ORIGINAL ARTICLE

Detection of FMS-Like Tyrosine Kinase 3 (FLT3) and Nucleophosmin 1 (NPM1) Mutations from Marrow Tissues in Patients with Acute Myeloid Leukaemia

Mansoor NM[a], Muhammad N[a], A. Talib N[a], Ismail R[b], Ibrahim I[c], Abd Aziz KH[d], Hamdan AH[a]

[a]Department of Pathology and Laboratory Medicine, Kuliliyyah of Medicine, International Islamic University Malaysia, Kuantan, Pahang, Malaysia
[b]Department of Pathology, Hospital Tengku Ampuan Afzan, Kuantan, Pahang
[c]Department of Internal Medicine, Kuliyyah of Medicine, International Islamic University Malaysia, Kuantan, Pahang, Malaysia
[d]Department of Community Medicine, Kuliyyah of Medicine, International Islamic University Malaysia, Kuantan, Pahang, Malaysia

ABSTRACT

INTRODUCTION: Acute myeloid leukaemia is a haematological malignancy with diverse cytogenetic abnormalities and molecular mutations. Amongst the important mutations are FMS-related tyrosine kinase 3 (FLT3) and nucleophosmin 1 (NPM1) gene mutations. These mutations have been shown to be of prognostic significance. A cross-sectional study to examine the frequency of these mutations and their association with the haematological and cytogenetic characteristics of the cases was carried out in Kuantan, Pahang, Malaysia. **MATERIALS AND METHODS:** A total of 43 cases were included in the study. Polymerase chain reaction-based assays were employed for mutation detection from the retrieved trephine biopsy tissue blocks. Mutation positivity was subsequently validated by Sanger DNA sequencing. **RESULTS:** Six of the 43 cases (14.0%) of the acute myeloid leukaemia were positive for FLT3-type internal tandem duplications (FLT3-ITD) and a similar proportion (6/43, 14.0%) were positive for NPM1 mutations. FLT3 mutations at codon D835 (FLT3-D835) mutation was identified in three of the cases (7.0%) while concurrent mutations of NPM1 and FLT3-ITD were seen in two of the mutation-positive cases (4.7%). The total white cell count was found to be significantly higher in patients with FLT3 mutations (p=0.001). Other haematological parameters and the cytogenetic results did not reveal any significant association with the mutational status. **CONCLUSION:** The frequency of FLT3-ITD, FLT3-D835, and NPM1 mutations among AML patients were 14%, 7%, and 14% respectively. Follow-up studies to include the clinical parameters and the treatment outcomes are advocated.

Keywords
Acute Myeloid Leukaemia, FLT3-ITD, FLT3-D835, NPM1

Corresponding Author
Dr. Asmah Hanim Hamdan
Department of Pathology and Laboratory Medicine, Kulliyyah of Medicine, International Islamic University Malaysia, Kuantan, Pahang, Malaysia
E-mail : drahanim@iium.edu.my

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INTRODUCTION

Acute myeloid leukaemia (AML) is a haematological malignancy involving the haemopoietic stem cells or early progenitor cells. By definition, a diagnosis of AML is made when there are ≥20% of myeloblasts in the bone marrow or peripheral blood.[1] It is characterised by clonal evolution with heterogeneous cytogenetic aberrations and molecular mutations.[2] Amongst the important and more common mutations in AML include FMS-related tyrosine kinase 3 (FLT3) and nucleophosmin 1 (NPM1) gene mutations. These mutations are of prognostic significance.[3]

The FLT3 gene located on chromosome 13q12 encodes for tyrosine kinase receptor (class III) that plays an important role in the regulation of haematopoietic progenitor cells.[4,5] In AML, mutations in the FLT3 gene cause continuous activation of FLT3 signalling thereby stimulating the proliferation and survival of the leukaemic cells.[3,4] The two predominant categories of FLT3 mutations include internal tandem duplications (FLT3-ITD) between exon 14 and 15 regions in the juxtamembrane domain of the receptor and point mutations affecting codon D835 of the tyrosine kinase domain (FLT3 D835) of exon 20.[6,7] FLT3-ITD is a negative prognostic factor associated with an increased relapse rate with inferior overall survival and remains to be of prognostic significance despite intensive chemotherapy and haematopoietic stem cell transplant.[8,9] The prognostic consequence of FLT3-D835 in AML is, however, still unclear with some studies indicating weak associations.[8,10]

NPM1 gene located on chromosome 5q35, codes for a multifunctional nucleocytoplasmic shuttling protein localized mainly in the nucleolus.[4,11] NPM1 mutations would result in abnormal expression and aberrant delocalisation of the NPM1 mutant proteins.[3] Although their role in the pathogenesis of AML is unclear, they most probably act through different cellular processes.[12] The World Health Organisation classification has included mutations in NPM1 as a distinct entity of AML and is associated with a favourable prognosis.[13]

Since both FLT3 and NPM1 mutations in AML carry prognostic and hence therapeutic implications, this study examined the frequency of these mutations. We also investigated the relationships between FLT3 and NPM1 mutational status and the haematological and cytogenetic characteristics of the cases.

MATERIALS AND METHODS

Sample collection

This cross-sectional study involved cases of AML diagnosed in the Sultan Ahmad Shah Medical Centre @ IIUM and Hospital Tengku Ampuan Afzan, Kuantan, Pahang, Malaysia. All AML cases with available diagnostic trephine biopsy tissue blocks, for the years 2016-2019 were included. The information collected for this study included age, gender, ethnicity as well as the full blood picture, bone marrow aspiration, trephine biopsy, and cytogenetic reports of patients at diagnosis.

DNA extraction and quantification

The extraction of DNA from the formalin-fixed paraffin-embedded (FFPE) trephine biopsy tissue block sections was performed using Maxwell® RSC DNA FFPE Kit (Promega, USA) and Maxwell® RSC Instrument (Promega Corporation, USA), as described by the manufacturer. The DNA concentration was quantified using the SimpliNano™ spectrophotometer (GE Health Care Life Sciences, UK). The extracted DNA was stored at -80 °C for subsequent use.

FLT3-ITD mutation detection

For FLT3-ITD mutation detection, the primer set used included forward primer 5'- GCA ATT TAG GTA TGA AAG CCA GC -3' (ITD_14F) and reverse primer 5'-CTT TCA GCA TTT TGA CGG CAA CC -3' (ITD_15R) (Integrated DNA Technologies, USA).[14] Polymerase chain reaction (PCR) assay was performed in a total volume of 25 μL of 12.5μL HotStarTaq master mix (Qiagen, Germany), 1 μL (10 μM) of forward and reverse primers respectively, 8.5 μL nuclease-free water, and 2 μL (10 ng/μL) genomic DNA. The PCR conditions included initial denaturation at 95°C for 7 minutes and followed by 35 cycles of denaturation (at 94°C for 1 minute), annealing (at 58°C for 45 seconds), and extension (at 72° C for 1 minute). The subsequent final extension was carried out at 72°C for 7 minutes (C1000 Touch Thermal Cycler, BIO-RAD, Singapore). The amplified products at a volume of 10 μl each were electrophoresed through 4% agarose gel, stained with FloroSafe DNA stain (First Base Axil Scientific, Singapore), and visualised under UV light (EndruroTM GDS Imaging System, China).

FLT3-D835 mutation detection

The primer set utilised for FLT3-D835 mutational analysis was 5'-CCG CCA GGA ACG TGC TTG-3' (D835_F) as the forward primer and 5'-GCA GCC TCA CAT TGC CCC-3' (D835_R) as the reverse primer (Integrated DNA Technologies, USA).[14] The total volume used for the PCR assay was 25 μL, including 12.5 μL Hot Star Taq master mix (Qiagen, Germany), 1 μL (10 mM) of forward and reverse primers respectively, 2.5 μL Q-Solution (Qiagen, Germany), 6 μL nuclease-free water and 2 μL DNA template (10 ng/ml). The PCR conditions were initial denaturation at 95°C for 9 minutes followed by 35 cycles of denaturation (at 94°C for 18 seconds), annealing (at 59°C for 1 minute), and extension (at 72°C for 1 minute). The subsequent final extension was carried out at 72°C for 7 minutes (C1000 Touch Thermal Cycler, BIO-RAD, Singapore).

Following the PCR amplification, restriction enzyme digestion of the products was carried out with the enzyme Eco321 (Thermo Fisher Scientific, Malaysia) which recognises the sequence 5'-GAT ATC-3', 3'-CTA TAG-5'. It was performed in a total volume of 31 µl including 1 µl of Eco321 enzyme, 2 µl restriction buffer (10X Buffer R), 18 µl nuclease-free water, and 10 µl PCR product. Each digestion reaction was incubated at 37°C for 1 hour, followed by inactivation at 85°C for 20 minutes (C1000 Touch Thermal Cycler, BIO-RAD, Singapore). A volume of 10 µl of each of the PCR products was subsequently electrophoresed through 4% agarose gel, stained with FloroSafe DNA stain (First Base Axil Scientific, Singapore), and visualised under UV light (EndruroTM GDS Imaging System, China).

NPM 1 mutation detection

For the detection of NPM1 mutation, PCR amplification was performed in a total volume of 25 µL reaction containing 12.5 µL HotStarTaq master mix (Qiagen, Germany), 1 µL (10 µM) of forward and reverse primers respectively, 8.5 µL nuclease-free water, and 2 µL (10ng/µL) DNA template. The primer set used included 5'-TTA ACT CTC TGG TGG TAG AAT GAA-3' (NPM1_F) and 5'- TGT TAC AGA AAT GAA ATA AGA CGG-3' (NPM1_R) (Integrated DNA Technologies, USA).14 The PCR cycle protocol included initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation (at 95°C for 1 minute), annealing (at 58°C for 27 seconds), and extension (at 72°C for 2 minutes). The final extension was also carried out at 72°C, for 7 minutes (C1000 Touch Thermal Cycler, BIO-RAD, Singapore). The amplified products (10 µl each) were electrophoresed through 4% agarose gel, stained with FloroSafe DNA stain (First Base Axil Scientific, Singapore), and visualised under UV light (EndruroTM GDS Imaging System, China).

DNA sequencing

Direct sequencing was performed on randomly selected samples that were positive for FLT3-ITD, FLT3-D835, and NPM1 mutations respectively. This was carried out for verification of each PCR product sequence and confirmation of the mutations. Those samples which were negative for the mutations were also subjected to normal reference sequence and validation for the absence of mutations. All PCR products of the selected samples (20 µl each) were sent to Apical Scientific Sdn Bhd (Selangor, Malaysia) for direct sequencing. The sequence data was presented in ABI and FASTA format. The data were subsequently analysed using DNA Baser Assembler v4 (Heracle bioSoft RSL). The sequence references used were: NG_007066.1 [Target FLT3-ITD (Exon 14-15)]; NG_007066.1 [Target FLT3-D835 (Exon 20)] and NC_000005.10 [Target NPM1 (Exon 12)]

Statistical analysis

All statistical analyses were performed using SPSS version 23 (SPSS Inc., IBM, Armonk, New York, USA). For the analysis of categorical data, Pearson's chi-square test/ Fisher Exact was used. For numerical data, a t-test was used. A p-value of less than 0.05 was considered statistically significant.

RESULTS

In all, there were 43 cases of AML included in this study. There were 16 (37.2%) male and 27 (62.8%) female patients. Their mean age at diagnosis was 44 years (range 16-83 years). Twenty-seven cases (62.8%) were less than 50 years of age. The patients were predominantly Malays (35 cases, 81.4%) while Chinese made up 16% (7 cases) with one categorised as other ethnicities.

FLT3 and NPM1 mutations

Nine out of the 43 AML cases (20.9%) were positive for FLT3 mutations, of which 6 cases exhibited FLT3-ITD mutation (14.0% of the total cases) while FLT3-D835 mutation was observed in the three other cases (7.0% of the total cases). NPM1 mutation was detected in 6 out of the 43 AML cases (14.0%). Of the cases that were positive for the mutations, two (4.7% of the total cases) exhibited concurrent mutations of FLT3-ITD and NPM1. The PCR amplification results of selected samples for FLT3-ITD, FLT3-D835, and NPM1 mutations are shown in Figure 1.

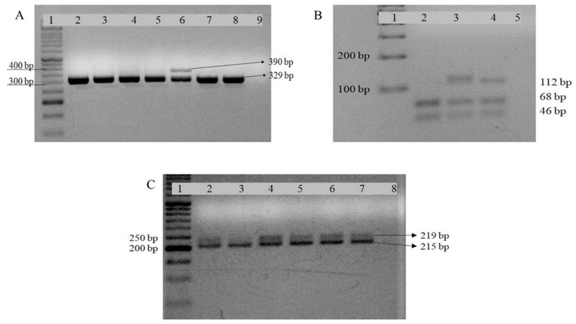

Figure 1: Agarose gel electrophoresis of PCR assays to identify FLT3-ITD (A), FLT3-D835 (B), and NPM1 (C) mutations. (A): PCR amplification results for FLT3-ITD gene mutation. Lane 1 is the DNA marker (50 bp ladder), lane 2 is the negative control, lanes 3-5, 7, and 8 are the negative results, lane 6 is the positive result while lane 9 is the no template control (NTC) which has no band. (B): PCR amplification results for FLT3-D835 gene mutation. Lane 1 is the DNA marker (100 bp ladder), lane 2 is the negative result, lanes 3 and 4 are the positive results while lane 5 is the NTC. (C): PCR amplification results for NPM1 gene mutation. Lane 1 is the DNA marker (50 bp ladder), lane 2 is a positive control, lane 3 is the negative result, lanes 4-7 are the positive results, and lane 8 is the NTC.

FLT3 mutations and the haematological profile

The mean and range of the full blood count values including haemoglobin concentration, white cell count, platelet count, and blast cell percentage in patients with and without FLT3 mutations are shown in Table I. The total white cell count was significantly higher in patients with FLT3 mutations (p=0.001). Other haematological parameters did not exhibit any significant difference between the two groups.

HB = Haemoglobin concentration; WBC = White cell count; PLT = Platelet count
Independent t test, values represent mean ±standard deviation (range). A p-value of <0.05 was considered statistically significant.

In Table II, the AML cases with and without the *FLT3* mutations were categorised into the presence or absence of hyperleukocytosis (white cell count of $>50\times10^9$/L), and severe thrombocytopenia (platelet count of $<50\times10^9$/L) and severe anaemia (haemoglobin concentration of <7 g/dL) respectively. There was no significant association seen between the mutation status and the haematological parameter categorisations (*p*-values >0.05).

HB = Haemoglobin concentration; WBC = White cell count; PLT = Platelet count
Fisher's exact test and a p-value of <0.05 was considered statistically significant.

Table I: FLT3 mutations and the selected full blood count parameters of the AML cases

Haematological parameters at diagnosis	**FLT3 mutations**		**Mean difference**	**t statistics**	**p-value**
	Present n=9	Absent n=34			
HB (g/dL)	8.4 ±2.1 (4.8-11.2)	7.7 ±2.1 (4.0-12.0)	0.74	0.95	0.35
WBC ($\times10^9$/L)	89.8 ±58.4 (32.5-220.9)	44.5 ±25.8 (2.2-87.2)	45.33	3.49	0.001
PLT ($\times10^9$/L)	99.0 ±160.9 (5.4-518.0)	88.2 ±157.1 (7.0-724.0)	10.83	0.18	0.86
Blast percentage	72.5 ±29.7 (4.0-94.0)	59.1 ±27.2 (5.0-98.0)	13.4	1.29	0.21

NPM1 mutations and the haematological profile

The mean full blood count values including haemoglobin concentration, the white blood cell count, platelet count, and blast cell percentage in patients with and without NPM1 mutation are shown in Table III. None of the haematological parameters in the two groups showed any significant difference.

HB = Haemoglobin concentration; WBC = White cell count; PLT = Platelet count
Independent t test, values represent mean ±standard deviation (range). A p-value of <0.05 was considered statistically significant.

Table II: FLT3 mutations and haematological parameter categorisations in the AML cases

Variables	*FLT3* mutations		*p*-value
	With mutation n (%)	Without mutation n (%)	
WBC			
$> 50\times10^9$/L	7 (77.8)	17 (50.0)	0.25
$<50\times10^9$/L	2 (22.2)	17 (50.0)	
PLT			
$>50\times10^9$/L	5 (55.6)	13 (38.2)	0.45
$<50\times10^9$/L	4 (44.4)	21 (61.8)	
HB			
>7 g/dL	7 (77.8)	21 (61.8)	0.45
<7 g/dL	2 (22.2)	13 (38.2)	

The AML cases were also categorised into hyperleukocytosis, severe thrombocytopenia, and severe anaemia respectively (Table IV). There was no significant association seen between the NPM1 mutation status and the haematological parameter categorisations (p-values >0.05).

HB = Haemoglobin concentration; WBC = White cell count; PLT = Platelet count
Fisher's exact test and a p-value of <0.05 was considered statistically significant.

For the two AML patients who had both the FLT3-ITD and NPM1 mutations, the haemoglobin levels were 7.6 g/dL and 8.9 g/dL respectively. One patient had hyperleukocytosis (126.2 ×109/L) while in another patient the white cell count was <50 ×109/L (47.9×109/L). None of the patients had severe thrombocytopenia.

Table III: NPM1 mutations and the selected full blood count parameters of the AML cases

Haematological Parameters at diagnosis	*NPM1* mutations		Mean difference	*t* statistics	*p*-value
	Present n=6	Absent n=37			
HB (g/dL)	8.8 ±1.9 (6.5-11.8)	7.6 ±2.2 (4.0-12.3)	1.17	1.29	0.21
WBC ($\times10^9$/L)	63.2 ±37.8 (12.3-126.2)	52.5 ±39.5 (2.2-220.9)	10.73	0.62	0.54
PLT ($\times10^9$/L)	53.8 ±33.4 (25.0-104.0)	96.4 ±167.7 (5.4-724.0)	-42.58	-0.614	0.43
Blast percentage	82.7 ±7.4 (70.0-90.0)	58.6 ±28.7 (4.0-98.0)	24.04	2.02	0.05

FLT3 and NPM1 mutations and cytogenetic characteristics

Of the 43 AML patients, 18 patients had cytogenetic results retrieved. In three of the 18 cases (16.7%) the cytogenetic results were abnormal. One of the abnormalities was trisomy 22 while in the other two cases the patients had hyperploidy. None of these three cases had *FLT3* or *NPM1* mutations. No association was observed between the cytogenetic results and the *FLT3* (p=1.00, Fisher's exact test) and *NPM1* (p=0.52, Fisher's exact test) mutational status.

Table IV: NPM1 mutation and haematological parameter categorisations in AML patients

Variables	*NPM1* gene		*p*-value
	with mutation n (%)	without mutation n (%)	
WBC			
> 50 $\times10^9$/L	4 (66.7)	20 (54.1)	0.68
<50 $\times10^9$/L	2 (33.3)	17 (45.9)	
PLT			
>50 $\times10^9$/L	3 (50.0)	15 (40.5)	0.68
<50 $\times10^9$/L	3 (50.0)	22 (59.5)	
HB			
>7 g/dL	5 (83.3)	23 (62.2)	0.40
<7 g/dL	1 (16.7)	14 (37.8)	

Sequencing results

The sequencing chromatograms of the wild type and mutant *FLT3-ITD, FLT3-D835,* and *NPM1* are illustrated in Figure 2.

DISCUSSION

In this study, we investigated the presence of *FLT3* and *NPM1* mutations in AML cases using FFPE marrow trephine biopsy-type samples. These mutations play a significant role in the diagnosis, risk assessment, and guidance to therapy of AML patients.[10, 15] We carried out validated in-house PCR assays reported by Mat Yusoff et al. (2019) for the detection of these mutations in our study.[14] The methods are deemed to be robust, cost-effective, and relatively less labour intensive. The mutation positivity in our study was subsequently validated by Sanger DNA sequencing.

The frequency of *FLT3*-ITD mutation in the AML patients in this study (14%) is comparable to an earlier study carried out in Kelantan, Malaysia (13%).[16] Our result also concurs with the findings of other published studies from other Asian countries with the *FLT3*-ITD mutation ranging from 9-20%.[17,18,19,20] The proportion of positivity for *FLT3*-D835 mutation (7%) in our study also concurs with that of others, ranging from 7-10%.[8] It is well established that *FLT3*-D835 mutation in AML occurs at a lower rate than that of *FLT3*-ITD mutation. However, in a study conducted by Mat Yusoff et al. (2019) among Malaysian patients with AML involving cytogenetically normal AML cases, a much lower frequency (2.5%) of *FLT3*-D835 mutation was reported.[14]

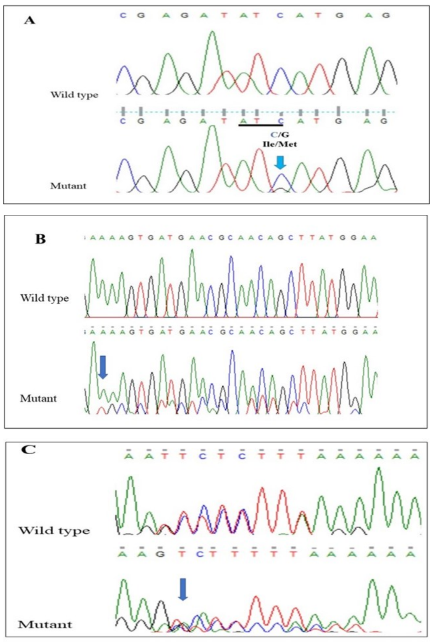

Figure 2: Electropheregram of *FLT3*-D835 (A) *FLT3*-ITD (B), and *NPM1* (C) mutations. (A): showing sequencing of *FLT3-D835* case which exhibited a heterozygous form of c.26I>M substitution missense mutation. (B): Nucleotide sequences showing duplication type mutation of *FLT3* gene. (C): Chromatograms show the mutant sequence of the *NPM1* gene. The arrows show the sites of the mutations.

As for the *NPM1* mutation frequency, our finding of 14% seems to be lower compared to others. The two studies conducted among Malaysians by Mat Yusoff et al. (2019) and Abdullah et al. (2020) reported higher detection rates of 27.1% and 22.2% respectively. [14,21] As aforementioned Mat Yusoff et al. (2019) included only cytogenetically normal AML cases while Abdullah et al. (2020) however, examined all cases of AML similar to our study. Other series reported *NPM1* mutation in the range of 25-30% and is deemed to be the most frequent mutation.[3]

Our patients with *FLT3* mutations exhibited significantly higher total white cell count as compared to those patients that did not harbour the mutations. *FLT3* mutations have been strongly associated with leucocytosis.[6] The haemoglobin concentration, platelet count, and blast percentage did not exhibit any significant differences between the groups with wild-type and mutated *FLT3*, as also shown by Rezaei et al (2017).[22] When categorized into hyperleukocytosis, severe thrombocytopenia, and severe anaemia we also did not observe any association with the mutation status. Nevertheless, hyperleukocytosis is a feature considered to be frequently observed with *FLT3* mutations.[3, 23]

As for the NPM1 mutation, our findings are consistent with that of others in which no significant differences were observed between the mean full blood count values and the mutation status.[22,24] Likewise, we did not find any significant association between hyperleukocytosis, severe anaemia, and severe thrombocytopenia, and the mutation. However, Abdullah et al (2020) showed that their patients with NPM1 mutation had significantly lower haemoglobin levels. They also reported significantly higher platelet count and bone marrow blast percentage in patients without the NPM1 mutation.[21]

NPM1 and *FLT3* mutations are among the most frequently recurring molecular genetic changes in AML, predominantly in cytogenetically normal AML. [3, 25,26] We, however, did not find any significant association between the cytogenetic results and these mutations. This finding might not be truly representative as almost two-thirds of our cases had no cytogenetic results.

CONCLUSION

The frequency of *FLT3*-ITD*, FLT3*-D835*,* and *NPM1* mutations among AML patients in this study were 14%, 7%, and 14% respectively. Two patients had concurrent mutations (*FLT3*-ITD and *NPM1* mutations). Patients with *FLT3* mutations exhibited a significantly higher total white cell count than those who did not harbour the mutation. Other haematological parameters and the cytogenetic results did not reveal any significant results concerning the mutational status. Follow-up studies should include a larger number of samples, clinical parameters, and treatment outcomes as these mutations are of therapeutic and prognostic significance.

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CONFLICT OF INTEREST

None

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