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ORIGINAL ARTICLE

CREBBP IS A MAJOR PROGNOSTIC BIOMARKER FOR RELAPSE IN CHILDHOOD B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA: A NATIONAL STUDY OF UNSELECTED COHORT

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ABSTRACT

Although the identification of disease subtypes conveying prognostic significance along with minimal residual disease (MRD) assessment represent cornerstones for stratification in childhood acute lymphoblastic leukemia (ALL), approximately half of the relapses occur in patients from standard-risk groups. Identification of the drivers of treatment failure is crucial for detection of high-risk clones at diagnosis. We evaluated clinical variables and the most common genetic alterations in an unselected cohort of 55 patients with B-ALL treated according to the ALL-IC-BFM 2002 protocol, with a median follow-up of 46 months. Matched diagnosis-relapse samples underwent screening for additional alterations using whole-exome sequencing. Mutations in the *CREBBP* gene were found in 80% (4/5) of the patients with relapse, either present from the disease onset or acquired at relapse, while none of the examined patients in remission presented alterations in this gene. Deletions in *TP53* and *EBF1* (present in 2/5 and 1/5 of the patients with relapse, respectively) were infrequent or absent in the patients in remission, respectively. Screening for alterations in the *CREBBP* gene at diagnosis and/or at multiple time-points during chemotherapy could be incorporated into treatment protocols, as it may contribute to the identification of significant number of patients with predefined or acquired chemoresistant clones.

Keywords: acute lymphoblastic leukemia, relapse, molecular biomarkers, *CREBBP*

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most prevalent form of cancer among children, comprising 25% of all childhood malignancies, with a consistently increasing incidence rate over the years [1, 2]. It arises following the clonal proliferation of immature B and/or T lymphoid cells, with around 80% of the cases being of B lineage origin [3, 4]. The most common initial genetic lesions are chromosomal loss (hypodiploidy), gain (hyperdiploidy), or fusion genes, leading to a pre-leukemic clone. A subsequent second hit, either a copy number alteration (CNA) or single nucleotide variant (SNV), is believed to be the cause of lymphoid arrest and the development of symptomatic disease [4, 5].

Conventional (karyotyping, fluorescence in situ hybridization - FISH) and molecular (reverse transcription quantitative polymerase chain reaction - qRT-PCR, multiplex ligation-dependent probe amplification - MLPA) tech-

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niques are routinely used for the identification of numerical and structural chromosomal abnormalities, allowing for the detection of several disease subtypes with different prognostic and therapeutic associations [3, 5, 6]. Among these, high-hyperdiploidy is the most common subtype in childhood B-ALL (25-30%), which is associated with favorable prognosis [6-8]. The structural chromosomal abnormalities involve genes that regulate hematopoiesis and lymphoid development (*RUNX1, ETV6*), activate oncogenes (*MYC*), or constitutively activate tyrosine kinases (*ABL1*). In particular, recurring translocations leading to different subtypes in B-ALL include t(12;21)(p13;q22) encoding ETV6::RUNX1 associated with favorable prognosis, t(1;19)(q23;p13) encoding TCF3::PBX1 with intermediate prognosis, t(9;22)(q34;q11) encoding BCR::ABL1, and rearrangements of *MLL* at 11q23 with different partner genes, both associated with poor prognosis [7, 8]. More recently, genomic profiling has led to the identification of new abnormalities that are not detectable by conventional methods, resulting in more than 20 disease subtypes [9, 10].

The risk classification at diagnosis of patients with ALL varies among different treatment protocols, but in general this includes the patient's age, white blood cell (WBC) count, and presence of specific disease subtypes. In general, patients are classified as standard-risk if diagnosed at the age 1-5, presenting a WBC count of < 20 x 10^9/L and the absence of iAMP21, *IKZF1* deletion or *CRLF2* overexpression. The high-risk group includes patients with either poor prednisolone response, hypodiploidy, BCR::ABL1, TCF3::HLF or MLL::AFF1 subtypes, or any other *MLL* rearrangement in patients younger than 1 year, while all other patients are considered intermediate-risk. Although the identification of disease subtypes conveying prognostic significance along with minimal residual disease (MRD) assessment represent cornerstones for disease stratification, approximately half of the relapses occur in patients from standard-risk groups [11]. Additionally, it has been shown that more than half of the relapse samples have at least one genetic alteration originating either from the leukemic or the pre-leukemic clone, potentially affecting disease progression and therapy response [12,13]. Many of these alterations affect transcription factors (*ETV6*, *PAX5*, and *IKZF1*), epigenetic regulators (*ATF7IP*, *SETD2*, *KMT2D*, and *CREBBP*), cell cycle regulators (*CDKN2A/B*, *BTG1*, and *RB1*), RAS pathway genes (*KRAS*, *NRAS*, and *PTPN11*), and the tyrosine kinase *FLT3* [14, 15]. Identification of the drivers of treatment failure is crucial for detection of high-risk clones at the time of diagnosis, which can also contribute to uncovering new therapeutic targets, personalization of the treatment protocol and reduction of the short- and long-term adverse effects of intensified chemotherapy.

In this prospective observational national study, we present the clinical variables, identify the most common molecular biomarkers and the individual therapy response (MRD) data, as well as their relation to the clinical status in a cohort of 55 children with B-ALL. Additionally, we conduct a more comprehensive analysis of the patients who experienced disease relapse using whole exome sequencing to detect other alterations that may prove useful in risk stratification and to potentially discover new altered pathways that could be targeted therapeutically.

MATERIALS AND METHODS

Clonal rearrangements detection and MRD analysis

All patients (a total of 55) included in the study were diagnosed with B-ALL at the University Clinic for Pediatric diseases in Skopje over a period of 6 years (2018-2023). For the molecular clonality analyses, nucleic acids were extracted from bone marrow mononuclear cells using an automated nucleic acid extractor (MagCore Super, RBC Bioscience, New Taipei City). The clonal immunoglobulin (Ig) gene rearrangements were identified using multiplex PCR according to the BIOMED-2 protocol [16]. The dominant rearrangements (from the diagnostic and relapse samples) were subsequently sequenced with the specific family primers using the Sanger sequencing method, and the sequences were analyzed using the IMGT/V-QUEST and IgBLAST tools. The initial clonal rearrangements were also detected using Next-Generation Sequencing (NGS) method with the LymphoTrack Dx IGH-FR3 assay (Invivoscribe Technologies, San Diego, CA, USA) according to the manufacturer's protocol. These clones were tracked for MRD analysis at two time-points, on day 33 and day 78 from therapy onset, with a sensitivity threshold at 10^(-4) [17].

Hybrid transcripts and copy number alterations

The most common gene rearrangements, including t(12;21)(p13;q22) ETV6::RUNX1, t(1;19)(q23;p13) TCF3::PBX1, t(9;22)(q34;q11) BCR::ABL1, and t(4;11)(q21;q23) MLL::AFF1 were detected using qRT-PCR according to the BIOMED-1 protocol [18]. Aneuploidy was assessed using the SALSA MLPA P036 and P070 subtelomeric probe-mix kits (MRC-Holland, Amsterdam, the Netherlands) following the manufacturer's recommended procedures. High-hyperdiploidy or hypodiploidy were defined if >50 or <44 chromosomes were present, respectively. Identification of copy number alterations in specific regions and genes with prognostic significance in B-ALL was performed using the P327, P329, P335, and P038 SALSA MLPA kits, which allowed for the detection of intrachromosomal amplification of chromosome 21 (iAMP21), *EGR* deletion, deletions or duplications in the

PAR1 region (*CRLF2*, *CSF2RA*, *IL3RA*), deletion of *TP53*, deletion of *IKZF1*, and deletions or duplications in genes associated with B-cell differentiation and cell cycle control (*CDKN2A/2B*, *PAX5*, *ETV6*, *RB1*, *BTG1*, and *EBF1*).

Whole exome sequencing

Targeted, massively parallel sequencing of exons in >99% of protein-coding genes (Whole Exome Sequencing, WES) was performed in all patients with relapse (samples obtained both at diagnosis and at relapse) and in five additional patients in remission. The reactions were conducted on an Illumina NovaSeq 6000 sequencer, using the Twist Human Core + RefSeq + Mitochondrial Panel kit (Twist Bioscience, San Francisco, CA, USA) with a mean read depth of targeted regions across samples of approximately 100X. Variant annotation, filtering, and classification of the detected variants were done according to ACMG guidelines, utilizing multiple databases and tools (ClinVar, HGMD, dbSNP, ExAC, gnomAD, OMIM, Varsome, Franklin Genoox).

The patients were treated according to the ALL-IC-BFM 2002 protocol, and the median follow-up was 46 months (range: 11-79). Since this was an observational study, the MLPA analyses and the NGS-MRD data were not used for patient stratification or clinical decisions. The study was approved by the Ethics Subcommittee of the Macedonian Academy of Sciences and Arts, and written informed consent was obtained from the patients' guardians in accordance with the Declaration of Helsinki.

RESULTS

Clinical characteristics and treatment response

All clinical data of the patients included in the study are summarized in Figure 1. The median age at diagnosis was 4 years (range: 0-11). The white blood cell (WBC) count at diagnosis was higher than 20 x 10^9/L in 34% of the patients, while CNS infiltration was a less frequent event (10%). The percentage of patients with poor prednisolone response (PPR; absolute blast count >1000 in pe-

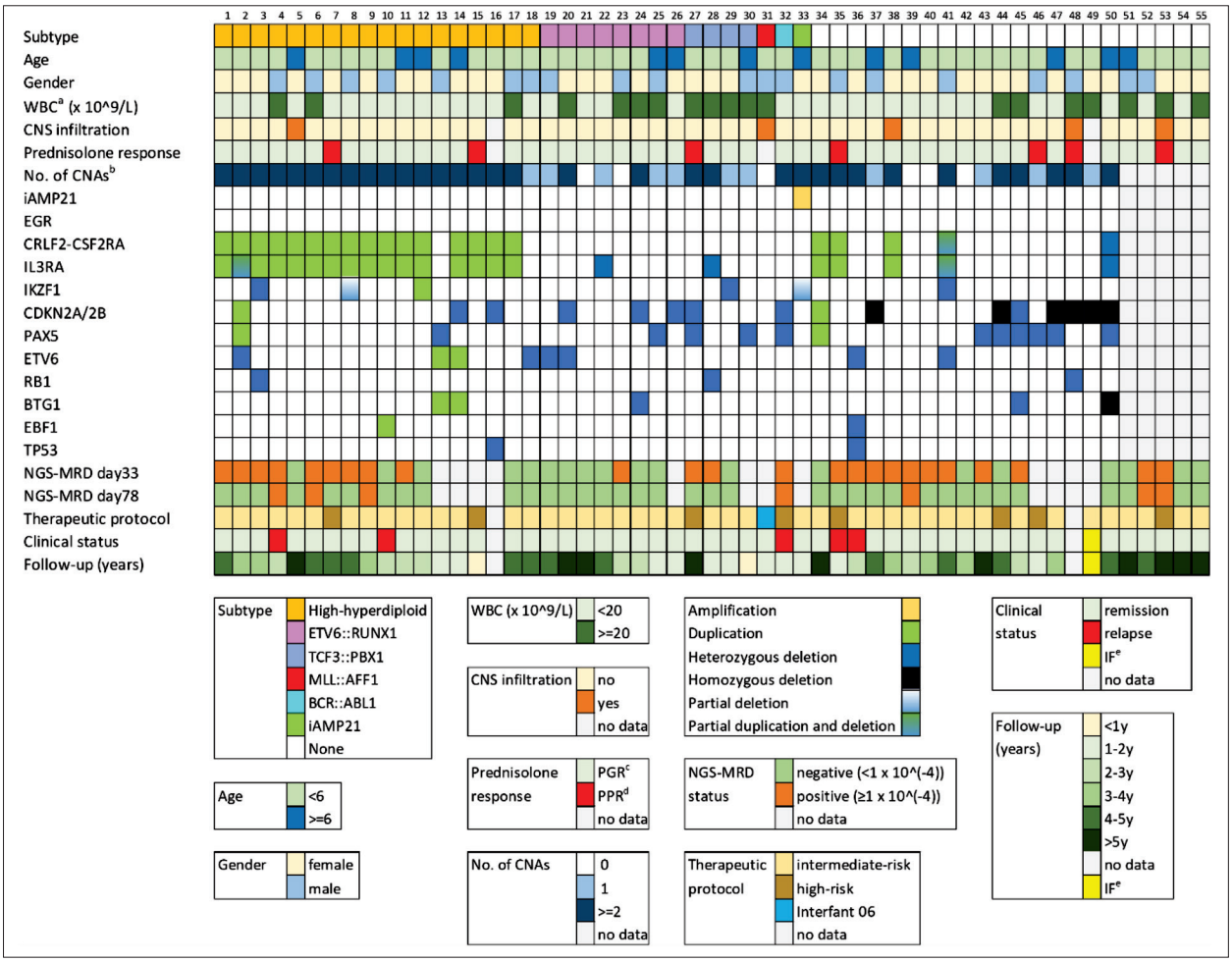

Figure 1: Heatmap of the clinical, molecular and treatment response data in a cohort of 55 pediatric patients with B-ALL
[a] white blood cell count; [b] copy number alterations; [c] prednisolone good response; [d] prednisolone poor response; [e] induction failure

ripheral blood on day 8) was 13%. Following the ALL-IC-BFM 2002 classification [19], 15% of the patients (8/52) were stratified into the high-risk group. One patient (aged <1 year) was enrolled in the Interfant-06 trial, while two patients moved out of the country after diagnosis and were lost for follow-up. The remaining patients received treatment according to the intermediate-risk protocol. NGS-MRD data for two time-points from therapy onset (day 33 and day 78) were available for 43 patients. A positive MRD status (MRD ≥ 1 x 10^(-4)) was detected in 24/43 (56%) and 7/43 (16%) of the patients on days 33 and 78, respectively [17]. During a median follow-up of 46 months, five patients relapsed. All relapses occurred between 17 and 32 months after diagnosis.

Molecular alterations at diagnosis

The most common genetics subtype in the study population was high-hyperdiploidy, present in 33% (18/55) of the patients, followed by the ETV6::RUNX1 subtype in 15% (8/55). Four other patients (7%) were identified with the TCF3::PBX1 subtype, and one patient each (1,8%) with the MLL::AFF1, BCR::ABL1 and iAMP21 subtypes. At diagnosis, at least one copy number alteration in the selected genes was detected in 88% (44/50) of the patients, with 75% of them showing more than two alterations (Figure 1). The most common alteration was the duplication of the PAR1 region (*CRLF2, CSF2RA, IL3RA*), while PAR1 deletion was observed in one patient. Deletions in the *IKZF1* gene were present in 10% (5/50) of the patients, in two of which partial, and none of them presented with the IKZF1^{plus} profile [20]. *CDKN2A/2B* gene deletion was detected in 28% (14/50) of the patients (8 heterozygous, 6 homozygous), while *PAX5* gene deletion in 22% (11/50), co-occurring in six patients. Deletion and duplication of the *ETV6* gene was detected in 6 (12%) and 2 (4%) patients, respectively. *BTG1* gene deletion was observed in three patients (one of them homozygous), while duplication was found in two other patients. *RB1* gene deletion was present in three patients, while the *EBF1* gene was deleted in one patient and duplicated in another. Deletion of *TP53* gene was present in two patients (4%).

CNAs and SNVs in the matched diagnosis-relapse samples

Clinically relevant alterations in the five patients with relapse (No. 4, 10, 32, 35 and 36 from Figure 1), including the findings from the WES analysis, are summarized in Table 1. Among them, two patients were identified with high-hyperdiploidy, one of which had an additional *EBF1* gene duplication; one patient had a BCR::ABL1 hybrid transcript along with *CDKN2A/2B* and *PAX5* gene partial deletions, and one patient was found to have *TP53*, *EBF1*, and *ETV6* gene deletions at diagnosis. The aneuploidy, hybrid transcript, and the *EBF1* and *ETV6* gene alterations were preserved in the matched relapse samples. The deletion of *TP53* was present in both diagnosis and relapse samples in one patient and gained at relapse in another. By contrast, the *CDKN2A/2B* deletions were either gained or lost in the relapse samples, and *PAX5* gene deletion was lost in the relapse sample. Additionally, single nucleotide variants in three genes were involved in the relapse samples, including the epigenetic regulator *CREBBP*, the RAS pathway gene *NRAS*, and the DNA mismatch repair gene *MSH2*. Notably, alterations in these genes were not observed in the five diagnostic samples from patients in remission that were analyzed by WES. Mutations affecting the histone acetyltransferase (HAT) domain of the *CREBBP* gene were persistent in the diagnostic and re-

Table 1. Clinically relevant alterations in the patients with relapse

			Copy number alterations					Single nucleotide variants			
No.[a]	**Sample type**	**Aneuploidy/ Hybrid transcript**	***TP53***	***EBF1***	***CDKN2A/2B***	***PAX5***	***ETV6***	***CREBBP***	***NRAS***	***MSH2***	***FLT3***
4	Dg	high-hyperdiploidy	-/-	-/-	-/-	-/-	-/-	-	-	-	+
	Relapse	high-hyperdiploidy	-/-	-/-	-/-	-/-	-/-	+	+	-	-
10	Dg	high-hyperdiploidy	-/-	-/dup	-/-	-/-	-/-	+	-	-	-
	Relapse	high-hyperdiploidy	-/del	-/dup	-/-	-/-	-/-	+	-	-	-
32	Dg	BCR::ABL1	-/-	-/-	-/del[b]	-/del[b]	-/-	-	-	-	-
	Relapse	BCR::ABL1	-/-	-/-	-/-	-/-	-/-	+	-	+	-
35	Dg		-/-	-/-	-/-	-/-	-/-	+[c]	+	-	-
	Relapse		-/-	-/-	-/del	-/-	-/-	+[c]	-	-	-
36	Dg		-/del	-/del	-/-	-/-	-/del	-	-	-	-
	Relapse		-/del	-/del	-/-	-/-	-/del	-	-	-	-

[a] Patient identification number from Figure 1
[b] Partial deletion
[c] Different amino acid change detected at Dg and relapse

lapse clones in a single patient, acquired at relapse in two patients or involved a change in the same amino acid in this domain in one patient (from R1446H in the diagnostic clone to R1446C in the relapse clone). The p.G12D mutation in the *NRAS* gene was the second most common alteration in these patients, either gained or lost in the relapse samples of two patients, while the mutation in *MSH2* was present in the relapse sample only, of one patient. Additionally, a mutation in *FLT3* was observed in the diagnostic clone in one patient who relapsed, but mutations in this gene were also found in 2 out of 5 patients in remission. The type of SNV for each gene is presented in Table S1.

Clonal evolution of the relapses

Fragment analysis and Sanger sequencing of the immunoglobulin heavy chain genes in the matched diagnosis and relapse samples revealed the presence of the same clonally rearranged sequences at relapse as those observed at diagnosis in four patients (No. 4, 10, 35 and 36) (Table S2). In two of these patients (No. 10 and 36), one of the clonal rearrangements was lost at relapse. Nevertheless, in these two patients, we observed the same genetic alterations in both the diagnosis and relapse samples, with additional gain of *TP53* deletion in one of them. In the other two patients with the same clonal rearrangements, we observed gains (in *CDKN2A/2B*, *CREBBP*, and *NRAS*) and losses (in *FLT3* and *NRAS*) of genetic alterations at relapse. The last patient (No. 32) retained only the BCR::ABL1 hybrid transcript at relapse, lost the *CDKN2A/2B* and *PAX1* deletions, and gained alterations in *CREBBP* and *MSH2*. This patient also showed different clonally rearranged sequences at relapse, presenting three rather than five clonal sequences detected at diagnosis, of which only one was same in both samples (Table S2).

DISCUSSION

This study presents data from all pediatric patients diagnosed with B-ALL in our country over a period of six years. All patients were treated according to the ALL-IC-BFM 2002 protocol, which was escalated to high-risk protocol in eight patients. After a median follow-up of 46 months, five patients (9%) experienced disease relapse. In general, the patients with relapse were diagnosed before the age of 6; none presented with CNS infiltration at diagnosis, and the WBC count was slightly higher than 20 x 10^9/L in only one patient. Initial high-risk features (poor prednisolone response and BCR::ABL1 hybrid transcript) were detected in only two of the five patients with relapse. These findings support the need for inclusion of new molecular biomarkers to help identify the high-risk clones at diagnosis and redefine the stratification [6,21].

We identified the presence of alterations in the *CREBBP* gene in 80% (4 out of 5) of the patients with relapse in our cohort, all of which occurred in the HAT domain of the gene. These alterations were found at the time of initial diagnosis in 2 and at relapse in another 2 patients. Notably, none of the specimens from patients in remission featured alterations in this gene. The *CREBBP* gene has been recognized as one of the most common relapse-enriched genes in ALL [22-24], and its association with relapse is particularly evident in the high-hyperdiploid subtype [10, 25]. Alterations in this gene affect the response to one of the key components of the treatment protocol, glucocorticoids, leading to a treatment failure [22]. The mechanisms through which CREBBP contribute to glucocorticoid resistance are considered to be associated with its activity as a transcriptional co-activator, which interacts with the glucocorticoid receptor (GR) and potentially modulates its transcriptional activity. When glucocorticoids bind to the GR, the receptor undergoes a conformational change allowing it to interact with co-activators like CREBBP, which in turn can acetylate histones and other chromatin regulators, promoting a more accessible chromatin structure and facilitating gene transcription of target genes involved in processes like apoptosis. Therefore, alterations in this gene can lead to disruption of normal cellular processes including transcriptional regulation, chromatin remodeling and apoptosis. Importantly, mutations in the *CREBBP* gene also have a therapeutic significance, as it has been found that different CREBBP inhibitors and histone deacetylase inhibitors can alleviate chemotherapy resistance and may become a successful approach for the treatment of relapsed ALL [26].

We also detected alterations in several other genes with potential prognostic value. These include deletions in *TP53* in two patients with relapse (in one of them relapse-specific) which were infrequent in the rest of the patients, and deletion in *EBF1* gene in one patient with relapse which was absent in the rest of the patients in our cohort. Previous studies have also associated these alterations with disease progression and reduced overall survival rates [3, 5, 22, 27, 28]. Furthermore, *NRAS* gene mutation was found at relapse only, in one of the patients. Mutations in this gene have been observed in high-risk ALL by others and have been reported as important biomarkers for poor relapse-free survival [23, 29, 30]. However, *NRAS* mutation was also lost in the relapse clone in another patient from our cohort, indicating its sub-clonal nature and uncertain role in clonal chemoresistance. By contrast, deletions of *IKZF1* and *CDKN2A/2B* genes, individually reported as high-risk markers for disease progression and correlated with poor outcome in several studies [31-34], were not found to independently influence prognosis in our study. Their prognostic significance has been further refined with the detection of the

IKZF1^{plus} profile [20] which, however, was not present in our cohort. Additionally, the occurrence of the *IKZF1* deletion in the high-hyperdiploid subtype, which was also associated with an increased relapse risk in a recent large prospective study [10], was detected in one patient in our cohort, who is still in remission after a follow-up of 46 months.

Concerning the evolutionary mechanisms of the clones from diagnosis to relapse, we found that none of the patients with relapse in our cohort experienced expansion of a novel clonal population completely distinct from the population present at diagnosis. Rather, in most of them (4 out of 5), in addition to the same clonal rearrangement and initiating genetic event (CNA, hybrid transcript), we observed novel alterations at relapse that were not detected in the primary clone/s. This either indicates that they were present in minor sub-clones, not detectable with the applied method, survived chemotherapy, and arose as dominant clone/s due to the presence of chemotherapy resistance mutations, or that they were acquired during chemotherapy (treatment-induced) [13, 22]. However, the absence of other alterations in three of these patients suggests that the clonal evolution from an ancestral sub-clone was probably the mechanism for relapse, which has also been described as the most frequent event by others [13, 24, 35]. Only one patient retained all the alterations within the diagnostic and relapse clones, suggesting a linear evolution [35].

The strengths of this study are that it includes an unselected cohort of pediatric patients with B-ALL who were uniformly treated, and that it provides comprehensive data for all patients along with detailed molecular characterization for those with relapse. The limitations of the study include the lack of complete knowledge of the frequency of SNVs at diagnosis in patients without relapse, the insufficient depth of the WES analysis to detect mutations present in minor sub-clones (<10%), and the relatively small patient cohort.

In conclusion, we identified that alterations in the epigenetic regulator *CREBBP* were the most frequent event in the patients with relapse, either appearing at diagnosis or being acquired at relapse. Screening for alterations in this gene at the beginning, and/or at multiple time-points during chemotherapy, could be incorporated into treatment protocols, as they may contribute to the identification of significant number of patients with predefined or acquired chemoresistant clones. In addition, detection of deletions in the *TP53* and *EBF1* genes in the *CREBBP*-negative patients could further help identify patients at increased risk for relapse. Finally, screening for clinically actionable alterations in these and other pathways and genes (RAS, MMR genes), could be of substantial significance for patients with relapse in the coming years by offering a more individualized targeted therapy or immunotherapy therapeutic approach.

SUPPLEMENTARY MATERIALS

Table S1: Type of the single nucleotide variants detected in the patients with relapse.

Table S2: The fragment lengths and Sanger sequencing details (V-D-J genes and CDR3 sequences) of the clonal immunoglobulin heavy chain gene rearrangements present in the matched diagnosis-relapse samples.

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Author Contributions

Conceptualization, A.D., S.K. and N.M.G.; Methodology, E.K.B., M.S.S. and E.G.; Software, E.K.B; Formal Analysis, E.K.B., M.S.S and E.G; Investigation, A.D., S.K., N.M.G. and E.K.B.; Resources, S.K. and A.J.; Writing – Original Draft Preparation, E.K.B.; Writing – Review & Editing, A.D., S.K. and N.M.G.; Supervision, A.D., S.K. and N.M.G.

Conflicts of Interest

The authors declare no conflict of interest.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Subcommittee of the Macedonian Academy of Sciences and Arts (date of approval 03.04.2023).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

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