Next-generation sequencing-based genomic profiling of pediatric acute myeloid leukemia and inherited myeloid malignancies

PhD thesis

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1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous malignancy caused by the malignant transformation of the stem cell precursors of the myeloid lineage, characterized by the impaired maturation and uncontrolled proliferation of the leukemic blast cells. Leukemia is the most common type of malignancy in childhood, accounting for 25 to 30% of cancers, of which acute lymphoblastic leukemia (ALL) is the most common subtype, as it constitutes 75% to 80% of cases. While pediatric AML is a rare disease as it is responsible for only 15% to 20% of cases, it accounts for a disproportionate more than 40% of childhood leukemic mortality. In Hungary, the annual incidence rate of AML among children is approximately ten, according to the Hungarian Childhood Cancer Registry.

The overall survival (OS) rate of children with AML has increased significantly in the past decades. In the 1970s, 5-year OS was about 10%, while nowadays, it reaches around 70% in developed countries. Still, compared to the treatment of ALL, where the 5-year OS of children now exceeds 90%, the survival rate of children with AML seems suboptimal.

Accurate prognostication is one of the key factors for the successful treatment of AML. The outcome of AML varies widely, and both patient-related and disease-related factors influence an individual's prognosis. The latter includes the cytogenetic and molecular genetic profile of the leukemic cells as well as the quality of response to treatment. Establishing the molecular characteristics of AML is crucial as both cytogenetics and molecular genetics are significant independent predictors of prognosis and are the mainstay of the World Health

Organization (WHO) classification of AML. With the advent of high-throughput genomic technologies, the identification of molecular alterations has revealed considerable molecular heterogeneity, both between and within individual patients, and expanded our knowledge of mechanisms underlying leukemogenesis, clonal expansion, and treatment resistance of leukemic cells. Comprehensive genomic characterization of the AML genome revealed that the pathogenesis of AML is more complex than previously appreciated. Genes recurrently mutated in AML belong to various functional groups or pathways.

The widespread utilization of next-generation sequencing (NGS) led to the comprehensive characterization of patients with AML and the discovery of novel, clinically relevant recurrent molecular aberrations, some of which represent important prognostic and predictive biomarkers. Identifying targetable lesions meant that the therapeutic arsenal for the management of AML has expanded significantly in recent years. However, these advances are mainly seen in adult patients, and they are not fully reflected in the treatment of children with AML. Although the genomic background of AML has been widely studied, pediatric AML is less characterized, as only a limited number of studies have focused on investigating the molecular landscape of children with AML. From these studies, it became evident that the spectrum of genetic alterations in pediatric and adult AML patients is remarkably different, suggesting that the pathogenesis of pediatric AML differs from that of adult AML. Consequently, not all findings related to adult AML patients apply to children, justifying the need to further investigate the mutation profiles of children with AML.

Familial predisposition to hematopoietic malignancies was initially described as a rare entity; however, several novel predisposition genes have been identified due to the increased use of NGS. Recently published studies using high-throughput sequencing revealed that germline predisposition to myeloid neoplasms is more common than previously appreciated, as an estimated 5% to 10% of patients with hematological malignancy have an underlying genetic predisposition. Since 2016, myeloid neoplasm with germline predisposition has been included in the WHO classification as a unique entity.

The diagnosis of hereditary predisposition to myeloid neoplasms has profound clinical significance for the patients and their relatives, as it influences therapeutic decision-making, the choice of transplant preparative regimens for the patient, proper genetic counseling, and cancer surveillance for affected family members. Inherited myeloid neoplasms show significant heterogeneity in penetrance, age of onset, and clinical characteristics. People carrying variants linked to germline predisposition have an elevated lifetime risk of developing myeloid malignancies. The clinical outcome of germline variant carriers can differ widely even within family members with the same variant, suggesting that acquiring somatic mutations influences individuals' risk for developing hematopoietic malignancy. Despite advances in the identification of germline variants, our understanding of the pathogenesis, including the additional somatic mutational landscape presumably responsible for variability in penetrance, and recognition of individuals at the highest risk for developing myeloid malignancies, remains incomplete.

2. Objectives

During my PhD research, we aimed to:

1. To perform comprehensive genetic characterization of pediatric patients diagnosed with AML using panel-based targeted next-generation sequencing for the first time in a Hungarian patient cohort.

2. To investigate the cytogenetic profiles of pediatric AML patients and the relationship between cytogenetic alterations and somatic mutations.

3. To evaluate the clinical impact of the identified genetic alterations on event-free and overall survival.

4. To provide a comparative analysis of genetic alterations detected in relapsed patients at the time of diagnosis and relapse.

5. To evaluate the mutational landscape of inherited myeloid malignancies with the aim of uncovering genetic lesions contributing to disease development

3. Methods

3.1. Patient samples

Seventy-five patients with pediatric AML were included in this study, with a female:male ratio of 1:1.2. Diagnostic bone marrow (n=70) or peripheral blood (n=2) samples from 72 children diagnosed with AML, as well as skin (n=1) or lymph node (n=2) samples from 3 children diagnosed with extramedullary AML, were analyzed. Diagnoses were established based on morphologic, immunophenotypical, and genotypical criteria at the Department of Pathology and Experimental Cancer Research, Semmelweis University, in the Department of Pathology, University of Pécs, or in the Department of Pathology, University of Debrecen, between 2003 and 2021 according to the classification system of WHO. Patients were risk-stratified and treated according to Berlin-Frankfurt-Münster (BFM) protocols. In addition to the diagnostic samples of 75 patients, nine samples drawn at the time of the first relapse and three samples drawn at the second relapse were also investigated. The median follow-up time was 23.8 months (range, 0.2 to 205.0 months). The study was approved by the Ethics Committee of the Hungarian Medical Research Council (IV/51-1/2022/EKU). Written informed consent from the patients and/or from the parents or guardians were obtained for the study, which was conducted in accordance with the Declaration of Helsinki.

In the frame of an international collaboration, DNA samples from well-characterized MDS/AML families ≥2 members were diagnosed with a hematological disorder, of which ≥1 case was

specified as MDS/AML were included in this study. Overall, DNA samples from 51 individuals from 33 families were available. Of the 51 individuals, 16 were diagnosed with AML, 22 MDS, 1 thrombocytopenia (TCP), 7 bone marrow failure (BMF), 1 lymphoedema, 1 had abnormal lymphocyte subsets, and 3 were asymptomatic carriers. Each individual carried a germline variant in 1 of 13 discrete loci known to predispose to familial MDS/AML. The median age of onset was 30 years (range, 1–76 years). The age of onset of MDS/AML was highly variable and dependent on the nature of the germline mutations. In addition to our cohort, we performed an integrative analysis of the acquired mutations in published families harboring germline variants in the same 13 predisposing loci. Overall, 395 MDS/AML patients from 64 publications were included in the analysis.

3.2. Cytogenetic analysis

Cytogenetic aberrations were determined by conventional G-banding and fluorescence *in situ* hybridization (FISH) to detect abnormalities related to AML. Probes for t(8;21)(q22;q22) *RUNX1::RUNX1T1*, inv(16)(p13.1q22)/t(16;16)(p13.1;q22) *CBFB::MYH11*, t(15;17)(q22;q12) *PML::RARA*, and 11q23 *KMT2A* rearrangements were used for FISH analysis. A complex karyotype was defined as three or more chromosomal aberrations in the absence of the recurrent AML genetic aberrations defined by using the WHO classification, including t(8;21)(q22;q22), inv(16)(p13.1q22)/t(16;16)(p13.1;q22), t(15;17)(q22;q12), t(6;9)(p23;q34.1), *KMT2A*/11q23 rearrangement, or t(9;22)(q34;q11.2).

3.3. Assessment of *FLT3*-ITD and *CEBPA* Mutational Status

Mutation analysis of *FLT3*-ITD was performed from genomic DNA using primers adapted from Kottaridis et al. Fluorescently labeled PCR products were analyzed using a capillary electrophoresis 3500 Genetic Analyzer (Thermo Fisher Scientific) and GeneMapper software 5 (Thermo Fisher Scientific). The mutant *FLT3*-ITD allelic burden was calculated as the ratio of area under the mutant versus wild-type *FLT3* peak. For the assessment of the mutation status of *CEBPA*, the entire coding sequence was amplified by using three overlapping PCR fragments with previously described primer pairs and analyzed by bidirectional Sanger sequencing.

3.4. Targeted next-generation sequencing and bioinformatic analysis

Targeted NGS was performed using the TruSight Myeloid Sequencing Panel (Illumina, San Diego, CA) covering 54 leukemia-associated genes for both the pediatric AML and inherited myeloid malignancy cohort. Individual libraries were prepared from 50 ng of genomic DNA according to the manufacturer's recommendations. DNA extracted from peripheral blood mononuclear cells of 15 healthy volunteers with normal complete blood profiles was used as negative control subjects. After quality control and equimolar pooling, libraries were sequenced on a NextSeq 550 platform (Illumina) using v2.5 chemistry with 150 bp paired-end configuration. For the pediatric AML cohort, raw sequencing data generated from TruSight Myeloid libraries were analyzed by using the TruSeq Amplicon app in BaseSpace Sequence Hub (Illumina). After demultiplexing and FASTQ file generation, reads were aligned

against the GRCh37 reference human genome with a custom banded Smith-Waterman aligner. Single-nucleotide polymorphisms and short insertions or deletions were identified using the Genome Analysis Toolkit. Variants were further processed by using a custom Snakemake pipeline. For reliable detection of high-confidence mutations for each sample, variants were kept if they met all variant filtering criteria.

3.5. Statistical analysis

Event-free survival (EFS) and overall survival (OS) were estimated using the Kaplan-Meier method and compared statistically using the log-rank test. Complete remission (CR) was defined as <5% blasts in the bone marrow, no evidence of leukemia at any other site, and evidence of regeneration of normal hematopoietic cells. OS was calculated from the date of diagnosis to exit or last follow-up. EFS was calculated from the date of diagnosis to the first event (induction failure, relapse, or death) or to the date of the last follow-up (death from early toxicity was excluded). Patients who failed to achieve CR on day 60 were considered treatment failures.

4. Results

4.1 Genomic characterization of patients with pediatric AML

4.1.1. Cytogenetic profiles of pediatric AML patients at diagnosis

Cytogenetic results were available in 71 out of 75 patients, with normal karyotype detected in 22.5% (n=16) of the patients. *KMT2A*-rearrangements were the most frequently observed (n=16, 22.5%) cytogenetic aberrations, followed by CBF-rearrangements (n=10, 14.1%): t(8;21) translocations (n=7) and inv(16)/t(16;16) alterations (n=3). Eleven patients had karyotypes associated with adverse prognosis (complex karyotype, monosomy 7, t(6;9), or inv(3)). Twenty percent of the patients (n=15) were characterized by 'other' aberrations (i.e. cytogenetic abnormalities not classified in the above-mentioned subgroups). The cytogenetic categories showed age-related distribution with younger children harboring *KMT2A*-rearrangement and 'other' aberrations more frequently, while the abundance of CBF-rearrangements and adverse karyotype increased with age.

4.1.2. Targeted NGS-based mutation profiling of pediatric AML at diagnosis

Targeted NGS with an average allelic depth of 4,960x revealed a total of 154 single-nucleotide variants (SNVs) and short insertions/deletions in the diagnostic samples of 74 patients. The number of mutations identified in patients at diagnosis showed uneven distribution across specific cytogenetic subgroups,

although these differences were not statistically significant. Median number of mutations per patient was 2.0 (range: 0-18), with the highest rate of mutations in CN-AML (3.0) and the lowest in *KMT2A*-rearranged AML (1.0). In terms of mutation classes, missense and frameshift mutations were the most commonly detected, followed by duplications and splice site variants. The most common class of mutations involved genes controlling kinase signaling (36.7%; n=65) and encoding transcription factors (20.9%; n=37), followed by chromatin modifiers (15.3%; n=27), tumor suppressors (9.6%; n=17), DNA methylation (5.6%; n=10), cohesion genes (5.6%; n=10) and RNA splicing (3.4%; n=6). Figure 1 depicts the pairwise co-occurrence of the mutations in the different functional subgroups. Mutations associated with activated signaling commonly emerged together with mutations of genes encoding transcription factors and epigenetic modifiers, while *NPM1* and epigenetic modifier mutations were mutually exclusive.

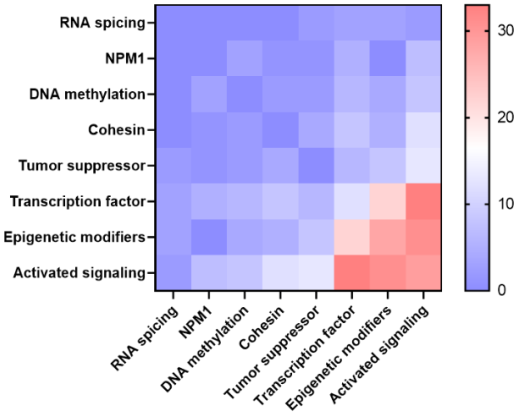
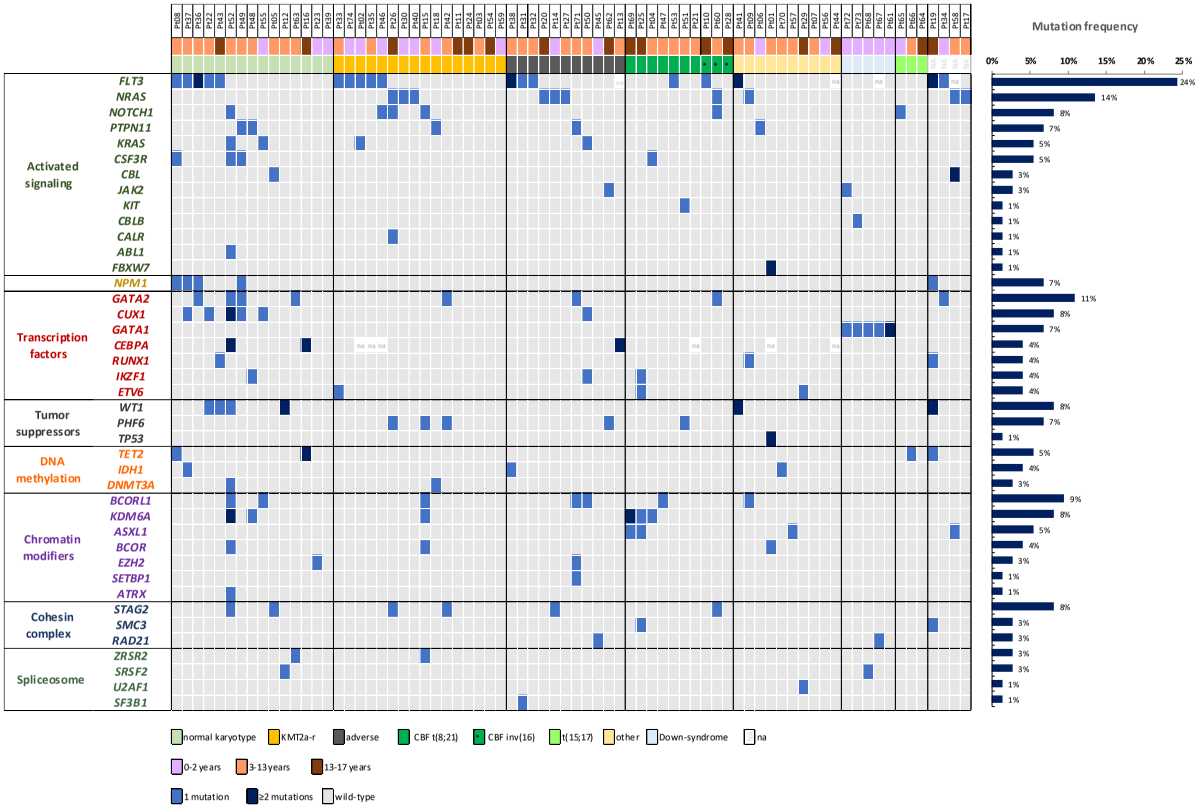


Figure 1. *Heat map illustrating the pairwise co-occurrence of molecular aberrations based on the functional groups.*

Overall, 83.8% of patients (62/74) carried at least one mutation in genes analyzed by NGS. Considering all genetic alterations detected by different modalities, including cytogenetics, aberrations were identified in 98.6% (73/74) of patients. Of the 54 genes examined, 40 genes were altered in our cohort, with 17 genes recurrently mutated in over 5% of patients. *FLT3* (24%, 18/74), *NRAS* (14%, 10/74), and *GATA2* (11%, 8/74) represented the most frequently mutated genes (Figure 2). *FLT3*-ITDs were detected in 14.9% (11/70) of patients, including a total of 17 *FLT3*-ITD mutations with a median allelic ratio of 0.09 (range: 0.02 - 4.91). *FLT3*-ITD mutations were predominantly present in patients with CN-AML (27%; 4/15) and CBF-AML (20%; 2/10).

KDM6A mutations were present in 8.1% of patients (6/74) at diagnosis, with a close association with t(8;21). Enrichment of *KDM6A* mutation has been described in adult CBF-AML patients; however, it has not been reported in pediatric patients before. Besides genes with known mutational hotspots (*NRAS*, *FLT3*, *IDH1*, *ASXL1*, *SRSF2*), recurrent variants were detected in *CUX1* and *BCORL1* genes. *CUX1* mutations were detected in 8% (6/74) of patients, predominantly associated with CN-AML (5/6; p=0.0013). Nine percent (7/74) of patients carried *BCORL1* mutations, including a recurrent frameshift mutation (c.2541del p.Ser848ValfsTer5) identified in 3 patients previously not reported. Novel mutations were identified in *NPM1* (c.869_873delinsCCCTTTCCC) and in *CEBPA* (c.950_953delinsACCTT p.Leu317HisfsTer4; c.691_701del p.Val232AlafsTer85) genes.


Mutation frequency
0%
5%
10%
15%
20%
25%
Activated signaling
FLT3
NRAS
NOTCH1
PTPN11
KRAS
CSF3R
CBL
JAK2
KIT
CBLB
CALR
ABL1
FBXW7
NPM1
Transcription factors
GATA2
CUX1
GATA1
CEBPA
RUNX1
IKZF1
ETV6
Tumor suppressors
WT1
PHF6
TP53
DNA methylation
TET2
IDH1
DNMT3A
Chromatin modifiers
BCORL1
KDM6A
ASXL1
BCOR
EZH2
SETBP1
ATRX
Cohesin complex
STAG2
SMC3
RAD21
Spliceosome
ZRSR2
SRSF2
U2AF1
SF3B1
24%
14%
8%
7%
5%
5%
3%
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3%
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8%
3%
3%
3%
3%
1%
1%
normal karyotype
KMT2a-r
adverse
CBF t(8;21)
CBF inv(16)
t(15;17)
other
Down-syndrome
na
0-2 years
3-13 years
13-17 years
1 mutation
≥2 mutations
wild-type

***Figure 2**. Heat map displaying the somatic variants detected in the 54 target genes analyzed in the diagnostic samples of 74 pediatric AML patients. Illustrated is the distribution of the somatic variants, age groups, and cytogenetic profiles as determined by karyotyping or FISH, as well as the mutation frequency of the individual genes for all cases.*

4.1.3. Comparative analysis of mutation profiles at diagnosis and relapse

To uncover progression-related changes in mutational profile, targeted NGS analysis was performed on matched diagnosis-relapse samples of 8 patients with pediatric AML. The relapse samples carried a slightly higher number of mutations compared to the diagnostic samples, with an average of 2.5 mutations (range: 1-6) per sample in the relapsed cohort (vs 2.0 at diagnosis). 61.5% (8/13) of initially detected mutations persisted at relapse, 38.5% (5/13) of mutations were detected only in the diagnostic sample, while 65.4% of mutations (17/26 relapse mutation) emerged during disease progression. Mutations that persisted from diagnosis to relapse had a higher VAF at diagnosis compared with those that were eliminated at relapse (median VAF at diagnosis: 30.7% vs 10.9%). However, due to the relatively limited number of samples analyzed, this did not reach statistical significance. At relapse, mutations were *identified* in 16 genes with *WT1* (42%; 5/12), *FLT3* (42%; 5/12), *NRAS* (33%; 4/12) and *NPM1* (25%; 3/12) representing the top four affected genes. Comparison of the function of the affected genes at diagnosis and relapse unveiled enrichment of mutations of genes affecting tumor suppression (44.4% vs. 16.2%) and transcription factors (55.6% vs. 35.1%) at relapse.

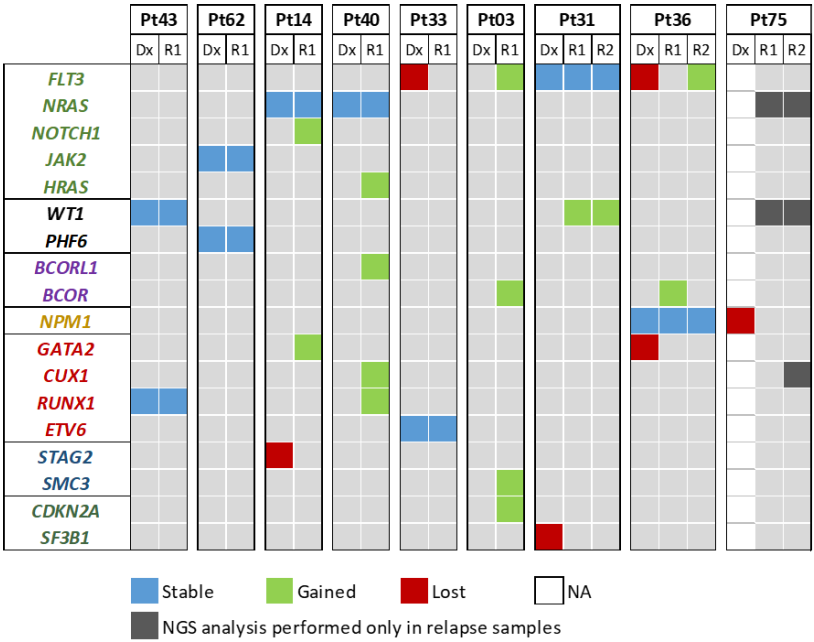

Figure 3. *Heat map displaying the mutational status of nine patients at the time of diagnosis and relapse. Stable mutations (i.e. present at diagnosis and relapse) and unstable mutations (i.e. present either only at diagnosis or at relapse) are shown with different colors.*

In five patients, relapsed AML evolved from one of the subclones detected at the initial diagnosis and was accompanied by several additional mutations that were absent or present at a lower allele frequency in the diagnostic sample, indicating the multistep process of leukemia recurrence. In contrast, two patients relapsed early with seemingly identical clones suggesting the incomplete eradication of leukemic cells by initial treatment.

4.1.4. Evaluation of clinical outcomes of patients based on molecular profiles

At 5 years, the EFS and OS for the whole cohort proved to be 50.0% and 56.2%, respectively, with a median follow-up time of 23.8 months (range: 0.2-205). Risk classification based on cytogenetics and mutational status of *NPM1*, *CEBPA*, *FLT3*-ITD, and *WT1* revealed that patients in the favorable-, intermediate, and adverse-risk categories had significantly different 5-year EFS and OS (EFS: 90% vs. 30% vs. 18%, $p<0.0001$; OS: 90% vs. 42% vs. 22%; $p=0.0014$). Multiparametric flow cytometry measurable residual disease (MRD) follow-up data were available from 61 patients. CR after 2 courses of intensive chemotherapy was reached by 91.8% (56/61) of patients, while three patients experienced fatal complications during the induction therapy, and two patients were non-responders. Due to the small number of patients, assessment of the prognostic significance of individual mutations was limited; only mutations in tumor suppressor genes (*TP53*, *PHF6*, and *WT1*) were significantly associated with induction failures ($p=0.0039$; Fisher's exact test) and shorter three-year EFS (22.2% vs 61.2%, $p=0.044$; Fisher's exact test) (Figure 4). Risk stratification based on AML-BFM protocols was the following: patients with *RUNX1::RUNX1T1*, *CBFB::MYH11*, *PML::RARA*, normal karyotype with *NPM1* or biallelic *CEBPA* mutations were categorized in the favorable subgroup. Patients with complex karyotype (i.e. >3 aberrations), monosomy 7, t(6;9), inv(3), t(6;11), t(10;11) or *FLT3*-ITD with *WT1* mutation belonged to the adverse risk group, with all remaining patients categorized as intermediate risk. The 5-year OS was 90.0% for patients with favorable prognosis, 42.3% for

patients classified in the intermediate risk group and 22.2% among patients with unfavorable prognosis (p=0.0014), while the EFS values in these risk groups proved to be 90.0%, 30.0 and 18.5%, respectively (p<0.0001).

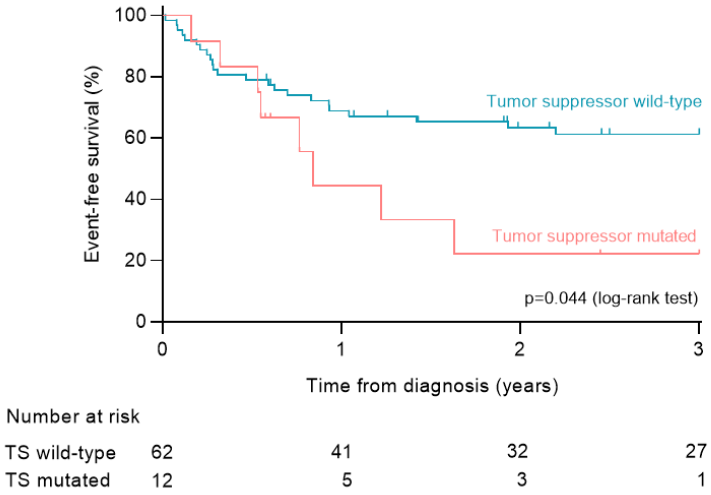

***Figure 4**. Three-year OS rates of patients with early (blue) and late (yellow) relapses. OS was calculated from the time of relapse. Patients with late relapses had significantly superior survival. Log-rank P value is indicated.*

In our cohort, 25.3% (19/75) of patients experienced relapse. Our cohort is representative of patients with relapsed AML in terms of time to relapse rates, as approximately half of the relapses (57.9%; 11/19) were early relapses, occurring within 1 year after diagnosis. Comparing the 3-year OS from the time of relapse of patients with early and late relapse unveiled that patients with early relapse have significantly worse prognosis

(9.1% vs. 87.5%; p=0.0004), suggesting that identification and quantification of MRD may be of great importance as early intervention and change of therapy may improve the clinical outcome of these patients.

4.2 Assessment of acquired mutations in inherited myeloid malignancies

Targeted NGS with a mean allelic depth of 1,300 revealed a total of 78 acquired mutations corresponding to 27 genes. At least one acquired mutation was identified in 28/51 individuals. The median variant allele frequency of the acquired mutations was 11.7% (range, 5.2–99.5%).

To better understand the spectrum of acquired mutations in inherited myeloid disorders, 395 MDS/AML patients from 64 publications were included in the analysis. The integrated results showed marked heterogeneity of acquired mutations in familial myeloid malignancies. We found a high frequency of second-hit *RUNX1*, *CEBPA*, and *DDX41* mutations in patients harboring these germline mutations. Monosomy 7, trisomy 8, *STAG2*, and *ASXL1* mutations were recurrently detected in *GATA2* families, while *CEBPA* families usually acquired secondary *GATA2* mutations. Acquired *TP53* mutations were present in a strikingly high 75% of patients with germline *SBDS* mutation, several of whom encountered multiple *TP53* mutations.

Our study reveals a distinctive mutational pattern in *TERT* families for the first time. Four families (FML) with germline *TERT* variants were included in our series (FML038, FML039, FML040, and FML041), with a total of six individuals with

aplastic anemia (AA) (n=2), MDS (n=3), and AML (n=1), and three asymptomatic carriers. A complex pattern of inheritance was observed in FML040, suggesting that germline variants at three discrete loci, including *TERT*, *NOTCH1*, and *GATA2*, may determine the clinical presentation in this family (Figure 5). Two offspring inherited *TERT* and *NOTCH1* (p.Pro2128Leu) variants from the asymptomatic mother, including the index case who developed secondary AML (II.4), and her sister diagnosed with AA (II.1). Sibling II.1 also inherited a germline *GATA2* variant (p.Pro14Ser) that was also present in her asymptomatic sister (II.2) which we assume was inherited from her father (I.II). Interestingly, in the index case with secondary AML (II.4) acquired mutations in *NOTCH1* (p.Glu1636Lys, Ala1634Asp) and *GATA2* (p.Phe400Leu) at low VAF could also be detected. Therefore, our current model proposes that the onset of overt symptoms may depend on the presence of both *NOTCH1* and *GATA2* variants since the symptom-free mother (I.1) harbors a single *NOTCH1* variant and the asymptomatic sibling (II.2) carries a single *GATA2* variant. In support of this model, we found that the index case in family FML039 (III.1), with AA and having a germline *TERT* variant, also retained two acquired *NOTCH1* mutations (p.Pro2551fs, p.Ser2499delinsSerPro) and a *GATA2* mutation (p.Glu398Lys) with low VAFs.

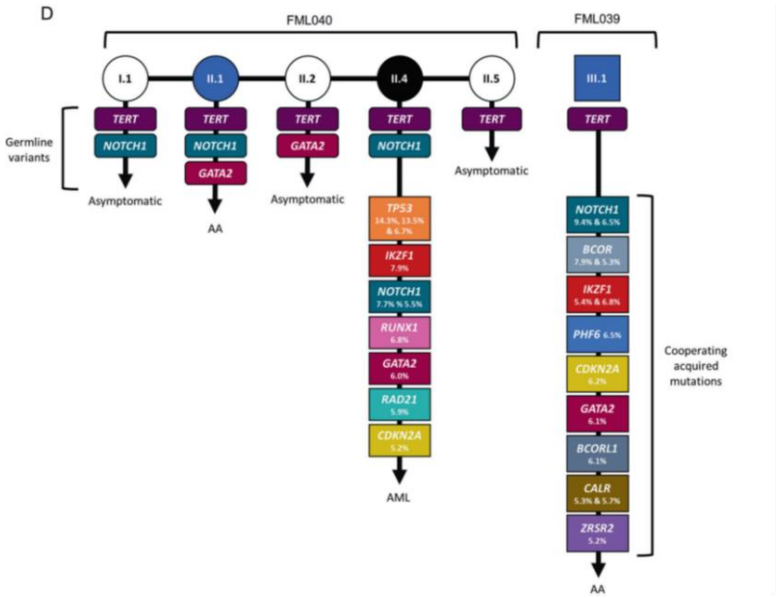

***Figure** 5. Variants identified in TERT families, FML040 and FML039. Germline variant pattern (TERT, GATA2, NOTCH1), cooperating acquired mutations with corresponding VAF and patient phenotype (AML, AA, or asymptomatic) are indicated.*

5. Conclusions

Novel findings of my thesis are the following:

1. We determined the mutational profile of a relatively large cohort of Hungarian children diagnosed with AML by targeted next-generation sequencing for the first time.
2. Three novel mutations in *NPM1* and *CEBPA* genes and a recurrent *BCORL1* mutation in three patients were identified.
3. *BCORL1*, *CUX1*, *KDM6A*, *PHF6*, and *STAG2* were found to be more frequently mutated in pediatric AML than previously appreciated. Low VAFs of these mutations suggest that these alterations are late events in leukemogenesis.
4. *CUX1* was recurrently mutated in cytogenetically normal AML, while *KDM6A* mutations are significantly enriched in AML with *RUNX1::RUNX1T1* fusion.
5. Mutations of tumor suppressor genes (*PHF6*, *TP53*, *WT1*) were found to be associated with induction failure and a trend towards shorter EFS.
6. Comparing samples from diagnosis and relapse revealed significant enrichment of mutations in tumor suppressor genes and genes involved in epigenetic modification at relapse.
7. Acquired mutational landscape of individuals with germline predisposition to myeloid malignancy showed marked heterogeneity.

8. Our study identified a novel mutational signature in *TERT* families for the first time.
9. The combination of *NOTCH1* and *GATA2* mutations may act as a risk factor for developing myeloid disease in patients with germline *TERT* variants.

6. Bibliography of the candidate's publications

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