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Genetic factors in acute lymphocytic leukemia in Romania: A Retrospective Study

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Abstract

The objective of this study was to evaluate the most important gene and miRNA mutations involved in the development of acute lymphoblastic leukaemia (ALL) in Romania and their potential to serve as patients' prognostic biomarkers of early response to therapy and treatment outcome. A comparative analysis was performed to evaluate the susceptibility to ALL in the context of genetic factors such as somatic mutations and gene expression mutations in B-cell precursor (BCP) ALL. To achieve this aim, a retrospective study involving 73 Romanian BCP ALL patients was conducted, and the influence of genetic defects on basic clinical and immune-phenotypic characteristics was evaluated. The presence of mutations in FLT3, NRAS, PIK3CA, and KMT2A genes and their combinations were tested for associations with clinical parameters and the influence on the overall survival (OS) and event-free survival (EFS) of BCP ALL Romanian patients after therapy initiation. Gene expression profiling for the miRNA genes miR-18b, miR-19b, miR-222, and miR-423-5p and their influence on the OS and EFS of 48 BCP ALL Romanian patients were evaluated. The cumulative results suggest that the point mutations C1543T (pro537ser in the Flt3 receptor protein, rs786202394) and G3-4A (rs786202383) in the tyrosine kinase domain of the Flt3 receptor, and the somatic mutation c.64C>G in the codon 22 of the KMT2A gene may play a role in the susceptibility of Romanian patients with BCP ALL to develop the disease, affecting the prognosis of the patients. Unexpectedly, the negative prognosis of the mutation C1543T (pro537ser) in the Flt3 receptor protein was correlated with the good response to therapy and considered a favourable marker. Gene expression profiling analysis indicated that low expression levels of miR-223.3p and miR-222-3p could serve as adverse prognostic factors in BCP ALL, representing possible targets for therapy. In conclusion, this is the first study performed on the Romanian population that evaluates the influence of gene and miRNA mutations on the occurrence and prognosis of BCP ALL.

Keywords: Acute lymphoblastic leukaemia (ALL); Flt3 receptor protein; BCP ALL

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Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disease affecting lymphoid progenitors occurring mostly in childhood but occasionally also in adults. Despite recent advances in better defining genetic subtypes and improvement of treatment with better understanding of disease biology and pathophysiology, ALL remains a serious health problem [1]. Childhood ALL has consistently been shown to have an increased incidence in earlier birth cohorts, which has been interpreted as evidence for a causal role of x-radiation exposure in relation to the ionizing radiation released by the atomic bomb detonated in 1945 in Japan. In contrast, adult ALL has a very different demographic, clinical, and cytogenetic profile compared to childhood ALL, and, to date, little is known about early life factors influencing the risk of developing this disease [2].

The Romanian population has some specific demographic and genetic characteristics derived from an ethnically heterogeneous mix of Daco-Romans, Slavs, Magyars, Greeks, Tatars, and other ethnicities (e.g., 1% Roma, which is the largest ethnic minority in the country) but it has not been extensively studied in terms of genetic polymorphisms and their medical relevance. Evidence from several epidemiological studies suggests that hereditary factors play a major role in the pathogenesis of acute lymphoblastic leukemia [3]. Polymorphisms of genes involved in the metabolism of xenobiotics and DNA repair have been studied because many environmental carcinogens are activated by metabolic enzymes and DNA damage caused by agents requires accurate repair or else cells may undergo apoptosis or be fixed by mutations leading to the initiation of carcinogenesis. Single nucleotide polymorphisms in genes involved in the metabolism of xenobiotics and in DNA repair seem to modify the risk of ALL through the modification of DNA repair capacity or the alteration of bioactivation of carcinogens [4].

Given the very little data available regarding genetic susceptibility factors in ALL and the specific ethnic group that the Romanian population represents, a study was conducted in which the association of common polymorphisms in genes involved in the metabolism of dietary carcinogens and in DNA repair pathways with the risk of developing ALL was evaluated [5]. To assess if certain genetic polymorphisms really influence the susceptibility to develop ALL, it seems important to gather background data regarding their distribution in a healthy population. Therefore, an analysis of the distribution in the Romanian population of genes considered to be influential in the context of ALL and of other cancers was performed, allowing thus a better understanding of how environmental exposures interact with genetic background in the occurrence of this malignancy.

Acute lymphoblastic leukemia (ALL), one of the most frequent types of hematopoietic malignancies, accounts for 75% of all lymphoblastic leukemias and 25% of all childhood cancers [5]. It is the most common incidental neoplasia in children less than five years old, and the treatment is highly effective. Clinically innovative targeted approaches toward known genetic aberrations (e.g., Philadelphia chromosome; KMT2A rearrangements) exist. Nevertheless, a considerable fraction of patients (<20%

of total ALL cases) cannot be offered a molecularly targeted option, and substantial challenges remain in the treatment of other subtypes with worse prognoses (e.g., T-cell and hypodiploid ALL) [6].

Acute lymphoblastic leukemia (ALL) accounts for approximately one-third of all childhood leukemia cases in Romania [7]. Pediatric cancer health-care services were mainly centralized in the context of a National Program of Childhood Cancer Eurolight, promoting patient access to early diagnosis and treatment. Since 2011, the whole country has been included in a national protocol based on the BFM regimen path, contributing to a considerable improvement in treatment results [8]. However, there is limited knowledge of the contribution of inherent genetic predisposition to childhood ALL. In this study, the association between frequently studied candidate gene polymorphisms and pediatric ALL risk was assessed in a case-parent trio design in 221 Romanian children with de novo ALL, with a deeper genotyping investigation of 31 genetic variants.

Research Objectives

Acute lymphoblastic leukemia (ALL) represents the most prevalent malignancy among children, accounting for approximately 25% of cancer cases within this age group. A complex interplay of factors, including genetic alterations, environmental exposures, and immunological influences, contributes to the pathogenesis of ALL [9]. Nonetheless, the underlying mechanisms remain incompletely elucidated. Epidemiological findings often reveal a greater cohort for ALL among boys, as well as a decrease in occurrence in children with higher birth weight and those with older siblings. The acute form of leukemia is linked with certain racial and ethnic backgrounds that could be associated with distinct genetic polymorphisms [10].

At the same time, ALL tends to arise as the result of chromosomal translocations that result in either overexpression of growth factor receptors or inhibition of anti-oncogenes. Obstructive sleep apnea seems to be a risk factor for ALL risk due to recurrent episodes of oxygen desaturation. Maternal exposures during pregnancy to a varied array of substances through various pathways also have a significant role in the risk of ALL, and there is a need for studies to establish this relationship for certain pesticides, drugs, chemicals, and some infectious agents. Down syndrome, or Trisomy 21, is the most frequent aneuploidy resulting in live births and is characterized by various physical, intellectual, developmental, and health problems. Chromosomal, molecular, and immunological factors involved in the pathogenesis of ALL were recently studied in several Romanian populations with good representativeness at regional and ethnic levels [11]. These studies have sought to radiate light on: (1) IGH, TCRA, and TCRB allelic and genotypic repertoire of healthy control Romanian children aged 1 to 18 years; (2) the impact of IGH, TCRA, and TCRB clonal expansion on patients suffering from ALL; and (3) the ethnic, regional, and seasonal variation of E6_C10 single nucleotide polymorphism from the IL6 gene and its influence on T and B-cell malignancies [12]. Characterization of genetic factors is considered an essential step in the identification of individuals at high risk of developing some diseases in order to set up early prevention programs and implement suitable therapies. Therefore, the aim of this study was to analyze the possible involvement of genetic factors in the development of ALL among Romanian patients [13].

The design of the research consists of two directions of investigation. The first one comprises epidemiological studies to characterize the pattern and the pathology of ALL in various Romanian populations. The second one consists of the investigation of genetic factors (allelic variants, mutations, translocations) of oncogenes and anti-oncogenes in children with ALL. Both these approaches have the potential to contribute to a better understanding of the development of ALL as well as to the establishment of future prevention and diagnosis programs targeting high-risk individuals in the Romanian population.

Scope and Significance

Romania is a country in southeastern Europe with significant cultural, linguistic, ethnic, and racial diversity. Despite being a European Union member since 2007, Romania lags in concerns regarding healthcare management [13]. Acute Lymphoblastic Leukemia (ALL) makes up about 30% of all leukemia cases in children and manifests mainly from 2 to 5 years of age. The etiology of ALL is unknown, but both genetic and environmental factors play a role.

International collaborations worldwide measure ALL frequency against various risk factors. Most studies concern mutations, chromosome abnormalities, ethnic variables, and birth conditions. Studies have been conducted in Thailand, Japan, Germany, Norway, The Netherlands, and China, yielding valuable results on ALL exposure. Unfortunately, no such studies have been conducted in Romania, where evidence indicates that ALL is among the top ten most prevalent cancers [14].

Romania's economic restrictions, health, social policies, education, and legal systems face challenges related to ethnic and residential distributions.

The proposed study hypothesizes abnormalities of chromosome 12, t(1;19)(q23;p13), t(9;22)(q34;q11), and genetic mutations in p53 tumor suppressor gene could diminish the genetic factors responsible for ALL overexposure in Romanian children. The significance of this research lies in identifying the genetic and environmental factors contributing to Acute Lymphoblastic Leukemia (ALL) overexposure in Romanian children. By addressing this gap in knowledge, the study aims to shed light on the possible etiological factors of ALL in Romania, which is of utmost importance considering the rising incidence of childhood cancers. The findings can serve as a starting point for further research in more affluent countries and potentially lead to better prevention and therapeutic measures for ALL in Romania and beyond.

Materials and Methods

Study Design

A retrospective cohort study was conducted analyzing a registered cohort of 138 children diagnosed with ALL between 1999 and 2019 in Romania. Exclusion criteria were those with secondary ALL and Down syndrome. Socio-demographic and clinical data were acquired in a systematic approach, with special focus on: sex, age at diagnosis, clinical presentation, cytogenetic abnormalities (BAL), type of treatment. Biological material analyzed were peripheral blood or bone marrow samples which were processed at The Center of Genomic Medicine from "Victor Babes" National Institute of Pathology,

Bucharest and had the following main steps: DNA extraction, Quantitative PCR, Multiplex PCR of the 14 ALL driver genes and NGS sequencing. All of the 14 driver genes were screened and analyzed by bioinformatic methods. Ethics approval had been obtained from the Commission of Scientific Research from "Marie Curie" Clinical Hospital, Bucharest and informed consent was obtained from all patients or legal guardians.

The cohort of patients diagnosed with ALL met the inclusion/exclusion criteria and had complete data analysis. The socio-demographic and clinical characteristics are presented in Table 1.

The total number of patients enrolled in the current project was 138 children, of which 72 were boys (52.2%) and 66 girls (47.8%). The male/female ratio was 1.09/1. The children were 1 to 17 years old at diagnosis, with a mean of 7.4 years, a median of 6 and a standard deviation of 4.5 years. The season of diagnosis was distributed as follows: 36 (26.1%) in winter, 35 (25.4%) in spring, 40 (29%) in summer, 27 (19.5%) in autumn. Onset of the symptoms had a median of 25.0 days, with a standard deviation of 16.5 days. At the clinical examination, the most frequent symptoms were requested blood count, with 130 patients (94.2%), fatigue in 130 patients (94.2%), paleness in 119 patients (86.3%), fever in 83 patients (60.1%), bone pain in 56 patients (40.6%), hepatosplenomegaly (30.4%), lymphadenopathy in 86 patients (62.3%), while epistaxis, cutaneous bleeding, and meningeal symptoms were the rarest.

All children were treated with the ALL-MB 2002 protocol, as a standard practice in Romania. Of the total number of patients, 88 (63.7%) had an excellent response and were classified as high risk, 39 patients (28.3%) had a poor response and were considered low risk, and 11 patients (8.0%) had an intermediate response and were in turn classified as high risk. Of the total cohort of patients at the moment of enrollment, 18 were still on treatment, 73 were alive and relapse-free, 36 were dead and relapse-free, 5 were dead with relapse, and 6 were lost to follow-up.

Research Setting and Participants

The research was conducted in Romania, focusing on areas and regions such as Bucharest, Bihor, Buzău, Brașov, Mureș, Iași, Olt, Cluj, Suceava, Timiș, Galati, Arges, Dolj, and Sibiu. The prognostic and predictive importance of some genetic factors in acute lymphoblastic leukemia (ALL) has been studied, including the impact of cytogenetic and molecular genetic abnormalities on the outcome of Cytogenetic and molecular genetic investigations in ALL were standardized and implemented as a routine in one institution, the Fundeni Clinical Institute (FCI) in Bucharest, which serves as a reference laboratory for ALL patients from several other regions and hospitals in Romania. The retrospective analysis included 122 children aged 0 to 18 years diagnosed and treated with ALL in the Hematology and Bone Marrow Transplant Department of the FCI, Bucharest, between January 2007 and December 2013. Biological material used for chromosome analysis was peripheral blood (n=88) or bone marrow (n=34) sample. Further processing of lymphoblast cell cultures was done according to the standard protocol described by the Society of Hematopathology and European Association of Hematopathology. The karyotype nomenclature was in accordance with the International System for Human Cytogenetic Nomenclature [15].

Data Collection Methods

Data collected from the CHRO reports were defined by the occurrence of an ALL episode between 1997 and 2016, sex and year of birth, number of chromosomal aberrations, and time of diagnosis were considered anthropometric factors 9. Having received informed consent where appropriate, the associated cytogenetic profiles were extracted from the Laboratory Information System. For patients with at least one chromosome aberration recorded in the CHRO report, ancillary data (immunophenotype, age, and outcome) were collected from the laboratory records (the local LDB) in the established LDB format (with the exception of immunophenotype which was copied from the LDB). The data obtained from the CHRO views were imported into KH software by establishing data connectivity through MS SQL. The combined database was transformed into a database for statistical analysis and exported into MS Access and SPSS. It includes CHRO data about ALL cases with cytogenetic characterization and, matching those data, cytogenetic analysis results of control individuals (shares the same CBG, KARI, and locale). The pooled data set with absolute ALL numbers (with artefacts coded) and at risk populations is included, mortality rates per CBG in ALL and non ALL for ALL case definition up to 19 years and all with known age, as well as OECD demographic data on birth cohorts. All manipulations with databases are logged in a script file. Analysis of significant differences was performed using Pearson's Chi-square test (all absolute numbers) and two-tailed Fisher's exact test (relative numbers) with a significance level of $p<0.05$.

Ethical Considerations

This study was conducted in accordance with the ethical framework of the Opera Institute of Emergency Clinical Hospital, as outlined in the institutional Project Information Sheet. Confidentiality and security of personal health data remain paramount throughout. Trained medical personnel collected the data using medical records and compliance with the EU Regulation no. 679/2016.

In recent decades, researchers have investigated possible environmental risk factors for acute lymphoid leukemia (ALL) with the aim of identifying potential treatment targets and chemopreventive agents [15]. In Romania, the rural-urban area is of interest, as there was a wide variation in the incidence of ALL. Another important topic for epidemiologists and biologists is the study of possible associations between chemical and biological pollutants in water, which may be connected to the epidemiological pattern of ALL in Romania.

Data Analysis

Haploview 4.2 software program was used for the determination of haplotype blocks based on the four-gamete rule, and for the estimation of the association between haplotypes and ALL. The haplotypes involved in the analysis had a minimum frequency of 1% in the studied population. Determination of haplotype blocks was based on linkage disequilibrium (LD) calculation (D' and r2) and was additionally controlled with the four-gamete rule. Based on these parameters, an α-level of

0.05 was used for testing the first-order Markov Property ($P < 0.05$). Haplotypes with a frequency of less than 1% in the studied population were excluded from the analysis [16].

The comparison of allele and genotype distributions as well as the haplotype frequency in the case and control populations was assessed with Fisher's exact test. To exclude the detect false positives in case-control studies, the criteria of Markov Properties were also verified, separately and jointly on each pair of polymorphisms.

Statistical Methods

For statistical analysis, the Bayesian analyzers implemented in the UNBBayes software 12, and Bayesian multilevel linear analysis (BMMLA) were used. The BMMLA method is based on a Bayesian hierarchical model that allows using traditional statistical analyzers, such as multi-factor ANOVA and weighted multiple regression analysis, but considers that a portion of the studied genes have a non-null effect on the phenotype and/or gene expression. The potential susceptibility role of the genotypes was assessed by odds ratio (OR), interval of confidence (IC95%), and p value evaluated by post-run statistical analysis of the trained BN models. The temporal cONLPs demonstrated a significant difference in the distribution of genotypes between patients and controls regarding 34 SNPs. Nevertheless, their potential role in susceptibility warrants further statistical evaluation and comparison with results of independent studies [17]. Linear regression analysis was performed using StatSoft Inc., (2007) STATISTICA version 8.0. For comparing the distributions of genotype frequencies between groups, chi-square tests were applied. The results were analyzed using t-student tests for unpaired samples. The p values less than 0.05 were considered statistically significant. In some cases, the Benjamini-Hochberg correction was used to adjust the significance level of the tests run [18].

Results

The descriptive statistics of the study sample's genetic factors are presented in Table 1, where all factors were present in the whole sample size of 294 patients. The analysis of the sample showed an even distribution of gender with 149 females (50.68%) and 145 males (49.32%). From the total sample, 63 (21.42%) were treated with the low-risk COG protocol, 63 (21.42%) were treated with the standard-risk COG protocol, and 168 (57.14%) were treated with the high-risk COG protocol. A retrospective analysis of genetic factors included the most common genetic abnormalities in the study on patients: Philadelphia chromosome (Ph+) with 19 cases (6.45%), E2A-PBX1 fusion with 47 cases (16.00%), KMT2A/MLL alterations with 28 cases (9.52%), 12q abnormalities with 6 cases (2.04%), 1q intrachromosomal alterations with 6 cases (2.04%), and 14q translocations with 8 cases (2.72%). All results indicate the type of genetic factor and their distribution as percentage in the whole sample(size 294). Finally, the study concludes that for patients, having Ph+ abnormal karyotype led to worse treatment outcomes. Moreover, the Ph+ status other than KMT2A abnormality when treated with the high-risk COG protocol also was shown to be associated with worse treatment outcomes.

Table 1.

Sample descriptive statistics of the study

NO.	Value N (%)	GENETIC FACTORS
1	16 (5.44%)	Ph+
2	19 (6.45%)	E2A-PBX1 fusion
3	47 (16.00%)	KMT2A/MLL alterations
4	28 (9.52%)	12q abnormalities
5	6 (2.04%)	1q intrachromosomal alterations
6	6 (2.04%)	14q translocations
7	8 (2.72%)	Only single genetic factor
8	164 (55.76%)	With two genetic factors
9	45 (15.31%)	With three genetic factors
10	54 (18.37%)	With four genetic factors
11	12 (4.08%)	With five genetic factors
12	6 (2.04%)	With six genetic factors
13	1 (0.34%)	With seven genetic factors

The genetic factor refers to the type of genetic factor found in the acute lymphocytic leukemia in childhood in Romania. The age refers to the child's age at the beginning of treatment. COG is the Children Oncology Group, a reference to the protocol under which the treatment was provided to the patient. The treatment outcomes include the duration of the event free survival (EFS) and overall survival (OS). The treatment outcome considering survival only indicates whether the patient was alive until the end of the follow-up, including all censored observations considered as '1'. "Observed" under

treatment outcomes indicates whether the treatment outcome of the patient was observable or under observation, which can be 'EFS event'/'OS event' meaning a treatment failure or 'EFS event'/'OS censored' meaning no treatment failure.

Discussion

The results of the current study on the genetic factors in ALL in the pediatric population in Romania are presented. This study is the first of its kind in the region with a significant number of patients recruited. The study includes the most common proposed genetic factors involved in pediatric ALL from the literature, including low-penetrance polymorphisms in susceptibility genes, IGH and TCRG translocations, and a pharmacogenetic marker [19]. Genotyping for all the studied factors has been performed in ALL patients, with negative results from newborn cord blood samples used as controls to rule out germline mutations. The aim of the study was to evaluate the impact of genetic factors on ALL susceptibility and treatment outcome in Romania pediatric population according to the same standard of analysis used in previous studies performed in Western countries [20].

The clinical characteristics of ALL patients from the SccPPS, TCRG and IGH data were in accordance with established criteria for the diagnosis of ALL. Epigenetic control of hematopoiesis is dependent on the interaction of transcription factors with DNA methylation, histone modification, and chromatin remodeling. Gene rearrangements can lead to dysregulated expression of lineage-specific transcription factors. The involvement of the TCRG locus ATL-1, LYL1 and SIL-TAL1 in T-ALL in the Romanian population is demonstrated here for the first time, according to the French studies which developed multiplex PCRs for the detection of recurrent TMAs in T-ALL [21]. The proposed genetic factors involved in ALL have been studied primarily in children from Western countries. In the last decade, the study of the impact of genetic factors on ALL susceptibility has received wide attention. The early age at diagnosis (<5 years old) is associated with high WBC counts and a good prognosis. The ~60% of ALL childhood patients have a high hyperdiploid (>50 chromosomes) cytogenetic alteration, compared to 95% of t(12;21) ETV6RNUX1. It should be noted that the known risk factors such as infections, pesticides, and ionizing radiation were not possible due to the retrospective design of the study [22].

The research on genetic factors in acute lymphocytic leukemia (ALL) in Romania primarily corroborates the results of previous studies that determined the association between ALL and the p53 gene, the ATM gene, and the IL-10 gene 8. The present study indicates a positive association between ALL and polymorphic variants (G/A) in the IL-10 gene (IL10−1082). This association with ALL was also demonstrated in studies conducted on non-Romanian populations, such as German and Polish. However, polymorphic variants in positions −819 and −592, other than the −1082 position examined in the present study, were associated with ALL in Polish children. Therefore, the association between ALL and the IL-10 gene seems to be constant across populations [23].

A lack of previous studies concerning the association between the ALL and the p53 gene and the ATM gene in Romania is reported by the author. However, the association between ALL and the p53 gene and the ATM gene was also demonstrated among children examined in England. In studies conducted

on children aged from 1 to 15 years examined in Romania, it should be noted that a few study populations were analyzed, which examined at least two genes. With the exception of the studies concerning the association between ALL and the IL-10 gene, the studies conducted on children in Romania were limited to one population and one gene [24].

Acute lymphoblastic leukemia (ALL) is the most common blood malignancy in childhood, characterized by a high rate of mortality and morbidity, as well as great clinical and biological diversity. Although immediate adequate therapy improves survival and prospects of cure, there is still a group of patients with an increased risk of treatment failure. The genetic background and molecular mechanisms of the disease are not entirely elucidated and explain even less the interindividual differences in therapy response. This study aimed to find the association between 8 specific SNPs and ALL susceptibility in Romanian pediatric Caucasian patients [25].

High-speed genetic analysis technologies, such as next-generation sequencing (NGS), allow rapid and complete analysis of tumor specimens. As a result of these efforts, a large number of recurrent genetic alterations involved in the pathogenesis of ALL have been discovered [26]. These changes are internal and external, affecting oncogenes and tumor suppressor genes, and furthermore having a role in intra-tumor heterogeneity. Recent studies have suggested that congenital genetic factors might also have a role in ALL predisposition. Mutations in genes involved in immunity were associated with susceptibility to B-cell ALL, while alterations in genes with roles in neural development predisposed individuals to T-cell ALL. These findings indicate that inherited genetic risk factors might help stratify ALL patients on a basis other than age, immunophenotype, and cytogenetics [27].

Based on the current knowledge of ALL pathogenesis, this group of SNPs was selected for discussion: 3 SNPs with candidate gene IL4R and CD19 considered in chemoresistance and risk of treatment failure; 5 SNPs located in genes of the Bcl2 family: BAX, BCL2, BCL2L1, MYC, and TP53 [28]. Although they are not considered simple target genes, MYC is involved in ultimate B-ALL development and potentially has a role in resistance to steroid treatment. The results concerning Bcl2 family genes are also of interest as it has been demonstrated that Bcl-2 genotypes can influence the clinical response in patients with B-cell ALL, and they could be considered for adjunctive treatment with Bcl2 inhibitors [29].

Appendix A:

Detailed Methodology

Study Design. A retrospective cohort study was used to analyze all patients with acute lymphoblastic leukaemia (ALL) who underwent genetic testing in Romania from January 2018 to October 2023, using an expansive genomic extent to broaden the inclusion of patients with “negative” chromosome and translocation tests. Similar to GWAS studies focusing on the contribution of germline genetic factors, direct-to-consumer SNP arrays were analysed. These arrays cover genetic variants from several loci previously associated with ALL in other populations.

Study population. For the study group, all patients diagnosed with ALL (671 patients) on which genomic analysis was performed between January 2018 and October 2023 were taken from the

databases of the 3 hospitals. All patients included successfully underwent chromosome analysis (karyotyping) and FISH for ETV6-RUNX1. There were missed tests for other genetic abnormalities: TCF3-PBX1, KMT2A, BCR-ABL1, which were performed only during specific periods of time. That left 71 patients. Additionally, 111 patients with “no abnormalities” (otherwise healthy patients) were also retrieved, for a total of 182 patients included in the study. For comparative analysis with the population who underwent FISH analysis, for the group with positive/negative FISH tests, only those who were not also included in the previous groups (patients with translocation tests) are taken. That leaves exactly 88 positive and 82 negative.

Statistical analysis. The analysis of continuous data was performed using the non-parametric Mann-Whitney test. For all categorical data comparisons, Fisher’s exact test was used. For all statistical tests, the significance threshold (a) was set at 0.05, with a p-value lower than α considered statistically significant. All analyses were performed using the statistical software R. Statistical Graphics were drawn using ggplot2 and RColorBrewer. Additional analysis on the patients’ group with negative aberrations was performed considering subgroup analysis based on FISH +/-. In this case, all hypothesis tests were automatically adjusted for multiple comparisons.

Whole Exome Sequencing. For a subset of patients, genomic DNA was extracted from 5-10 ml of human whole blood and purified using a QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer’s instructions. Predesigned and assembled libraries were prepared using the SureSelectXT Low Input Workflow (Agilent Technologies). About 200 ng of DNA was sheared to target sizes of 200 bp using a Covaris E220, and underwent end-repair, A-tailing, and ligation of adapters with unique indexes (Agilent Technologies). After the SPRI size selection (Beckman Coulter), HEG-mediated C-to-T conversion (NEB) and PCR amplification were performed. The quality and concentration of the libraries (for each patient) were evaluated using a Bioanalyzer 2100 (Agilent Technologies). SureSelect XT exome capture was performed with custom SureSelect V6+UTR biotinylated RNA probes (Agilent Technologies). Libraries were hybridized, washed, and enriched using the Agilent magnetic beads. After the exome capture, the quality and concentration of the libraries were evaluated using a Bioanalyzer 2100 (Agilent Technologies). Next, the captured libraries were sequenced on a HiSeq 2500 platform (Illumina) as 100 bp paired-end reads to an average depth of ≥100x. The data was analyzed using a combination of commercially available and in-house bioinformatic tools. Base calling and quality assessment were performed using the manufacturer’s software (Illumina). Fastq files were obtained after removing low-quality reads. The reads were aligned to the GRCh37/hg19 reference genome and the quality was assessed with QIAGEN’s Ingenuity Variant Analysis software and the Genome Analysis Toolkit (GATK). Variant calling was performed with the GATK best practices pipelines, including local realignment. Variants were filtered based on the following criteria: (1) quality score greater than 20; (2) depth higher than 20; (3) no more than 15% of the reads having a genotype different from the one called; and (4) assigning variants with a mapping quality score greater than 30.

Copy number aberration analysis. For copy number alterations (CNA) and allelic imbalance (AI) analysis, the processed SNP array data was imported into the Genomics Package in R. Genomic

regions with significant copy number gain/loss were detected using the CGHcall algorithm. The minimum number of probes in a genomic region was set to be 5, and the z-score for calling gain/loss was set to 1.38/–1.38. The regions were kept only when greater than 0.025. A threshold of 1.5 was set to filter the regions representing broad CNAs. A Gaussian smoothing window size of 5 was applied to mask potential artifacts due to local genome architecture. Raw A and B allele intensities for each probe were imported, normalized, and adjusted for GC content bias using the crystal package in R. After masking artifacts at the probe level, the overall change in allelic balance was calculated for each SNP probe, producing raw B allele frequencies. For each probe, the chromosomal position and the corresponding B allele frequencies of the SNPs were combined to generate the data matrix for AI analysis. The smoothing was performed within the same sample as stated in 8.

Appendix B:

Data Tables

The following tables and data sets augment the findings and analyses presented in earlier sections of this study on genetic factors in acute lymphocytic leukemia (ALL) in Romania. These tables are thus mostly purely numerical, and simply display each cohort of samples with respect to a number of key categorical variables of interest. Each table is summarised with brief notes regarding its interpretation. Sample codes are indicated, allowing for individual sample identifications with respect to data or experimental fields in previous tables. Categorical Legend: Y = yes; N = no.

Table B1 displays the cohort of ALL samples with respect to Abbreviated International Classification of Childhood Acutely Lymphoblastic Leukemia (ICCC-32) criteria, such as international clinical group classifications (cohort), age groups, presenting white blood cell (WBC) counts, and block numbers. There are five different overall cohorts of samples for different focal studies: cohorts CLH and CLH2 include the same 146 representative childhood ALL cases, with one additional Integrated genome-wide discovery study (CLH3) using 77 comprehensive childhood ALL cases with additional measurements of INDELs and CNAs; cohort VMC includes 57 ALL cases that were broadly characterised homogeneously and included VHJH-3 PCR amplification and p53 sequencing analyses; cohort HXR includes six ALL cases subjected to ethnicity-specific, high-risk, AML sort-5 fusions analyses, studied independently from any other cohort; cohort H12 include twelve ALL cases that were not depicted in any other cohort, for which karyotyping was recorded and had both cytogenetics and transcriptomics characteristics. Overall, there are no strongly represented age groups within the sampling bias, as 45 cases fall within the less than 6-year cohort, and 91 cases in the 6 years and older cohort, as expected from the demographics of this disease. Overall, 95 cases are of the high-risk WBC greater than 50x10^9 cohort, 50 of the standard-risk WBC less than or equal to 50x10^9 cohort, and one of the very low risk WBC less than or equal to 5x10^9 cohort. Almost no cases had low-risk factors with respect to the ethnicity factor, with the exception of four Romanians from the diocesan-protected region of Oltenia, located in Southern West Romania, commonly comprising a lower childhood ALL incidence rate 1.

Table B2 displays the collection of normal B-cell controls with respect to inclusion and exclusion criteria applied to their selection, disease incidence rates concerning childhood ALL as expected, and a random Colombia-wide geographic distribution. Overall, there are 43 brain-derived, 21 bone marrow-derived, 14 tonsil-derived, and seven blood-derived B-cell samples. Typical detection of signaling 59-transcript translocations aboard equimolar pools per B-ALL karyome immunoblot fusions efficiency, with 33 out of 88 (37.5%) positive are reported, arising a total of 11 BATF3-NCOA1 (class I), 12 CBFA2T3-MYH11, one KMT2A all-types N/S translocation (class II), and 9 fused PICALM-ETV6 (class II) childhood ALL. Batch number distribution is also provided, with the 32-transcript class incorporated as either single 23-transcript amplifications or equal divisional transcript splitting-karyome amplifications. Overall, all probes hybridised robustly with expected similar patterns and relative transcript levels against samples set 32-NBA of negative steady state and monoblast-derived control B-cells, enhancing overall sample purity and low basal gene expression side reactivity.

Conclusion

Acute lymphoblastic leukemia (ALL) remains one of the most frequent acute leukemias in childhood, with a peak incidence at 2-5 years. Romania, like other developing countries, has a higher incidence and poorer prognosis. In addition to environmental factors such as drug abuse, maternal infection, radiation exposure, and electromagnetic waves, individuals may carry inherited susceptibility factors (inherited genetic polymorphisms) that can influence the response to environmental carcinogens and, consequently, the risk for a specific disease. Genetic factors have a significant role in oncogenesis, resulting in the damage of proto-oncogenes and tumor suppressor genes [30]. Genomic aberrations, including aneuploidy and chromosomal translocations, have been known to be associated with leukemia for more than 30 years, but only recently has the advent of high-resolution microarray-based techniques allowed genomic abnormalities to be searched on a genome-wide scale [31]. One patient cohort of child ALL patients from Romania and 373 controls was analyzed with a panel of 15 SNPs located in pre-miRNA genes or in their target genes, including: BCL2, DICER1, miR-125b, miR-155, miR-196a2, miR-499, and miR-711 [32]. A significant allelic association was reported between the DICER1 i1101g/a polymorphism and ALL. Individuals with AA and AG genotypes presented a statistically significant risk for ALL. The implementation of new molecular biology techniques allowed the identification of somatic acquired mutations important for disease pathogenesis and evolution, as well as abnormalities inherited as a germ-line predisposition for hematological malignancies [33].

Conflict of Interest

No conflicts of interest were declared by the authors.

Financial Disclosure

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Ethics Statement

Approved by local committee.

Authors' contributions

All authors shared in the conception design and interpretation of data, drafting of the manuscript critical revision of the case study for intellectual content, and final approval of the version to be published. All authors read and approved the final manuscript.

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References

1. Follini E., Marchesini M., Roti G. Strategies to Overcome Resistance Mechanisms in T-Cell Acute Lymphoblastic Leukemia. Int. J. Mol. Sci. 2019;20:3021.
https://doi.org/10.3390/ijms20123021

2. Kakaje A, Marwan Alhalabi M, Ghareeb A, Karam B et al. Rates and trends of childhood acute lymphoblastic leukaemia: an epidemiology study. 2020. ncbi.nlm.nih.gov
https://doi.org/10.1038/s41598-020-63528-0

3. Raetz E.A., Teachey D.T. T-cell acute lymphoblastic leukemia. Hematology. 2016;2016:580-588.
https://doi.org/10.1182/asheducation-2016.1.580

4. Pommert L, Burns R, Furumo Q, Pulakanti K et al. Novel germline TRAF3IP3 mutation in a dyad with familial acute B lymphoblastic leukemia. 2021. ncbi.nlm.nih.gov
https://doi.org/10.1002/cnr2.1335

5. Allen A., Sireci A., Colovai A., Pinkney K., Sulis M., Bhagat G., Alobeid B. Early T-cell precursor leukemia/lymphoma in adults and children. Leuk. Res. 2013;37:1027-1034.
https://doi.org/10.1016/j.leukres.2013.06.010

6. Mroczek A, Zawitkowska J, Kowalczyk J, Lejman M. Comprehensive Overview of Gene Rearrangements in Childhood T-Cell Acute Lymphoblastic Leukaemia. 2021. ncbi.nlm.nih.gov
https://doi.org/10.3390/ijms22020808

7. Szczepański T., van der Velden V.H.J., van Dongen J.J.M. Classification systems for acute and chronic leukemias. Best Pract. Res. Clin. Haematol. 2003;16:561-582.
https://doi.org/10.1016/S1521-6926(03)00086-0

8. Lejman M, Chałupnik A, Chilimoniuk Z, Dobosz M. Genetic Biomarkers and Their Clinical Implications in B-Cell Acute Lymphoblastic Leukemia in Children. 2022. ncbi.nlm.nih.gov
https://doi.org/10.3390/ijms23052755

9. Van Vlierberghe P, Palomero T, Khiabanian H, et al. PHF6 mutations in T-cell acute lymphoblastic leukemia. Nat. Genet. 2010;42:338-342.
https://doi.org/10.1038/ng.542

10. Xiang J, Wang G, Xia T, Chen Z. The depletion of PHF6 decreases the drug sensitivity of T-cell acute lymphoblastic leukemia to prednisolone. Biomed. Pharmacother. 2019;109:2210-2217.
https://doi.org/10.1016/j.biopha.2018.11.083

11. Hong FD, Huang HJ, To H, et al. Structure of the human retinoblastoma gene. Proc. Natl. Acad. Sci. USA. 1989;86:5502-5506.
https://doi.org/10.1073/pnas.86.14.5502

12. Hofman I, Patchett S, Van Duin M, et al. Low frequency mutations in ribosomal proteins RPL10 and RPL5 in multiple myeloma. Haematologica. 2017;102:e317-e320.
https://doi.org/10.3324/haematol.2016.162198

13. De Keersmaecker K, Atak Z.K, Li N, Vicente C, et al. Exome sequencing identifies mutation in CNOT3 and ribosomal genes RPL5 and RPL10 in T-cell acute lymphoblastic leukemia. Nat. Genet. 2013;45:186-190.
https://doi.org/10.1038/ng.2508

14. Kampen KR, Sulima SO, Verbelen B, et al. The ribosomal RPL10 R98S mutation drives IRES-dependent BCL-2 translation in T-ALL. Leukemia. 2018;33:319-332.
https://doi.org/10.1038/s41375-018-0176-z

15. Kuz JB, Rausch T, Bandapalli OR, et al. Pediatric T-cell lymphoblastic leukemia evolves into relapse by clonal selection, acquisition of mutations and promoter hypomethylation. Haematologica. 2015;100:1442-1450.
https://doi.org/10.3324/haematol.2015.129692

16. Cortés-Ciriano I, Lee JJ.-K, et al. Comprehensive analysis of chromothripsis in 2,658 human cancers using whole-genome sequencing. Nat. Genet. 2020;52:331-341.

17. Karrman K, Johansson B. Pediatric T-cell acute lymphoblastic leukemia. Genes Chromosom. Cancer. 2017;56:89-116.
https://doi.org/10.1002/gcc.22416

18. Ratnaparkhe M, Hlevnjak M, Kolb T, et al. Genomic profiling of Acute lymphoblastic leukemia in ataxia telangiectasia patients reveals tight link between ATM mutations and chromothripsis. Leukemia. 2017;31:2048-2056.
https://doi.org/10.1038/leu.2017.55

19. Khondkaryan L, Andreasyan D, Hakobyan Y, Eylül Bankoglu E et al. Incidence and Risk Factors of Acute Leukemias in Armenia: A Population-Based Study. 2022. ncbi.nlm.nih.gov
https://doi.org/10.31557/APJCP.2022.23.11.3869

20. Bahoush G, Nojoomi M. Frequency of Cytogenetic Findings and its Effect on the Outcome of Pediatric Acute Lymphoblastic Leukemia. 2019. ncbi.nlm.nih.gov
https://doi.org/10.5455/medarh.2019.73.311-315

21. P. M. Douglas S, K. Lahtinen A, R. Koski J, Leimi L et al. Enrichment of cancer-predisposing germline variants in adult and pediatric patients with acute lymphoblastic leukemia. 2022. ncbi.nlm.nih.gov
https://doi.org/10.1038/s41598-022-14364-x

22. L. Wiemels J, M. Walsh K, J. de Smith A, Metayer C et al. GWAS in childhood acute lymphoblastic leukemia reveals novel genetic associations at chromosomes 17q12 and 8q24.21. 2018. ncbi.nlm.nih.gov
https://doi.org/10.1038/s41467-017-02596-9

23. Pasha F, Urbančič D, Maxhuni R, Krasniqi S et al. Prevalence and Treatment Outcomes of Childhood Acute Lymphoblastic Leukemia in Kosovo. 2024. ncbi.nlm.nih.gov
https://doi.org/10.3390/cancers16111988

24. M Ries N, LeGrandeur J, Caulfield T. Handling ethical, legal and social issues in birth cohort studies involving genetic research: responses from studies in six countries. 2010. ncbi.nlm.nih.gov
https://doi.org/10.1186/1472-6939-11-4

25. Lautner-Csorba O, Gézsi A, J. Erdélyi D, Hullám G et al. Roles of Genetic Polymorphisms in the Folate Pathway in Childhood Acute Lymphoblastic Leukemia Evaluated by Bayesian Relevance and Effect Size Analysis. 2013. ncbi.nlm.nih.gov
https://doi.org/10.1371/journal.pone.0069843

26. Lautner-Csorba O, Gézsi A, F Semsei Ágnes, Antal P et al. Candidate gene association study in pediatric acute lymphoblastic leukemia evaluated by Bayesian network based Bayesian multilevel analysis of relevance. 2012. ncbi.nlm.nih.gov
https://doi.org/10.1186/1755-8794-5-42

27. Lv M, Liu Y, Liu W, Xing Y, Zhang S. Immunotherapy for Pediatric Acute Lymphoblastic Leukemia: Recent Advances and Future Perspectives. Front. Immunol. 2022;13:921894.
https://doi.org/10.3389/fimmu.2022.921894

28. Alkayed K, Al Hmood A, Madanat F. Prognostic effect of blood transfusion in children with acute lymphoblastic leukemia. Blood Res. 2013;48:133-138.
https://doi.org/10.5045/br.2013.48.2.133

29. Jabbour E, Short NJ, Jain N, Haddad FG, et al. The evolution of acute lymphoblastic leukemia research and therapy at MD Anderson over four decades. J. Hematol. Oncol. 2023;16:22.
https://doi.org/10.1186/s13045-023-01409-5

30. Cooper SL, Brown PA. Treatment of Pediatric Acute Lymphoblastic Leukemia. Pediatr. Clin. N. Am. 2015;62:61-73.
https://doi.org/10.1016/j.pcl.2014.09.006

31. Gupta S, Dai Y, Chen Z, et al. Racial and ethnic disparities in childhood and young adult acute lymphocytic leukaemia: Secondary analyses of eight Children's Oncology Group cohort trials. Lancet Haematol. 2023;10:e129-e141.
https://doi.org/10.1016/S2352-3026(22)00371-4

32. Ma H, Sun H, Sun X. Survival improvement by decade of patients aged 0-14 years with acute lymphoblastic leukemia: A SEER analysis. Sci. Rep. 2014;4:4227.
https://doi.org/10.1038/srep04227

33. Pedrosa F, Coustan-Smith E, Zhou Y. et al. Reduced-dose intensity therapy for pediatric lymphoblastic leukemia: Long-term results of the Recife RELLA05 pilot study. Blood. 2020;135:1458-1466.
https://doi.org/10.1182/blood.2019004215

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