Polymorphisms of Asparaginase Pathway and Asparaginase-Related Complications in Children with Acute Lymphoblastic Leukemia

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Abstract

Purpose: Asparaginase (ASNase) is a standard and critical component in the therapy of childhood acute lymphoblastic leukemia (ALL), but it is also associated with several toxicities.

Experimental design: We recently reported the results of an association study between ASNase pathway genes and event-free survival (EFS) in childhood patients with ALL. The same polymorphisms were interrogated here in relation to allergies, pancreatitis, and thrombotic events following treatment with E. coli ASNase.

Results: Among patients of the discovery group, allergies, and pancreatitis were more frequent in individuals who are homozygous for the triple-repeat allele (3R) of the asparagine synthetase (ASNS) gene, resulting in remarkably higher risk of these toxicities associated with 3R3R genotype [OR for allergies, 14.6; 95% confidence interval (CI), 3.6–58.7; P < 0.0005 and OR for pancreatitis, 8.6; 95% CI, 2.0–37.3; P = 0.01]. In contrast, the ASNS haplotype “I” harboring double-repeat (2R) allele had protective effect against these adverse reactions (P ≤ 0.01). The same haplotype was previously reported to confer reduction in EFS. The risk effect of 3R3R genotype was not replicated in the validation cohort, whereas the protective effect of haplotype “I” against allergies was maintained (P ≤ 0.002). Analysis with additional polymorphisms in ASNS locus in lymphoblastoid cell lines showed that haplotype “I” is diversified in several subtypes of which one was associated with reduced in vitro sensitivity to ASNase (rs1048609, P = 0.01) possibly explaining an association seen in clinical setting.


Introduction

Over the past four decades, treatment of childhood acute lymphoblastic leukemia (ALL) has improved importantly such that approximately 80% to 85% of patients are cured with current therapy regimen. Up to 20% of patients experience treatment failure whereas treatment-related toxicities are often life-threatening and are the primary cause of interruption or discontinuation of chemotherapy. Asparaginase (ASNase) is a standard component in the childhood ALL treatment (1). Asparaginase is required by all cells for survival and is normally produced by the enzyme asparagine synthetase (ASNS). Malignant lymphoblasts are thought to have low ASNS levels and, thus, depend on extracellular sources of asparagine for their rapid growth. Depletion of asparagine by ASNase selectively kills leukemia cells by decreasing protein biosynthesis (2). Associations between success of ALL treatment and ASNase dose intensity or formulation have been reported in several clinical studies (3–5). E. coli–derived enzymes are more potent, it is associated with higher efficacy, but also with higher toxicity (5–7). Side effects related to ASNase treatment include allergic reactions that occur in 20% to 40% patients and require change of drug formulation; Two most serious and most frequent dose-limiting ASNase-related toxicities are pancreatitis and thrombotic events reported in up to 18% and 5% of patients with ALL, respectively (8, 9). Pancreatitis usually develops after the first few doses of ASNase, suggesting that it may occur as a result of an underlying predisposition rather than as a cumulative drug effect (9).

We recently analyzed relationship between event-free survival (EFS) in childhood ALL patients and genes in the ASNase pathway (10), which were selected on the basis of differential expression between ASNase-resistant and -sensitive cells (11–13). We showed that promoter variant of transcriptional factor ATF5 involved in ASNase regulation, is associated with higher promoter activity and confers higher risk of ALL relapse in patients who received E. Coli ASNase (10). Association with lower EFS has been also found with tandem repeat (14) in the ASNS gene and with
Thrombotic events) in two independent childhood ALL cohorts. Here, we report the analysis of the same set of polymorphisms in ASNS, ATFS, and ASS1 (argininosuccinate synthase 1) in relation to ASNS-related acute complications (allergies, pancreatitis, and thrombotic events) in two independent childhood ALL cohorts.

**Patients and Methods**

Study population and endpoints in the analysis

The study population consisted of 285 Caucasian children (98% of French-Canadian origin) diagnosed with ALL at the Hospital Sainte-Justine, (HSJ; Montreal, QC, Canada) between January 1989 and July 2005 (QcALL cohort or test group) who received *E. coli* ASNase as a part of Dana-Farber Cancer Institute (DFCI) group (Table 1) was composed of a 248 patients who received *E. coli* ASNase within the DFCI 95-01 and 00-01 ALL treatment protocol in remaining (without HSJ) consortium institutions (5, 6, 15).

**Cellular proliferation assay**

*In vitro* sensitivity to ASNase was assessed in lymphoblastoid cell lines (LCL) from 89 individuals of Northern and Western Europe (CEU), as described by Chen and colleagues (16) The drug concentration resulting in 50% inhibition of cell growth (IC50) during 48 hours incubations time was estimated using several *E. coli* ASNase concentrations ranging from 0.01 to 10 IU and the GraphPad software by fitting sigmoid dose–response curves. Obtained values were correlated with genotypes using the Mann–Whitney or Kruskal–Wallis test.

Informed consents were obtained from parents or guardians before enrolment into the study. The study was approved by institution ethics committees.

**Results**

Allergies, pancreatitis, and thrombotic events occurred in the discovery group (QcALL) with the frequency of 15.8%, 5.6%, and 3.5%, respectively. Pancreatitis was in most cases severe (in 13 of 16 cases) and systemic allergies also occurred more frequently (in 37 of 45 subjects with allergic reactions). Analysis between these toxicities and SNPs in ASNS, ATFS, and ASS1 genes revealed an association of tandem repeat polymorphism (rs3832526) in the ASNS gene with both pancreatitis and allergies (*P* = 0.008 and *P* < 0.0005, respectively, Fig. 1A). These complications were more

**Table 1. Baseline characteristics of patients with ALL in the test (QcALL) and validation (DFCI) cohort**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>QcALL (n = 285)</th>
<th>DFCI (n = 248)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>130 (45.6)</td>
<td>114 (46.0)</td>
</tr>
<tr>
<td>Male</td>
<td>155 (54.4)</td>
<td>134 (54.0)</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>224 (78.6)</td>
<td>203 (81.9)</td>
</tr>
<tr>
<td>≥10</td>
<td>61 (21.4)</td>
<td>45 (18.1)</td>
</tr>
<tr>
<td>WBC, x10^9/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>242 (84.9)</td>
<td>202 (81.5)</td>
</tr>
<tr>
<td>&gt;50</td>
<td>45 (15.1)</td>
<td>46 (18.5)</td>
</tr>
<tr>
<td>Cell type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>267 (93.6)</td>
<td>227 (91.5)</td>
</tr>
<tr>
<td>T</td>
<td>18 (6.4)</td>
<td>21 (8.5)</td>
</tr>
<tr>
<td>Risk groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>137 (48.1)</td>
<td>153 (61.7)</td>
</tr>
<tr>
<td>High</td>
<td>148 (51.9)</td>
<td>95 (38.5)</td>
</tr>
<tr>
<td>Treatment protocol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>87-01</td>
<td>20 (7.0)</td>
<td></td>
</tr>
<tr>
<td>91-01</td>
<td>57 (20.0)</td>
<td></td>
</tr>
<tr>
<td>95-01</td>
<td>92 (32.3)</td>
<td>73 (29.4)</td>
</tr>
<tr>
<td>00-01</td>
<td>116 (40.7)</td>
<td>175 (70.6)</td>
</tr>
</tbody>
</table>

**NOTE:** Protocol distribution is different between the QcALL and DFCI groups (*P* = 0.001), whereas the remaining characteristics do not differ significantly. Abbreviation: WBC, white blood cell count.
frequent among patients that were homozygous for the tripe-repeate allele (3R) resulting in 8- to 14-fold risk elevation (OR for allergies, 14.6; 95% CI, 3.6–58.7; and OR for pancreatitis, 8.6; 95% CI, 2.0–37.3). The association with allergy remained significant with FDR of lower than 1%, whereas association with pancreatitis remained significant only with FDR of 16%. We further analyzed ASNS haplotypes composed of the tandem repeat polymorphism and promoter C-181T substitution (rs3757676). Two haplotypes were associated with allergies and pancreatitis: Homozygosity for haplotype ‘1’, uniquely tagged by 3R allele conferred higher risk of these toxicities (high-sensitivity haplotype), whereas haplotype ‘1’ defined by C-181T and 2R alleles had protective effect (low-sensitivity haplotype; OR for allergies, 0.4; 95% CI, 0.2–0.8; and OR for pancreatitis, 0.2; 95% CI, 0.07–0.7; P < 0.01; Fig. 1B). In our previous analysis the low-sensitivity haplotype ‘1’ conferred reduction in EFS in the QcALL cohort (10).

We further performed analyses of the ASNS gene in the replication (DFCI) cohort. Pancreatitis and allergies occurred in this group with the frequency of 8.5% and 23%, similar to the frequencies reported for the 00-01 clinical trial (15). Distribution of severe/moderate pancreatitis and systemic/local allergies differed from the discovery group, 33.3% pancreatic cases had moderate form and among patients with allergies, 50.1% had local manifestation. The risk effect of 3R3R genotype (or haplotype ‘2’) was not seen, whereas protective effect of haplotype ‘1’ against allergies was maintained, particularly against local allergic manifestation and in patients assigned to the high-risk group (P < 0.002 and P < 0.0005, respectively, Fig. 1B).

To further understand the variability of the ASNS gene, we retrieved an information on all SNPs in coding and regulatory regions with minor allele frequency higher than 5%, that might have been identified (19) after our first analysis of this gene (10). We also included SNPs within 5 kb upstream and downstream from the gene boundaries. Sixteen additional tag SNPs were
analyzed, which were all except one (Val210Glu) located beyond the coding region (Fig. 2A). Given high number of resulting haplotypes and low frequency of adverse events, detailed stratified analysis would have limited power in patients. We analyzed instead whether polymorphisms defining particular haplotype subtypes might have functional role as estimated by in vitro sensitivity assay in lymphoblastoid CEU cell lines. Protective haplotype *1 was diversified in five subtypes (defined by SNPs at 5 different positions, Fig. 2B). Two polymorphisms (rs10486009 and rs6971012, positions 4 and 5 in Fig. 2B) correlated with in vitro sensitivity to ASNSase. (P = 0.01 and P = 0.002, respectively; Fig. 2C). The TT genotype of rs6971012 had lower sensitivity, which was further reduced by the G allele of rs10486009 (Fig. 2C, P = 0.001), suggesting that it may contribute to protective effect of haplotype *1 observed in clinical setting. In contrast, only one haplotype defined by 3R allele remained after addition of other ASNS polymorphisms and it did not affect in vitro sensitivity to ASNSase.

Discussion

Differences in susceptibility to ASNSase have been attributed to variable levels of ASNS expression in number of studies: ASNS levels in leukemia cell lines, patients lymphoblasts, and surrounding mesenchyme cells suggested that elevated ASNS levels may counteract the ASNSase effect and underlie the resistance to treatment (11, 20, 21). Lower ASNS expression might then be expected to mediate higher sensitivity to treatment and possibly higher frequency of ASNSase-related complications. We found that the 3R3R genotype of tandem repeat polymorphism correlated more frequently with pancreatitis and allergies in the discovery group. Tandem repeat polymorphism is located in intron 1, but upstream from translation initiation site and was reported to act to counteract the ASNase effect and underlie the resistance to treatment (14). The 3R allele increased ASNS promoter activity in the embryonic kidney cell line (14). We did not observe relationship between 3R3R genotype with mRNA levels (10), or in this study, with in vitro sensitivity to ASNSase. The effect of 3R allele on therapeutic responses to ASNSase is also ambiguous. Recent study reported that 3R allele can affect early response to ALL treatment, as defined by the number of leukemic blasts following one ASNSase dose (22). We did not find an association of 3R3R genotype with reduced EFS (10) and in this study, we did not replicate an association of 3R3R genotype with adverse reactions of ASNSase. Other polymorphisms/haplotypes in the ASNS gene cannot explain this discrepancy; there was only one haplotype defined by 3R allele when additional polymorphisms were included in the analysis. The differences can nevertheless be due to low frequency of 3R3R genotype, different...
distribution of severe/moderate pancreatitis and systemic/local allergies, or different geographical location of discovery and replication groups. Other genes beyond those studied here, as well as disease and treatment characteristics might also play a role. For example, it has been shown that leukemic cells carrying the TEL/AML1 fusion gene are more sensitive to treatment with ASNase compared with other subtypes of ALL (23); several polymorphisms of the aspartate metabolic pathway have been associated with ASNase sensitivity in vitro using ALL cells and LCL cell lines (24); top ranking SNPs for ASNase-related allergies have been identified in the gene coding for glutamate receptor in the genome-wide association study (16). Distribution of treatment protocols differed between discovery and replication groups ($P = 0.001$, Table 1) and patients might have received different ASNase doses. An association with pancreatitis and allergies with 3R3R genotype was nevertheless maintained in the discovery group when analysis was limited to patients treated with DFCI 95–01 and 00–01 protocols ($P \leq 0.02$, not shown). Other baseline characteristics between two groups did not differ significantly (Table 1). Further and larger studies are needed to confirm the role of 3R3R genotype and to explore whether this finding is applicable to other protocols and other populations.

The results obtained for haplotype *1J, harboring 2R allele, seems more consistent and correlated with lower frequency of allergies in both discovery and replication patient cohorts. This is in agreement with previously reported association of the same haplotype with reduced EFS (10), suggesting its lower sensitivity in response to ASNase treatment. Analysis of additional polymorphisms in ASNS locus revealed diversification or haplotype *1 in several subtypes; one of them (defined by minor allele of rs10486009) seems particularly interesting because it was associated with reduced sensitivity to ASNase in vitro, possibly explaining lower sensitivity of haplotype *1 seen in clinical setting. Our finding might be as well of interest for the treatment of other cancers because asparagine depletion strategies using ASNS inhibitors and ASNase have been suggested in pancreatic and ovarian malignancies (25, 26). Tumor-specific upregulation of ASNS was also reported in castration-resistant prostate cancer and correlated with the progression to a therapy-resistant disease state (27).

In conclusion, we reported an association of 3R3R genotype of the ASNS gene with a higher frequency of ASNase-related adverse reactions. The association was not seen in the replication group suggesting limited study power or possible modulating role of other genes and/or disease and treatment features or patient origin. Haplotype harboring 2R allele seems to have protective role against ASNase allergies in both discovery and replication patient sets. Extension of the analysis to additional polymorphisms and cellular proliferation assay in response to ASNase treatment, identified variants possibly explaining lower sensitivity of this haplotype observed in clinical setting.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Study supervision: M. Krajnovic

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References