The potential role of glucocorticoid receptor gene polymorphisms regarding the efficacy and toxicities of the ALL therapy in paediatric patients

PhD thesis outline

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I. Introduction

Pediatric acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood. The overall survival rate of this disease has been dramatically improved in the last decades and reached approximately a 85% cure rate. Due to this improvement in the therapy regarding the overall survival, new aim must have to be the decreasing of the toxicities occuring during the therapy for ALL. In this point of view one of the most important tools is the individualized therapy based on the pharmacogenomics.

Glucocorticoids (GC) are key drugs in the treatment of pediatric ALL, however, they have numerous well known side effects developing during the chemotherapy. These toxicities in some cases require extra care such as dietary, insuline, hepatoprotecive or anxyolytic therapy. Several studies have already investigated a variety of glucocorticoid receptor polymorphisms such as N363S, ER22/23EK, BCLI polymorphisms as potential pharmacogenetic risk factors of the development of glucocorticoid related toxicities. Only few studies investigated these SNP-s as potential factors having influences on some very important prognostic factors regarding the outcome of pediatric ALL such as day 8. prednisone response or 5-year event-free survival (EFS) or 5-year overall survival (OS) rate.

Prednisolone response on day 8. is one of the most important prognostic factor regarding the outcome of paediatric ALL. Till day 8. of the chemotherapy beyond one dose of inthrathecal methotrexate (MTX) patients recieve only high doses of prednisolone (60mg/m2/day). This prednisolone treatment is responsible for the effective blast reduction. If the blast count in the peripherial blood is higher than 1G/L, patients are consider to be poor prednisone responders and when it is lower than 1G/L, patients are good prednisone responders. The 5-year event free survival (EFS) and overal survival (OS) rate are important indicators of the outcome of the paediatric ALL. There is only few articles that have already investigated the possible relation of glococorticoid receptor polymorphisms and day 8. prednisone response or 5- year EFS or 5-year OS.

Several investigations on association of glucocorticoid receptor (GCR) gene polymorphisms and glucocorticoid related toxicities in childhood acute lymphoblastic leukemia have yielded conflicting results. Nucleotide sequence variants of glucocorticoid receptor gene are reported to modify the sensitivity to the glucocorticoids. They may increase (N363S/BCL1) or decrease (ER22/23EK) the sensitivity to glucocorticoids

N363S GR gene polymorphism increases the sensitivity of the glucocorticoid receptor. This polymorphism causes unfavorable metabolic profile due to the higher glucocorticoid sensitivity. N363S receptor was found to be associated with higher occurance of hypertension, glucose metabolism abnormalities. This polymorphism was reported to cause higher BMI values and higher subsceptibility to coronary artery disease (CAD). According to some investigations the carriers of the 363S genotype have higher insuline, triglicerid, cholesterine levels. As a result of the elevated sensitivity N363S polymorphism has protective role in patients with mild form of congenital adrenal hyperplasia (CAH). Patients with N363S polymorphism may have higher sensitivity to secunder ocular hypertension after treatment of topical prednisolone-acetate eye drops.

ER22/23EK glucocorticoid receptor polymorphism is considered to decrease the sensitivity to the glucocorticoids. ER22/23EK polymorphism has a beneficial effect on glucose metabolism abnormalities and causes more favorable metabolic profile such as better HDL/LDL ratio, higher insuline sensitivity, lower fasting glucose level. Patients with ER22/23EK polymorphism have higher muscle strenght and they are taller than the non carrieres. Despite of its beneficial effects ER22/23EK polymorphism due to the relative glucocorticoid insensitivity results more aggressive sclerosis multiplex and more severe manifestation of some autoimmune diseases. Patients with ER22/23EK polymorphism may have more severe depression episodes or post traumatic stress disease (PTSD) symptomes.

The BCLI polymorphism of the GCR gene have been reported to be associated with an enhanced sensitivity to the glucocorticoids. Therefore this polymorphism is associated with increased abdominal fat mass, higher BMI values and unfovorable metabolic profile like N363S polymorphism (higher insuline and trigliceride levels). BCLI polymorphism was found to be associated with hypertension as well. Among the BCLI carriers the occurance of the major depression was higher than in non carriers.

We supposed that the N363S, ER22/23EK, BCL I glucocorticoid receptor polymorphisms upon the alteration of the glucocorticoid sensitivity may modify the glucocortiocid induced toxicities during the therapy for paediatric ALL, the day 8. prednisone response and the 5-year EFS and OS rate.

II. Objectives

1. Are there any associations between the N363S glucocorticoid receptor gene polymorphism and the glucocorticoid induced toxicities in patients recieving high doses of glucocorticoids during the chemotherapy for paediatric ALL?

1. Are there any associations between the ER22/23EK glucocorticoid receptor gene polymorphism and the glucocorticoid induced toxicities in patients recieving high doses of glucocorticoids in the chemotherapy for paediatric ALL?

2. Can be any correlations observed in patients with BCL1 polymorphism regarding the glucocorticoid induced toxicities in chemotherapy for ALL containing high doses of glucocorticoids?

3. Are there any changings in the above observed results if the different polymorphisms are combined?

4. Are there any associations between the N363S, ER22/23EK, BCLI glucocorticoid receptor gene polymorphisms and the very important prognostic factor: the day 8. prednisone response?

5. Are there any associations between the N363S, ER22/23EK, BCLI glucocorticoid receptor gene polymorphisms and the 5-EFS or the 5-OS?

III. Methods

III.1. Study population

346 children with ALL were involved (aged 1-18) who had undergone chemotherapy for ALL between 1989 and 2004. The sex ratio was 207 boys and 139 girls. These children were treated according to the ALL BFM 90/95 protocols. Each patient enrolled in this study received high dose glucocorticoid therapy during the protocol I., phase1. (prednisolone, 60mg/m2/day) and protocol II.. phase 1. (dexamethasone, 10mg/ m2/day). Prednisolone was started with the 25% of the calculated dose and rapidly increased till day 8. then the maximal dose was given until day 28. and from day 29. tapered over 9 days. Dexamethasone was given in the maximal dose between day 1-21 and was tapered down stepwise over 9 days.

Due to some technical problem we could perform the detection of this polymorphism in the case of 257 patients. 140 were boys and 117 were girls.

III.2. Genetic analysis

III.2.1.DNA isolation

DNA was extracted from peripherial blood taken in heamatologic remission. We used the HighPure PCR Template Preparation Kit according to the manufacturers' instruction.

III.2.2. N363S polymorphism

N363S/A1220G polymorphism was established by allele-specific polymerase chain reaction (PCR). Allele-specific PCR is a commonly applied method for the speedy detection of known single-base polymorphisms in DNA utilizing specially designed oligonucleotides. 5µl of extracted DNA was amplified in 150 µl of composition of supplied of "ImmoMix 250" containing DNA polymerase, 32 mM $(NH_4)_2SO_2$, buffer (125 mM Tris-HCl, pH=8.3), 0.02% Tween 20, 2 mM dNTP-s, stabilizer and 3 mM $MgCl_2$. The two internal control primers for PCR were: E2/4 foward primer: 5'-CCA GTA ATG TAA CAC TGC CCC-3', E2/ 4 reverse primer: 5'-TTC GAC CAG GGA AGT TCA GA-3'. The sequence of the wild primer was:

5’-ATC CTT GGC ACC TAT TCC AAT-3’, the mutant primer was: 5- ATC CTT GGC ACC TAT TCC AAC-3’. After an initial incubation at 95 °C for 7 min, amplification was performed by 10, 15 and 20 cycles. In the first 10 cycles: incubation at 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min. In the second 15 cycles: incubation at 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min. The third 20 cycles: 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min. Then followed the final extension at 72 °C for 30 min. PCR products were subjected to gelelectrophoresis through 1% agarose gel followed by ethidium bromide staining. For making it visible we applied ultraviolet light and took a photo of each of them. In a pair of PCR units containing DNA of heterozygous patient 2 PCR products were seen, contrary to this in a pair of PCR units containing DNA of homozygous patient only one product was seen in one unit of the pair.

III.2.3. BCLI polymorphism

The detection of the BCLI polymorphism was performed as described at the N363S polymoprhism. Allele specific PCR was applied. Foward primer: 5’-AGA GCC CTA TTC TTC AAA CTG and reverse primer: 5’-GAG AAA TTC ACC CCT ACC AAC. The sequence of the normal primer: 5’-CAA TTC CTC TCT TAA AGA GAT TG, the sequence of the mutant primer was: 5’-GAC AAG TTA TGT CTG CTG ATG. 5µl of extracted DNA was amplified in 150 µl of composition of supplied of “ImmoMix 250” containing DNA polymerase, 32 mM $(NH_4)_2SO_2$, buffer (125 mM Tris-HCl, pH=8.3), 0.02% Tween 20, 2 mM dNTP-s, stabilizer and 3 mM $MgCl_2$. PCR: after an initial incubation at 95 °C for 7 min, amplification was performed by 34 cycles. Incubation at 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min. Then followed the final extension at 72 °C for 30 min. PCR products were subjected to gelelectrophoresis through 1% agarose gel followed by ethidium bromide staining. For making it visible we applied ultraviolet light and took a photo of each of them.

III.2.4. ER22/23EK polymorphism

For the detection of ER22/23EK polymorphism we applied melting point analysis process. Here the different meltpoints between the DNA strands caused by the polymorphism was used for detection of the ER22/23EK polymorphism. We used an M-primer (ACATCTCCCCTCTCCTGAGCAA-P) containing a strain that can be triggered by the

anchor primer ligated to the M-primer. Anchor-primer: (GTAGCTCCTCCTCTTAGGGTTTTATAGAAGTCCA-fluorescein). At a certain grade of temperature (Light Cycle machine warms the system constantly) the M-primer leaves the sequence of the site of polymorphism and then the anchor triggers the M-primer and due to that a photoemission occurred. This photoemission could be detected by the software of the light cycle machine. M-primer does not have completely the complementer sequence of the mutant variant only of the wild variant. For that reason, if the polymorphism is present the photoemission is occurred at a lower point of temperature and due to that the a peak of the photoemission can be detected in lower temperature values. If patients are heterozygous then 2 peaks can be detected. The melting point analysis was preformed after the PCR. Foward primer: 5'-GAT TCG GAG TTA ACT AAA AG. Reverse primer: 5'- TAC TGA GCC TTT TGG AAA AT. 5µl of extracted DNA was amplified in 150 µl of composition of supplied of "ImmoMix 250" containing DNA polymerase, 32 mM $(NH_4)_2SO_2$, buffer (125 mM Tris-HCl, pH=8.3), 0.02% Tween 20, 2 mM dNTP-s, stabilizer and 3 mM $MgCl_2$. PCR: an initial incubation at 93 °C for 7 min, amplification was performed by 34 cycles. Incubation at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Then followed the final extension at 72 °C for 30 min. After that occurred the melting point analysis elevating the temperature from 45°C to 90°C (Light Cycler Software Version 4.05). Melting curves were designed by the software.

The study was approved by the Ethics Committee of the Hungarian Medical Research Council and all participants signed informed consent form.

III.3. The analyzed glucocorticoid-induced toxicities

These toxicities were analyzed during protocol I., phase 1 and protocol II., phase 1 when high doses of glucocoticoids (prednisolone: 60mg/m2/day and dexamethasone: 10mg/m2/day) were administered.

The following clinical and laboratory data were collected retrospectively and scrutinized for potential adverse effects of GC:

Hepatotoxicity was defined by elevated alanine transaminase (ALT), aspartate transaminase (AST) and total bilirubin (TBIL). Severe toxicity was defined by AST or ALT, higher than ten fold and/or TBIL higher than five fold of the normal values.

Glucose metabolism abnormalities were defined by diabetes requiring insulin treatment, significant glucosuria for at least 2 days or requiring medical intervention or hyperglicaemia (fasting glucose >6.5 mmol/L).

CNS (central nevous system)-/behavioral abnormalities were defined by epileptic seizures, coma, paresis, seriously altered behavior, or steroid psychosis.

Hypertension was defined by blood pressure above the 95th percentile for age.

We also investigated the combination (two or three) of the glucocortiod indiced toxicities in each patient.

III.4. Day 8 prednisone response and 5-years EFS and OS

Good prednisone response was defined by less than 1,000 blasts/μL and poor responders were characterized by 1,000 or more than 1,000 blasts/ μL in the peripherial blood on day 8.

5-years EFS was calculated as the interval between first documented complete remission and relapse or death in 5 years. 5-OS was investigated as well.

III.5. Statistical analyses

Allele frequencies were tested for Hardy Weinberg equilibrium (HWE). Allele frequencies were compared by chi-square test or Fisher's exact test where appropriate. The significance level used for testing was 5%. Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) software. Odd ratio (OR) was estimated with 95% confidence interval. Kaplan-Meier and Cox regression modell were calculated for the EFS and OS.

IV.Results:

IV.1. N363S glucocortiocid receptor polymorphism

The frequency of the N363S polymorphism was 9.24% (32/346 patients). All of the carriers were heterozygous for this SNP and no homozygous carriers were identified. The allele frequency was in Hardy Weinberg equilibrium (p=0,36>0,05).

IV.1.1. The glucocorticoid-induced toxicities

Hepatotoxicity was more frequent among carriers than in non carriers (p=0.004).

Glucose metabolism abnormalities were also significant more frequent among carriers than in non-carriers (p=0.001).

There was no significant difference regarding hypertension between the carriers and the non-carriers (p=0,171).

The incidence of CNS/behavioral abnormalities were not different between the carriers and the non carriers (p=1.0).

Carriers were more likely to suffer from glucocorticoid related toxicities as a whole.

Significantly more carriers had at least one toxicity (65.7% vs. 34.1%, p=0.001).

In addition, carriers were more likely to suffer from a combination of two toxicities than non-carriers. (p=0.009). Carriers were more likely to have three toxicities than non-carriers (p=0.02).

None of the patients had all the four toxicities.

IV.1.2. The day 8. prednisone response

The number of prednisone good responders among carries were higher than non-carriers. All the 363S-carriers had good prednisone response (32/32, 100%). Poor prednisone response among non-carriers occurred (26/314, 8.28%).

IV.1.3. The 5-year EFS and 5-year OS

The 5-years EFS for patients with 363S genotype was significantly better than for patients with the wild type (p=0.012).

The 5-years OS for patients with 363S genotype was significantly better than for patients with the wild type (p=0.013).

IV.2. ER22/23EK polymorphism

The frequency of the ER22/23EK polymorphism was 3.46% (10/346 patients), and no homozygous carriers were identified. The allele frequency was in Hardy Weinberg equilibrium (p=0.7>0.05).

IV.2.1. The glucocorticoid-induced toxicities

No statistical significant association was found between this polymorphism and hepatotoxicity (p=0.6185).

Regarding the glucose metabolism abnormalities we didn't find any carriers who had this toxicities (0/10 vs.14/322, p=0.510).

In the case of the CNS/behavioral abnormalities we neither found anybody among the carriers who had behavioral or CNS abnormalities (0/10 vs. 29/307, p=0.695).

No association was found between the carriers and the non carriers regarding the occurance at least one toxicity (p=0.06).

We neither found any significant associations in the case of the occurance of at least two or three toxicities, however a tendency could be observed. No one in the carrier-group had two (p=0.716) or three toxicities (p=0.645).

None of the patients had all the four toxicities.

IV.2.2. The day 8. prednisone response

All the carriers had good prednisone response (10/10, 100%). Poor prednisone response among non-carriers occurred (26/336, 7.74%).

IV.2.3. The 5-year EFS and 5-year OS

The 5-year EFS for patients with ER22/23EK polymorphism did not differ significantly from patients without this polymorphism (p=0.18).

The 5-years OS for patients with ER22/23EK polymorphims did not differ significantly from patients without this polymorphism (p=0.27).

IV.3. BCL1 polymorphism

At this polymorphism we found homozygous patients as well for this SNP: GG. Therefore we investigated the above mentioned toxicities, the day 8. prednisone response, the 5-year EFS and OS always on association with the GG genotype (GG vs.CC+GC) and with the G-allele (CC vs. GC+GG) at as well. We also investigated patients having both the BCL1 and the N363S polymorphism at the same time.

The allele frequencies were in Hardy Weinberg equilibrium (p=0,33>0,05). 30/257 patients were GG homozigous, 105/257 were GC heterozygous and 122/257 were CC homozygous. 9 patients had the combination of the two SNP-s (Bcl1-N363S).

IV.3.1. The glucocorticoid-induced toxicities

In the case of the glucocorticoid induced toxicities there was no statistically significant association between nor hepatotoxicity nor glucose metabolism abnormalities nor CNS/behavioral abnormalities nor hypertension and the investigated groups (CC vs. GC+GG, GG vs.CC+GC, Bcl1-N363S vs. no combination) (table1). See p-values in table 1.

Hepatotoxicity			
Genotype	Occurred	Not occurred	P-value
G/G+G/C	27	108	P=0.455
CC	20	102	
G/G	10	20	P=0.23
C/C+G/C	37	190	
N363S-BCL1	2	7	P=0.671
NO N363S-BCL1	45	203	

CNS-/ behavior changings			
Genotype	Occurred	Not occurred	P-value
G/G+G/C	18	117	P=0.785
CC	15	108	
G/G	6	24	P=0.209
C/C+G/C	27	201	
N363S-BCL1	1	8	P=1.00
NO N363S-BCL1	32	217	

Glucose metabolism abnormalities			
Genotype	Occurred	Not occurred	P-value
G/G+G/C	7	128	P=0.332
CC	10	112	
G/G	1	29	P=0.7
C/C+G/C	16	211	
N363S-BCL1	2	7	P=0.113
NO N363S-BCL1	15	233	

Hypertension			
Genotype	Occurred	Not occurred	P-value
G/G+G/C	33	102	P=0.167
CC	39	82	
G/G	7	23	P=0.534
C/C+G/C	65	161	
N363S-BCL1	3	6	P=0.714
NO N363S-BCL1	69	178	

Table 1. Associations of the glucocorticoid induced toxicities with the Bcl1 polymorphism and with Bcl1-N363S combination.

In the case of the cumulations of the toxicities our results showed that no patients with the GG genotypes had 3 toxicities. There was no any association between the G-allele and the cumulations of the toxicities (CC vs. GC+GG, GG vs.CC+GC). Similary there was no any correlation found between the amount of the toxicities and the BCL1-N363S combination. (Table 2.) See the the p-values in table 2.

Combinations of the glucocorticoid induced toxicities– BCL1						
	At least one toxicity	No toxicity	At least two toxicities	No two toxicity	At least three toxicities	No three toxicities
G/G+G/C	64	71	16	119	130	5
CC	69	54	11	112	119	4
G/G	18	12	6	24	0	30
C/C+G/C	115	113	21	207	9	219
N363S + BCL1	4	5	2	7	2	7
NO N363S-BCL1	129	120	25	224	7	242

Combinations of the glucocorticoid induced toxicities – BCL1- P-values			
	At least one toxicity	At least two toxicities	At least three toxicities
G/G+G/C CC	P=0.163	P=0.543	P=1.000
G/G C/C+G/C	P=0.325	P=0.104	P=0.563
N363S + BCL1 NO N363S-BCL1	P=0.743	P=0.24	P=0.34

Table 2. Associations between the combinations of the glucocorticoid induced toxicities with the Bcl1 polymorphism and with BCL1-N363S combination.

IV.3.2. The day 8. prednisone response

In the case of the BCL1 polymorphism there was no patient with GG genotype who had poor prednisone response. Similary, no patient with BCLI-N363S combination had poor prednisone response. In the case of the group CC vs. GC+CC we didn't find any associations regarding the prednisone response and the G-allele (p=0.559).

IV.3.3. The 5-year EFS and 5-year OS

Regarding the 5-year EFS we didn't find any correlations between the investigated groups (CC vs. GC+GG p=0.535; GG vs.CC+GC p=0.445; BCLI-N363S vs. no combination p=0.3) and the EFS.

In the case of the 5-year OS we neither found any associations between the investigated groups (CC vs. GC+GG p=0.816; GG vs.CC+GC p=0.849; Bcl1-N363S vs. no combination p=0.681) and the OS.

We found that hepatotoxicity occurred more often in the case of N363S glucocorticoid receptor polymorphism. No association was found between the ER22/23EK polymorphism and hepatotoxicity. Carbohydrate metabolism abnormalities occurred more often by the N363S polymorphism while no one with the ER22/23EK polymorphism had this toxicity. No patient with hypertension nor with behavior changing nor with neuropathy was observed in the group of the ER22/23EK carriers. The combinaton of the toxicities in one patient occurred significantly more often in the 363S carriers. No patient with two or three glucocorticoid toxicities occurred in the case of the ER22/23EK polymorphism. None of the 363S and ER22/23EK carriers had poor prednisone response. Both the 5-year EFS and 5-year OS in the 363S carriers were significantly better than in the non-carriers. Patient with N363S polymprhism could expect to more severe toxicities but more favorable prednisone response, 5-year EFS and 5-year OS. The carriers of the ER22/23EK polymorphism had less severe or frequent toxicities –however the results were not significant - but their 5-year EFS and 5-year OS were not significantly better. In the case of the BCL1 SNP among the G/G homozigous carriers no one had poor prednisolone response being consistent with elevated glucocorticoid sensitivity. In patients with GG genotype no one had 3 toxicities at the same time. We didn't find neither poor prednisone responder in the case of having the BCL1-N363S combination that underlines the fact that these SNP-s increase the sensitivity to the glucocorticoids.

Based on our data the extension of the number of the investigated patients is required to be found more significant correlations. It would be important as well to invesigate further relevant glucocorticoid receptor polymorphism may having significant effect on steroid response such as thtIII of N766N polymorphisms. In the case of the N363S and ER22/23EK SNP-s an individual dosing of glucocrticoids and/or more alertness of the glucocorticoid toxicities can be raised up in the future.

V. Conclusions

1. Most of our results support the data in the literature regarding the N363S polymorphism. In the case of this polymorphism the steroid induced hepatotoxicity and glucose metabolism abnormalities occurred more often proving a higher glucocorticoid sensitivity. Similar results was observed in the prednisone response. None of the carriers had poor prednisone response due to the elevated receptor senitivity. Among the carriers better 5-years EFS and 5-years OS were seen.

2. Our results regarding the ER22/23EK polymorphism support the data in the literature as well. This polymorphism decreases the sensitivity to the glucocorticoids. We found a tendency that the investigated glucocorticoid induced toxicities occurred less in the case of this polymorphism. However our results were not significant maybe the low number of the carriers. ER22/23EK polymorphism seems to be protective against the glucocoticoid induced toxicities. No carriers had poor prednisone response. Between the carriers and non carriers no significant difference can be seen regarding the 5 year EFS and OS.

3. According to the literature BCLI polymorphism elevates the sensitivity to the glucocorticoids. Our results show that among the GG mutant homozygous patients no one had poor prednisolone response that can be consistant with a higher glucocorticoid sensitivity. Among GG pateints no one had at the same time three toxicities which can be controversal regarding the elevated sensitivity, however, the number of the patients in this group wasn't high enough for a statistical reliable analysis. In the case of the combination of BCLI-N363S polymorphisms no poor prednisone response could be observed that underlines the elevated steroid sensitivity caused by both polymorphisms.

4. Till that time in the literature there aren't any investigations that showed significant association between the N363S, ER22/23EK, BCLI and the day 8. prednisone response. According to our results no one with the N363S polymorphism and the GG genotype of the BCLI polymorphism had poor prednisonr response. However these results are not statistically significant maybe due to the low number of the patients.

5. In the literature there are only few articles that found significant association between the the glucocorticoid receptor polymorphisms and the 5-year OS. According to our results in the case of the N363S polymorphism both the 5-years EFS and OS were significant better.

Our results regarding the glucocorticoid induced toxicities may raise up the necessity in the future to select a risk group who are more prone to have glucocorticoid indiced toxicities requiring closer monitory.

In the case of the extention of the number of the patients regarding the investigations of the glucocorticoid receptor polymorphisms we may get more reliable data that may help predict in a certain groups the 5-year OS and EFS.

VI. Bibliography

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