk for acute toxic encephalopathy at the same period. Our clinical observations verify in vitro results of other groups finding that the 3435T>C polymorphism itself and not the linked 2677G>T,A and 1236C>T variations or their haplotypes have functional relevance.

The rare *ABCG2* 421C>A polymorphism could only be examined with low statistical power. It doesn't seem to be a relevant factor in the immunosuppression, however, our data suggest that it may play a role in acute toxic encephalopathy. We were not able to sufficiently test the clinical relevance of the *ABCG2* 34G>A polymorphism.

A multiplicative synergistic interaction was observed between the *ABCB1* 3435T>C and the *ABCG2* 421C>A variants regarding central nervous system toxicity. This way a small patient group can be identified (*ABCB1* 3435TT together with *ABCG2* 421AA/CA genotype, approximately 7% of the population), who are extremely sensitive to this adverse reaction, and are likely to have great benefit from hopeful future therapy individualization.

In our study, the *ABCB1* 3435T>C and *ABCG2* 421C>A polymorphisms did not have an impact in susceptibility to childhood ALL. Our unpublished survival analysis based on a biased sample shows that the *ABCB1* 3435T>C genotype is associated with altered survival in case of the given BFM chemotherapy protocols.

List of publications

RELATED TO THE THESIS:

Erdélyi DJ, Kámory E, Zalka A, Félné-Semsei Á, Csókay B, Andrikovics H, Tordai A, Borgulya G, Magyarosy G, Galántai I, Fekete Gy, Falus A, Szalai Cs, Kovács GT: The role of ABC-transporter gene polymorphisms in chemotherapy induced immunosuppression, a retrospective study in childhood acute lymphoblastic leukaemia. *Cell Immunol* 2006; 244: 121-4. IF=1.709

Erdélyi DJ, Kámory E, Csókay B, Andrikovics H, Tordai A, Kiss C, Félné-Semsei A, Janszky I, Zalka A, Fekete G, Falus A, Kovács GT, Szalai C.: Synergistic interaction of ABCB1 and ABCG2 polymorphisms predicts the prevalence of toxic encephalopathy during anticancer chemotherapy. *Pharmacogenomics J* 2008; 8: 321-7; IF=5.435

F. Semsei Á, Erdélyi DJ, Ungvári U, Kámory E, Csókay B, Andrikovics H, Tordai A, Cságoly E, Falus A, Kovács GT, Szalai Cs: Association of some rare haplotypes and genotype combinations in the MDR1 gene with childhood acute lymphoblastic leukaemia. *Leukemia Research* 2008; 32: 1214-20; IF=2.390

OTHER PUBLICATIONS

Erdélyi D., Daróczi K., Haltrich I., Kálmánchey R.: A case study and overview on ataxia teleangiectasia. [In Hungarian] Gyerm*ekgyógyászat* 2002, 53:443-447.

Erdélyi D.: Do the disposable diapers have effect on the later fertility of male babies? [In Hungarian] *Tudományos szemelvények a gyermekgondozási gyakorlatból*, 2003, Procter&Gamble RSC Kft.

Erlaky H., Tóth K., Szabolcs J., Horváth E., Kemény V., Müller J., Csóka M., Jókúti L., Erdélyi D., Kovács G.: Prevention of

cardiotoxicity caused by anthracyclines in childhood. [In Hungarian] *Magyar Onkológia* 2006, 50(1):25-32.

Kovács GT, Erlaky H, Tóth K, Horváth E, Szabolcs J, Csóka M, Jókúti L, Erdélyi D, Müller J: Subacute cardiotoxicity caused by anthracycline therapy in children: can dexrazoxane prevent this effect? *Eur J Pediatr* 2007; 166: 1187-8; IF=1.277

Kovács G, Erdélyi D, Csóka M, Müller J, Szalai Cs, Fekete Gy: Possibilities for individualized therapy in haematology/oncology. [In Hungarian] *Gyermekgyógyászat* 2008; 59: 143-7

Majoros X, Székelyhidi Z, Csóka M, Erdélyi D, Zubreczky-Hegyi M, Cságoly E, Kovács G: Early and late nephrotoxicity of cytostatic therapy. [In Hungarian] *Gyermekgyógyászat* 2008; 59: 214-8.

BOOK CHAPTERS:

Erdélyi D.: Somatic and mental development in infants and children. [In Hungarian] In: Gy Fekete (ed): Tabularium Paediatriae. Melania kft., 2000.

Erdélyi D: Infectious diseases and vaccinations. [In Hungarian] In: Gy Fekete (ed): Tabularium Paediatriae. Melania kft., 2000.

Erdélyi D: State of the art in pharmacogenetics of cytostatic drugs; some results of our workgroup. [In Hungarian] In: S Tury (ed): Tiszaparti esték 2004-2005. Gyermekgyógyászati továbbképző előadások. Humán Rt. 57-62. 2005.

Falus András, Erdélyi Dániel: Introduction to pharmacogenetics. [In Hungarian] In: K Gyires, Zs Fürst (ed): Farmakológia és Farmakoterápia I., Farmakológia. Medicina, Budapest, 2007, 87-94.