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Perforin polymorphism A91V and susceptibility to B-precursor childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group

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Abstract

Perforin plays a key role in the cytotoxicity of natural killer and cytotoxic T cells. Genetic mutations in the perforin gene (PRF1) give rise to approximately 30% cases of familial hemophagocytic lymphohistiocytosis. A frequent polymorphism, A91V (C to T transition at position 272), may impair processing of perforin protein to the active form, and has been suggested to increase susceptibility to childhood acute lymphoblastic leukemia (ALL). To investigate the role of A91V in ALL, we genotyped 2272 children with *de novo* ALL registered on the Pediatric Oncology Group ALL Classification study P9900 and 655 normal controls. Allele frequencies in the controls showed a very low frequency of the variant allele in blacks, 0.7% compared to 4% in white controls. In light of this, analysis was restricted to a comparison of white cases and controls only. Overall genotype frequencies were similar in white ALL cases and normal white controls ($P = 0.58$), indicating that in contrast to the previous report, A91V polymorphism is not associated with increased risk of childhood ALL. PRF1 A91V frequency was significantly increased in children with BCR-ABL positive ALL (24 vs 8.5%; $P = 0.0048$); however, this observation includes a relatively small number of cases and needs further exploration.

Keywords

perforin; polymorphism; acute lymphoblastic leukemia

Introduction

Perforin, a membrane-disruptive protein of 555 amino acids, secreted by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, is essential for killing virus-infected or transformed cells targeted for destruction through the granule exocytosis pathway.[1–6] A syndrome of perforin deficiency has recently been described in humans. As many as 30% of children (typically younger than 2 years) presenting with the rare autosomal recessive disorder

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familial hemophagocytic lymphohistiocytosis (HLH) carry mutations in both their perforin (*PRF1*) alleles.[7–9]

A C>T change at position 272 in exon 2 of perforin gene, replaces alanine with valine at position 91 (A91V). A91V is the most common amino-acid substitution identified in perforin, with an allele frequency ranging between 3 and 17% in the general population.[9,10] The role of this substitution in disease pathogenesis remains unclear, and is the subject of continuing debate.[10–12] The A91V substitution has generally been regarded as a functionally unimportant polymorphism of the *PRF1* gene. However, recent data suggest that A91V results in conformational change that may impair processing of perforin protein to the active form,[13] and expression of this human mutant perforin in cell lines leads to reduced cytotoxicity compared to wild-type perforin.[14]

We recently studied two boys who developed HLH while being treated for pre-B acute lymphoblastic leukemia (ALL). Both showed decreased NK cell function along with decreased perforin expressing CD8 cells, and were found to be heterozygous for PRF1A91V.

Also, Santoro *et al*.[15] have reported A91V substitution in 12% of a cohort of Caucasian children with ALL. This prevalence exceeded that of 3.9% observed in a fully comparable control population from Italy, suggesting that the single amino-acid change A91V in the perforin is associated with the risk of developing childhood ALL.

In this study we show that, in contrast to the Italian data, PRF1 A91V does not influence overall susceptibility to childhood ALL, but may increase susceptibility to BCR-ABL positive disease.

Patients and methods

ALL patients

The study population included 2272 children with *de novo* ALL enrolled on Pediatric Oncology Group (POG) ALL Classification study P9900 between 1999 and 2005. All patients provided informed consent to submit a sample to the ALL repository for investigation of leukemia biology. Cases were classified on the basis of established criteria by central pathology review. All ALL subtypes were eligible for enrollment and were treated with the predefined chemotherapy regimens to which patients were assigned based on risk stratification.

Six hundred and fifty five normal blood donors (507 white; 148 black) were randomly selected from the Cincinnati area to determine control genotype frequencies. The majority of donors were of northern European ethnic origin and represented similar sex and racial distribution comparable to the US population subgroups.

Genotyping

Gene-specific polymerase chain reaction (PCR) primers and fluorogenic probes for allelic discrimination, selected using Primer Express software (Perkin-Elmer/Applied Biosystems Inc., Foster City, CA, USA), are described in Table 1.

For each 25 μl PCR reaction, 10 ng DNA template was added to the reaction mixture containing wild-type VIC and variant FAM probe, PCR mastermix (Applied Biosystems, CA, USA) along with forward and reverse primer. Thermocycling was performed with an initial 50°C incubation for 2 min followed by a 10-min incubation at 95°C. Forty cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min were performed. Results were analyzed by the automated TaqMan allelic discrimination assay using sequence detection system 1.2.3 software (ABI TaqMan 7300, Applied Biosystems).

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DNA from normal controls was extracted using standard techniques and genotyped as described for cases. Genotyping results were duplicated in 10% of samples; concordance between repeats was 100%. Furthermore, 10% of the samples were also genotyped using direct sequencing; concordance with TaqMan genotyping was 100%.

Statistical analysis

The significance of observed differences in proportions was tested using the χ^2-test and Fisher's exact test when data were sparse. Genotype frequencies were compared between cases and controls. Further comparisons were also made between the controls and known genetic subtypes of ALL. All analyses were performed at the 5% significance level.

Results

Genotype frequencies for cases and controls are shown in Table 2. Allele frequencies in the normal US blood donors showed significant variation by race, with a very low frequency of the variant allele (T allele, representing the valine for alanine substitution in A91V) in black controls (0.7%) compared to whites (4%). Also, there were no black cases with a T allele; hence, the comparison of genotype frequencies was restricted to white cases and controls only. Overall, T/T genotype was found in one normal control and three patients with ALL. C/T and T/T genotypes were combined for all analyses because of the low frequency of the homozygous genotype. Genotype frequencies were similar in white cases and normal white controls (90.7% CC, and 9.3% CT/TT in cases vs 91.5% CC, and 8.5% CT/TT in controls; $P = 0.58$) (Table 1). Also, allelic frequency of the 272T allele between the normal controls and patients with ALL were comparable (3.5 and 3.8%). The distribution of PRF1 A91V genotypes was consistent with the Hardy–Weinberg equilibrium.

Children with BCR-ABL positive ALL were significantly more likely to carry at least one variant T-allele than controls or children with other sub types of ALL (24% CT/TT vs 8.5% CT/TT $P = 0.0048$; Table 2). This difference should be interpreted cautiously due to the small number of patients with BCR-ABL positive ALL. Stratification of cases by other ALL subtypes revealed no difference in genotype frequencies (Table 2).

Discussion

Perforin deficiency was the first disordered genetic cause of familial HLH, a reactive process resulting from hyperactivation, proliferation and migration of macrophages and type I T cells. Clinically, patients with HLH present with fever, hepatosplenomegaly, pancytopenia, hypertriglyceridemia, hypofibrinogenemia and frequent central nervous system involvement. Patients typically have decreased NK cell function, and a proportion will have pathogenic mutations in the perforin gene. Patients with HLH on the basis of perforin mutations usually demonstrate decreased perforin expression in the CTL.

To date, most mutations in perforin result either in loss of a functional mRNA and complete loss of perforin protein, or production of a nonfunctional protein. Additional mutations in the amino terminal domain of perforin have been identified but their function is not understood.

A single nucleotide change C272T in the perforin gene leading to replacement of an alanine residue with valine (A91V) has been reported in several patients with HLH.[7,9,12,16] Different reports have tried to address the controversial pathogenic role of A91V. We have previously reported that this amino-acid change was present in seven of 202 controls (3%), suggesting that A91V should be considered a neutral polymorphism.[9] Stadt *et al.*[10] and Santoro *et al.*[15] concurred with our finding, reporting 17.5 and 3.9% of normal controls being A91V heterozygous (both relatively small samples of German and Italian populations respectively).

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However, recently Trambas *et al.*[13] and Risma *et al.*[14] reported that A91V perforin undergoes conformational changes and impaired cleavage, likely explaining the reduced cytotoxicity in CTL and NK cells contributing to the pathogenesis of HLH and also suggesting that A91V should be considered a functional variant that affects perforin function.

Murine and human data suggest a possible link between *PRF1* mutations and lymphoid malignancy. Smyth *et al*.[17] showed that perforin deficient mice have a high incidence of malignancy in distinct lymphoid cell lineages (T, B, NKT), indicating that perforin mediated cytotoxicity is critical for lymphoma surveillance. Clementi *et al*.[16,18,19] have reported biallelic perforin mutation in tumor cells from four of 29 patients with lymphoma, suggesting that reduced perforin activity may play a role in susceptibility to lymphoma. Two out of these four patients had A91V as one of their defects along with a second mutation.

In a recent paper, Santoro *et al*. screened 100 children with ALL and reported 11 being heterozygous and one homozygous for A91V. This incidence (12%) was significantly higher than found in controls (3.9%) (OR: 3.4; 95% CI: 1.15–9.95), suggesting that A91V is associated with increased risk of ALL in children.[15] The number of children with ALL in the Santoro study was too small to allow exploration of genetic subtypes of ALL.

In contrast to the findings of Santoro *et al*., our study did not demonstrate any differences in frequency of A91V genotype between children with ALL overall and normal controls. Our hypothesis for these divergent results is that the Italian study analyzed only 100 ALL patients – it is difficult to generalize the findings from studies with small sample size. Our study analyzed a significantly larger and better characterized patient population compared to the Italian study. Genetic characterization of ALL is important as genetic subsets of ALL may have different etiologies. The difference between the two studies highlights the need for confirmation of results from association studies in independent larger data sets. Our larger study allowed exploration of different genetic subtypes of ALL and the PRF1 A91V variant was seen more frequently in children with BCR-ABL positive ALL. However, this observation includes a relatively small number of cases and the potential association should be explored further, perhaps in an adult ALL series in which the frequency of BCR-ABL positivity is likely to be high. In our study, we also report a very low frequency of the variant allele in black controls. This finding has not been reported before.

Conclusion

In summary, our study concludes that A91V polymorphism is not associated with increased risk of childhood ALL. Role of this polymorphism in causation of BCR-ABL positive leukemia needs further confirmation. We are continuing to investigate a potential role of A91V in susceptibility to secondary HLH in response to chemotherapy. Further mechanistic studies defining the functionality of A91V polymorphisms will help clarify the importance of this sequence change. In addition, as data from the clinical study of ALL become mature we will investigate the impact of the polymorphism on the outcome of therapy.

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Table 1

Allele specific primers and probes

Primers
Forward primer – CACCCTCTGTGAAAATGCCCTAC
Reverse primer – TTCCAGTCGTTGCGGATGCTAC
Probes
VIC (wild type) – CTC TGG TGC TCA CCA A
FAM (variant) – CTC TGG TGC TCA CCA A

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Table 2

Genotype analyses ALL cases and subtypes

	Perforin genotype CC (*n* (%))	Perforin genotype CT or TT (*n* (%))	*P*-value (cases vs controls)
Controls (*n* = 507)	464 (91.5%)	43 (8.5%)	—
Cases (all; *n* = 1321)	1198 (90.7%)	123 (9.3%)	0.58
BCR-ABL	22 (76%)	7 (24%)	0.0048
Trisomy 4,10	238 (93.7%)	16 (6.3%)	0.29
TEL-AML1	250 (90.6%)	26 (9.4%)	0.66
E2A-PBX	24 (92.3%)	2 (7.7%)	0.89
MLL abn.	40 (87%)	6 (13%)	0.29
Hyperdiploid	244 (92.4%)	20 (7.6%)	0.66
Hypodiploid	8 (100%)	0 (0%)	0.4