Hyperleucocytosis grading score and NPM1 gene mutation among patients with acute myeloid leukemia: Malaysian experience

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Abstract

This is the first study evaluating the frequencies of nucleophosmin (NPM1) gene mutation according to the hyperleukocytosis (HL) grading score. The current two cut off values of this score in acute myeloid leukaemia (AML) patients are [1] those with $50\text{--}100 \times 10^9/\text{L}$ and [2] those with $\geq 100 \times 10^9/\text{L}$ total WBC count. The score represents the current clinically based definition of HL in patients with AML. A total of 90 patients with AML were included. AML patients were stratified into three groups: [1] those with a WBC count below $50 \times 10^9/\text{L}$ ($n = 33$), [2] those with a WBC count $50\text{--}100 \times 10^9/\text{L}$ ($n = 29$) and [3] those with a WBC above $100 \times 10^9/\text{L}$ ($n = 28$). Complete blood cell count, immunophenotyping and PCR of the exon 12 of NPM1 gene followed by sequencing analysis were done. NPM1 mutations were detected in 20/90 (22.2%) of patients with AML involving exon 12 of NPM1 gene and showed significant correlations with some hematologic characteristics. Contrary to expectation, no statistically significant difference was found in the WBC counts according to the NPM1 gene the mutation status among the studied groups. Also, there were no significant differences in the WHO classification categories according to the HL grading score. The difference in the frequency of NPM1 mutation in Asian compared to Western patients might be attributed to biology and aetiology variation of the disease in different population. The current two cut off values defining the HL are molecularly unreliable in spite of being clinically defined.

Keywords Hyperleucocytosis · Grading score · NPM1 gene · Mutation · Acute myeloid leukaemia · Malaysia

Introduction

Reliability of hyperleukocytosis (HL) grading scores and its correlation with the frequency of the NPM1 gene mutation in acute myeloid leukaemia (AML) has not been verified to date. AML is a phenotypically and genetically heterogeneous clonal disorder of hemopoietic myeloid stem cells characterized by the inhibition of differentiation, accumulation of cells at various stages of incomplete maturation, reduced production of normal hemopoietic elements and variable responsiveness to therapy [1]. Genetic-based analyses and molecular markers are now established as the central component for accurate diagnosis, prognostic stratification, monitoring minimal residual disease and developing targeted therapies [2]. NPM1, also known as nucleolar phosphoprotein B23, numatrin1 or NO38 is a ubiquitously expressed nucleolar phosphoprotein that shuttles between the nucleus and cytoplasm with predominant nucleolar localization. In humans, NPM1 gene is implicated in the genesis of different cancers and its role in AML was well

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reviewed [3, 4]. As a general rule, the presence of an NPM1 mutation is associated with a more favourable clinical outcome [2]. The NPM1 mutation was discovered in 2005 and represents the most frequently observed molecular abnormalities in about 50% of AML cases [3, 5, 6].

NPM1 was first identified as a nucleolar phosphoprotein that has a role in the regulation of cell growth, proliferation and transformation. Translocations of the mutated NPM (NPMm) protein into the cytoplasm are the most specific and frequent events found in AML with a normal karyotype that will have a good response to induction chemotherapy [5]. Cytogenetic location of NPM1 is 5q35.1 with a size of 25 kbp. Exon 12 of NPM1 with a size of 650 bp encodes a ubiquitously expressed 37 kDa protein that is localized at the nucleolus [5, 6]. Other functions for this protein include regulation of centrosome duplication; ribosomal function; stress-induced regulator of p53 in response to hypoxia, UV irradiation or cytotoxic drugs [7]; molecular chaperone that have role in preventing both proteins and nucleic acids aggregation in the nucleolus; regulation of the assembly; transport of preribosomal particles through the nuclear membrane; progression of cell cycle; regulation of the p53 tumour suppressor pathway and stabilization and nucleolar localization of p14ARF [8, 9]. The cytoplasmic displacement of NPM1 and the mutation of exon 12 were described as being a hallmark of a large AML subgroup [10]. AML with mutated NPM1 shows distinctive biological and clinical features, and is therefore, categorized as a provisional entity in the World Health Organization classification of leukemias. To date, the presence of this genetic lesion does not define specific disease entities [4]. AML with NPM1 gene mutation encompasses all the WHO categories, and it represents leukemic entity accounting for approximately one-third of all AML [7, 10–14].

HL in acute leukemias has been variably defined as a total white blood cells (WBC) count of more than $50 \times 10^9/L$ [15]. This definition is based on clinical parameters which took into consideration the WBC level at which leukostasis occur more frequently [16]. To date, the reliability of the correlation of these grading scores of HL with the NPM1 gene mutation was not verified. The WBC count significantly affects blood viscosity in the microcirculation of the lung, brain and heart at a level exceeding $50 \times 10^9/L$ and the risk increases significantly with $WBC \geq 100 \times 10^9/L$. HL with leukostasis is a medical emergency caused by the blast cell aggregates and thrombi formation in the microcirculation resulting in clinically observed signs and symptoms. The underlying mechanisms leading to the development and the progression of HL are still unclear [17, 18].

The overall objective was to evaluate the reliability of the two grading scores of HL based on the frequencies of NPM1 gene mutation. Specific objectives were to assess the types and frequencies of NPM1 gene mutation in AML patients with WBC count $< 50 \times 10^9/L$, compared to frequencies of

the NPM1 gene mutation in patients with WBC count between 50 and $100 \times 10^9/L$ and those with WBC count $> 100 \times 10^9/L$. Mutations in exon 12 of NPM1 are the most frequently acquired molecular abnormality in AML occurring with an overall frequency of 25–35%. [1, 2]. NPM1 mutations have been reported in 50–60% of adults with de novo AML, especially those with a normal karyotype [5, 6, 19, 20]. Generally, patients with NPM1 mutation express substantially higher WBC count than those with the wild type. Additionally, those patients were found to possess significantly lower CD34 antigen expression on flow cytometry [21]. Among NPM1-mutated AML, the antigen expression pattern of HLADR(+) CD34(+) CD7(+) is associated with a poor prognosis [22]. A variety of molecular methods for detecting NPM1 mutations have been employed including PCR amplification and direct sequencing, high-resolution fragment analysis, melting curve analysis on the Light Cycler system, denaturing high-performance liquid chromatography (DHPLC), LNA-mediated PCR clamping, flow cytometer, a western blot assay using polyclonal antibodies, FAST PCR assay and quantitative PCR [22–25].

Methodology

Ethical approval was obtained from UniSZA (UniSZA/C/2/CRIM/628-1 [25]) and National Medical Research Register (NMRR-13-1487-1712).

This is a cross-sectional study which enrolled a total of 90 patients with newly diagnosed (de novo) or relapsed AML with or without abnormal karyotypes. Patients with secondary acute leukaemia were excluded. All blood and bone marrow samples were obtained at the time of initial diagnosis. The classification of AML followed the WHO classification 2008. Diagnosis of AML and its subtype were confirmed by cytological examination and immunophenotyping of blood and bone marrow. Briefly, blast cell determination was expressed as the percentage out of all the bone marrow nucleated cells. The differential WBC count was typically based on a 200-cell count in peripheral blood and 500-cell count in the bone marrow. The total 90 patients with AML enrolled in this study were stratified into three groups: group 1 were those with a WBC count below $50 \times 10^9/L$ ($n = 33$), group 2 were those with a WBC count between 50 and $100 \times 10^9/L$ ($n = 29$) and group 3 were those with a WBC above $100 \times 10^9/L$ ($n = 28$). Peripheral blood or bone marrow samples or unstained bone marrow smears were used for genomic DNA (gDNA) extraction. The unstained bone marrow smears were scraped off the surface of the microscope slides in a laminar flow hood using a sterile scalper blade. Separate blade was used for each patient's smears. The scraped material was hydrated in a sterile 3.0 ml tube contained 2.0 ml phosphate-buffered saline (PBS; 0.02 M, pH 7.4). Complete blood cell count (CBC)

was done using modern, automated haematology system (Sysmex XE5000) (Sysmex Corp., Chuo-ku, Kobe, Japan) at HKL, Malaysia. The CBC parameters and immunophenotyping results of the patients were obtained from the patient's records.

The gDNA was extracted and purified from the peripheral blood mononuclear cells by the fully automated DNA purification instrument, QIASymphony (Qiagen) using the LaboPass Genomic DNA isolation kit (Qiagen Biotechnology Malaysia Sdn. Bhd., Kuala Lumpur, Malaysia) according to the manufacturer's recommendations. gDNA quantification was based on the optical density measurement to assess the purity of extracted DNA. The concentration of DNA in solution was measured by reading the absorbance at a wavelength of 260 nm. The gDNA was stored at -20°C for 2–3 months till the analysis of the exon 12 of NPM1 gene mutation was done as described [19]. Polymerase chain reaction (PCR) amplification of the exon 12 of NPM1 gene was carried out as described using forward primer (5'-TTAACTCTCTGGTGGTAGAATGAA-3') and the reverse primer (5'-CAAGACTATTTGCCATTCTAAC-3') [19]. The total reaction volume was 25 μL contained approximately 5 ng DNA, 10 mM dNTP mix each, 2.5 μL of $10\times$ PCR Buffer, 0.125 μL Master Mix (HotStarTaq DNA Polymerase (5 U/ μL)), 0.25 μL of each primer and 16.5 μL distilled water. gDNA samples were amplified using the following PCR conditions: initial denaturation, 95°C for 5 min, 40 cycles of denaturation at 94°C for 30 s, annealing 55°C for 1 min and extension 72°C for 1 min.

PCR products were purified by the standard methods using PCR purification Kit (Qiagen, Kenlo, Netherlands), and directly sequenced with forward primer NPM1: 5'-TTAACTCTCTGGTGGTAGAATGAA-3' and the reverse primer NPM1: 5'-CAAGACTATTTGCCATTCTAAC-3' (Fig. 1). The amplified DNA product was sequenced using the same primers via automated DNA sequencer according to the standard procedure with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on ABI Prism 3700 DNA Analyser (Applied Biosystems, Foster City, Calif). Further analysis was done using the 310 Genetic Analyser (Sanger sequencing; Applied Biosystems) (Fig. 1). DNA sequencing is the standard practice for determination of mutations in human genes.

Fig. 1 Examples of the PCR products from patients' gDNA samples. Qiagen HotStarTaq DNA Polymerase amplify the targeted DNA at expected size of 358 bp

Multiple sequence alignment and analysis were conducted using Bioinformatics tool to interpret the alignments of multiple sequences (UGENE, version 1.25). All sequencing amplicons were screened for quality prior to analysis. Regions with erroneous base-calling were disregarded in the downstream analyses. Samples with highly incongruous sequences despite acceptable base-calling quality were removed from the multiple alignments. Multiple sequence alignment was performed using the ClustalOmega plugin. As a reference, the NPM1 gene sequence was acquired from the NCBI database [*Homo sapiens* nucleophosmin (nucleolar phosphoprotein B23, numatrin) (NPM1), RefSeqGene (LRG_458) on chromosome 5 > gi|269954735/ref/NG_016018.1].

Statistical analysis

Haematological data of patients were statistically evaluated using SPSS version 13.0 for Windows, SPSS, Chicago, IL. Sample size calculation was done using Power & Sample Size Program version 3.0.43 which indicated the need to enrol 29 experimental subjects and 29 control subjects in order to be able to reject the null hypothesis that the failure rates for experimental and control subjects are equal with probability (power) 0.8. p values < 0.05 were considered statistically significant.

Results

The mean platelet count ($\times 10^9/\text{L}$) value revealed significant intergroup differences. The platelet count was found to be significantly lower in group 1 compared to those of group 3 (ANOVA test; p value = 0.03). However, the platelet count values in group 2 were not significantly different from both group 1 and group 3 (p value 0.39, 0.39 respectively). Mean MCV (fl) values showed significant higher level in group 3 (ANOVA test: p value = 0.0001). Mean MCH (pg) and MCHC (g/L) values were significantly lower in group 3 compared with group 1, while their values compared to group 2 showed no significant difference (Table 1).

No correlation was found between the other immunophenotyping variables (CD45, CD34, CD33, cMPO, CD13, CD117, HLADR, CD14, CD11b, CD11c, CD64, CD16, CD15, CD3, CD7, CD2, CD56, CD79a, CD19, CD71, CD38 and CD4) and mutation in exon 12 of the NPM1 gene in patients with AML.

Overlapping peaks in the DNA sequence chromatogram indicate the presence of NPM1 gene mutations. SNPs identified in exon 12 of the NPM1 gene were at positions 27806 (rs772573379, rs200860746), 27898 (rs1059404), 27998, 28027 (rs34351976) and 28193 (rs117701982). Two of the mutations were located upstream (C27806T) and downstream (A28193G) of the exon12 region. These two mutations were removed from the analysis to rule out false or low-confidence

Table 1 Basic haematological parameters in the studied groups

Haematological parameters	Groups			<i>p</i> value ^a
	Group 1 (<i>n</i> = 33)	Group 2 (<i>n</i> = 29)	Group 3 (<i>n</i> = 28)	
Total WBC count ($\times 10^9/L$)	19.1 $\pm$ 12.5 1.6–42.5	66.7 $\pm$ 14.8 44.5–89.4	181.7 $\pm$ 77.6 100–375.6	0.0001
Haemoglobin (g/L)	80 $\pm$ 1.8 4.3–13.4	68 $\pm$ 2.2 3.1–13.2	77 $\pm$ 2.5 3.8–13.3	0.1
Platelet count ($\times 10^9/L$)	52.8 $\pm$ 58.5 6–269	77.8 $\pm$ 60.8 16–223	105.4 $\pm$ 104.0* 15–490	0.03
Bone marrow blast (%)	51.4 $\pm$ 26.2 6–49	60.4 $\pm$ 24.7 12–97	60.6 $\pm$ 25 13–97	0.26
MCV (fl)	78.4 $\pm$ 31.5 70–123	95.8 $\pm$ 13.7 75–132	100.9 $\pm$ 9.7* 79–123	0.0001
MCH (pg)	39.1 $\pm$ 22.9 23–33	29.4 $\pm$ 2.8 26–36	31.3 $\pm$ 2.7* 26–36	0.019
MCHC (g/L)	33 $\pm$ 2.5 27–38	31 $\pm$ 2.5 27–36	30 $\pm$ 4.3* 13–35	0.001

Group 1 = AML with a WBC count below $50 \times 10^9/L$, Group 2 = AML with a WBC $50\text{--}100 \times 10^9/L$, Group 3 = AML with a WBC above $100 \times 10^9/L$

MCV mean corpuscular volume, MCH mean corpuscular haemoglobin, MCHC mean corpuscular haemoglobin concentration

**p* value less than 0.05 compared to group 1

^a ANOVA test: values represent either numbers (percentages) or as mean $\pm$ S.D. (minimum-maximum)

mutations resulting from noise (the noisy data occurred after the mutation point in the erroneous samples). Failed sequencing in some samples was due to homopolymer region (Fig. 2).

AML patients with mutation of exon 12 of *NPM1* gene showed significant differences in the hematologic characteristics compared to the counterpart. The mean RBC count ($\times 10^{12}/L$), Hb (g/L) and Hct (l/l) levels were significantly lower in the group with mutations of the exon 12 of *NPM1* gene (independent *t* test: *p* value = 0.01). On the other hand, the mean platelet count ($\times 10^9/L$) and bone marrow blast percentage showed significantly lower level in the group with mutations of the exon 12 of *NPM1* gene (Independent *t* test: *p*-values = 0.006 and 0.001, respectively (Table 2).

No statistically significant difference in the type and frequencies of mutation of the *NPM1* gene among the studied groups according to the WHO classification (HL grading score) in patients with AML (Table 3).

Discussion

To the best of our knowledge, the relevance of the mutation status of *NPM1* gene with WBC count according to the current clinical definition of the HL grading scores in patients with AML was not evaluated to date. A cohort of Malaysian patients with AML was investigated for the presence of mutations in exon 12 of *NPM1* gene using direct sequencing approaches.

A significantly higher mean platelet count ($\times 10^9/L$) value in patients with WBC count higher than $100 \times 10^9/L$ compared with those with WBC count less than $50 \times 10^9/L$ (Table 1) as well as higher platelet count in patients with *NPM1* mutation (Table 2) were detected. These findings might reflect the inability of the modern automatic blood cell analyser to identify and differentiate the small particles resulting from shedding of cytoplasmic fragments from the leukemic cells despite the use of two approaches of electrical impedance and the optical scatter in the calculation of the platelets. This finding indicates the need for clinicians' awareness of the occurrence of spuriously higher platelet count level in the situation of analysing blood samples from AML patients with HL particularly those with *NPM1* mutation.

The finding of significantly lower mean MCH (pg) and MCHC (g/L) values in group 3 compared with group 1 (Table 1) seems to indicate the more severe suppression of the erythropoiesis associated with increased leukemic burden as reflected by the higher WBC counts in the blood.

AML patients with mutation of *NPM1* gene showed a statistically significant lower levels of the hematologic characteristics including the mean RBC count ($\times 10^{12}/L$), Hb (g/dL) and HCT (l/l) compared to those without mutation at *p* value of 0.01. The bone marrow blast percentage showed significant higher values in the group with exon 12 of *NPM1* gene mutation than those without at *p* value 0.006 and 0.001, respectively (Table 2). These findings might explain the more aggressive nature and behaviour of the leukemic process in patients with *NPM1* gene mutation particularly in younger age

Fig. 2 Example of failed sequencing due to homopolymer region

group and reflect a variation in cellular behaviour and leukemic mechanisms in these patients with mutation of *NPM1* gene.

Screening of *NPM1* gene mutations may improve risk stratification of patients with AML in the context of genetic analysis [26]. This study is the first to present a comparison of the exon 12 *NPM1* mutation rate in Asian and Western patients with AML. Evidence of discrepancy in frequency of exon 12 *NPM1* mutation between Asian and Western patients with AML might be attributed to variations in biology and aetiology of the disease in different population.

In studies involving Western patients, a low incidence (6.5%) of *NPM1* mutation in paediatric patients was reported [27]. *NPM1* mutations were significantly less frequent in adult patients younger than 35 years [28]. A study reported that the frequencies of *NPM1* mutation in relation to WBC count below or equal to $20 \times 10^9/L$ compared to those with WBC count more than $20 \times 10^9/L$ was statistically significant [29]. Significant correlation of *NPM1* gene mutation with WBC count below or equal to $80 \times 10^9/L$ compared to those with WBC count more than $80 \times 10^9/L$ was reported [11]. In another study, mutated *NPM1* was found in 19% of AML patients with WBC count of $< 20 \times 10^9/L$, 35% of patients with WBC count between 20 and $100 \times 10^9/L$ and in 44% patients with WBC count $> 100 \times 10^9/L$ [9]. *NPM1* mutations were investigated in 400 Southeast Asian leukaemia patients and were detectable in 105 cases (26.25%) of AML [30].

NPM1 mutations was reported as significantly associated with higher peripheral WBC count ($p < 0.05$), and the WHO categories (AML without maturation and Acute monoblastic and monocytic leukaemia), but negatively associated with expression of CD34 ($p < 0.05$) and CD117 ($p < 0.05$) [31]. Contrary to expectation, no significant differences in the WHO classification categories according to the studied groups with HL and the *NPM1* gene mutation were found (Table 3).

The number of the WHO categories of AML studied were AML with minimal differentiated 1.0 (1.1%), AML without maturation 1.0 (1.1%), AML with maturation 4.0 (4.4%), acute promyelocytic leukaemia with t(15; 17) 11.0 (12.2%), acute myelomonocytic leukaemia 5.0 (5.6%), acute monoblastic and monocytic leukaemia 12.0 (13.3%), acute erythroid leukaemia 2.0 (2.2%), acute megakaryoblastic leukaemia (0.0) and myelodysplasia-related AML 54.0 (60%). The reported frequency of HL among the WHO categories is lower in AML with maturation (20%) and higher in acute myelomonocytic leukaemia (40%–50%), acute monoblastic and monocytic leukaemia (40–90%) [14, 19, 20, 32]. In the current work, the WBC count of $50\text{--}100 \times 10^9/L$ was found in acute promyelocytic leukaemia with t(15; 17), acute myelomonocytic leukaemia, acute monoblastic and monocytic leukaemia categories 9/29 (31%) and WBC count more than $100 \times 10^9/L$ found in 9/28 (32.1%) of acute promyelocytic leukaemia with t(15;17) and acute monoblastic and monocytic leukaemia categories (Table 3).

Table 2 Comparison of the haematological variables between the patients group with mutations of the exon 12 of NPM1 gene and those without

Variable	Exon 12 of NPM1 gene mutation		Mean difference	<i>t</i> statistic (df)	<i>p</i> value ^a
	Present 20 (22.2)	Absent 70 (77.8)			
WBC count (X10 ⁹ /L)	72.1 ± 69.6 1.60–270.0	80.6 ± 81.6 1.3–375.5	– 8.56	31.7	0.38
RBC count (X10 ¹² /L)	2.3 ± 0.4 1.6–3.1	2.5 ± 1.0 1.0–5.4	– 0.13	74.2	0.019 ^a
Hb (g/L)	71 ± 11 54–100	76 ± 22 31–134	– 0.52	56.1	0.018 ^a
Hct (l/l)	0.22 ± 0.04 0.16–0.33	0.24 ± 0.07 0.10–0.39	– 0.02	48.1	0.014 ^a
MCV (fl)	91.7 ± 9.1 75.4–109.7	93.5 ± 11.9 72.1–132.4	– 1.81	34.9	1.37
MCH (Pg)	33.3 ± 15.3 24.4–97.8	33.9 ± 16.1 22.8–112.9	– 0.54	29.0	0.66
MCHC (g/L)	329 ± 23 266–366	316 ± 33 130–376	103	38.83	0.08
Platelet count (X10 ⁹ /L)	116.4 ± 158.6 8.0–700.0	73.1 ± 76.8 4.0–490.0	43.28	21.01	0.006 ^a
BM Blast (%)	75.4 ± 14.1 34–90	60 ± 27 6.0–97	15.94	54.70	0.001 ^a

Hct haematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular haemoglobin, *MCHC* mean corpuscular haemoglobin concentration

^a Independent *t* test, Values represent either numbers (percentages) or as mean ± S.D. (minimum-maximum)

The incidence of all forms of *NPM1* mutations varied between 25 and 35% in the literature while the reported *NPM1*-A type of mutation was 19.1–20.3% [7, 21, 30]. Mutation in the exon-12 of the *NPM1* gene was reported in 28.2% of the overall population, including 1/1 (100%) of AML, with minimal differentiated, 11/27 (40.7%) of AML without maturation, 11/46 (23.9%) of AML with maturation, 0/29 (0%) of acute promyelocytic leukaemia with t(15; 17), 2/9 (22.2%) of acute myelomonocytic leukaemia, 18/39 (46.2%) of acute monoblastic and monocytic leukaemia and 1/5 (20.0%) of acute erythroid leukaemia [33].

In agreement with previously reported frequencies [25], the *NPM1* gene mutations in this study was detected in 20/90 (22.2%) AML patients with or without abnormal karyotypes (Table 3). Three types of exon 12 mutation were identified at positions 27898 (rs1059404), 27998 and 28027 (rs34351976). Two of the mutations fall upstream (C27806T) and downstream (A28193G) of the exon12 region were removed from the analysis to rule out false or low-confidence mutations resulting from noise (Fig. 2).

In a study conducted in Japan, *NPM1* mutations were reported in 24.9% adults with de novo AML and 47.4% AMLs with a normal karyotype, in seven patients (11.7%) among another group of 60 Japanese patients and in Taiwan; the incidences were 19.1% and 40.3% in two local groups [13, 34, 35]. It was reported that among the Korean patients, 38.3% had *NPM1* mutation [30] [35]. Most studies for *NPM1* mutation status in Asians have recruited AML patients with or

without abnormal karyotypes [20, 36]. The mutation in the *NPM1* gene in Western population occurs with an overall frequency of 25–35% [1, 2]. *NPM1* mutations have been reported in 50–60% of adults with de novo AML, especially in those of a normal karyotype.

Unexpectedly, the lack of relationship between the WBC count and the mutation status (Table 2) was consistent with the result from another study in Malaysia and with another study conducted among the Western population [37]. The difference in the incidence of *NPM1* mutation between the populations suggests that leukaemia in Asian patients may differ from that of Western patients in pathogenesis and biological characteristics [24, 38, 39]. Based on these findings, this study carries a significant impact both at the local and the regional levels.

Unlike prior research findings [21, 22], this study revealed no correlation between the immunophenotyping variables (CD45, CD34, CD33, cMPO, CD13, CD117, HLADR, CD14, CD11b, CD11c, CD64, CD16, CD15, CD3, CD7, CD2, CD56, CD79a, CD19, CD71, CD38 and CD4) and the mutation status in exon 12 of the *NPM1* gene in patients with AML.

Although 90 patients with de novo AML were enrolled in this study, the sample size was not sufficiently large to reflect the actual population of adults and paediatric patients with AML in Malaysia. This study is the first to investigate the correlation of the HL grading score with the frequency of the *NPM1* gene mutation in Malaysian patients with AML with or without abnormal karyotypes.

Table 3 Distribution of WHO categories of AML according to HL grading scores ($\times 10^9/L$) and the NPM1 gene mutation status

WHO categories	No. (%)	Groups			Mutation status of NPM1 gene		p value ^a
		Group 1	Group 2	Group 3	Present	Absent	
AML with recurrent genetic abnormalities: Acute promyelocytic leukaemia with t(15; 17)	90 (100%)	33 (36.7%)	29 (31.1%)	28 (32.2%)	20 (22.2%)	70 (77.8%)	NS
AML not otherwise specified (NOS)	11.0 (12.2%)	5 (15.1%)	4 (13.8%)	2 (7.1%)	2 (10.0%)	10 (14.2%)	
AML, with minimal differentiated	55.0 (61.1%)	19 (57.6%)	18 (62.1%)	18 (64.3%)	13 (65%)	41 (58.6)	
AML without maturation	1.0 (1.1%)	0.0	1 (3.4%)	0.0	3 (15.0%)	2 (2.8%)	
AML with maturation	4.0 (4.4%)	3 (9.0%)	0.0	1 (3.5%)	1 (5.0%)	4 (5.7%)	
Acute myelomonocytic leukaemia	5.0 (5.6%)	2 (6.0%)	3 (10.3%)	0.0	0 (0.0%)	5 (7.1%)	
Acute monoblastic and monocytic leukaemia	12.0 (13.3%)	3 (9.2%)	2 (6.8%)	7 (25.0%)	1 (5.0%)	7 (10.0%)	
Acute erythroid leukaemia	2.0 (2.2%)	1 (3.0%)	0.0	1 (3.5%)	0 (0.0%)	2 (2.8%)	
Acute megakaryoblastic leukaemia	0.0	0.0	0.0	0.0	0 (0.0%)	0 (0.0%)	

Values represent either numbers (percentages), Group 1 = AML with a WBC count below $50 \times 10^9/L$, Group 2 = AML with a WBC count $50-100 \times 10^9/L$, Group 3 = AML with a WBC above $100 \times 10^9/L$. No significant difference found in the WHO categories of AML according to the HL grading score (WBC count). NPM1 mutation was found in: M0 0 (0.0%), M1 3 (15.0%), M2 1 (5.0%), M3 2 (10.0%), M4 0 (0.0%), M5 1 (5.0%), M6 0 (0.0%), M7 0 (0.0%) and AML-NOS 54.0 (60%) the WHO categories of AML. No significant difference found in the WHO categories of AML according to the mutation status in exon 12 of the NPM1 gene in patients with AML found

^a ANOVA test

Conclusions and recommendations

In spite of being clinically defined, the current two cut off values defining the HL (total WBC count of $50-100$ and $\geq 100 \times 10^9/L$) seem to be molecularly unreliable, based on NPM1 gene mutation. Contrary to expectation, no statistically significant difference was found in the WBC counts; according to the NPM1 gene, the mutation status among the studied patients. Also, there were also no significant differences in the WHO classification categories according to the HL grading score. The association between the WBC count and the mutation status was absent. The difference in the incidence of NPM1 mutation among different populations suggests that the cellular behaviour and pathogenic mechanisms of leukaemia in Malaysian patients might vary from that of Western patients in pathogenesis and biological characteristics. There is still much to be investigated about the correlation of the current HL grading scores with the underlying genetic alteration and interaction with other mutations such as FLT3, N-RAS, JAK 2 and C-KIT.

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Compliance with ethical standards

Ethical approval was obtained from UniSZA (UniSZA/C/2/CRIM/628-1 (26)) and National Medical Research Register (NMRR-13-1487-1712).

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Limitations of this study: this study would still have a limited influence at the global level.

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