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Original article

The influence of polymorphisms in the drug transporter, ABCB1 on the toxicity of glucocorticoids in Saudi children with acute lymphoblastic leukaemia

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ABSTRACT

Background: Glucocorticoids play essential roles in the treatment of childhood acute lymphoblastic leukaemia (ALL); however, treatment with these agents can result in severe side-effects. This study, the first of its kind in a Saudi population, investigates associations of ABCB1 gene polymorphisms (pharmacodynamics and pharmacokinetic) with the development of toxicity and side effects (glucose abnormality, liver toxicity and infection) in a small population of Saudi children with ALL.
Methods: Three single nucleotide polymorphisms (SNPs) of the ABCB1 gene (rs 3213619 T129C, rs 2032582 G2677T and rs1045642 C3435T) were analysed in 70 Saudi children with ALL and 60 control subjects. Participants were treated according to the ALL 2000 study protocol. Toxicities were assessed and associations with genotypes were evaluated according to Common Toxicity Criteria (NCI-CTC).
Results: Significant associations were observed among carriers and the mutated genotype C3435T (ABCB1), which had an incidence of infection ($p = 0.05$). Although no correlations were found between liver toxicity and glucose abnormalities for patients carrying ABCB1 SNPs, risk factors for liver toxicity were elevated by a factor of three for patients carrying the SNP G2677T, OR 3.00 (1.034–8.702). The risk factor of glucose abnormality toxicity for the patients carring T129C were increased three times OR 3.06 (0.486–19.198).
Conclusions: In terms of infection incidence, polymorphism C3435T may contribute to potential life-threatening infections during paediatric ALL therapy, through glucocorticoid usage.

Introduction

Acute lymphoblastic leukaemia (ALL) is a severe cancer affecting children and adolescents and is characterised by the overproduction of immature white blood cells in bone marrow. Glucocorticoids (GCs) play a significant role in paediatric ALL treatment. Treatment with GCs induces apoptosis and the expression of P-gp, a key transporter protein for many anticancer agents [1].

By 1998, recovery rates for ALL patients had reached 85%. In spite of intensified treatments, however, the process can often be accompanied by critical short and long-term side-effects, and about 25% of patients are associated with treatment failure. Treatment with GCs can cause severe and sometimes life-threatening toxicities [2].

ABCB1 is a member of the superfamily of adenosine triphosphate (ATP)- Binding Cassette (ABC) transporters [3]. The gene encodes a transporter channel protein called P-glycoprotein (P-gp) which functions as an energy dependent efflux pump [4]. ABCB1 has two primary transcriptional regions: a proximal promotor in exon1 for constitutive expression and a cryptic distal promotor, active for drug selection [5]. P-gp is the most important protein of the ABC superfamily. The protein also plays important roles in protective mechanisms against a variety of drugs and xenobiotics

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[6]. The ABCB1 gene carries several SNPs. Seven synonymous mutations are located in its introns, four SNPs are at wobble positions with no amino acid changes in exon 12;21 and two in exon 26. There are also two rare mutations G2677A: Ala893Thr; and A3320C: Gln1107Pro. Mutations can affect posttranscriptional modifications or affectkey sequences for mRNA processing [7]. While there is no clear consensus regarding the contribution of ABCB1 variation to the development of ALL risk, there is an inter-individual variability in ABCB1 expression and function [8].

The most common SNPs in the protein coding regions are rs1128503 (C1236T) Gly412Gly, rs2032582 (G2677T) Ser893Ala Thr and rs1045642 (C3435T) Ile1145Ile [9]. These SNPs have been the focus of many pharmacokinetic, disease association and treatment outcome investigations [9]. Among the different SNPs in ABCB1, the mutation at position 3435 in exon 26 (C3435T) is the most important SNP across different races. The homozygous allele-T (TT) and heterozygous CT allele show 2-fold lower expression of P-gp at the intestine when compared to the CC homozygous allele but the precise molecular mechanisms of these associations are poorly understood [10]. However,mechanisms have been proposed,the linkage of C3435T SNP with other mutations in ABCB1, such as in the promotor/enhancer or intrinsic regions like T-129C or G2677T/A, leads to silent mutation of this gene [11,12].

The SNP (G2677T/A) has been well studied and documented because it is a common amino acid change in P-gp. The serine bearing 2677T allele frequency varies as much as 2–65% among populations (international Hap Map project). Some articles have reported associations between this SNP and increased P-gpexpression [13,14] decreased expression [15] and no change in expression [16]. Also, it was shown that the G2677A/C, 3435C haplotypes may help in ABCB1 polymorphism predictability and function [15].

ALL child patients are more sensitive to retrograde episodes related to GC treatment, even when they are adjusted for weight-based dosing. The risks of using GCs have been known for a long time; among the complications of GC toxicity are diabetes mellitus, hypertension, peptic ulcer disease, hypothalamic-pituitary-adrenal suppressions (HPA), fatty liver, osteoporosis and increased infection susceptibility [17]. Nevertheless, glucocorticoids still essential drug in the treatment of acute lymphoblastic leukaemia. Supra-physiological doses of GCs may however, suppress the Hypothalamus-Pituitary-Adrenal (HPA) axis and may lead to impaired stress responses and inadequate host defences against infections, potentially leading to morbidity and death [18].

Recent studies have suggested that low GC doses are associated with some adverse effects and that these effects are diverse in different patients [19]. High GC doses are well knownin increasing the risk of infection susceptibilities and complications. In a prospective cohort study, involving 2108 patients at the Mayo Clinic, hospitalisation incidences for infections in patients treated with GCs were more than 2.5 times those of the general population [20]. Indeed, another study showed the adverse effects of GCs and the associated development of hospitalised pneumonia; the study reviewed 16,000 patients from the USA national databank for rheumatic disease, and showed that patients treated with GCs had hospitalised pneumonia rates 1.7 times greater than those not receiving GCs.The increased rate of serious infection was greater during the first 90 days after initiation of treatment by GCs, OR (2.99,95% CI 1.60–5.60) [21].

Studies exploring the clinical and toxicological impact of ABCB1 SNPs in childhood ALL are few. Therefore, this study was performed in a Saudi Arabian population to assess the impact of ABCB1 polymorphisms on the incidence of ALL and associated GC toxicity developed during treatment. Accordingly, the aim was to improve individualisation of medication and improve GC treatment regimens for individual ALL patients. The target was to enhance drug activity and safety through a better understanding of the individuals' genetic makeup.

Subjects and methods

The subjects in this study were 70 Saudi children patients, comprising 43 males and 27 females. All were diagnosed with Pre-B ALL between 2009 and 2013. Fifty-one patients were under 10 years old and 19 were over 10 years of age. Sixty control subjects within the same age range, matched sex and no previous developing ALL or other cancers was also included in this study. This study was approved by the Medical Ethics.

Diagnostic measures involved the following: detailed medical history and routine laboratory investigations during induction and re-induction phases. Patients were eligible for treatment protocols if they fulfilled the following inclusion criteria:

(1) Under 18 years old;
(2) Bone marrow blast cells $\geq$ 20%; and
(3) Absence of other active malignancies.

Data collected included a complete blood profile (differential counts, platelet counts, and haemoglobin levels), routine biochemical profiles (serum creatinine, serum bilirubin, and liver enzymes) and bone marrow aspiration. Clinical data were obtained retrospectively, were reviewed by a paediatric oncologist, and were analysed blindly, separate from the genotype results. Toxicity data were collected from patient's records and graded retrospectively, according to NCI common toxicity criteria scales included in ALL 2000 protocols [22]. Patients were classified according to NCI-CTC for incidences of infection (localised infection requiring *IV* antibiotics, antifungal or antiviral therapy).

Genetic analysis

PCR amplification of DNA and sequencing

DNA was extracted using commercial DNA extraction kits (Wizard® Genomic DNA Purification kit, Promega, WI, USA). Additionally, genomic DNA was also extracted from blood samples which had been anti-coagulated with EDTA using the Norgen blood DNA isolation mini kit (NORGEN Biotek Corporation). SNPs sequences were amplified by the polymerase chain reaction (PCR) using specifically designed DNA primers. The forward and reverse primers for each SNP were:

For T-129C;
F: 5`-TGATGCGCGTTTCTCTACTTG-3` and
R: 5`-TGAAAGCCTGACACTTGGGAA-3`,
For G2677T;
F: 5`-TGCAGGCTATAGGTTCCAGG-3` and
R: 5`-AAGGCATGTATGTTGGCCTC-3`,
and C3435T;
F: 5`-TGTTTTCAGCTGCTTGATGG-3` and
R: 5`-AAGGCATGTATGTTGGCCTC-3`.
The PCR parameters were;

i Denaturation at 94 °C for 3 min, followed by
ii 35 cycles of 30s at 94 °C, 55 °C for 30s, 72 °C for 30s,
iii and extension for 1 cycle at 72 °C for 7 min [23].

Once the amplification was complete, the DNA was purified and genotyped by direct DNA sequencing at Macrogen Inc., Seul, Rebublic of Korea.

Statistical analyses

The distribution of genotypes of ALL patients and control subjects were compared using Chi-square tests or Fisher's exact

tests where appropriate. Any possible associations between glucose metabolism abnormalities, liver toxicities and susceptibility to infection, and ABCB1 SNPs was investigated using the Chi-square test. Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% confidence intervals (CI) were calculated using two-sided Fisher's exact test, using the statistical software package SPSS version 23.0 (SPSS, Inc., Chicago, IL, USA). The value of $p < 0.05$ (two-tailed) was considered statistically significant.

Results

Three ABCB1 polymorphisms were analysed with respect to GC pharmacogenomics and/or pharmacokinetics. For all ABCB1 polymorphisms, there were no significant differences between ALL patients and control subjects. Table 1 and Fig. 1 shows the genotype distributions of the ABCB1 SNPs for (a) T129C, (b) G2677T and (c) C3435T.

The sequenced data for the polymorphism T129C SNP of ALL patients were represented in Table 1. For T129C, the frequency of allele T (wild type) was 88% while for allele C, it was 12% (mutated) as the lowest ratio. The results also showed that the most frequent genotype was TT (84.3%) rather than the TC (15.7%) heterozygous genotype, and that no CC genotype pattern (0%) was recorded in the T129C SNP for all ALL patients and control subjects.

Table 1 shows the sequence data for the G2677T SNP in ALL patients. As listed, for the G2677T SNP, two different alleles were exhibited in ALL patients and control subject with different allelic frequencies. The frequency of genotype GG (wild type) accounted for 31%, whereas it was 47% for the heterozygous GT. The data for the G2677T SNP demonstrated that a moderate ratio of ALL patients and control subjects 22% and 20% respectively were genotyped as TT (mutated).

The comparisons of C3435T SNP of ALL patients and control subjects are shown (Fig.1). The frequency of genotype CC (wild type) accounted for 25% and 23% of ALL and control subjects respectively. The heterozygous genotype CT was a highly frequent genotype in ALL and control subjects; 57% and 47% respectively. The results demonstrated that genotype TT (mutated type) occurredin 18% and 30% of ALL patients and control subjects respectively (Table 1).

The incidence of hepatic toxicity are represented in Table 2. There was not significantly different between the three SNPs. Also, no significant relationship was reported between liver toxicity and age and also between gender $p = 0.865$ and age and $p = 0.74$ between gender.

No significant relationship was reported between glucose metabolism abnormalities and ABCB1 polymorphisms (Table 3)

The progression of infection was significantly more frequent among carriers of SNP C3435T (Table 4). Five of the 18 wildtypes and 29 of the 52 heterozygous and mutated genotypes had incidences of infection (27.77% *vs.* 55.76%) $p = 0.05$, OR = 3.278 CI 1.020–10.573, 95%. The other two SNPs showed non-significant differences in the incidence of infection among patients carrying wild type genes *vs.* heterozygous and mutated genotypes (Table 4).

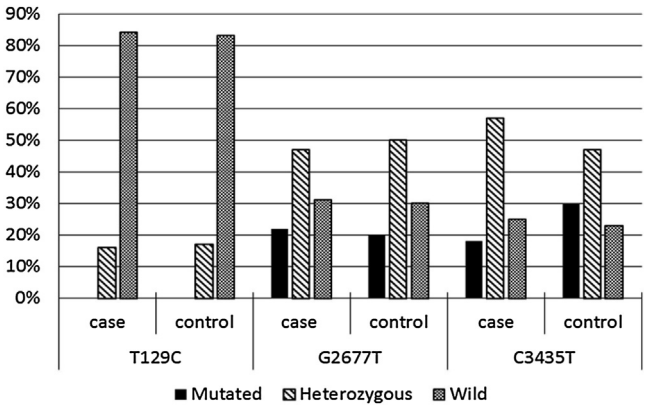

Fig. 1. Genotype Distribution of Different SNPs in ABCB1 of Control and ALL Patients.

Discussion

The most important prognostic factor in the treatment of ALL is GC response in the prophase stage of treatment. There are varying responses between patients, accompaniedby various side effects and psychodynamic changes. The most important side effects are altered fat distribution, temporary diabetes mellitus, hepatomegaly and immune suppression leading to increased risk of infection [18,24].

Several studies exploring diseases in populations have shown that single nucleotide polymorphism (SNPs) of the multi drug resistance (ABCB1) drug transporter, P-glycoprotein are associated with steroid responses, resistance, side effects and toxicity. Detecting and surveying SNP frequencies in certain populations is fundamental for the detection of inter-individual variations in disease treatment outcomes and drug efficacy. From this perspective, we sought to determine the allelic frequencies of the SNPs T-129C, G2677T, and C3435T in ABCB1 in a Saudi Arabian population. Similarly, we sought to determine associations (if any) between these SNPs and GC toxicity.

All 130 subjects (70 ALL patients and 60 controls) in this Saudi Arabia population were either homozygous TT genotype (84%) or heterozygous TC (16%). No mutated genotype CC had been determined in this study population. So, this mutated SNP was too low in the Saudi population to be detected in our 130 subjects. The allelic frequency of C was approximately 9%, which was similar to the allelic frequency in Japanese (8%) and Turkish populations (5%). The T allele was very important for transcription, because it was located in the transcription initiation site of the promotor region of ABCB1. It was recorded that heterozygous carriers'

Table 1
Allelic frequencies and genotype distribution of ABCB1 SNPs in childhood ALL participants (n = 70) and control subjects (n = 60).

		ABCB1								
SNP		Allelic Frequency		OR (95%CI)	*p* value	Genotype			OR (95%CI)	*p* value
		WT	MUT			WT	HET	MUT		
T-129C	Case	118 (88%)	22 (12%)	1.886 (0.749–4.745)	0.183	59 (84.3%)	11 (15.7%)	0		
	Control	110 (93%)	10 (7%)			50 (83.3%)	10 (16.7%)	0		
G2677T	Case	77 (55%)	63 (45%)	1.000 (0.716–1.397)	1.000	22 (31%)	33 (47%)	15 (22%)	1.071 (0.468–2.493)	0.700
	Control	66 (55%)	54 (45%)			18 (30%)	30 (50%)	12 (20%)		
C3435T	Case	76 (54%)	64 (46%)	0.857 (0.636–1.154)	0.356	18 (25%)	40 (57%)	12 (18%)	0.571 (0.270–1.211)	0.183
	Control	56 (47%)	64 (53%)			14 (23%)	28 (47%)	18 (30%)		

Table 2
Genotype (wild type *vs.* hetero mutated) in ALL children (n = 70) related to glucocorticoid "induced" liver toxicity during therapy.

SNP	% liver toxicity in wildtype	% liver toxicity in SNP carrier	*p* value (0.05)	OR (CI 95%)
T 129C	45.8%	72.7%	0.188	1.36 (0.425-3.110)
G2677	31.8%	58.3%	0.07	3.00(1.034-8.702)
C3435T	33.3%	55.8%	0.171	2.522 (0.821-7.748)

Table 3
Genotype (wild type *vs.* hetero mutated) in ALL children (n = 70) related to glucocorticoids "induced" glucose abnormalities during therapy.

SNP	% glucose abnormality in wildtype	% glucose abnormality in SNP carrier	*p* value (0.05)	OR (CI 95%)
T129C	10.5%	18.2%	0.215	3.056 (0.486-19.198)
G2677	14.1%	8.3%	0.916	0.909 (0.154-5.379)
C3435	8.6%	9.6%	0.596	1.809 (0.197-16.661)

Table 4
Genotypes (wild type *vs.* hetero mutated) in ALL children related to glucocorticoid "induced" infection during therapy.

SNP	% infection incidence in wildtype	% infection incidence in SNP carrier	*p* value (0.05)	OR (CI 95%)
T129C	46.7%	60%	0.508	1.710 (0.439-6.699)
G2677	48%	49%	0.567	1.045 (0.385-2.837)
C3435	27.8%	55.8%	0.05*	3.278 (1.020-10.537)

* Significant difference $p \leq 0.05$.

subjects (TC) showed a lower P-gp placental expression [25]. Interestingly, another study showed that T-129C SNP was more frequent in acute myeloid leukaemia (AML) and chronic lymphocytic leukaemia (CML) patients,where drug resistant phenotypes were more acute, in contrast to ALL and CLL patients. These data suggest that this polymorphism may have a functional role in ABCB1 expression [26].

The G2677T SNP is found on the intracellular side of the ABCB1 protein. The SNP converts a serine to an alanine at position 2677, resulting in conversion from a lipophilic to a hydrophilic protein form. It was reported that low placental P-gp protein expression was detected in subjects carrying TT genotypes of this SNP [27]. In this study, the allelic frequency were similar to those reported in German and Japanese populations, but were much lower than frequencies reported in Turkey [13,28]. In this study, both patient and control subjects had similar percentages of TT genotypes for the G2677T SNP, therefore it can be concluded that the mutated TT SNP did not contribute to the development of childhood ALL. Toxicology data also showed there were no significant differences between patients carrying TT genotypes and other genotypes, regarding glucose abnormality and incidence of infection. The toxicity results also showed that this mutated SNP may have contributed to liver toxicity, as there was a difference between patients carrying the TT SNP and other patients. However, it did not reach significance ($p = 0.07$), but the risk factor is tripled in TT patients, OR 95%CI = 3.00 (1.034–8.702). This observation may be explained by the substitution of serine to alanine, which is a neutral amino acid. It may change the geometric secondary structure of the P-gp interaction site and thereby affect protein function [29].

The ABCB1 C3435T polymorphism is a silent SNP. However, different authors have reported that subjects carrying a mutated TT SNP genotype, exhibit lower expression of P-gp in different tissues [30,31]. In this study, the allelic C frequency was approximately 53% and the frequency of the T allele was 47%. There was an obvious difference in the allelic frequency and genotype of the C3435T polymorphism in different populations when compared to the Saudi population. The frequency of the C allele in a Saudi population was much lower when compared to African and black populations (African American, Ghanaian, and Kenyan 84%, Sudanese 75%), however it was similar to Caucasian populations (48% British, 49% German, and 48% Portuguese) and Asian populations (51% Japanese, 53% Chinese, and 59% Filipino). From our data, it was obvious that the allelic frequency and genotype of the C3435T polymorphism in a Saudi population were significantly different to those in African populations [31]. This study showed that the C3435T SNP of ALL patients was lower than the control group; 18% and 30% respectively, and that there was no significant difference between both groups, $p = 0.7$ and the risk factor was OR= 1.071 (95% CI). This meant that, this SNP did not contribute to the development of childhood ALL. This result was similar to other population studies; Hispanic populations [32], Mexican populations [33] and Chinese populations [34]. Also, our study compared well with a recent study from Denmark, in which, in a population of more than 750 individuals, investigators found no associations between C3435T and ALL. In an updated meta-analysis published in 2015 [35], the authors showed no significant associations between C3435T and ALL risk in the overall population.

Homogeneity of the C3435 allele (wild type) has been associated with decreases in the risk of developing ALL when compared to TT homozygous subjects, whereas, the CC homozygous genotype was related to poor outcomes [36]. The association of the CC genotype with clinical variables in ALL may be due to high P-gp expression, which may eliminate anti-leukaemic drugs, leading to low intracellular drug diffusion and poor prognoses. However, controversially, the data from this study disagrees with other studies in Poland [37], Japan [23], Turkey [28] and India [38]. These authors reported that mutated TT genotypes of C3435T SNP carriers are more at risk of developing leukaemia than other individuals whereas the wild types of these SNPs are assumed to have a worse prognosis.

Toxicity results showed that homozygous mutated C3435T (TT) did not contribute to glucose toxicity ($p = 0.596$) nor liver toxicity

($p = 0.171$), however, the risk factor for liver toxicity was approximately 2.5 times higher than CC and CT genotypes, OR (95% CI) =2.522 (0.821–7.748). For glucose toxicity, it was approximately 2 times higher, OR (95%CI) =1.809 (0.197–16.661). The data also showed that this SNP was contributing to the incidence of infection in ALL patients ($p = 0.05$) and that the risk factor was approximately three times higher than the CC and CT genotypes, OR (95% CI) =3.278 (1.020–10.537).

The progression to infection data in this study is in agreement with other investigators [39], who reported strong associations between ALL patients with the 3435 SNP TT genotype and the requirement for intravenous antimicrobial therapy, when compared to patients carrying CC + CT genotypes. The study also reported the haplotype of this SNP with other ABCB1 gene SNPs (G2677T and T1236C). They had weaker associations with the progression of infection. Other authors also reported this phenomenon for ABCB1, and indicated that the C3435T polymorphism itself was responsible for variation in gene expression [40].

Toxicity data from this study was also concordant with recently published data [41]. These authors not only reported on the infection protective effects of heterozygous 3435 CT in patients treated with glucocorticoids, but they reported on protection from the adverse effect of the drugs [41]. This phenomenon can be explained by NK cells, macrophage and T-lymphocytes (CD4 and CD8); these cells express high P-gp levels in the body. Patients undergoing treatment with prednisolone are expressing more P-gp transporter protein than others, so the over-activity of ABCB1 in immune cells might be associated with the increasing efflux of this drug the reduced side effect of the GC.

P-gp is also involved in the release and transport of cytokines such as IL-2, IL-4, IFNγ, and TNF-α, which may activate immune cells and reduce infection incidences [42]. It has been reported that the TT genotype of C3435T was associated with lower secretion of cytokines in H-P-A-stimulated lymphocytes. Conversely, in HIV-infected individuals, P-gp expression and function were decreased and attained normal function after IL-15 stimulation. Therefore, secretion of IL-15 might alternate in which affects cellular immune responses and treatment outcome consequently [43].

The toxicity data generated in this study is, however, in disagreement with a study by Marino [1]. These authors did not find any correlations between the ABCB1 SNPs (T129C, G2677T, and C3435T) and the incidence of infection in childhood ALL patients. The molecular mechanisms associating C3435T with other SNPs are poorly understood. However, some explanations for this association have been reported in cervical cancer patients: the most popular one is linkage of C3435T and other mutation within the ABCB1 gene, such as in the promotor/enhancer or intrinsic regions like T-129C or in another exon like G2677T/A [44].

In conclusion, these data support the need for adequate prediction of anti-leukaemic drug responses before treatment commences. This prediction occurs through the investigation of polymorphisms in candidate SNPs T129C, C3435T and G2677 of ABCB1 gene that influence anti-leukaemic therapies, in response to glucose abnormality, incidence of infection and liver toxicity, respectively. Even though, the risk factor (OR) increased by three folds for these SNPS it was only significant for C3435T and the incidence of infection. Prospective studies will be conducted to confirm these data in larger population to establish relationships between these polymorphic genotypes and GC side-effects. Through these investigations, significant clinical insights will allow clinicians to adjust individualising GC dose therapies, based on inherited genetic predispositions, in specific genes. These steps will generate bespoke strategies that will diminish the risk of serious toxicity, increase treatment effectiveness and permitting more individualising therapies for patients.

Conflict of interest statement

No conflict of interest to disclose.

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