Original Research Article

Routine Diagnostic Immunophenotypic Profiling in AML Identifies Chemoresistant Patients and Predicts Treatment Outcomes

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ABSTRACT

Aim: This study aimed to examine different phenotypic stages of arrest observed in Acute Myeloid Leukemia.

Material and Method: A total of 220 patients with Acute Myeloid Leukemia (AML) were categorized into five subgroups (HCP, MPP, CMP, GMP, GP/MP) based on their pattern of stage of leukemia arrest (SLA) using an Immunophenotypic profile with routine myeloid markers (MPO, CD13, CD33, CD14, HLADR, and CD34) at diagnosis and were correlated with clinicohematological parameters, molecular and cytogenetic characteristics, and disease status.

Results: The distribution of the SLA phenotypes showed 21% with MPP, 26% with CMP, 21% with GMP, and 37% with GP/MP and none of the patient showed HCP phenotype. In relation to clinicohematological parameters, male patients had maturation arrest at an earlier stage of differentiation (MPP/CMP), whereas female showed in a later stage (GP/MP). High white blood cell (WBC) and blast counts exhibited maturation arrest at the later stage (GP/MP. In relation with FAB subtypes, 83% AML-M3, 42% of AML-M4, and 50% of AML-M5 are associated with the GP/MP phenotype, while 67% of AML-M0 and 23% of AML-M1 are associated with the MPP phenotype. Further, maturation arrest at the MPP level was associated with aberrant CD7 expression. In relation with mutations in FLT3-ITD/ FLT3 D835 were associated with MPP (27%) and CMP (40%), C-kit mutations with CMP stage (67%), whereas NPM1 mutations (75%) and CEBPA mutations (50%) were showed arrest at the progenitor stage (GP/MP). In relation to treatment response, 75% with the MPP phenotype, 13% with CMP, 27% with GMP, and 19% with GP/MP showed persistent leukemic activity. This suggests that the MPP group is the most resistant to chemotherapy.

Conclusion: AML Immunophenotyping can identify a new SLA phenotype that effectively correlates with leukemic cell behavior and helps predict major genetic subgroups at diagnosis, along with their response to standard intensive chemotherapy**.**

Keywords: [Immunophenotyping, AML, Stage of Leukemia arrest, Chemo resistance]

1. INTRODUCTION

Leukemia is characterized by abnormal cell differentiation leading to an increased number of blast cells with impaired functionality. It constitutes approximately 5% of all human cancers, with about half of these cases categorized as Acute Leukemia (AL). Acute leukemia includes

both Acute Lymphoid Leukemia (ALL) and Acute Myeloid Leukemia (AML), affecting an estimated 4 million individuals annually in developing countries (1-2).
During normal hematopoiesis, precursor cells originate from Hematopoietic Stem Cells (HSCs). These cells have the capacity for indefinite self-renewal and differentiate into non-self-renewing Multipotent Progenitors (MPPs) which loses self-renewal capacity but differentiate in to precursor cells. From MPPs, two main branches emerge: Common Lymphoid Progenitors (CLPs), which finally differentiated into T lymphocytes, B lymphocytes, and natural killer (NK) cells; and Common Myeloid Progenitors (CMPs), which differentiate into Granulocyte/Macrophage Progenitors (GMPs) responsible for producing monocytes, macrophages, and granulocytes, and Megakaryocyte/Erythroid Progenitors (MEPs) that generate megakaryocytes, platelets, and erythrocytes (Red blood cells) (3-7).
Precursor cells (blast cells) from in AML, express cell surface markers (CD markers) specific to their developmental stage. These markers serve as phenotypic indicators used to identify the stage of cell differentiation arrest and Multiparametric Flow cytometry is helpful to characterize these blasts in AML patients, resembling the organized structure seen in normal blood cell formation (7-11).

In this study, we conducted an analysis of a AML patients to investigate their phenotype arrest at diagnosis. Our goal was to identify distinct AML surface profiles influenced by the stage of leukemia arrest (SLA). We assumed that understanding of leukemic arrest could help in existing AML classifications by identifying unique phenotypic subtypes linked to specific clinical and molecular features. Furthermore, we aimed to gain insights into treatment outcomes following standard therapies. Ultimately, integrating this phenotypic classification with morphological analysis available within 24 hours of diagnosis could greatly facilitate the planning of therapeutic strategies in everyday clinical settings.

2. MATERIAL AND METHODS

2.1 Patients characteristics

This retrospective study was conducted at the Immunohematology Laboratory, The Gujarat Cancer and Research Institute (GCRI), Ahmedabad, involving 220 patients diagnosed with AML between January 2022 and May 2024. Diagnosis of AML was done through morphological examination along with cytochemitry and flow-cytometric Immunophenotyping. Hematological data, including WBC count, FAB subtypes, gender, age, clinical outcomes, and follow-up information, were retrieved from the Institute's records Department. Cytogenetic and Molecular testing were carried out by Cytogenetic and Molecular diagnostic laboratories of within the institute and data were subsequently correlated.

2.2 Immunophenotyping of AML: Multi-parameter flow cytometry (MFC) was conducted with standardized 8-color EURO Flow panel on either bone marrow (BM) or blood samples (1×10^5 cells per tube) using a standard stain-lyse-wash (BD FACS lysing) procedure using antibodies against CD13, CD33, CD34, CD45, CD117, HLA-DR, CD14 and cytoplasmic MPO. Then samples were run on FACSCanto II cytometers with FACSDiva software (BD Biosciences) and data were acquired on at least 1×10^4 of total when specimen quality allowed. A blast gate was defined, encompassing CD45 dim blast population, on which Myeloid and immature marker expression was analysed. Further, blasts were categorised by their phenotypic expression in to Multipotent progenitor (MPP), Common Myeloid Progenitor (CMP), Granulocytic Myeloid Progenitor (GMP), Granulocyte/Monocyte Progenitor (GP/MP).

2.3 Molecular and Cytogenetic analysis:

Molecular analysis for FLT3-ITD/FLT3D835 was done as per standard protocol with PCR in 91 patients and extended mutation analysis was done in 22 patients on the iron torrent oncomine Myeloid assay GX on fully automated Genexus NGS system (Thermoscientific) at Molecular diagnostic lab of Institute. This panel identify 45 DNA genes and 34 RNA fusion driver genes. Including FLT3-ITD, CEBPA, JAK2, CALR, TP53, NPM1, IDH1/2, and PML-RARA, along with many other important biomarkers. Cytogenetics analysis was carried out by FISH technique at Cytogenetic lab of Institute to detect the cytogenetic abnormalities like, t (15;17)], Inv (16;16)] and Deletion of 7q, deletion of 11q, t (8;21).

2.4 Statistical analysis:
Statistical analysis was carried out using SPSS statistical software version 20 (SPSS Inc, USA). Pearson's Chi-square test with Pearson's correlation coefficient (r) was used to assess correlation and significance between two parameters. Disease-free survival was measured from the date of achieving complete remission until the date of relapse. P values ≤ 0.05 were considered to be statistically significant.

3. RESULTS

3.1 Stages of Leukemia Differentiation Arrest in the Study Cohort.

To define leukemic stage arrest we used expression of routinely used key myeloid markers MPO, CD13, CD33, CD117 along with immature marker CD34 and HLADR. Hematopoietic Stem cell (HSC) and Multipotent Progenitor (MPP) were characterized by CD34 and CD117 expression and absence of MPO. Expression of CD13 and CD33 started at the MPP stage till full maturation. MPO expression was started Common myeloid precursor (CMP) stage and Granulocyte/Monocyte precursor (GMP) had the highest MPO expression (>70%). Expression of HLA-DR was useful to distinguish Monocyte progenitor (MP, HLA-DR positive) from Granulocyte progenitor (GP, HLA-DR negative).
These six markers (CD34, CD117, CD13, CD33, MPO, and HLA-DR) used in routine AML diagnosis are differentially expressed in six stages of normal myelopoiesis. Based on these markers, all AML patients can be categorized in to their leukemia differentiation arrest. Thus, all 220 AML patients were divided into Group 1 Hematopoietic Stem Cells (HSC): $CD34^{+}$, $CD117^{-/+}$, $CD13^{-}$, $CD33^{-}$, MPO^{-}, $HLA\text{-}DR^{+,}$ Group 2 Multipotent Progenitors (MPP): $CD34^{+}$, $CD117^{+}$, $CD13^{+}$, $CD33^{+}$, MPO^{-}, $HLA\text{-}DR^{+}$, Group 3 Common Myeloid Progenitors (CMP): $CD34^{+}$, $CD117^{+}$, $CD13^{+}$, $CD33^{+,}$ MPO^{+} (10-70%), $HLA\text{-}DR^{+}$, Group 4 Granulocyte/Monocyte Progenitors (GMP): $CD34^{+}$, $CD117^{+}$, $CD13^{+}$, $CD33^{+}$, MPO^{+} (>70%), $HLA\text{-}DR^{+}$, Group 5 Granulocyte Progenitors/Monocyte Progenitors (GP/MP): $CD34^{-}$, $CD117^{+/-}$, $CD13^{+}$, $CD33^{+}$, $MPO^{+/-}$, $HLA\text{-}DR^{+/-}$ (11).

In the studied cohort, the most abundant Immunophenotypic group was Group 5 (GP/MP) with 81 cases (37%), followed by Group 3 (CMP) with 57 cases (26%), Group 2 (MPP) with 47 cases (21%), and Group 4 (GMP) with 35 cases (16%). Group 1 (HSC) had 0 cases.

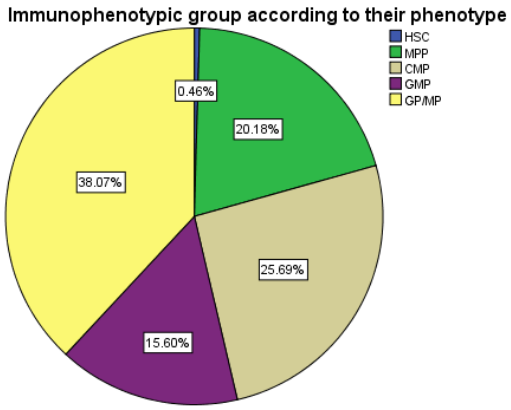


Figure:1 AML subgroups based on their leukemic arrest using Immunophenotypic marker expression

3.2 Correlation of SLA subgroups with Clinicopathological parameters

In relation with Clinical parameters, male patients had maturation arrest at initial stage of differentiation MPP (28%), CMP (21%) as compared to females, showing maturation arrest at later stage GP/MP (44%) (P=0.013). No such correlation was observed with age.

In relation to pathological parameters, high WBC was observed in advance stage of maturation GP/MP (44%) as compare to earlier stage MPP/CMP (25%) (P=0.05). Similarly, high blast count was observed in advance stage of maturation GP/MP (44%) as compare to earlier stage MPP/CMP (22%) (P=0.04)

In relation with FAB subtype, significant correlation of FAB subclass of AML was observed with Immunophenotypic maturation arrest. 67% of AML-M0 patients exhibited MPP phenotype, 30% of AMLM1 with GP/MP phenotype, 33% of AML-M2 with CMP, 83% AML M3 patients with GP/MP and 42% AML-M4 and 50% AML-M5 patients with GP/MP phenotype (P=0.01). Moreover, initial stage of maturation was MPP (24%) was associated with expression of aberrant CD7 positive AML as compared to CD7 negative AML (15%).

Table 1: The distribution of various clinical and pathological parameters among different SLA groups (MPP, CMP, GMP, GP/MP) in the study cohort

Parameters	**N (%)**	**Immunophenotypic marker expression**				**P**
Clinical parameters		**MPP**	**CMP**	**GMP**	**GP/MP**	
Age	**220 (100%)**	**47 (21%)**	**57 (26%)**	**35 (16%)**	**81 (37%)**	
Pediatric (< 14 years)	37 (17%)	9 (24%)	12 (31%)	2 (5%)	14 (38%)	0.26
Adult (> 14 years)	183 (83%)	38 (21%)	45 (25%)	33 (18%)	67 (37%)	
Gender	**220 (100%)**	**47 (21%)**	**57 (26%)**	**35 (16%)**	**81 (37%)**	**0.01**
Male	118 (54%)	33 (28%)	34 (21%)	14 (12%)	37 (31%)	
Female	102 (46%)	14 (14%)	23 (23%)	21 (21%)	44 (43%)	
Pathological parameters						
WBC count (cells/µl)	**220 (100%)**	**47 (21%)**	**57 (26%)**	**35 (16%)**	**81 (37%)**	**0.05**
< 30150/ µl	110 (50%)	27 (25%)	27 (25%)	23 (21%)	33 (30%)	
> 30150/ µl	110 (50%)	20 (18%)	30 (27%)	12 (11%)	48 (44%)	
Blasts (%)	**220 (100%)**	**47 (21%)**	**57 (26%)**	**35 (16%)**	**81 (37%)**	**0.04**
< 77	112 (51%)	25 (22%)	37 (33%)	17 (15%)	33 (30%)	
> 77	108 (49%)	22 (20%)	20 (19%)	18 (17%)	48 (44%)	
FAB subtypes	**220 (100%)**	**47 (21%)**	**57 (26%)**	**35 (16%)**	**81 (37%)**	
M0	3 (1%)	2 (67%)	0 (0%)	0 (0%)	1 (33%)	
M1	53 (24%)	12 (23%)	12 (23%)	13 (24%)	16 (30%)	
M2	96 (44%)	23 (24%)	32 (33%)	17 (18%)	24 (25%)	

M3	24 (11%)	0 (0%)	2 (8%)	2 (8%)	20 (83%)	
M4	31 (14%)	7 (23%)	9 (29%)	2 (6%)	13 (42%)	
M5	10 (5%)	2 (20%)	2 (20%)	1 (10%)	5 (50%)	**0.001**
M7	3 (1%)	1 (33%)	0 (0%)	0 (0%)	2 (67%)	
Aberrant Lymphoid marker expression						
CD7	**220 (100%)**	**47 (21%)**	**57 (26%)**	**35 (16%)**	**81 (37%)**	**0.003**
Negative	60 (27%)	9 (15%)	14 (23%)	4 (7%)	33 (55%)	
Positive	160 (73%)	38 (24%)	43 (27%)	31 (19%)	48 (30%)	

3.3 Correlation of SLA subgroups with molecular parameters

Out of 220 patients, FLT3-ITD/FLT3-D835 mutation was carried out in 91 patients and extended NGS panel was run in 22 patients, of which some gene mutations (ABL1, ASXL1, BCOR, CREBBP, EZH2, KMT2A, MECM, MRTFA, NUP214, PDGFRB, SF3B1, SRSF2, U2AF1, ZRSR2) not detected in any analyzed patients. While STAG2, TP53, BCOR was detected in only single patient., therefore these mutations were not included in further analysis.

In relation with FLT-ITD maturation arrest at initial stage of differentiation MPP (27%), CMP (40%) as compared to FLT3-ITD negative patients, Also, patients with FLT3-D835 mutation showing maturation arrest at MPP (40%) and CMP (20%) stage. Similar results were observed with patients with C-kit mutation showing maturation arrest at early stage CMP (67%). In contrast, majority of patients with NPM mutation (75%) showed differentiation arrest at progenitor stage (GP/MP) stage as compared to NPM negative patients (1%).

Table 2: The correlation of mutational status with different SLA groups (MPP, CMP, GMP, GP/MP) in the study cohort

Parameters	**N (%)**	**Immunophenotypic marker expression**				**P**
Molecular mutation		**MPP**	**CMP**	**GMP**	**GP/MP**	
FLT-ITD	**91 (100%)**	**19 (21%)**	**32 (35%)**	**16 (18%)**	**24 (26%)**	0.65
Negative	76 (84%)	15 (20%)	26 (34%)	15 (20%)	20 (26%)	
Positive	15 (16%)	4 (27%)	6 (40%)	1 (7%)	4 (27%)	

FLT-D835	**85 (100%)**	**18 (22%)**	**30 (35%)**	**15 (18%)**	**22 (26%)**	0.46
Negative	80 (94%)	16 (20%)	29 (36%)	15 (19%)	20 (25%)	
Positive	5 (6%)	2 (40%)	1 (20%)	0 (0%)	2 (40%)	
NPM1	**22 (100%)**	**0 (0%)**	**1 (25%)**	**0 (0%)**	**3 (75%)**	0.10
Negative	18 (81%)	2 (12%)	10 (52%)	2 (13%)	4 (23%)	
Positive	4 (18%)	0 (0%)	0 (00%)	0 (00%)	4 (100%)	
CEBPA	**20 (100%)**	**0 (0%)**	**0 (0%)**	**2 (50%)**	**2 (50%)**	**0.01**
Negative	16(80%)	2 (12%)	9 (57)	0 (0%)	5 (31%)	
Positive	4 (20%)	0 (0%)	0 (0%)	2 (50%)	2 (50%)	
C-KIT	**32 (100%)**	**4 (13%)**	**17 (53%)**	**5 (16%)**	**6 (18%)**	
Negative	29 (91%)	4 (14%)	15 (52%)	5 (17%)	5 (17%)	
Positive	3 (9%)	0 (0%)	2 (67%)	0 (0%)	1 (33%)	0.70
RUNX-1	**30 (100%)**	**5 (17%)**	**10 (33%)**	**3 (10%)**	**12 (40%)**	0.86
Negative	17 (57%)	2 (12%)	6 (35%)	2 (11%)	7 (41%)	
Positive	13 (43%)	3 (23%)	2 (30%)	1 (08%)	5 (38%)	

3.4 Correlation of SLA subgroups with cytogenetics

In relation with cytogenetics, patients with t (15;17) showed maturation arrest at later stage of differentiation GP/MP (79%), as compared to t(15;17) negative patients (P=0.001), Also, patients with inversion 16 showing maturation arrest at GP/MP (67%) stage. In contrast t(8;21) AML1-ETO was observed at arrest at early stage MPP (40%) and CMP (20%). Similarly, 7q deletion was observed at MPP stage (100%). FISH11q was also performed in 28 cases but all the cases were negative for abnormality of 11q chromosome.

Table 3: The correlation of cytogenetics status with different SLA groups (MPP, CMP, GMP, GP/MP) in the study cohort

Parameters	N (%)	Immunophenotypic marker expression				P
		MPP	CMP	GMP	GP/MP	
t(8;21)	**141 (100%)**	**32 (23%)**	**44 (31%)**	**26 (18%)**	**39 (27%)**	0.37
Negative	126 (89%)	26 (21%)	41 (33%)	24 (19%)	35 (28%)	
Positive	15 (11%)	6 (40%)	3 (20%)	2 (13%)	4 (27%)	
dele (7q)	**7 (100%)**	**6 (86%)**	**0 (0%)**	**0 (0%)**	**1 (14%)**	0.49
Negative	5 (71%)	4 (80%)	0 (0%)	0 (0%)	1 (20%)	
Positive	2 (29%)	2 (100%)	0 (0%)	0 (0%)	0 (0%)	
Inv (16)	**137 (100%)**	**31 (23%)**	**41 (30%)**	**27 (20%)**	**38 (27%)**	0.31
Negative	134 (98%)	31 (23%)	41 (31%)	26 (19%)	36 (27%)	
Positive	3 (2%)	0 (0%)	0 (0%)	1 (33%)	2 (67%)	
t(15;17)	**58 (100%)**	**10 (17%)**	**11(18%)**	**10 (17%)**	**27 (47%)**	**0.001**
Negative	34 (59%)	10 (29%)	8 (23.5%)	8 (23.5%)	8 (23.5%)	
Positive	24 (41%)	0 (0%)	3 (13%)	2 (8%)	19 (79%)	

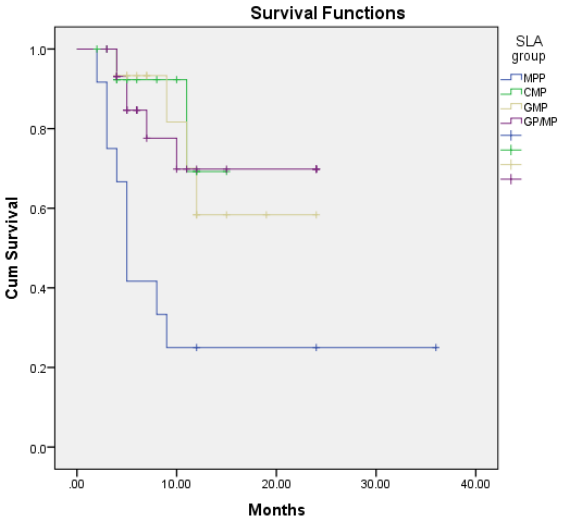
3.5 Correlation with treatment outcome and disease status

Out of 218 patients, detail treatment history was available in 175 patients, of which 73 patients has received standard chemotherapy with Ara-C and had follow-up up to 24 months. Therefore, disease status and early treatment response of Ara-C (post induction remission) was correlated with SLA groups in these 73 patients. Out of 73 patients, 75% patients with MPP, 13% with CMP, 27% with GMP and 19% with GP/MP phenotype had persistent leukemic activity (P=0.003).

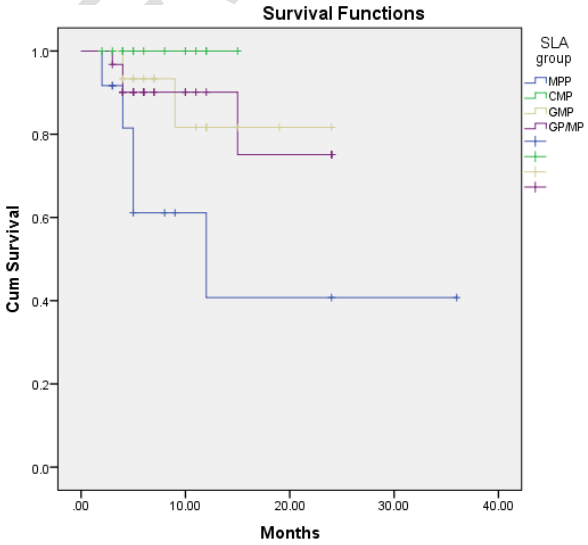
Similarly, patients with MPP phenotype showed significantly reduced overall survival as compared with other groups (P=0.026)

Table 4: Assessment of standard chemotherapy response following the completion of induction therapy in SLA group

SLA Group	N (%)	Persistent Leukemic activity	Post induction Remission	
MPP	12 (100%)	9 (75%)	3(25%)	0.003
CMP	15 (100%)	2 (13%)	13(87%)	
GMP	15 (100%)	4 (27%)	11(73%)	
GP/MP	31(100%)	6 (19%)	25(81%)	



(a)



(b)

Figure:2 (a) Correlation of SLA phenotype with standard Ara(C) treatment (b) Correlation of SLA phenotype with overall survival of AML patients

4. Discussion

Hematopoiesis is the process through which blood cells develop progressively from immature precursors to more specific mature blood cells. Initially, hematopoietic stem cells have the potential to develop into a diverse range of blood cell types. However, as these cells mature, their differentiation potential becomes more restricted, and each stage in this

hierarchical process generates increasingly specialized cells. In a similar hierarchical manner, leukemic blasts in acute myeloid leukemia (AML) also follow a structured development path. It begins with leukemia stem cells (LSCs) that have the capacity for self-renewal and differentiation into precursor cells. When mutations cause a disruption at any stage of this maturation process, it leads to an abnormal proliferation of leukemic blasts (12-14).

In this study, we aimed to understand the pathogenesis of acute myeloid leukemia (AML) within an immunophenotypic context for stage of leukemic arrest (SLA) during maturation process, focusing on five distinct subgroups of AML (HSC, MPP, CMP, GMP, GP/MP) based on routinely used surface markers (MPO, CD34, CD13, CD33, CD117, HLA-DR, CD14, CD15). We correlated these subgroups with clinicohematological parameters, mutational status, and therapy response.

In our study, none of the patients exhibited the HSC phenotype. The distribution of the other phenotypes showed 21% with MPP, 26% with CMP, 21% with GMP, and 37% with GP/MP. In relation to clinicohematological parameters, male patients displayed maturation arrest at an earlier stage of differentiation (MPP/CMP), whereas female patients showed maturation arrest at a later stage (GP/MP). Patients with high white blood cell (WBC) and blast counts exhibited maturation arrest at the later stage (GP/MP). Regarding the FAB classification, these subgroups correlate well with the FAB subtypes, 83% AML-M3, 42% of AML-M4, and 50% of AML-M5 are associated with the GP/MP phenotype, while 67% of AML-M0 and 23% of AML-M1 are associated with the MPP phenotype. However, no such correlation was observed with AML-M2. Additionally, maturation arrest at the MPP level was associated with aberrant CD7 expression.

Gene mutations play a significant role in hematopoietic differentiation and are linked to specific maturation stages. In this study, mutations in FLT3-ITD and FLT3 TKD are associated with early differentiation stages, including MPPs (27%) and CMPs (40%). C-kit mutations are also connected to early maturation arrest, primarily at the CMP stage (67%). In contrast, NPM1 mutations (75%) and CEBPA mutations (50%) are related to differentiation arrest at the progenitor stage (GP/MP). Our analysis did not find a correlation between RUNX1 mutations and SLA stages, which differs from the findings of François Vergez et al. They observed a significant association between RUNX1 mutations and the MMP phenotype. However, like our study, their research also found that NPM1 and CEBPA mutations are associated with the progenitor stage (GMP, GP/MP level). However, this results should be confirming on larger sample size. In relation to cytogenetic analysis, the t(15;17) translocation and inversion 16 are associated with the GP/MP phenotype, whereas the t(8;21) translocation is correlated with early maturation arrest at the MPP and CMP levels.

To examine the correlation between SLA groups and treatment response, we analyzed disease status in relation to SLA in 73 patients who were followed for 24 months and treated with standard chemotherapy along with Ara-C. Among these patients, 75% with the MPP phenotype, 13% with CMP, 27% with GMP, and 19% with GP/MP showed persistent leukemic activity. This suggests that the MPP group is the most resistant to chemotherapy,

followed by CMP, GMP, and GP/MP. Additionally, patients with the MPP phenotype had significantly lower overall survival compared to other groups. Both in vitro chemosensitivity studies and clinical data indicate that the SLA phenotype can predict responses to the main AML therapies. Specifically, genetic subgroups such as CEBPA, and NPM1 mutations, which fall under GMP-L/GPL/MP-L, tend to be more chemosensitive and benefit more from intensive chemotherapy compared to HSCL/MPP-L/CMP-L. Exploring the impact of new therapeutic combinations, such as azacitidine and venetoclax, in this context would be highly interesting. (18)

Conclusion : AML Immunophenotyping can identify a new SLA phenotype that effectively correlates with leukemic cell behavior and helps predict major genetic subgroups at diagnosis, along with their response to standard intensive chemotherapy. This could help in clinical management strategies. However, the molecular complexity of AML indicates the possible existence of sub clones at diagnosis (15-17). Therefore, further research using additional genetic and immunophenotypic markers is needed to fully understand the relationship between SLA and AML.

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