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Prevalence of *FLT3*, *NPM1* and *CEBPA* Mutations and Correlation to Haematological Parameters in Newly Diagnosed Adult Acute Myeloid Leukaemia Patients in North and Northeast Regions of Peninsular Malaysia

***Abstract* –** Acute myeloid leukaemia (AML) is a clinically heterogeneous haematological cancer with increased incidence in the elderly. Genetic mutations remain important prognostic factors for treatment outcome. Our study determined the prevalence and clinical significance of *FLT3, NPM1*, and *CEBPA* mutations of AML in the Northern and East Coast of Peninsular Malaysia. Forty-seven de-novo adult patients were analysed for 3 common classical mutations in AML; *FLT3* (exons 14-15 and 20), *NPM1* (exon 12), and *CEBPA* (exon 1) by polymerase chain reaction (PCR), Conformation Sensitive Gel Electrophoresis (CSGE) and Sanger sequencing. The CSGE analysis revealed the following mutation combinations; $NPM1^{+}/FLT3^{-}/CEBPA^{-}$ (17.0%), $NPM1^{-}/FLT3^{-}/CEBPA^{+}$ (10.6%), $FLT3^{+}/NPM1^{-}/CEBPA^{-}$ (4.3%), $NPM1^{-}/FLT3^{+}/CEBPA^{+}$ (4.3%), and $NPM1^{+}/FLT3^{+}/CEBPA^{-}$ (2.1%). c.71666delC, c.71674T>A and c.71683insA mutations were identified in 80%, 60% and 40%, respectively, in addition to *FLT3*-ITD; while c.28026delA (n=3) and c.27837insCAGA (n=4) in *NPM1*; c.5699_5700insACCCGC (n=2) and c.5711C>G (n=2) in *CEBPA*. *FLT3*-ITD and *CEBPA* mutations were significantly correlated with higher blast percentage (p=0.045), higher WBC count (p=0.005), and higher platelet count (p=0.001). $NPM1^{-}/FLT3^{+}/CEBPA^{-}$ (1.0%), $NPM1^{-}/FLT3^{-}/CEBPA^{+}$ (0.8%) and $NPM1^{+}/FLT3^{-}/CEBPA^{-}$ (0.5%) have the longest one-year cumulative overall survival (OS), but not significant (p>0.05). The different types of molecular variants found within the FLT3, *NPM1* and *CEBPA* hotspots, hinting intraclonal diversities which their translocated proteins display distinct features for future prognosis of AML.

***Keywords* –** AML, *FLT3*, NPM1, CEBPA, mutational analysis

1. INTRODUCTION

Acute myeloid leukaemia (AML) is a heterogeneous group of malignant clonal disorder of early haemopoietic progenitor cells in the bone marrow, blood, and other tissues [1]. It is morphologically defined as the presence of at least 20% of myeloblasts (monoblast or megakaryoblast) in the bone marrow or blood at clinical presentation, in exception for AML with specific chromosomal abnormalities, such as (inv16), t(8;21) and t(15;17), and exceptional cases of erythroleukemia [2,3]. AML accounts for 30% of all leukaemias in adults and children, and it represents approximately 20% of acute leukaemias [3,4]. AML is curable in 35-40% of patients 60 years or younger, but lower in 60 years and above with about 5-15% [2]. The low survival rate, about 5-10 months in older patients, is pertinently due to inadequate treatment response and poor outcomes during intensive treatment regimen leading to treatment-related mortality [4]. AML with age 60 years and above have relatively decreased overall survival (OS) and a low rate of complete remission (CR) status [5]. The treatment armamentarium for AML was continuously

expanded over time. The mutation-specific therapies, such as FLT3 inhibitors (e.g., midostaurin, quizartinib, sunitinib, crenolanib and gilteritinib)[6], IDH1 (e.g., ivosidenib), IDH2 (e.g., enasidenib) inhibitors, RAS pathway inhibitors (e.g., MEK1/MEK2 inhbitors (selumetinib and trametinib), AKT inhibitor (GSK2141795-phase II clinical trial) KIT inhibitors (NCT02013648- phase III clinical trial) and TP53 inhibitor (APR-246-NCT03745716-phase III clinical trial), have greatly improved treatment outcomes and OS. A BCL2 inhibitor (venetoclax) have been approved by FDA to use in combination therapy with either low-dose cytarabine (LDAC), azacitidine, or decitabinein older AML patients (≥ 75 years at diagnosis). Gemtuzumab ozogamicin (GO), an antibody–drug conjugate (ADC) of a recombinant IgG4 humanized mAb against CD33 has reapproved by the FDA due to pronounced efficacy in improving median OS rate [7]. A comtemprorary treatment approachs have been recently introduced which focused on refractory or relapsed cases of AML, i.e., (1) Immune-Based Treatment Approach (Checkpoint inhibitors; e.g., CTLA-4 (Ipilimumab), PD-1 (Nivolumab, Pembrolizumab), TIM-3 (MBG453) and CD47 (Magrolimab); (2) Antibody-Drug Conjugate; e.g., CD33 (GO, Vadastuximab) and CD123 (IMGN632), (3) Bispecific-T-Cell Engager; CD123 (Vibecotamab, Flotetuzumab), as monotherapy or in combination [8]. Some investigating agents, i.e., Menin inhibitors (SNDX-5613- in early phase clinical trial) [9] and chimeric antigen receptor T-cells (CAR T-cells) [10]. At present, decisions on therapy are based mainly on prognostic factors identified at the time of diagnosis. Cytogenetics and molecular genetics are the most important independent and reliable prognostic factors in AML. Among the important genetic mutations are FMS-like tyrosine kinase 3-(internal tandem duplication) (*FLT3*-ITD), nucleophosmin (*NPM1*), and CCAAT/enhancer-binding protein α (*CEBPA*) mutations. The combination of conventional cytogenetics and genetic mutations detection is essential as a screening tool and prognostic indicator following the 2010 European LeukemiaNet (ELN) recommendations [11].

It is generally recognised that AML is the result of a multistep process [12] which required three genetic alterations to develop into AML; the class I, II and III mutations. Class I mutation incorporating the mutations that activate signal transduction pathways, leading to increased proliferation and existence of haemopoietic progenitor cells. This group include genes such as *RAS, c-KIT,* and *FLT3* [13]. Class II mutation, which consists of mutations that affect transcription factors and impairing haematopoietic differentiation such as *CEBPA*, MLL (mixed-lineage leukaemia gene) and the *NPM1*. Whereas class III mutation mainly promote epigenetic modifications and this class include genes such as *EZH2, DNMT3A, TET3, ASXL1, IDH1,* and *IDH2* [14,15]. The mutation diversities of *FLT3* gene such as the allelic ratio of mutant to a wild-type, mutation length, insertion site and concomitant mutation impacted the prognosis of AML patients [16-18]. In addition,—the frequency of *NPM1* mutation variant allele was significantly correlated with leukaemic burden albeit with insufficient data [19], are correlated with minimal-residual disease (MRD) at the first remission [20] and harboured unfavourable outcomes [21]. In cases of *CEBPA* mutation, the N-terminal region of the gene was predominantly associated with germline mutation confer complete penetrance. The mutation accompanied by C-terminal mutation represents an independent leukaemic episode [22]. Even though available treatment and chemotherapy induced CR in about 80% cases, 40-50% relapsed cases occurred due to emergence of a resistant clone [1]. Previous studies have elucidated that AML subclones were identified in 83% of relapsed patients with *FLT3*-ITD mutant [23]. The subclones, although minor at diagnosis, would expand at the recurrence of the disease, latter represents the prominent leukaemic features [24,23].

In this study, AML patients at diagnosis were evaluated for *FLT3*-ITD, *NPM1* and *CEBPA* mutations by CSGE. *FLT3*-ITD and *NPM1* were the commonest mutation observed in adulthood AML and when exist in combination with *CEBPA* might carry prognostic implications. We identified the sequence variations indicated by multiple bands size deviated from the wild-type band representing mutation of *FLT*3, *NPM*1 and *CEBPA* genes confirmed by sequencing analysis. The mutation signatures of *FLT*3, *NPM*1 and *CEBPA* generated from this study cohort might harbour prognostic significance. The association of prominent mutations in AML with the clinical parameters (i.e., WBC count, blast count, and platelet count) and the OS analysis represent an insightful finding that lead to a new understanding in the architecture of *FLT3*, *NPM1* and *CEBPA* mutations of AML.

2. MATERIALS AND METHODS

2.1 AML Patients and Routine Laboratory Tests

A cross-sectional study of a two-year duration from September 2016 until August 2018 was carried out on all eligible newly diagnosed adult patients with AML admitted to Hospital Pulau Pinang and Hospital Universiti Sains Malaysia (USM), in Northern and East Coast of Peninsular Malaysia, respectively (Inclusion criteria; newly diagnosed AML, not in chemotherapy regimen, no comorbidities; Exclusion criteria: presented with critical illnesses, relapsed cases). Sample size was calculated by using this formula, $n= (Z/\Delta)^2 P(1-P)$ [(where n= sample size, Z = Z statistic for a level of confidence, (95%, Z=1.96), P = expected prevalence or proportion, (23.1%, P = 0.231), and Δ = precision, (10%, Δ = 0.10), with 5% of dropout rate to the total n=71] to estimate the prevalence of *FLT3*, *NPM1* and *CEBPA* mutations in AML population using a random sample. However, only 47 samples were available. Genomic DNA from patients' peripheral blood (PB) and/or bone marrow (BM) aspirate were extracted using the GeneAll® ExgeneTM DNA extraction kit (GeneAll Biotechnology, Seoul, South Korea), according to the manufacturer's protocols. Normal control peripheral blood samples were collected from screened healthy blood donors from Blood Transfusion Unit, Hospital USM, following informed consent. The procedures involved in the study of human subjects were according to ethical standards of the Universiti Sains Malaysia's (USM) Human Research Ethics Committee (USM/JEPeM/16030102), Ministry of Health Medical Research & Ethics Committee [NMRR-14-1902-21613 (IIR)], and with the Helsinki Declaration of 1975, as revised in 2008.

2.2 PCR Amplification of FLT3, NPM1 and CEBPA

The primers for *FLT3*, *NPM1*, and *CEBPA* mutation hotspots were designed by Primer 3 software, available online (http://bioinfo.ut.ee/primer3). The primer sequences for *FLT3*-ITD (exon 14-15) [25], *FLT3*-tyrosine kinase domain (TKD) (exon 20) [25], and *NPM1* (exon 12) [26,27] were adapted from Yunus et al., 2015 and Döhner et al., 2005. The primers for *CEBPA* were designed at 2 locations: i) N-terminal region consisting of transactivated domains (TAD 1 and TAD 2) (*CEBPA-A*), and ii) C-terminal region (*CEBPA-B*). The primer sequences were then applied on PCR Primer stats, available at (https://www.bioinformatics.org/sms2), which shown properties of each primer, including the percentage of GC content, PCR suitability, and melting temperature. The primers sequences and details were shown in Table 1.

The reaction mix containing 12.5 µL of 1× GoTaq green master mix, 1 µL of 10 µM of forward and reverse primers, 2 µL of 100 ng/ µL DNA samples which made up with deionised water to a total of 25 µL reaction, was amplified in ABI Veriti™ Thermal Cycler (Applied Biosystem, Foster City, USA). The reaction conditions were set up as follows; 95 ºC for 2 min, 35 cycles of 95 ºC for 45 sec, annealing for 45 sec and extension at 72 ºC for 30 sec with a final extension at 72 ºC for 5 min. The PCR products were visualised by 1.5% - 2.0% agarose gel electrophoresis and viewed under UV light to confirm the presence of PCR products of appropriate size and purity.

Table 1. Primers details for mutation detection

Genes	Chromosome / Exon Number	NCBI Reference (Location)	Primer Sequence 5'-3'	PCR Product (bp)
CEBPA-A	19q13.1/exon 1	NG_012022.1 (5081 to 5628)	F:tcgccatgccgggagaactctaac R:agctgcttggcttcatcctcctcg	480
CEBPA-B	19q13.1/exon 1	NG_012022.1 (2453 to 3157)	F:cggccgctggtgatcaagcagga R:acggtctgggcaagcctcgagat	645
NPM1	5q35/exon 12	NG_016018.1 (27815 to 28214)	F:ctctctggtggtagaatgaa R:caagactatttgccattccta	400
FLT3-ITD	13q12/exon 14-15	NG_007066.1 (71354 to 71682)	F:gcaatttaggtatgaaagccagc R:ctttcagcattttgacggcaacc	329

FLT3-TKD	13q12/exon 20	NG_007066.1 (86894 to 87180)	F:cctactgaagttgagtctag R:gtgagtgcagttgtttacca	284

Abbreviation: F, forward; R, reverse

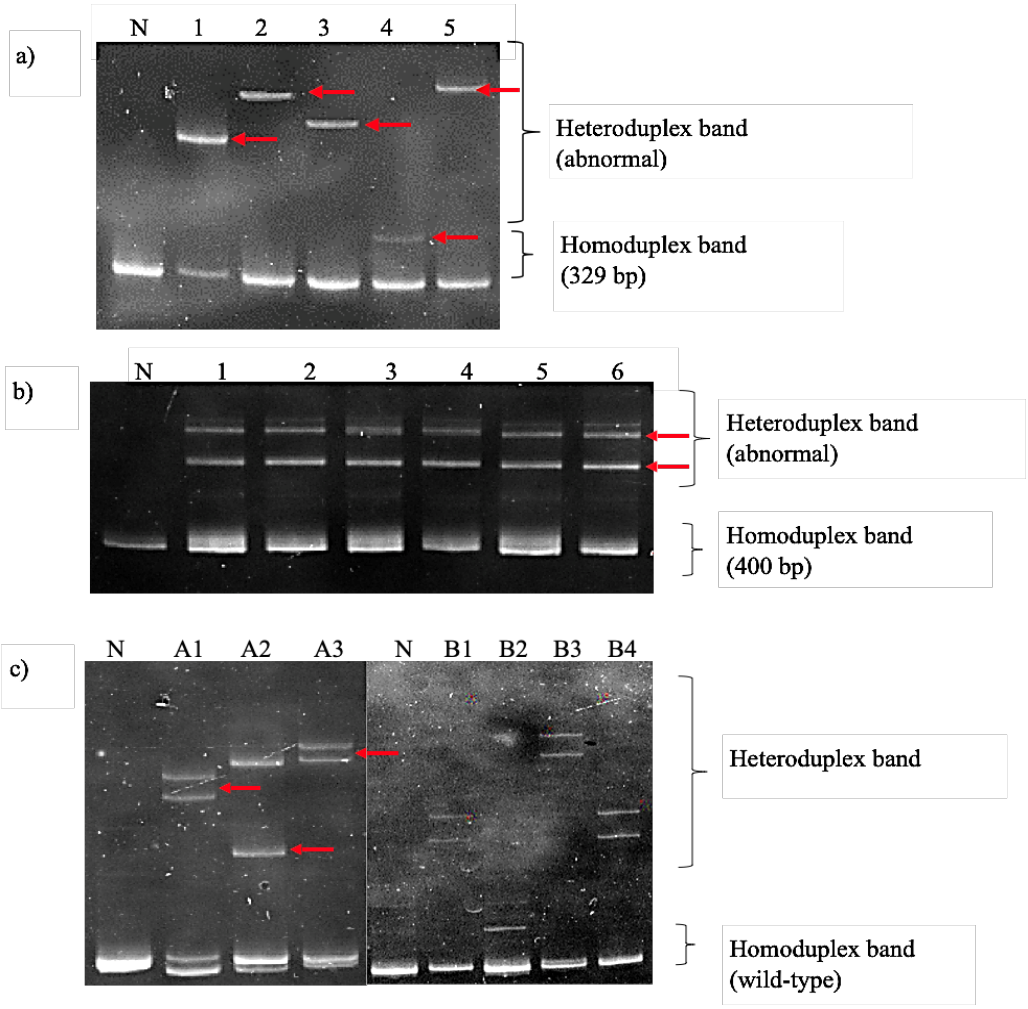


Figure 1. A representative of CSGE analysis of *FLT3*-ITD, *NPM1* and *CEBPA* mutations. (a) Abnormal band pattern was seen on CSGE gel for test samples 1-5 (Case no. 2, 18, 22, 24, and 37, respectively) related to heteroduplex formation between wild-type (homoduplex band) and a different size of mutant alleles (arrows) of *FLT3*-ITD gene. (b) Abnormal band pattern is seen on CSGE gel for test samples 1 to 6 (Case no. 1, 2, 5,34, 35 and 36, respectively) related to heteroduplex formation between wild-type and mutant *NPM1* gene. There was a uniform size of heteroduplex products (arrows). (c) Abnormal band pattern was seen on CSGE gel for test samples A1, A2 and A3 (Case no. 7, 22 and 28, respectively) and test samples B1, B2, B3 and B4 (Case no. 7, 22, 28 and 33, respectively) amplified using *CEBPA-A* and *CEBPA-B* primers, respectively, observed above the wild-type (480 bp). N= normal control

2.3 Mutation Detection by CSGE

Conformational Sensitive Gel Electrophoresis (CSGE) method was implemented according to Ganguly (2003) protocol to screen polymorphism and mutations in the PCR products [28]. This technique is based on differential migration of DNA heteroduplex (abnormal) compared to homoduplexes (wild-type) under mildly denaturing

conditions during polyacrylamide gel electrophoresis [29]. For heteroduplexing, 5 µL of individual PCR amplicons were mixed with 5 µL of healthy control amplicons, whilst 10 µL of healthy control amplicons were used for homoduplexing and were incubated at 95 °C for 5 min, and 65 °C for 30 min to give samples consisting largely of a single species molecule. A gel size of 41 × 33 × 0.1 cm 10 % polyacrylamide gel was prepared in the total of 175 mL (2 gels), consisting of 44.0 mL of 40% acrylamide 29:1, 4.38 mL of 20 × Tris-Taurine-EDTA (TTE) buffer, 26.26 mL of formamide, 17.50 mL of ethylene glycol, 1.76 mL of 10% APS (volume/volume) and 100 µL of tetramethylethylenediamine (TEMED). During gel loading, 3 µL of homoduplexed and heteroduplexed amplicons were used for each sample and electrophoresed at 400 V for 16 hours. The gel was then pre-stained with 1 µg/mL of ethidium bromide solution for 30 min, washed with deionised water for 5 min and viewed under Chemilmager 4400 (Alpha Innotech, San Leandro, CA, U.S.A.).

The remaining PCR products of the respective genes that showed abnormal CSGE profiles were sent for Sanger Sequencing service at Apical Scientific, Selangor, Malaysia. DNA sequencing peak was analysed using FinchTV software (Geospiza Inc., Seattle, WA, U.S.A.). The sequence was aligned by BioEdit sequence alignment editor software (Tom Hall Ibis Therapeutics, Carlsbad, CA, U.S.A.) using ClustalW Multiple Alignment accessory application to find sequence similarity and difference between the tested patient sample with the wild-type sequence. The translated amino acid was retrieved from ExPASy software (https://web.expasy.org/translate/) to look for changes in amino acids sequence. Sequence variant nomenclature was described from a guideline provided by Human Genome Variation Society (http://www.hgvs.org/content/guidelines).

2.4 Statistical Analysis

Statistical Package for the social science (SPSS) version 24 software was used for statistical analyses. Comparisons between patient characteristics were performed by Fishers Exact for categorical variables (gender and age) and Mann-Whitney test for continuous variables; haemoglobin, WBC count, platelet count and blast count with significance different, $p < 0.05$. Kaplan-Meier test was used to analyse the OS for 1-year between the different mutational groups and cytogenetical. OS describes the percentage of the people in the study cohort who were alive for the specific period after diagnosed of AML and death to any cause (censored), and the p-value < 0.05 were considered significant.

3. Results

3.1 Patients' Profiles

Forty-seven newly diagnosed AML patients (age ranged 15-78 years old) was recruited following informed consent. The patients were classified according to the French-American-British (FAB) criteria as: M0 (n=12), M1 (n=1), M2 (n=2), M3 (n=12), M4 (n=8), M5 (n=3), M6 (n=0), M7 (n=0), AML secondary to myelodysplastic syndrome (MDS) (n=3) and six undefined (not reported). The median value of total WBC, Hb and Plt were 11.4 x 10^9/L, 8.0 g/dL and 53.0 x 10^9/L, respectively. The flow cytometric immunophenotyping results revealed the most common myeloid marker expression was CD33 (93.6%), followed by MPO (89.4%), CD117 (89.4%), CD13 (89.4%), CD64 (68.1%) and the immature marker, CD34 (57.4%) (Supplementary 1).

3.2 FLT3, NPM1 and CEBPA Mutational Profiles

The representative of CSGE of *FLT3*-ITD*, NPM1, CEBPA*-A*,* and *CEBPA*-B were shown in Figure 1. The mutation analysis of CSGE positive cases of *FLT3*, *CEBPA* and *NPM1* mutations were illustrated in Figures 2, 3, and 4.

FLT3, *NPM1* and *CEBPA* mutations were detected in 5 (10.6%) (Figure 1a), 9 (19.2%) (Figure 1b), and 7 (14.9%) (Figure 1c), cases, respectively, showed by heteroduplex (abnormal) band above the homoduplex (wild type) band. The summary of the mutation analysis *of FLT3*-ITD*, NPM1 and CEBPA* were summarised in Table 2. No mutation was detected within the non-coding region of *FLT3*-TKD exon 20. Sequencing analysis discovered a frameshift mutation and an in-frame mutation between 15 to 140 base pairs (bp) in *FLT3-ITD*. The duplication was found in different location in each case. There were three common in-frame mutations involving a deletion of cytosine (C) at nucleotide (nt) position c.71666, substitution of thymine (T) to adenine (A) at nt position c.71674, and A insertion at nt position c.71683.

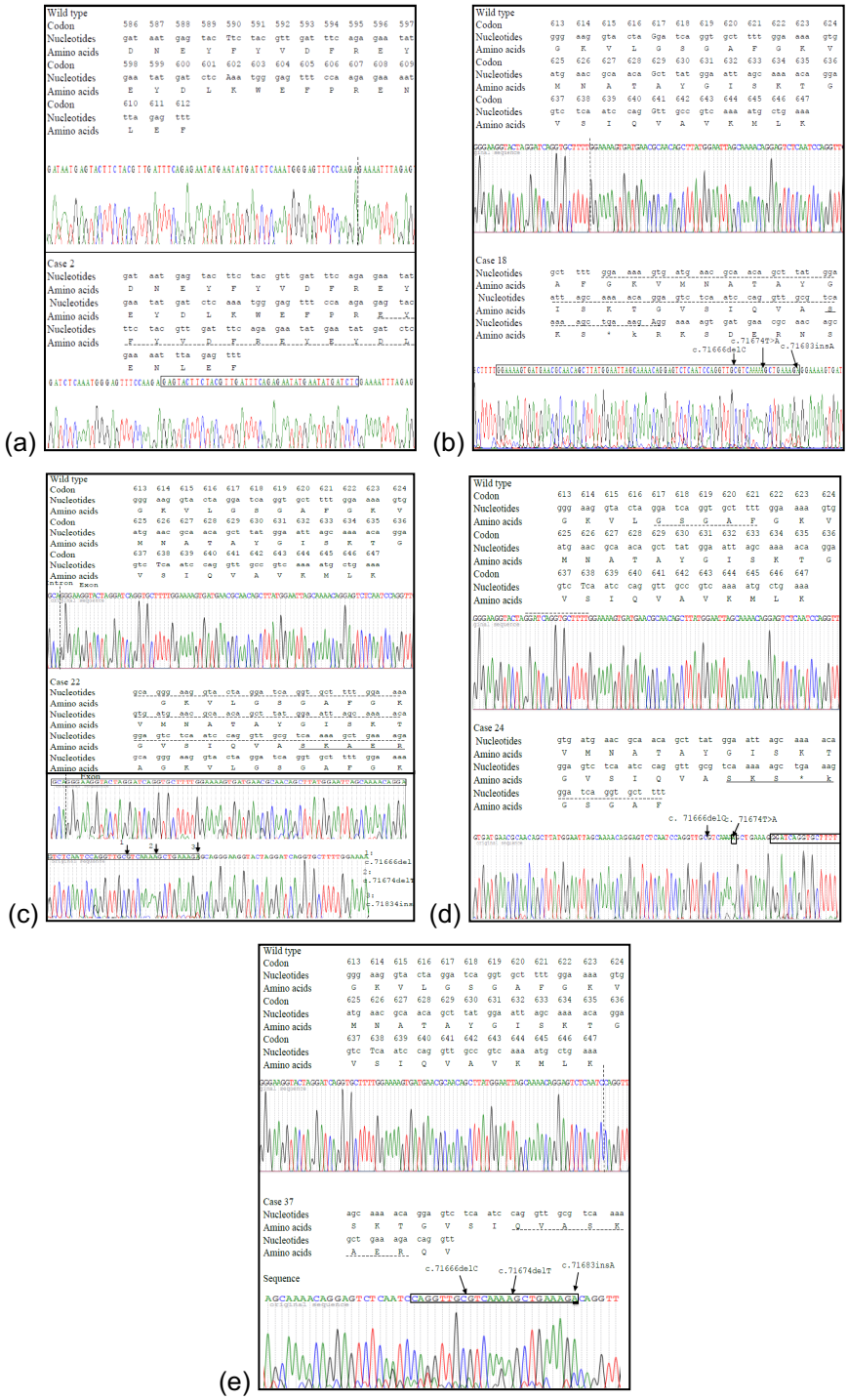


Figure 2. Sequencing analysis of *FLT3*-ITD. The *FLT3* wild-type nucleotide sequence and their translated amino acid within *FLT3* exon 14 was indicated above the studied samples. (a) Case 2: The internal duplication (ITD) of EYFYVDFREYEYDL amino acids in exon 14 was identified. (b) Case 18 representing patient 18: ITD was identified in exon 15 after codon 621. Within the duplication (inframe mutation), there were three different mutations observed: deletion of C at c.71666, T>A substitution at c.71674 and A insertion at c.71683. (c) Patient 22: ITD started from 3 nucleotides before exon 15, and the whole exon was duplicated. Within the duplication, three mutations similar to patient 18 were found. (d) Patient 24: ITD of GSGAF observed at the end of exon 15. Detected together were deletion of C at c.71666 and A substitution at c.71674. (e) Patient 37: ITD was identified just after codon 639. There were three mutations observed at position c.71666delC, c.71674delT, and c.71683 insA within ITD duplication

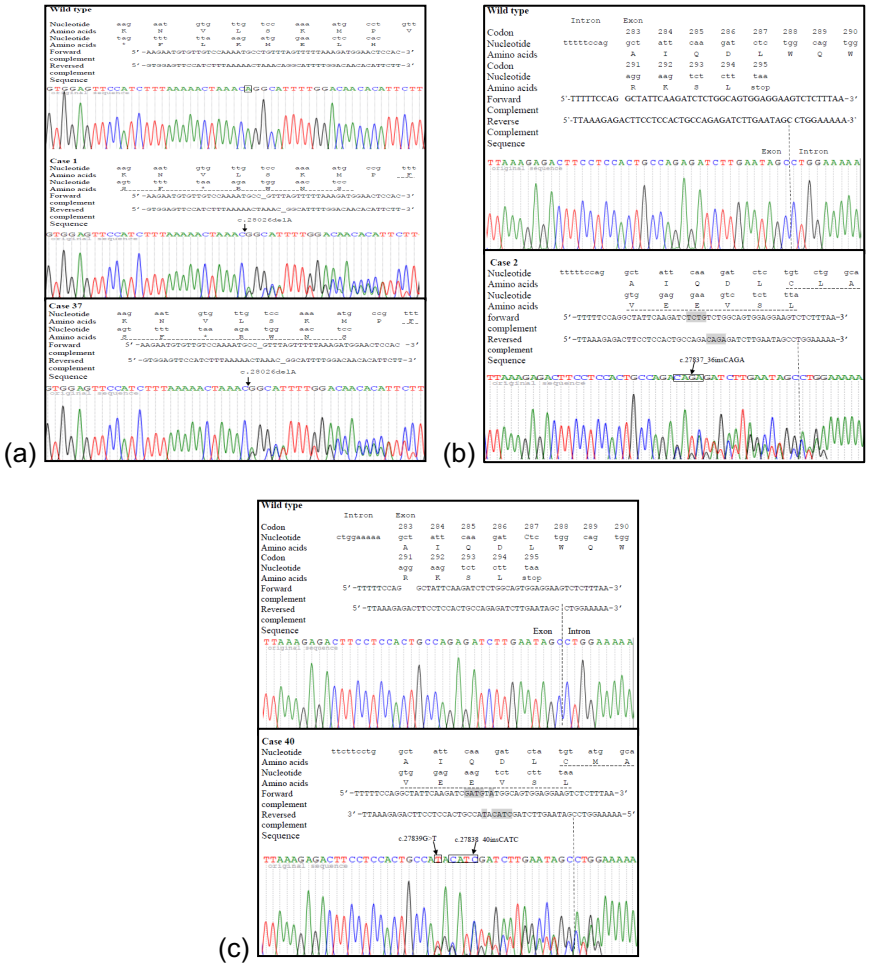
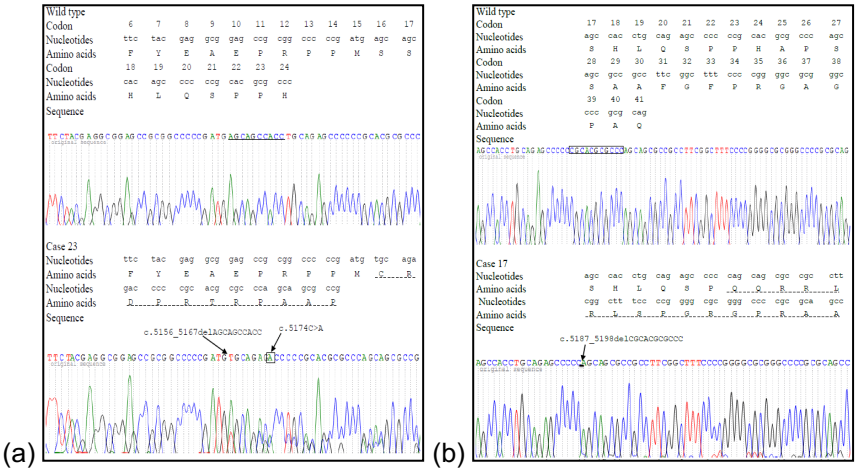


Figure 3. Sequencing analysis of *NPM1*. The *NPM1* wild type (WT): nucleotide sequence and their translated amino acids was indicated above the studied samples. (a) Case 1 and 37 representing patients 1, 36 and 37: deletion at c.28026. (b) Case 2 representing patients 2, 5 and 34: CAGA insertions at c.27837_27836 resulted in frameshift translation (c) Case 40 representing patients 40: G>T substitution at c.27839 and CATC insertion at c.27838_27840



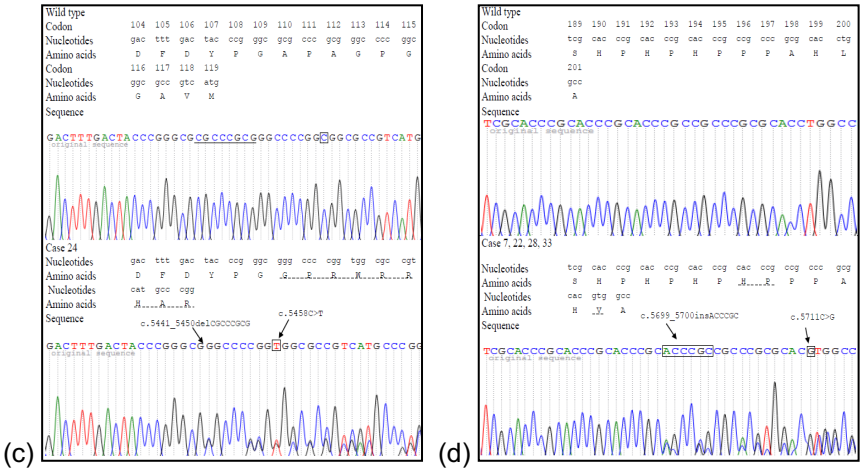


Figure 4. Sequencing analysis of *CEBPA* leading to frameshift translation. The analysis was in comparison to *CEBPA* wild-type sequence. (a) Patient 23 showed a AGCAGCCACC deletion at c.5156_5167, and a C>A substitution between codons 6-24 (b) Patient 17 presented with 11 nt. deletions between c.5187 and 5198 between codons 17-41. (c) Patient 24 showed a CGCCCGCG deletions between c.5441 and 5450, C>T substitution at c.5458 in codons 104-119. (d) ACCCGC insertions between c.5699 and c.5700, and C>G substitution were shown in patients 7, 22, 23, 28 and 33

Table 2. Summary of *FLT3*-ITD, *NPM1* and *CEBPA* mutations analysis

Mutations	Types of Alterations	Patient No.
FLT3-ITD	The *ITD* of EYFYVDFREYEYDL amino acid in exon 14 was identified, followed by the insertion of glutamic acid (E) just after amino acid arginine codon no. 608.	2
	The *ITD* was identified in exon 15 just after codon 621. Within the duplication (inframe mutation), there were three different mutations observed; deletion of C nucleotide (nt) at position c.71666, the substitution of T nt by A at position c.71674 and insertion of A nt at position c.71683.	18
	The *ITD* was observed in exon 15 for patient no. 22. The duplication was started from 3 nucleotides before exon 15, and then the whole exon was duplicated. Within the duplication, the mutation was found similar to case 18.	22
	The *ITD* of GSGAF amino acid was observed at the end of exon 15. Detected together were; deletion of C nt at position c.71666 and substitution of A nt at position c.71674 resulted in five amino acid S.K.S.*K.	24
	The *ITD* was identified just after codon 639. There were three mutations observed at position c.71666delC, c.71674delT, and c.71683 insA within *ITD* duplication.	37
NPM1	There was one deletion of nucleotide A nt at position c.28026.	1, 36, 37
	Four insertions (CAGA) were observed at nt position c.27837_27836 resulted in a frameshift translation.	2, 5, 34
	There was one deletion of A nt at position c.28026, insertion of G nt at c.27089_27088, and deletion of A nt at c.27084	35

	NPM1 mutation was identified by substitution of G nt by T at position c.27839 and insertion of four nt (CATC) at position c.27838_27840.	40
CEBPA-A	The mutation was identified in exon 1 just after codon 22. 11 nt (CGCACGCGCCC) were deleted between nt position c.5187 and c.5198 resulted in a frameshift mutation.	17
	The mutations were observed in two places; deletion of 10 nt (AGCAGCCACC) at position c.5156_c.5167, and substitution of C nt by A nt just after codon 15.	23
	The mutations were observed in two places: deletion of 8 nt (CGCCCGCG) between nt position c.5441 and c.5450 just after codon 109, and substitution of C nt by T nt at position c.5458.	24
CEBPA-B	The same mutation was found in cases 7, 22, 28, and 33, which was the insertion of nucleotide ACCCGC at between nt position c.5699 and c.5700 and one substitution of C nt by G nt.	7, 22, 28, 33

Five cases with mutations in *NPM1* C-terminal of the translated region were found: 3 cases with insertion of four nucleotides, CAGA at a position between c.27836 and c.27837, while two cases with insertion of CATC at the position between c.27838 and c.27840 and one substitution of guanine (G) nucleotide by T at position c.27839. Four mutations were found in the non-coding region of exon 12. All these cases had the same A nucleotide deletion at position c.28026, while case 35 had one more deletion of A nucleotide at position c.27084 and one insertion of G nucleotide between c.27088 and c.27089. Three patients presented with *CEBPA* mutation at both N-terminal region (*CEBPA*-A*)* and C-terminal region *(CEBPA*-B*)* manifested the same number of two heteroduplex bands, albeit in different position. One patient harboured only *CEBPA*-B mutation, presented with one heteroduplex band. All cases with *CEBPA*-B mutation bear the same type of mutation, which involved insertion of 6 nucleotides (ACCCGC) and substitution of G nucleotide by C nucleotide. The types of *FLT3*-ITD, *NPM1* and *CEBPA* mutations analysis were summarised in Table 2.

3.3 Significance of FLT3-ITD, NPM1 and CEBPA Mutational State with Clinical Parameters, Gender, and Age Group

The clinical characteristics of patients with and without *FLT3*-ITD*, NPM1,* and *CEBPA* mutations were described in Table 3. FLT3-*ITD* mutation was significantly associated with higher WBC count and blast count with p-value of 0.045 and 0.005, respectively. However, no significant difference was found related to platelet count, haemoglobin level, gender, and age groups. *CEBPA* mutation was significantly correlated with higher platelet count (p=0.01), and not significant with the other parameters. There is no association between *NPM1* mutation with the stated parameters.

3.4 Significance of FLT3-ITD, NPM1 and CEBPA Mutational State with Overall Survival

There were 44 patients with complete survival data for Kaplan-Meier's OS analysis. The patients were classified into six groups of different mutation combination according to ELN risk groups. Cumulative OS of AML with mutation combination of *NPM1*+/*FLT3*-/*CEBPA*- (favourable), *NPM1*-/*FLT3*+/*CEBPA*- (intermediate) and *NPM1*-/*FLT3*-/*CEBPA*+ (favourable) were 0.5 (50%), 1.0 (100%) and 0.8 (80%), respectively, but with insignificant value, $p > 0.05$ (Figure 5). Mutation of *NPM1*+/*FLT3*+/*CEBPA*- (adverse) presented with the worst OS because none survived in one year. However, the analysis showed no significant difference, p=0.476 probably due to small sample size. The relationship between genetic mutations, cytogenetics, and survival, were summarised in Table 4.

4. DISCUSSION

AML is a heterogeneous disease with a wide range of genetic abnormalities. *DNMT3A, IDH2, ASXL1, TET2, RUNX1, NRAS* mutations were frequent, especially *NPM1, FLT3*-ITD *and CEBPA*, accounted for 22.83%, 18.48% and 13.04%, respectively [30]. Mutations in *FLT3* and *NPM1* are the most frequent genetic alterations in AML,

especially in adult patients [30]. Although *CEBPA* monoallelic mutation is less common, it was associated with favourable clinical outcomes in AML patients with *NPM1*/*FLT3*-ITD wild-type in intermediate cytogenetic risk [31], an independent risk factor for good prognosis in *NPM1* mutated AML cases [32] and a favourable prognostic in non-M3 AML with wild-type *NPM1*/*FLT3*-ITD [33]. Mutation changes in leukaemic blast provide a target for a leukaemic inhibitor, such as *FLT3* inhibitors (i.e cytarabine and anthracycline), and is a sensitive measure in the determination of MRD [34].

Among the combined mutations studied, cases with *NPM1+/FLT3-/CEBPA-* have a higher prevalence compared with other mutation combinations, as reported previously [35,36]. In this study, *FLT3*-ITD mutation was detected in 3 cases co-existing with *CEBPA* and *NPM1* mutations. Alone, *FLT3*-ITD was seen in 2 cases. *FLT3*-ITD is one of the most common co-mutation found in AML besides *DNMT3A*, *IDH1,* and *NRAS* [37]. There were no cases of *NPM1+/FLT3+/CEBPA+* and *NPM1+/FLT3-/CEBPA+* combined mutations detected in this study. However, a study showed that their combinations had significantly low OS [38]. *FLT3* mutation is the most prominent gene mutation in AML proven by the extended stages of *FLT3* inhibitor development. *FLT3*-ITD accounted for 25-30% of all AML (of all FAB subtypes), while 5% presented with *FLT3*-TKD [39]. As recommended by the 2017 ELN, the significance of the prognosis is based on the allelic ratio of *FLT3*-ITD to *FLT3* wild-type (high allelic ratio >0.5) [11]. *FLT3* mutation screening has been implemented as a frontline due to its negative prognostic indicator for relapsed [40], low OS and adulthood malignancy [41]. The study also reported no significant differences in the OS and relapse-free survival of *FLT3* numerical variations, single *FLT3* mutation, two mutants or three mutants [42], as well as *FLT3*-ITD length.

NPM1 is the commonest mutation found in about 30% of adult AML cases, present in about 50% of cytogenetically normal (CN) patients, and confers a good prognosis in the absence of *FLT3* mutation [41]. *NPM1* mutation was commonly co-mutated with *DNMT3A* (54%), *FLT3*-ITD (39%), *NRAS* (19%), *TET2,* (16%) and *PTPN11* (15%) while was less common in *CEBPA* mutation [43], and associated with poor prognosis [44]. It was dictated in the previous studies that *NPM1* is frequent co-mutation followed by *CEBPA* and *FLT3* mutations in CN AML cases [45,46]. Compared to *FLT3*, *NPM1* mutation has been validated as a dependent marker for assessment of MRD and in relapsed case predictor [47]. In this study, *CEBPA* mutation was detected in 7 cases (14.9%), while in all AML cases, it was detected in 10-15% [48,49], which can be monoallelic or biallelic. Most patients with monoallelic *CEBPA* mutation occurred at the N-terminus, which result in N-terminus frameshift mutation. The frameshift mutation resulted in the loss of 42 kDa full-length CEBPA protein, leading to a 30 kDa protein overexpressed, a shorter isoform of *CEBPA* (Figure 6) [50,51]. Biallelic *CEBPA* mutation occurred in two locations; one in the N-terminal part and another one in the basic zipper (bZIP) domain, located at the C-terminus [52]. In the C-terminus domain, the in-frame insertions or deletions usually occurred in the DNA binding or basic-leucine zipper domain [53]. *CEBPA* mutation has been specified to be prognostically favourable in the intermediate-risk group of AML [50].

As shown in this study, *FLT3*-ITD was highly correlated with high WBC count and high blast count ($p<0.05$), which was supported by the previous studies [25,54]. Age distribution and gender have no effects on the occurrence of all mutations ($p>0.05$). However, few studies reported a significant difference of *FLT3* and *NPM1* mutation occurrence that increases with age [55]. Age turns out to be the most significant relative contribution to adverse prognosis in AML modified by clinical, cytogenetic, and molecular features [47]. In regards to gender difference, the previous study has shown a positive association of *CEBPA* mutation with female adult AML ($p=0.04$), but not significant with age ($p=0.69$) and platelet count ($p=0.09$) [56].

5. CONCLUSION

In this study cohort, *NPM1* mutation is the largest contributor to genetic aberrancy in AML, followed by *CEBPA* and *FLT3*. It was primarily known that *NPM1* exon 12, *FLT3* exon 14-15 and *CEBPA* exon 1 are the eminent mutation hotspots in AML, but not much information reported on mutation types/variants found within. Signature mutation variants have been found in this study which predicted their stable state and homogeneity. These mutation signatures are likely pathogenic and may harbour prognostic significance, for example, in the monitoring of MRD, remission status, treatment outcomes and survival rate.

Table 3. Relationship of AML patients with *FLT3-ITD*, *NPM1* and *CEBPA* mutants and wild-type with the clinical parameters, gender, and age groups

Parameters	*FLT3-ITD*			*NPM1*			*CEBPA*		
	M	**WT**	***p***	**M**	**WT**	***p***	**M**	**WT**	***p***
Haematological									
Median, Hb (g/dL)	6.46	7.97	0.108	7.60	7.86	0.219	7.36	7.89	0.701
Median WBC ($x10^9$/L)	133.90	46.49	0.045*	90.178	47.64	0.729	68.39	53.57	0.519
Median blast	79.80	49.64	0.005*	61.89	50.71	0.368	51.57	53.08	0.913
Median Plt ($x10^9$/L)	39.26	84.26	0.206	88.89	77.24	0.679	33.04	87.60	0.001*
Gender (%)			0.654			0.715			0.102
Male	3 (13.7)	19 (86.3)		5 (22.8)	17 (77.2)		1 (4.6)	21 (95.4)	
Female	2 (7.0)	23 (92.0)		4 (15.4)	21 (84.6)		6 (24)	19 (76)	
Age groups (%)			0.811			0.882			0.196
15-30	1(12.5)	7(87.5)		2(25)	6(75)		0	8(100)	
31-40	0	7(100)		1(14.3)	6(85.7)		0	7(100)	
41-50	1(11.1)	8(88.9)		1(15.7)	8(88.9)		1(11.1)	8(88.9)	
51-60	1(8.3)	11(91.7)		2(16.7)	10(83.3)		4(33.3)	8(66.7)	
>61	2(18.2)	9(81.8)		3(27.3)	8(72.7)		2(18.2)	9(81.8)	

*Statistically significant. Abbreviation: M, mutation; WT, Wild type; *p*, p-value

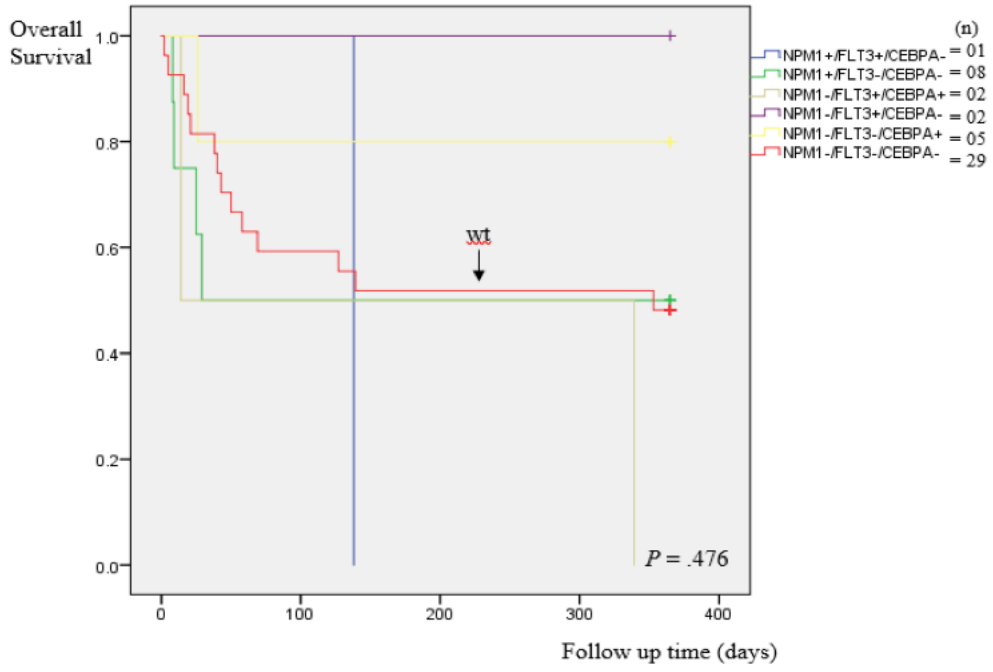


Figure 5. Overall survival (OS) analysis by Kaplan-Meier survival curve

Table 4. Summary of mutation profile, cytogenetics and 1-year OS

Mutations	Incidence (n=47)	Cytogenetics (n=41)		Survival (n=45) (1 year after diagnosis)	
	(%)	(%)	(%)	Alive (%)	Dead (%)
NPM1+/FLT3-/CEBPA-	8 (17.0)	5 (62.5)	2 (25.0)*	4 (50.0)(NK=3)	4(50.0)(NK=2)
NPM1+/FLT3+/CEBPA-	1(2.1)	1(100.0)	0	0	1(100.0)(NK=1)
NPM1-/FLT3+/CEBPA+	2 (4.3)	0	2 (100.0)¶	0	2 (100.0)
NPM1-/FLT3-/CEBPA-	29 (61.7)	13 (44.8)	12(41.4)^	13 (44.8)(NK=4)	14 (48.3)(NK=8)
NPM1-/FLT3+/CEBPA-	2 (4.3)	1(50)	1(50)ϕ	2 (100.0)(NK:2)	0 (0.0)
NPM1-/FLT3-/CEBPA+	5 (10.6)	-	4 (80.0)‡	4 (80.0) (NK:3)	1 (20.0)
Total	47	20	21	23	22

* 1: 48, XY, t (6;14) (p25; q11.2), +12, +19[8]/46, XY[7] 2: no further data
¶ 1: inv16(p13q22), 2: t(8;21)
^ 1: 46,XY,t(8;21)(q22;q21)[1]/46,XY[7], 2: 46,XY,t(15;17)(q22;q22)[12], 3: 46,XX,i(7)(q10)[11]/46, 4: 47,XX,+X[9]/46,XX, 5: t(8;21) (7 cases with complex karyotype pattern)
ϕ no further data
‡ 1: 46,xx[25], 2: complex karyotype; 3 &4 no further data
NK = Normal karyotype

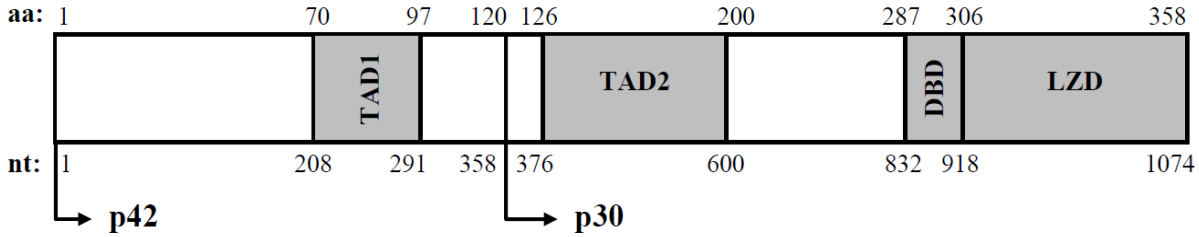


Figure 6. Functional domains of *CEBPA* and translational start sites for the two main isoforms p42 and p30, (aa) amino acid and (nt) nucleotide numbering are indicated

A continuous study using larger sample cohort need to be conducted for better evaluation and analysis. Combining with other sequencing techniques such as next-generation sequencing (NGS) and non-sequencing techniques, such as spatial transcriptomic technology would provide a better genomic information. This comprehensive approach is rendering personalized therapy for better disease outcomes.

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