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Research paper

Perforin gene variation influences survival in childhood acute lymphoblastic leukemia

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ABSTRACT

Although a growing body of data links mutations in the perforin gene with increased susceptibility to hematologic malignancies, no studies discuss their influence on the clinical course of such diseases. The present study examines the impact of perforin gene variation on the clinical outcome in acute lymphoblastic leukemia (ALL) patients. The study enrolled 312 children aged 1–18 years, treated for ALL. *PRF1* gene variants were analyzed through direct DNA sequencing. Variation in rs885822 was found to be associated with overall survival: patients carrying the *GG* genotype demonstrated a significantly increased risk of death compared to those carrying the *A* allele, independently of ALL risk groups (HR 3.13, 95%CI 1.16–7.8, p = 0.014). The effect was even more pronounced in high-risk ALL patients (p = 0.006). On the other hand, the presence of the rs35947132 minor *A* allele was slightly protective with regard to overall prognosis (p = 0.047). No differences in relapse-free survival were observed with regard to genotypes. The results of the study may imply that perforin gene variation has a role in modifying mortality in childhood ALL.

1. Introduction

Perforin is a pore-forming protein stored in the acidic secretory granules of cytotoxic lymphocytes [1]. Perforin-mediated cytotoxicity is crucial in killing transformed cells and cells harboring intracellular pathogens; it is also involved in downmodulation of the immune response by inducing apoptosis of effector lymphocytes and antigen-presenting cells [2–4].

The belief that perforin is critical for immune homeostasis and tumor immune surveillance is supported by the spectrum of pathologies associated with perforin deficiency [5]. Perforin-depleted ($Prf1^{-/-}$) mice are highly immunosuppressed and susceptible to diverse intracellular pathogens [6], as well as to transplanted [7], virus-induced [8] and spontaneous malignancies, primarily to aggressive B-cell lymphomas [9]. Perforin has also been shown to play an important role in NK-cell mediated suppression of tumor metastasis [10] and in controlling the growth of carcinogen-induced sarcoma [11]. In humans, biallelic *PRF1* gene mutations are well recognized as the cause of 20–50% of familial heamophagocytic lymphohistiocytosis (FHL2) [12–15], a severe hyperinflammatory syndrome with uncontrolled activation of antigen-presenting cells, CD8+ T cells and massive cytokinaemia [16]. Recent studies also describe the presence of perforin-dependent immune surveillance in humans. Carriers of mutant *PRF1* were shown to suffer more frequently from relatively early-onset melanoma – a malignancy in which CD8+ T cells may play a role in the prevention of disease progression and metastatis [17]. Both mono- and biallelic *PRF1* gene mutations have also been found in a proportion of patients with Hodgkin and non-Hodgkin lymphomas [18–20]. A limited number of studies on acute lymphoblastic leukemia (ALL), the single most common pediatric malignancy [21], have provided contradictory results: the A91V polymorphism was identified as an ALL-predisposing factor in a relatively small group of children [22], but was not confirmed on a larger cohort [23]. It was also suggested that perforin mutation may be more prevalent in ALL with *BCR-ABL1* translocation [24].

As perforin plays a pivotal role in maintaining immune homeostasis,

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Table 1
Clinical characteristics of ALL patients.

Clinical variables	n = 312
Age at diagnosis, yrs, median (range)	5.28 (0.96–17.9)
Gender, n (%)	
Male	174 (55.8)
Female	138 (44.2)
Immunophenotype, n (%)	
B-ALL	267 (85.6)
T-ALL	45 (14.4)
Risk group, n (%)	
SR	75 (24.0)
IR	169 (54.2)
HR	68 (21.8)
WBC at diagnosis, 10^9/L, median (range)	14.45 (0.1–851)
Prednisone poor response, n (%)	31 (9.9)
MRD d15, %, median (range)	0.30 (0.0–93.0)
High risk genetic aberrations, n (%)	
BCR-ABL1 positive	4 (1.3)
MLL rearrangements	16 (5.1)

WBC, white blood cell count; HR:, high risk group; IR, intermediate risk group; SR, standard risk group according to BFM ALL IC2002 or 2009 protocol; MRD d15, minimal residual disease on treatment day 15.

not only regarding tumor immune surveillance, but also pathogen defense and immune regulation, the aim of the present study is to examine the impact of perforin gene variation on the clinical outcome in pediatric ALL patients. As such, this is the only study so far to address the role of perforin in the clinical course of ALL.

2. Patients and methods

2.1. The study population

The study enrolled 312 children aged 1–18 years, treated for ALL in reference centers of the Polish Pediatric Leukemia/Lymphoma Study Group between April 20 and September 2015. The diagnosis, risk group assignment and treatment were performed according to the guidelines of BFM ALL-IC 2002 and BFM ALL-IC 2009 Protocols, as described before [25]. Clinical variables are presented in Table 1. The Ethics Committee approved the research protocol and informed consent was obtained from all participants and/or their parents.

2.2. DNA extraction, amplification and sequencing analysis

DNA was extracted from EDTA venous remission blood or bone marrow samples (according to ALL IC2002 protocol) using QIAamp DNA Blood mini kits (Qiagen, Hilden, Germany) and quantified by NanoDrop 8000 (Thermo Fisher Scientific Inc., Waltham, USA). All coding exons as well as the exon–intron boundaries of *PRF1* gene were amplified in polymerase chain reactions, using the following primers for Exon 2: 5′-GTC ATC CTC CAT CCC TCC ACC C-3′ and 5′-AGA CCA CCC AGA GTT TCC CGC-3′, and Exon 3: 5′- GAA GGA GTT ATT TGA TTG AAT GGG G-3′ and 5′-AAC CCC TTC AGT CCA AGC ATA CT-3′. Amplification of 100 ng DNA was performed in 30 µl assay of 1x PCR buffer, 1x Q-Solution, 0.2 mmol dNTP, 0.4 µmol of each primer, and 2.5U HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). Thermal cycling conditions were as follows: 15 min at 95 °C (initial activation step), followed by 35 cycles of denaturation at 95 °C for 30s, annealing at 64 °C and 62 °C (for exons 2 and 3, respectively) for 30s, and extension at 72 °C for 90s, with final extension at 72 °C for 10 min. The PCR products were purified and subjected to DNA sequencing using the Big Dye sequencing kit (Applied Biosystems, Foster City, USA) on an −ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA). The primers used for sequencing were the same as those for amplification, with the addition of internal exon 3 primers: 5‘-GTG GAC TAC ACC CTG GAA CCC-3′ and 5′-AGT CCC TCC AGC GAG CCC-3′. Sequences were compared with the *PRF1* reference sequence (NCBI GenBank accession number NM_001083116.1) using Sequencher software (Gene Codes Corp., Ann Arbor, MI, USA).

2.3. Control group

Genetic variants in the *PRF1* gene found among ALL patients were compared with DNA sequences of 212 children of Polish origin with no symptoms of ALL or any perforin-related morbidity, who were subjected to whole exome sequencing (WES) and collected in the regional registry. Additionally, to increase sample size of controls, 236 healthy anonymous adult blood donors from regional Blood Service Center were studied. All individuals from control cohort came from the region of Central Poland (Mazowieckie and Lodzkie Voivodoship) and were of Polish Caucasian origin. Median age of subjects in the whole control group was 22.3 years (25–75% 14.1–28.4; range 3.1–32.2 years), 241 (53.8%) were male, 207 (46.2%) were female. WES was performed on HiSeq 1500 using TruSeq Exome Enrichment Kit (Illumina, USA), as described previously [26]. After a standard 2 × 100 Illumina run, the results were processed by CASAVA. Generated reads were aligned to the reference human genome (UCSC hg19) with the Burrows-Wheeler Alignment Tool and processed further by Genome Analysis Toolkit. Base quality score recalibration, indel realignment, duplicate removal, and variant calling were performed. The detected variants were annotated using Annovar and converted to MS Access format for final manual analyses. Alignments were viewed with Integrative Genomics Viewer.

2.4. Statistical analysis

Univariate comparisons were performed using the two-tailed Fisher’s exact test or the Kruskall-Wallis test. The relapse-free survival (RFS) time was calculated from the date of diagnosis to the first relapse (either bone marrow or extramedullary or combined), leukemia-related death or to the date of last follow-up or to the day of bone marrow transplantation (BMT). The overall survival (OS) was calculated as the period from diagnosis to death or last contact or BMT. Patients lost from the follow-up (n = 4) were censored at the time of the last clinical examination. Survival analyses were performed using Kaplan-Meier method with log-rank test, and Cox proportional hazards regression. Variables, which reached a p value < 0.10 in univariate analysis were chosen for multivariate survival model. A p-value lower than 0.05 was considered statistically significant. Statistica 12.0 software (Statsoft, Tulsa, USA) was employed for data analysis and presentation of the results. Haploview 4.0 software was used to assess linkage disequilibrium between SNPs [27].

3. Results

3.1. Distribution of PRF1 allele frequencies in ALL patients and in controls

The sequencing of coding exons and exon-intron boundaries of *PRF1* gene revealed many genetic variants (see Supplementary Table S1).

Out of these, three polymorphic sites with MAF (minor allele frequency) > 0.05: rs885822, rs885821 and rs35947132 were found in the study group. All three SNPs were in the Hardy-Weinberg equilibrium in the control group (p = 0.15, p = 0.29 and p = 1.0, respectively). Moreover, rs885822 and rs35947132 SNPs were in complete linkage disequilibrium (D’ = 1, in that *A* allele in rs35947132 coexists only with *G* allele in rs885822).

For SNPs rs885821 and rs35947132, there were no differences in allele frequency between the study population and controls (p = 0.25 and p = 0.46, respectively). In rs885822, however, the minor *G* allele was significantly less frequent in ALL patients (OR 1.44, 95%CI