ORIGINAL ARTICLE

Molecular and Immunophenotyping Characteristics of non-APML, CD34 and HLA-DR Negative Acute Myeloid Leukaemia (HLA-DRneg non-APML)

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ABSTRACT

Introduction: Non-APML, CD34 and HLA-DR negative (HLA-DRneg non-APML) differ from acute promyelocytic leukaemia (APML). Morphology and blast immunophenotype alone cannot differentiate between the two; molecular confirmation is necessary (1). Nucleophosmin 1 (NPM1) is mainly found in the earlier type, while the PML-RARA mutation is characteristic of APML. This study aimed to characterise HLA-DRneg non-APML and APML through immunophenotyping, molecular findings and post-induction chemotherapy remission rate. **Materials and methods:** A cross-sectional study was conducted at HSAJB, involving all cases diagnosed as acute myeloid leukaemia (AML) for 3 years. The proportions of APML and HLA-DRneg non-APML and their immunophenotypes, molecular mutations and prognosis were compared. **Results:** A total of 182 AML cases were analysed. There was no statistically significant difference in immunophenotypes between the two types of AML. However, more APML cases were negative for CD11c and CD14 compared to HLA-DRneg non-APML cases. Only 69 samples had positive molecular mutations. Four cases of HLA-DRneg non-APML had NPM1 mutations; another four cases had NPM1 with FLT3 ITD mutations. HLA-DRneg non-APML cases with NPM1 mutations had a relatively higher post-induction haematological remission rate than HLA-DRneg non-APML NPM1 with FLT3 ITD mutations. **Conclusion:** A more extensive antibody panel in flow cytometry and a larger sample size may yield better results in distinguishing the two types of AML.
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INTRODUCTION

Acute promyelocytic leukaemia (APML) has unique morphological and immunophenotypic features. Morphologically, APML is recognised as having abnormal promyelocytes, which have kidney-shaped or bilobed nuclei associated with multiple bundles of Auer rods and heavy cytoplasmic granulation. Immunophenotypically, most APML blasts are negative for CD34 and HLA-Dr markers (2, 3). Diagnostically, almost 95% of APML cases displayed t (15;17), leading to a fusion gene of promyelocytic leukaemia retinoic acid receptor alpha (PML-RARA) (4, 5).

Most acute myeloid leukaemia (AML) cases exhibit CD34 and HLA-Dr positivity. However, a small proportion of AML cases display CD34 and HLA-DR negativity (HLA-DRneg non-APML). This group of cases is usually diagnosed as APML, especially in the presence of characteristic morphological findings (1). These AML cases often have morphologic features resembling those of hypogranular APL and belong almost exclusively to the myeloid AML subtypes (1, 2, 6). This group is separate from APML, although some immunophenotyping findings may overlap, raising the possibility of diagnostic challenges. The incidence has been reported to range from 1.5% to 25% (7). Therefore,

cytogenetic and molecular characterisation is necessary to establish an accurate diagnosis (1, 7). The common molecular mutations seen in this group are mainly NPM1 with or without FLT3 ITD (6, 8).

The distinction between APML and HLA-DRneg non-APML is important because they have different treatment modalities and prognoses. APML is a haematological emergency and needs to be treated urgently due to the significant risk of coagulopathy. The most important drug in the initial treatment of APL is all-trans-retinoic acid (ATRA), which induces the differentiation of abnormal promyelocytes (5). This drug is usually combined with arsenic trioxide or other chemotherapy. A more than 95% cure rate was achieved when ATRA was accompanied by arsenic trioxide (4). Whereas HLA-DRneg non-APML is treated with the standard AML chemotherapy protocol, which involves a combination of cytosine arabinoside and anthracycline (e.g., daunorubicin) (9). Recent findings showed that a treatment approach focusing on differentiating leukemic cells with ATRA may benefit HLA-DRneg non-APML cases with NPM1 mutations (9, 10, 11).

Following induction chemotherapy in AML, achieving haematological complete remission (CR) is a crucial milestone. It helps evaluate the effectiveness of induction therapies and treatment strategies (12). This involves examining the cellular bone marrow (BM) to ensure normal erythropoiesis, granulopoiesis and megakaryopoiesis with no more than 5% blast cells in the marrow smear. Additionally, peripheral blood neutrophils and platelets should recover to at least > 1.0 x109/l and 100 x109/l, respectively (12). The study revealed that BM blasts exceeding 5% after induction chemotherapy were associated with a gradual decline in the overall survival rate (12).

MATERIALS AND METHODS

Study population and patient classification

A retrospective, cross-sectional study was conducted in the Pathology Department at Hospital Sultanah Aminah, Johor Bahru from 2018 to December 2020. The estimated sample size was 118. The study included all confirmed cases of de novo AML, with complete data on immunophenotyping, bone marrow aspirate and molecular reports. Cases of secondary AML were excluded, such as AML transformed from myelodysplastic syndrome, myeloproliferative neoplasms or therapy-related cases. The cases were further classified into APML and HLA-DRneg non-APML, based on the immunophenotyping and molecular findings.

Immunophenotyping

Immunophenotyping was performed on samples using an eight-colour flow cytometry Facs Canto II (Becton Dickinson, USA). A standard panel of antibodies was used to diagnose acute leukaemia (13). The lysed-wash method was used using the previously outlined protocol (13). Antigen expression of the leukaemia cells was analysed as positive or negative with a cut-off set at 20% of the event.

Molecular and Cytogenetic Studies

The G-banding technique was utilised for cytogenetic analysis, and fluorescence in situ hybridisation (FISH) analysis was performed using PML-RARα probes to detect the t(15;17). Additional molecular studies were carried out using the RT-PCR method for mutational analysis. All tests were outsourced to the Institute of Medical Research and Hospital Ampang. The cytogenetic and molecular results were obtained from our record system.

Haematological complete remission (CR)

Post-induction haematology parameters were identified to evaluate haematological CR. The parameters include: i) The percentage of blasts in the bone marrow (BM) should be less than 5% after the first cycle of induction chemotherapy. The patient is considered not in remission if the BM blast percentage is greater than 5% after the first cycle. ii) The absolute neutrophil count (ANC) >1.0 x 10^9/L. iii) The platelet count is ≥100 x 10^9/L. The neutrophil and platelet counts were analysed simultaneously as the bone marrow aspiration and biopsy were done (12).

Statistical analysis

A descriptive analysis was conducted, with categorical data displayed as frequency and percentage. A chi-square or Fisher exact test was performed to determine the correlation between categorical variables. A p-value of less than 0.05 was considered significant.

Ethics

The Malaysian Ministry of Health's Medical Research and Ethics Committee granted ethical approval (NMRR ID-21-890-59126).

RESULTS

Patients' characteristic

A total of 182 confirmed AML cases were included in the study. Immunophenotyping and molecular analysis identified 18 APML and 8 cases of HLA-DRneg non-APML. Most of the AML patients were Malay 98 (53.8%), followed by Chinese 58 (31.9%), Indians 11 (6.0%), and others 15 (8.2%). Males constituted 96 (52.7%) of the patients, while females accounted for 86 (47.3%). Most patients were 41- 60 years old 55 (30.2%) or more than 60 years old 54 (29.0%). This was followed by the age groups of 21- 40 years old 39 (21.4%) and 0-20 years old 34 (18.7%). Table I displays the characteristics of the patients.

Table I: Characteristics of the patients

		AML	APML	Non APML*
Total patients (n)		182	18	8
Gender	Male	96	8	3
	Female	86	10	5
Ethnic	Malay	98	8	4
	Chinese	58	6	4
	Indian	11	3	0
	others	15	1	0
Age group	0 - 20	34	0	0
	21 - 40	39	11	3
	41 - 60	55	7	5
	>61	34	0	0

*HLA-DRneg non-APML

Immunophenotyping

Out of 18 cases of APML, only 13 cases of APML and 8 cases of HLA-DRneg non-APML had retrievable immunophenotyping results. The remaining five cases of APML, the immunophenotyping results could not be retrieved. Both groups (APML and HLA-DRneg non-APML) showed positive expressions of MPO, CD33, CD13 and CD117 while CD34, HLA-DR, CD19, CD7, CD3, CD2, CD16 and CD35 were all negative. No significant antigen characteristics were found in either group. CD11c and CD11b were negative in APML by 92.3% and 76.9%, respectively (Table II). In HLA-DRneg non-APML, these antigens were negative in 62.5% and 50% of cases, respectively. Although no statistical significance was found, APML cases showed relatively higher negativity for both antigens than HLA-DRneg non-APML. CD64 was positive in 100% of HLA-DRneg non-APML cases, whereas only 84.6% of APML cases were positive. CD117 was positive in 100% of all APML cases but only positive in 87.5% of HLA-DRneg non-APML cases.

Table II : Percentage of APML and non-APML* with a given immunophenotypic

	CD117pos (%)	CD10neg (%)	CD11c neg (%)	CD11b neg (%)	CD33 pos (%)	CD64 pos (%)	CD14 pos (%)	HLA Dr neg (%)	CD34neg (%)
Non APML*(n=8)	87.5	100	62.5	50	100	100	62.5	100	100
APML (n=13)	100	92.3	92.3	76.9	92.3	84.6	92.3	100	100
p value	0.381	1	0.253	0.346	1	0.505	0.253	1	1

*HLA-DRneg non-APML

Cytogenetic and molecular mutations

Table III displays the molecular mutations identified in AML, APML and HLA-DRneg non-APML. In AML, the mutations included RUNX1 RUNX1T1 (26.4%), FLT3 ITD (19.9%), NPM1 (15.4%) and FLT3 ITD NPM1 (15.4%). All APML cases exhibited positive results for PML-RARA while HLA-DRneg non-APML cases were associated with NPM1 (2.2%) and FLT3 ITD NPM1 (2.2%).

Table III: Molecular mutation in AML, APML and non-APML*

Molecular Mutation	AML(n=156)	APML(n=18)	non- APML* (n=8)
	n (%)	n (%)	n (%)
NPM1	28(15.4%)		4(2.2%)
FLT3 ITD NPM1	28(15.4%)		4(2.2%)
PML RARA		18(9.9%)	
RUNX1 RUNX1T1	48(26.4%)		
FLT3 ITD	36(19.9%)		
FLT3 ITD CBFB MYH11	4(2.2%)		
FLT3 ITD SET NUP214	4(2.2%)		
CBFB MYH11	8(4.4%)		

*HLA-DRneg non-APML

Haematological CR

Haematology CR post-induction chemotherapy in APML and HLA-DRneg non-APML was analysed. The criteria include i) an absolute neutrophil count (ANC) > 1 x 109. ii) a platelet count ≥ 100 x 109/L. iii) a less than 5% bone marrow blast in the aspirate (12). A bone marrow blast assessment was done after cycle 1 induction chemotherapy. The neutrophil count and platelet count were analysed at approximately the same time as the BMAT was done.

Table IV shows that all cases with HLA-DRneg non-APML and positive for NPM1 had a bone marrow blast count ≤ 5% after induction chemotherapy with an ANC >1.0x109/L. Whereas those with HLA-DRneg non-APML and positive for NPM1 with FLT3 ITD, 3 out of 4 cases (75%) had a bone marrow examination blast count ≤ 5% and an ANC>1.0x109/L after induction chemotherapy. However, one case (25%) of HLA-DRneg non-APML positive for NPM1 with FLT3 ITD did not achieve remission as evidenced by a bone marrow examination blast count of 25% and an ANC<1.0x109/L. All APML cases achieved haematological remission following induction chemotherapy.

Table IV: Haematological remission in APML and non-APML* with their associated molecular mutation

	BM# blast<5%	ANCε>1	PLTα >100
Non-APML* with NPM1	100% (n=4)	100%(n=4)	100%(n=4)
Non-APML* with NPM1 with FLT3 ITD	75% (n=3)	75% (n=3)	100%(n=4)
APML with PML-RARA	100%(n=18)	100%(n=18)	100%(n=18)

*HLA-DRneg non-APML
#bone marrow
εabsolute neutrophil count >1.0 x 10^9/L
αplatelet ≥100 x 10^9/L

DISCUSSION

In our study, both groups displayed MPO, CD33 and CD13 positivity, while CD34 and HLA-DR were negative. No significant antigen characteristics were observed in either group. However, we found the expressions of CD11c and CD11b negative relatively higher in APML than HLA-DRneg non-APML although this difference was not significant, possibly due to the small sample size. Several studies have demonstrated that the negativity of HLA-Dr and CD34 alone is insufficient for diagnosing APML, other antigens should be considered for an accurate diagnosis (2, 7, 14). The sensitivity and specificity for diagnosing APML are higher when CD11c and CD11b are negative (14). The combination of negative CD11b, CD11c, and HLA-DR identifies nearly 100% of APML cases (14).

A few studies have suggested that the triple-negative antigens involving CD34, HLA-DR and CD11b (CD34-/CD11b-/HLA-DR-) are more consistent with a diagnosis of APML and are only observed in a small percentage (6.7%) of HLA-DRneg non-APML cases (2, 14). These triple-negative antigens have a specificity of 93.3% and a sensitivity of 90% for diagnosing APML (2). Dong et al. proposed using surrogate markers for APML such as the negativity of CD11a and CD18, which were unavailable in our study (14, 15). They also identified the negativity of CD11c expression as another important marker for the diagnostic specificity of APML (14).

Mosleh et al. identified that a unique blast immunophenotype in APML showed a dim to partial expression of CD64 with CD117 which was not observed in HLA-DRneg non-APML (16). Another study found that CD9 was expressed in both APML and HLA-DRneg non-APML, but with distinct patterns. The authors observed a "homogeneous, moderate to a bright expression of CD9" in all APML patients, whereas in HLA-DRneg non-APML (62.7%), the expression of CD9 was "heterogeneous with dim to moderate" (1). Incorporating CD9 in antibody panels could enhance specificity and sensitivity for detecting APML.

The nucleophosmin gene (NPM1) mutation is detected in approximately 30% of AML patients, making it the most prevalent mutation (17, 18, 19). These mutations are often associated with a normal karyotype and a favourable prognosis (20, 21). However, when FLT3-ITD co-mutates with NPM1, the favourable prognosis is lost (20, 21). In HLA-DRneg non-APML, the most common mutation observed is also NPM1 (20, 22). Our study also identified similar molecular mutations in HLA-DRneg non-APML which were NPM1 (50%) and FLT3 ITD NPM1 (50%).

Comparing HLA-DRneg non-APML-NPM1 mutations and AML-NPM1 positives, Mason et al. found that the first group tends to occur in older individuals with distinct phenotypes and has longer relapse-free and overall survival (22). However, when comparing with FLT3-ITD mutations, no significant difference was found (22).

They also suggested further evaluation of the isocitrate dehydrogenase (IDH) mutation since it might have prognostic implications (22). They discovered that HLA-DRneg non-APML-NPM1 positive patients who had a co-mutation with IDH were less likely to relapse after undergoing induction therapy, resulting in an improved overall survival rate (22).

We identified four cases of HLA-DRneg non-APML with NPM1 positive that met the criteria for haematological remission. In contrast, among the HLA-DRneg non-APML cases with NPM1 co-mutations FLT3 ITD, three cases achieved haematological remission, while one experienced relapse post-induction chemotherapy. Although this study did not yield statistically significant results, a higher rate of haematological remission was observed in HLA-DRneg non-APML cases with NPM1 positivity. All these cases were treated with the standard AML protocol. All APML cases achieved haematological remission. APML cases with PML-RARA are well known for achieving a high percentage of complete remission and positive survival outcomes (4).

The absence of CD34 and HLA-DR markers in both groups suggests that the leukaemic cells are arrested at the promyelocyte stage (10). A few studies have investigated a similar method focusing on leukemic cell differentiation beyond the promyelocyte stage, which may benefit HLA-DRneg non-APML with NPM1 positivity (9, 10). These studies have shown that treatment with ATRA leads to the degradation of the NPM1 gene, resulting in the differentiation of the leukemic cells (9, 10). These findings are supported by Ramesh et al., who found that knocking down NPM1 in leukemic cells induces cell differentiation (11). There may be biological similarities between APML and HLA-DRneg non-APML with NPM1 positive, which can be utilised in designing future therapeutic options. Further research is needed to understand the mechanisms behind these diverse molecular mutations and their impact on treatment response and prognosis.

CONCLUSION

It is important to differentiate between APML and HLA-DRneg non-APML due to the differences in treatment modalities and prognosis. Additional immunophenotyping markers and molecular testing are necessary to identify additional mutations, which may affect prognosis.

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