Original Article

CHARACTERISATION OF MUTATIONS IN CYTOGENETICALLY NORMAL ACUTE MYELOID LEUKAEMIA PATIENTS WITH TRIPLE-NEGATIVE *FLT3-ITD/ NPM1/CEBPA* SUBGROUP USING TARGETED NEXT GENERATION SEQUENCING

Zahidah **Abu Seman**[1], Nor Rizan **Kamaluddin**[1], Noor Atiqah **Fakharuzi**[1], Ermi Neiza **Mohd Sahid**[1], Ezalia **Esa**[1], and Yuslina **Mat Yusoff**[1*]

1. Haematology Unit, Cancer Research Centre, Institute for Medical Research, National Institutes of Health, Ministry of Health Malaysia, 40170 Shah Alam, Selangor, Malaysia

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***Corresponding author:**
Yuslina Mat Yusoff
yuslina.my@moh.gov. my
Tel: +603-33627869

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ABSTRACT

Genetic aberrations play a pivotal role in acute myeloid leukaemia (AML) pathogenesis and clinical outcomes, with mutations in key genes such as *NPM1, FLT3*, and *CEBPA* well-established markers for the disease prognosis. Despite advancements in molecular diagnostics, a comprehensive analysis of the mutational landscape in AML is still needed using advanced techniques. This study aimed to analyse the mutational landscape of cytogenetically normal AML (CN-AML) with triple-negative *FLT3-ITD/NPM1/CEBPA* subgroup using targeted NGS techniques. Targeted next-generation sequencing (NGS) was employed to comprehensively explore the mutational spectrum of CN-AML in a cohort of 14 patients. In this study, six putative pathogenic or likely pathogenic variants, as well as seven variants of uncertain significance (VUS), were detected in nine CN-AML patients. Among these patients, a single mutation was observed in six cases, two mutations in one case, and three mutations in two cases each. *DNMT3A* and *RUNX1* were present in three cases each, followed by *SRSF2* and *NRAS*, which were found in two cases each. Additionally, *CEBPA, TET2, TP53,* and *U2AF1* were each observed in one case. Targeted NGS analysis revealed a heterogeneous mutational spectrum in CN-AML patients with recurrent alterations, highlighting their potential impact on disease progression. These findings improve our understanding of the genetic landscape of CN-AML and may contribute to refining prognostic classification and risk stratification.

KEYWORDS: acute myeloid leukaemia, next-generation sequencing, *NPM1* gene, *FLT3* gene, *CEBPA* gene

INTRODUCTION

Acute myeloid leukaemia (AML) arises from genetic alterations disrupting normal haematopoietic processes, leading to uncontrolled proliferation and impaired apoptosis within the bone marrow microenvironment (1). Cytogenetic and molecular markers are potent prognostic factors in AML, guiding treatment decisions (2, 3). In recent years, the identification of several genetic mutations has led to the categorisation of AML into subtypes with distinct prognostic implications, particularly in those with cytogenetically normal AML (CN-AML) (4). Among these mutations, alterations in nucleophosmin 1 (*NPM1*), fms-like tyrosine kinase 3 internal tandem duplication (*FLT3*-ITD), and CCAAT/enhancer-binding protein alpha (*CEBPA*) genes have emerged as key determinants of disease outcome (5-8).

FLT3-ITD is identified in approximately 25% of AML and 18%–38% of CN-AML patients and are associated with adverse outcomes (9-13). *NPM1* mutations confer a favourable prognostic outcome and are present in approximately 30% of adult AML cases, with a higher prevalence of 50-60% observed specifically in cases of cytogenetically normal AML (CN-AML) (14-15). *CEBPA* mutations are observed in 10% to 15% of AML cases and *CEBPA* mutations within the basic leucine zipper domain (bZIP domain) are linked to favourable outcomes (16-18). CN-AML patients with either *FLT3-ITD* mutations alone or both *FLT3*-ITD and *NPM1* mutations are classified as intermediate risk. In contrast, CN-AML patients without *FLT3-ITD* mutations but with *NPM1* or *CEBPA* bZIP domain mutations have a favourable prognosis (19).

An unresolved issue pertains to the prognostic significance of the triple-negative group within CN-AML patients, characterised by the absence of *NPM1* mutations, *FLT3*-ITD mutations, and *CEBPA* mutations. Despite being classified as intermediate risk, the clinical course and response to therapy in this subgroup may vary widely, highlighting the need for further investigation into the molecular mechanisms underlying their disease pathogenesis and prognosis (20-21). Technological advancements, particularly next-generation sequencing (NGS), have revolutionised the interrogation of AML's mutational landscape (22). Targeted NGS panels offer comprehensive profiling of genetic alterations, providing valuable insights for clinical decision-making (22-25). This study aimed to analyse the mutational landscape of CN-AML with triple-negative *FLT3*-ITD/*NPM1*/*CEBPA* subgroup using targeted NGS techniques.

MATERIALS AND METHODS

Sample selection

This cross-sectional study was conducted from March to November 2015. Samples were obtained from CN-AML patients whose specimens were submitted to the Diagnostic Laboratory of Haematology at the Institute for Medical Research (IMR) between January and December 2014. As a national referral centre for mutational analysis of *NPM1*, *FLT3*, and *CEBPA*, the laboratory receives samples from across the country. DNA samples from CN-AML patients were initially extracted and tested for these mutations during a routine diagnostic procedure. For this study, only CN-AML samples confirmed as triple-negative for *NPM1*, *FLT3*, and *CEBPA* were selected, resulting in a total of 14 patient samples. However, the selection of samples from referred patients at IMR may introduce selection bias and limit the generalizability of the findings to the broader CN-AML population in Malaysia. Additionally, budget constraints further restricted the sample size.

Sample size estimation

Due to budget constraints and the high cost of NGS, this preliminary study included 14 samples. As this is a preliminary study, no formal power calculation was conducted. The primary aim was to explore initial findings and generate hypotheses for future larger studies, particularly regarding key mutational profiles in the CN-AML triple-negative subgroup.

Targeted NGS

a) Library preparation

Target enrichment was performed on 50 ng of input DNA using the HaloPlex^{HS} system (Agilent Technologies, Santa Clara, CA), which is a high-sensitivity, amplicon-based targeted sequencing method incorporating the molecular barcoding system (MBS) in the DNA library. Library was prepared based on the manufacturer's protocol for ClearSeq AML^{HS}, ILM, which was designed to target 48 selected exons in 20 genes (*ASXL1, CSF3R, CEBPA, CBL, DNMT3A, EZH2, FLT3, IDH1, IDH2, JAK2, MPL, NPM1, NRAS, SETBP1, SF3B1, SRSF2, RUNX1, TET2, TP53, and U2AF1*) found to be commonly mutated in AML and to be associated with myelodysplastic syndromes and myeloproliferative neoplasms. Sequencing was performed using the MiSeq sequencer using the MiSeq Reagent Kit v2 (Illumina, San Diego, CA, USA).

b) NGS data analysis

Primary data analysis was performed using MiSeq Reporter to generate a pair of FASTQ files for each sample. FASTQ files were then analysed using Agilent SureCall software, which is used exclusively with the HaloPlex^{HS} system. Briefly, the SureCall software that incorporates Burrows-Wheeler Aligner (BWA), BWA-MEM, Sequence Alignment/MAP(SAMtools), and SNPPET (Agilent Technologies) analyses the molecular barcodes to remove duplicate reads and correct sequencing or PCR amplification errors. The human reference genome build hg19 was used as the reference.

The limit of detection was set at 5%. Variants with allele frequency in gnomAD (>0.1%) and annotation in ClinVar as benign or likely benign were excluded from further analysis. Non-intronic and non-synonymous variants with allele frequencies exceeding 5% were included, while intronic variants and those within the UTR region were omitted from the analysis. Variant pathogenicity was assessed based on data from Catalogue of Somatic Mutations in Cancer (COSMIC) and ClinVar databases. If any variants are not reported in these databases, the pathogenicity of these variants was assessed using computational prediction tools like SIFT, PolyPhen2, and FATHMM. Variants of uncertain significance (VUS) were subjected to additional search using ClinVar and VarSome databases to ascertain their significance.

RESULTS

Patient characteristics

The study cohort included 14 adult AML patients (10 males, 4 females) with a median age of 48.5 years (range: 18–63 years). The median white blood cell (WBC) count was 14.6 × 10^9/L (range: 4.3–56.6 × 10^9/L), and the median blast percentage was 28.5% (range: 3–85%). Median haemoglobin and platelet counts were 8.6 g/dL (range: 5.7–15.2 g/dL) and 79 × 10^9/L (range: 30–438 × 10^9/L), respectively.

Mutation profiling

NGS analysis identified 48 variants, including 32 exonic variants (17 missense, 10 silent, 3 nonsense, and 2 frameshift) and 12 intronic and 2 untranslated region (UTR) variants. Among these, 13 clinically significant variants were classified as pathogenic, likely pathogenic, or VUS, while benign and likely benign variants were excluded from further analysis. Clinically significant variants were identified in 9 out of 14 CN-AML patients. Among these variants, six were classified as pathogenic or likely pathogenic, while seven were categorised as VUS.

Within this patient cohort, there were six cases of single mutations (Patient 1, Patient 13, Patient 74, Patient 89, Patient 91 and Patient 94), one case with two mutations (Patient 138), and two cases with three mutations each (Patient 5 and Patient 141). *DNMT3A* and *RUNX1* were each present in three cases, followed by *SRSF2* and *NRAS* in two cases each. Additionally, *CEBPA*, *TET2*, *TP53*, and *U2AF1* were each observed in one case (Table 1).

Table 1. List of pathogenic variants and VUS identified in 9 patients

Patients	Gene	Codon and protein change	ClinVar	Cosmic ID	SNP ID
1	*DNMT3A* (NM_022552.5)	c.2645G>A (p.Arg882His)	Pathogenic	COSV53036153	rs147001633
13	*TP53* (NM_001276761.1)	c.742C>G (p.Arg248Gly)	Likely pathogenic	COSV52797251	rs121912651
74	*CEBPA* (NM_004364.4)	c.169G>T (p.Glu57Ter)	VUS	N/A	N/A
89	*RUNX1* (NM_001754.5)	c.1036_1037insC (p.Arg346fs*137)	Pathogenic	COSV55873034	rs1601333612
91	*NRAS* (NM_002524.5)	c.203G>T (p.Arg68Ile)	VUS	N/A	N/A
94	*RUNX1* (NM_001754.5)	c. 1272_1275del (p.Ser424fs)	VUS	N/A	N/A
138	*SRSF2* (NM_003016.4)	c.283C>G (p.Pro95Ala)	VUS	COSV57970391	N/A
138	*TET2* (NM_001127208.2)	c.1892C>G (p.Ser631Ter)	VUS	N/A	N/A
5	*DNMT3A* (NM_022552.5)	c.2644C>T (p.Arg882Cys)	Pathogenic	COSV53036332	rs377577594
5	*RUNX1* (NM_001754.5)	c.454A>T (p.Lys152Ter)	VUS	N/A	N/A

5	*SRSF2* (NM_003016.4)	c.283C>A (p.Pro95Thr)	VUS	COSV57970203	N/A
141	*DNMT3A* (NM_022552.5)	c.2645G>A (p.Arg882His)	Pathogenic	COSV53036153	rs147001633
141	*NRAS* (NM_002524.5)	c.38G>A (p.Gly13Asp)	Likely pathogenic	COSV54736416	rs121434596
141	*U2AF1* (NM_006758.3)	c.101C>T (p.Ser34Phe)	Likely pathogenic	COSV52341059	rs371769427

DISCUSSION

This study highlights the complexity and heterogeneity of genetic alterations in AML, emphasizing the importance of employing comprehensive genomic profiling techniques such as targeted NGS. The absence of detectable mutations in the study cohort highlights the limitations of conventional diagnostic approaches. The current study focused on AML patients lacking FLT3-ITD, NPM1, and CEBPA mutations, and utilised NGS to identify other potential genetic mutations for risk stratification.

The current study identified mutations in several genes implicated in tumour suppressors (TP53); transcription factors (RUNX1, CEBPA); RNA splicing (SRSF2, U2AF1); DNA methylation (DNMT3A, TET2) and cell signalling (NRAS). According to the European LeukemiaNet (ELN) 2022 classification criteria, mutations in the RUNX1, SRSF2, U2AF1, and TP53 genes are associated with adverse risk in AML (19). The present study observed 9 out of 14 CN-AML patients harboured clinically significant variants. This highlights the mutational landscape of adult CN-AML cases, aligning with previous studies that have reported distinct genetic profiles in this subgroup (26). However, given the small sample size, further studies with larger cohorts are needed to validate these findings.

TP53 is a tumour suppressor gene crucial for regulating the cell cycle and apoptosis (27, 28). In this study, the variant c.742C>G (p.R248G) was identified and has been classified as likely pathogenic according to ClinVar. *TP53* R248G is a hotspot mutation located within the DNA-binding domain (DBD) of the *TP53* protein (29). Mutations in the DBD region of *TP53*, including the identified variant, are believed to lead to loss of function by impairing the transactivation of p53-dependent genes. Subsequently, these mutations can be categorised as loss-of-function mutations due to their impact on the ability of *TP53* to regulate downstream gene expression (27, 28). The TP53 R248G variant has been detected in patients with AML (30, 31). According to the WHO classification of haematologic tumours, *TP53* alterations serve as prognostic biomarkers in AML with myelodysplasia-related changes. Large-scale studies indicate that patients harbouring *TP53* mutations, including R248G, generally have a less favourable prognosis (32).

RUNX1 is a transcription factor involved in haematopoiesis, and mutations in this gene have been associated with adverse outcomes in AML (33). There were three variants in *RUNX1* identified in this study. One of the variants c.1036_1037insC (p.R346fs*137) has been classified as pathogenic by ClinVar. This mutation was reported to be observed in AML patients. This frameshift alteration is expected to result in the loss of several functional domains of the 480-amino acid Runx1 protein, further intensifying its unfavourable effects on haematopoiesis and contributing to the pathogenesis of AML (33, 34). Moreover, two variants in *RUNX1* were identified, c.454A>T (p.K152Ter) and c.1272_1275del (p.S424fs). Notably, neither of these variants has been previously reported in ClinVar and other databases. These variants have the potential to induce alterations in the protein sequence

and are likely to disrupt the normal structure and function of the *RUNX1* protein, consequently contributing to aberrant haematopoiesis and AML development.

SRSF2 is a critical component of the cellular machinery responsible for RNA processing (35). The present study identified two variants located at position Pro95 (P95) of *SRSF2*, a known mutational hotspot. Both c.283C>G (p.P95A) and c.283C>A (p.P95T) have not been documented in ClinVar but have been reported in COSMIC. Alterations at P95 can affect the functional properties of *SRSF2*, including its RNA binding affinity and splicing activity. Dysregulation of RNA splicing can produce abnormal mRNA isoforms, contributing to the pathogenesis of AML (35, 36).

Another component involved in RNA processing is *U2AF1*, which plays a role in recognizing splice sites during RNA splicing (37). The variant c.101C>T (p.S34F) identified in *U2AF1* has been classified as likely pathogenic and previously associated with AML. Ser34 is a hotspot for mutation in *U2AF1*, and mutations at this location have been widely reported in haematologic malignancies and some solid tumour types (37, 38). Disruption of *U2AF1* function can impair proper splice site recognition, leading to aberrant splicing events and the generation of abnormal mRNA transcripts (37, 38).

DNMT3A and *TET2* are crucial epigenetic regulators involved in DNA methylation/demethylation regulators. Mutations in these genes are frequently detected in pre-leukaemia states and considered early leukemogenesis events (39 - 41). This study identified two variants in *DNMT3A*, namely c.2645G>A (p.R882H) and c.2644C>T (p.R882C), both of which were classified as pathogenic by ClinVar. These alterations have been reported to inactivate *DNMT3A*, leading to diminished methyltransferase activity compared to wild-type *DNMT3A* (40, 42). Additionally, the present study identified one variant in the *TET2* gene, namely the variant c.1892C>G (p.S631Ter). This variant has not been reported in ClinVar but has been previously observed in patients with myelodysplastic syndromes (MDS) (43). Despite numerous investigations into mutations in *DNMT3A* and *TET2*, their impact on the prognosis of AML remains a topic of debate due to conflicting evidence from various studies. Therefore, the current evidence does not justify their classification into a distinct prognostic group according to the ELN 2022 criteria.

Two variants in *NRAS* were identified, c.38G>A (p.G13D) and c.203G>T (p.R68I). The variant c.38G>A (p.Gly13Asp) has been classified as likely pathogenic by ClinVar and has been identified in AML. Extensive studies have investigated this variant as an oncogenic mutation in haematologic malignancies, particularly in specific types of leukaemia. Its expression has been associated with the activation of ERK and Stat5 signalling pathways, contributing to leukaemogenesis (44-46). In contrast, the variant c.203G>T (p.R68I) has not been documented in ClinVar and was identified in a previous study involving patients diagnosed with colorectal cancer (47). Several studies have suggested that *NRAS* mutations are associated with adverse outcomes, others have reported conflicting results (46, 48-50). The impact of *NRAS* mutations on prognosis may depend on various factors, including co-occurring genetic alterations, disease subtype, and treatment response.

The recent ELN 2022 classification has highlighted the importance of specific mutations within the *CEBPA* gene (19). Instead of biallelic mutations, only in-frame mutations in the bZIP domain of *CEBPA* are currently considered favourable prognostic indicators. However, mutations outside this domain, such as those in the N-terminal region identified in this study (c.169G>T (p.E57*)), are not considered to have prognostic significance according to ELN 2022 (16, 17, 19). It is worth noting that the *CEBPA* mutation detected by NGS in this study was not identified in our earlier mutational screening using Sanger sequencing, emphasizing the increased sensitivity of NGS in mutation detection.

The limited number of patients represents a key limitation of this study. A larger cohort would provide a more comprehensive understanding of the genetic heterogeneity in CN-AML. Despite this limitation, this study highlights the importance of expanding genetic investigations to detect less-defined genetic variants in CN-AML, which will enhance the understanding of the disease's genetic landscape. Further exploration of these variants is essential to understand their functional roles and potential influence on disease activity and progression.

CONCLUSION

In conclusion, this study provides insights into the mutational landscape of CN-AML and highlights the importance of NGS detection in improving genetic classification and risk assessment. Future studies are needed to explore the functional significance of identified mutations and their potential impact, which could contribute to more precise prognostic evaluations and therapeutic strategies.

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