Acute Lymphoblastic Leukemia

A single amino acid change A91V in perforin: a novel, frequent predisposing factor to childhood acute lymphoblastic leukemia?

We screened 100 children with acute lymphoblastic leukemia (ALL) to assess the incidence of single amino acid change A91V in perforin. Heterozygous A91V was found in 12/100 patients and 5/127 controls (OR, 3.4; 95%CI: 1.15-9.95; p=0.014). A91V is a novel and frequent predisposing factor for childhood ALL.

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Perforin plays a pivotal role in the cytotoxicity of natural killer (NK) and cytotoxic T lymphocytes (CTL). Perforin gene (*PRF1*) mutations have been associated with familial hemophagocytic lymphohistiocytosis (FHL, HLH).[1] The pathogenic role of the DNA variant C272T, resulting in a single amino acid change, A91V, has been recently questioned by reports of 4 and 17% prevalences among controls, suggesting this is only a neutral polymorphism.[2,3] To address this issue, we studied the prevalence of A91V in our control population.

Furthermore, a possible link between *PRF1* mutations and lymphomagenesis was suggested by reports of: lymphoma or HLH in two siblings with *PRF1* mutations;[4] autoimmune lymphoproliferative syndrome and lymphoma in a patient with heterozygous Fas and *PRF1* mutations;[5] and 29 patients with lymphoma sharing clinical characteristics with HLH harboring *PRF1* mutations.[6]

To assess whether the impaired cytotoxic function resulting from A91V *PRF1* might be associated with increased risk of developing childhood ALL, we screened 100 consecutive, unselected patients with ALL (<18 years). We also analyzed constitutional DNA from 127 Caucasian controls (61 healthy subjects and 66 consecutive neonates). To search for the C272T DNA variant by *minisequencing*, we amplified *PFR1* exon 2. Polymerase chain reaction products were used to perform a primer single base extension reaction (SNaPshot Kit Applied Biosystems). The reaction is based on a specific minisequencing primer, which is exactly one base short of the 272 position, and fluorescent ddNTP. The product obtained is separated by electrophoresis and the color of the peaks obtained makes it possible to identify the genotype (GeneScan Analysis Software, Applied Biosystems).

Heterozygous A91V was found in 2 of 61 healthy controls (3.2%) and 3 of 66 (4.5%) neonates. Thus, overall, heterozygous A91V was observed in 5 of 127 controls, with a prevalence of 3.9%. Five 272T alleles were observed in 254 chromosomes. Of the 100 children with ALL, 11 were heterozygous, and one homozygous, for A91V. This incidence was significantly superior to that in controls (OR, 3.4; 95% CI: 1.15-9.95). Thirteen 272T alleles were observed in 192 chromosomes (p=0.014) (Table 1). The main presenting features of the 12 ALL patients with A91V are described in Table 2.

Among 22 patients enrolled in the National HLH registry,[7] in whom a *PRF1* mutation was identified, A91V was present in 11 (50%), always in the heterozygous state, associated with another mutation (*Aricò M., unpublished data*). These data suggest that A91V is the most relevant single PRF1 protein change in Italian patients with HLH. The pathogenic role of A91V was questioned by two recent reports: ZurStadt found that 15 of 86 (17.5%) healthy Caucasians were heterozygous for A91V,[3] while Molleran Lee found the amino acid change in 7 of 202 controls (3%).[2] Both suggested that A91V should be considered only a neutral polymorphism. Our finding of A91V in 3.9% of controls is in keeping with the results of Molleran Lee.[2] Is A91V only a neutral polymorphism? At least two recent studies suggest that this is not the case. Voskoboinik reported that A91V perforin was expressed at lower levels in rat basophil leukemia cells than in wild-type cells, resulting in partial loss of lytic capacity.[8] Trambas *et al.* demonstrated that A91V perforin from a patient with HLH is not recognized using an antibody raised against native perforin (δG9), but is readily detected using an antibody raised against a peptide epitope (2d4), suggesting that the epitope recognised by δG9 is destroyed by A91V. A91V perforin undergoes conformational changes and impaired cleavage, likely explaining the reduced cytotoxicity in CTL and NK cells contributing to the pathogenesis of HLH.[9] Altogether these data suggest that A91V cannot be considered only as a neutral polymorphism but rather as a functional polymorphism – or a mutation – which affects perforin function.

Is HLH the only clinical condition in which A91V has

Table 1. Allele distribution for the 272T DNA variant in children with leukemia and in control subjects.

Category	*Number of subjects*	*Heterozygous subjects (%)*	*Number of alleles*	*272T alleles (%)*
Controls	127	5 (3.9)	254	5 (1.9)
Neonates	66	3 (4.5)	132	3 (2.2)
Healthy controls	61	2 (3.2)	122	2 (1.9)
Children with acute lymphoblastic leukemia	100	12* (12.0)°	200	13 (6.5)#

**One patient homozygous; °Odds Ratio: 3.4; 95% CI: 1.15-9.95; #χ^2 of Pearson without Yates' correction: 6,03 (p=0.014).*

Table 2. Presenting features of 12 patients with childhood acute lymphoblastic leukemia and the single amino acid change A91V.

Patient	*Age/Sex*	*WBC count (mm³)*	*Immunophenotype*
1.	5/F	3.200	Common
2.	9/F	3.430	Common
3.	4/M	43.890	T
4.	9/M	3.390	Common
5.*	2/M	10.980	Common
6.	3/F	6.660	Common
7.	0.7/M	243.000	Pre-B°
8.	12/M	9.310	B Mature
9.	4/F	82.000	Common
10.	2/F	4.360	Pre-B
11.	14/M	151.000	Common
12.	11/F	4.600	Pre-B

**This patient was homozygous for A91V. °t(9;11) at diagnosis; prednisone good responder. At relapse immunophenotype shifted from Pre-B to Pro-B.*

implications? We found A91V in 12% of a cohort of Caucasians children with ALL. This prevalence exceeded that of 3.9% observed in our fully comparable control population from the same geographic area. Since the patients with ALL were not selected for any additional criteria, including HLH-like clinical features, this finding suggests that the single amino acid change A91V in the perforin is significantly associated with the risk of developing childhood ALL. The presenting features of children with A91V and ALL were not different from those of the remaining ALL patients. In conclusion, we suggest that impaired function of the cytotoxic machinery, as induced by the A91V transition, may result in different clinical manifestations and may predispose to ALL, the most common type of childhood cancer.

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Key words: A91V, childhood acute lymphoblastic leukemia, perrforin.

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Chronic Lymphoproliferative Disorders

Reversal of bone marrow angiogenesis in chronic lymphocytic leukemia following fludarabine therapy

We evaluated bone marrow (BM) angiogenesis in 12 responding patients with Binet stage B chronic lymphocytic leukemia who received either chlorambucil or fludarabine. Microvessel density (MVD) was compared between pre-treatment marrow samples and those obtained after at least 4 cycles of chemotherapy. BM angiogenesis decreases in all patients but one, although subset analysis revealed the decrease of BM angiogenesis was significant only in patients who received fludarabine. Even if based on a small number of patients, these results point out the potential *in vivo* anti-angiogenic activity of fludarabine.

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Research over the past two decades has unravelled the importance of abnormal, increased angiogenesis in the development and spread of both solid and hematologic tumors.[1-6] In the context of hematologic malignancies, increased bone marrow (BM) angiogenesis has been demonstrated in multiple myeloma, chronic myeloid leukemia, acute myeloid or lymphocytic leukemia, chronic lymphocytic leukemia (CLL) as well as myelodysplastic syndromes and has been found to have prognostic value.[1-6] However, changes in BM angiogenesis after therapy have not been systematically evaluated.

With this in mind we analyzed the effect of chemotherapy on BM angiogenesis in responder B-cell CLL patients. The study included 12 symptomatic Binet stage B patients (median age 54 year; range, 28-67; M,7; F,5) who received intermittent chlorambucil (5 patients) or fludarabine (7 patients) as up-front therapy. Paraffin-embedded BM biopsy blocks were used to prepare slides for microvessel density (MVD) determination according to previously described methods.[6] BM evaluations were performed at the time of diagnosis and after at least 4 courses of chemotherapy (median, 8; range 4-12). According to the criteria proposed by the National Cancer Institute (NCI)[7] 7 patients were considered in complete remission (CR) and 5 in good partial remission (G-PR). Minimal residual disease (MRD), assessed in flow cytometry as the percentage of $CD19^+/CD5^+$ BM cells, closely reflected the type of therapy: chlorambucil, 21.8% (range, 11-29.9%); fludarabine, 5.2% (range, 0.1-11%; p=0.006; Mann-Whitney test). BM MVD decreased in all but one patient (Table 1). Specifically, the median microvessel area was 2.616 $mm^2 \times 10^{-2}$ (range, 0.545-4.126) before therapy and 0.644 $mm^2 \times 10^{-2}$ (range, 0.383-1.914) after therapy (p=0.003; Mann-Whitney test). Finally, we wondered whether different therapies could affect the amount of MVD reduction. Separate comparisons carried out in patients treated with chlorambucil and fludarabine revealed a significant decrease of MVD only in patients who received the latter treatment (p=0.04). Interestingly, no correlation was found between the amount of MVD reduction related to the therapy and MRD as defined by the percentage of BM $CD5^+/CD19^+$ cells (Spearman r= -0.042; p=0.907).