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Original Article

Value of flow cytometry for differential diagnosis between refractory cytopenia with multilineage dysplasia and aplastic anemia

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Abstract: Objective: The aim of this study was to estimate the diagnostic efficiency of aberrant immunophenotypes by flow cytometry (FCM) and generate a new scoring system of FCM for differential diagnosis between refractory cytopenia with multilineage dysplasia (RCMD) and aplastic anemia (AA). Methods: This prospective study with immunophenotypes of RCMD (including HRCMD) and AA bone marrows was analyzed by FCM, in a blinded fashion and compared with normal controls, to identify aberrant immunophenotypes. Diagnostic efficiency of aberrant immunophenotypes was evaluated by single and multi-parameter diagnostic tests. Based on comprehensive analysis of the diagnostic values of each aberrant immunophenotype, a new scoring system of FCM was generated. Results: In single parameter diagnostic tests between RCMD and AA, the specificity was 75~100%, but showed a very low sensitivity from 5.4%-50%. Only the parameters of $CD34^+$ ≥1% and myeloblasts ≥3% in myeloblasts showed diagnostic significance, with an AUC >0.7. Similar results were observed between HRCMD and AA. In multi-parameter diagnostic tests, the optimal combination was $CD34^+$ cells ≥1%, myeloblasts ≥3% in myeloblasts, and CD117 aberrancy in granulocytes with less parameters and with a comparatively better diagnostic value of sensitivity of 63.1%, specificity of 92.2%, and AUC of 0.79. AUC of the new scoring system of FCM was 0.836±0.02 (95% CI: 0.79-0.88) for differential diagnosis between RCMD and AA, with 0.8129±0.03, 95% CI: 0.69-0.86, between HRCMD and AA. Conclusion: The immunophenotypes of $CD34^+$ cells ≥1%, myeloblasts ≥3%, and CD117 aberrant expressions were the most important in differential diagnosis between RCMD and AA. The new scoring system of FCM was an independent predictor for differential diagnosis between RCMD and AA and between HRCMD and AA.

Keywords: Myelodysplastic syndrome (MDS), refractory cytopenia with multilineage dysplasia (RCMD), aplastic anemia (AA), diagnostic tests, scoring system of flow cytometry

Introduction

Myelodysplastic syndrome (MDS) comprises a heterogeneous group of clonal myeloid neoplasms, dysplastic features in one or more myeloid lineages, and increased risk of acute myeloid leukemia (AML) transformation. Refractory cytopenia with multilineage dysplasia (RCMD) is one of the most common subtypes of MDS, constituting about 65.5%-69.5% in Chinese populations [1, 2] and 17.7%-56% in foreign populations [3, 4]. Hypoplastic or hypocellular myelodysplastic syndrome (HMDS) accounts for 10%-15% of MDS [5, 6], while 11.1% of RCMD (41/369) have hypoplastic proliferative bone marrow (HRCMD) [5]. HMDS can occur in any subtype of MDS, but it is difficult to diagnose HRCMD as other subtypes have distinct characteristics of ringed sideroblasts, increased blasts, or increased monocytes, respectively. HRCMD nucleated cells display highly reduced blasts and less frequently show karyotypically abnormal dysplastic marrow cells. Aplastic anemia (AA) sometimes has dysplastic marrow cells. Only 33% of RCMD have karyotypic abnormalities [7], while the percentages in HRCMD are less, making it difficult to distinguish HRCMD from AA. Assessing BM cellularity in patients with BM failure can be difficult, particularly when the cytopenia is not severe. When BM examinations show hyper- or normal cellularity, the attending physician does

Table 1. Combinations of monoclonal antibodies to analyze dysplasia in flow cytometry

	FITC	PE	Per-CP	APC	PE-cy7
1	IgG1a	IgG1a	IgG1a	IgG1a	IgG1a
2	CD3	CD8	CD45	CD4	
3	CD15	CD117	CD45	CD10	CD34
4	CD7	HLA-DR	CD45	CD56	CD33
5	CD14	CD64	CD45	CD38	CD19
6	CD16	CD13	CD45	CD11b	
7	CD71	CD36	CD45	CD20	
8	cIgG1a	cIgG1a	cIgG1a	cIgG1a	CD45
9	cTdT	cMPO	CD45	cCD3	

Abbreviation: FITC: fluorescein isothiocyanate; PE: phycoerythrin; Per-CP: Peridinin-Chlorophyll-Protein Complex; APC: allophycocyanin; PE-cy7: phycoerythrin-cyanine 7.

not generally consider differential diagnosis of AA. As a result, confusion may arise between AA and low-risk MDS, where many patients with immune-mediated BM failure have been treated inappropriately with azacitidine and stem cell transplantation from unrelated donors. Therefore, distinguishing MDS from other immune-mediated BM failure diseases, such as AA, is very important.

Analysis of abnormal immunophenotypes of bone marrow cells by flow cytometry (FCM) has been introduced as an important co-criterion in the diagnosis and differential diagnosis of MDS [8-11]. In many studies, FCM scoring of myeloid aberrancies for diagnosing MDS have been reported with a sensitivity range from 59% to 98% and a specificity of 93%~100% [12-16]. The Ogata score [14, 17] and FCM scoring system (FCSS) [18] have been used for diagnosis and prognosis evaluation in MDS patients, focusing on correlation of scoring systems and prognosis evaluation. Eline M.P. Cremers et al. [10] reported that multi-parameter FCM was instrumental in distinguishing MDS from non-neoplastic cytopenias. They analyzed all subtypes of MDS but did not mention HRCMD.

Therefore, the present study tested the hypothesis that FCM and FCM scoring systems are ideal methods for differential diagnosis between RCMD and AA, especially for HRCMD and AA. This study aimed to estimate the diagnostic value and accuracy of aberrant immunophenotypes in RCMD, selecting the most important immunophenotypes to make a new FCM scoring system as a supplementary diagnostic indicator for differential diagnosis between RCMD and AA.

Materials and methods

Patient information

This study prospectively reviewed medical records and bone marrow samples of 168 RCMD patients (including 39 HRCMD patients) and 77 AA patients. All patients were diagnosed at eight hospitals in Shanghai of the People's Republic of China from January 2009 to December 2015. Each participant provided written informed consent. The study was in accordance with the Ethics Committees of all participating hospitals. RCMD was diagnosed according to criteria of World Health Organization (WHO), 2008 revision [19]. If the bone marrow cellularity was <30% (or <20% in patients >70 years), then the patients were diagnosed with HRCMD [20]. AA was diagnosed according to the guidelines of adult aplastic anemia [21]. All patients were comprehensively analyzed, including medical history and laboratory examinations, such as morphology, biopsy, cytogenetics, and FISH analysis of bone marrow aspirates, as reported before in previous studies [22-24]. Patients were divided into three categories: (1) RCMD patients (n=168), (2) HRCMD patients (n=39), and (3) AA patients (n=77). The median age of patients was 57 (21-80) years, 52 (19-81) years, and 32 (18-82) years in these three groups, respectively. RCMD and HRCMD groups had 33.3% and 38.4% abnormal karyotypes, respectively, while the AA group had no aberrant karyotypes. Immunophenotypes of bone marrow aspirates in all patients were performed centrally, using flow cytometry analysis.

Flow cytometry analysis of bone marrow aspirate specimens

Methods for processing and handling the samples were performed in accordance with the "European Leukemia NET" recommendations for standardization of cytometric procedures [8, 25]. Bone marrow aspirates were collected using heparin and samples were processed within 24 hours after aspiration. Erythrocytes were lysed by ammonium chloride and were washed with phosphate-buffered saline with 0.5% bovine or human serum albumin before staining. Cell suspensions were divided into

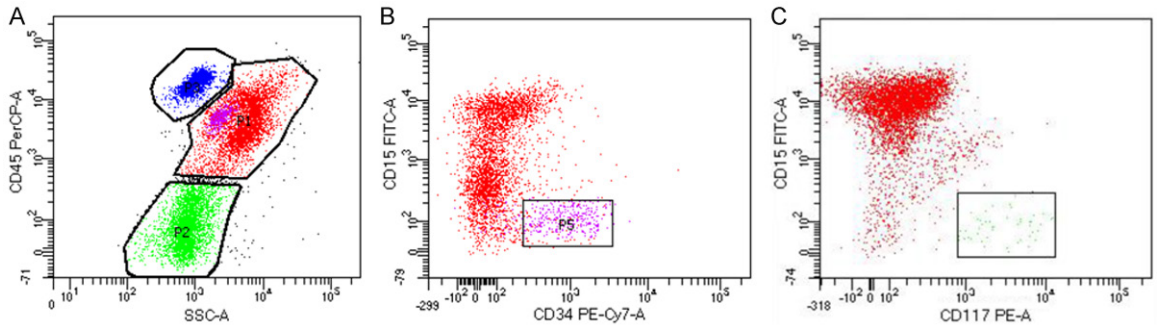

Figure 1. Immunophenotypic patterns in the myeloblast cell population in an RCMD bone marrow. In RCMD bone marrow, the immature cell compartment was very heterogeneous. Myeloblasts often overlapped with B-cell precursors, maturing granulocytes, and monocytes, with no clear boundaries. (A) Heterogeneity of all myeloid cell population ($CD45^{dim}$ and $SSC^{int\text{-}high}$) are gated as P1 (including myeloblasts, B-cell precursors, maturing granulocytes, and monocytes). (B) Cells gated in P1 were displayed on a CD15 versus CD34 plot, the $CD15^{-}CD34^{+}$ cells represented $CD34^{+}$ myeloblast cell cluster (P5, Carmine in the B). (C) Cells gated in P1 were displayed on a CD15 versus CD117 plot, the $CD15^{-}CD117^{+}$ cells represented myeloblast cell cluster.

100-μL aliquots, each containing $3*10^5$ nucleated cells, and were stained with antibodies conjugated with fluorescence, as presented in **Table 1**. All antibodies were purchased from Becton Dickinson, Franklin Lakes, NJ. The panels of antibodies were selected by well-known or proposed combinations of antibodies for MDS dysplastic analysis. Antibodies which were frequently used to differentiate myeloid or lymphoid malignancies from non-clonal hematologic disorders are shown in **Table 1**. Daily instrument quality controls, including fluorescence standardization, linearity assessment, and spectral compensation, were performed according to manufacturer recommendations (Becton Dickinson) to ensure identical operations on a daily basis. Flow data was analyzed using a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA). Immunophenotypic data was acquired via FACSDiva software (Becton Dickinson, San Jose, CA, USA) and were compared with normal bone marrow data to analyze aberrant immunophenotypes.

Analysis of aberrant immunophenotypes in the myeloblasts and mature granulocytes

FCM analysis of MDS should focus on the cells. Quantification of myeloblasts by FCM requires a definition of both reagents and gating procedures. Progenitor cells were selected based on the $CD45^{dim}SSC^{low/int}$ in a CD45 versus Side Scatter (SSC) plot (**Figure 1A**). However, the myeloblasts compartment is very heterogeneous. Antibody combinations, such as CD45/CD34/CD117/CD15 and CD45/CD34/CD19/CD11b, were recommended for distinguishing myeloblasts from other populations, such as B-cell precursors, monocytes, and mature granulocytes, which might show overlapping features of CD45 and SSC. Thus, multiple strategies must be applied to identify and enumerate the myeloblasts present in MDS: (a) $CD45^{dim}SSC^{low/int}$; (b) $CD45^{dim}SSC^{low/int}CD34^{+}$ (B-cell precursors expresses $CD19^{+}/CD11b^{-}/CD34^{+}$); (c) $CD45^{dim}SSC^{low/int}CD34^{+}CD117^{+}CD15^{-}$ ($CD34^{-}/CD19^{-}/CD11b^{+}/CD15^{+}$ were monocytes or mature granulocytes). Percentages of myeloblasts that were obtained with these definitions should be correlated, unless the aberrant myeloblasts lack an antigen (e.g., loss of HLA-DR, CD34, CD13, or occasionally CD45) or aberrantly gain expression, for example, CD56, CD7, or CD19.

Aberrant immunophenotypes in the myeloblasts were: (1) Percentage of myeloblasts ≥3% of all nucleated cells (myeloblasts ≥3%); (2) Percentage of $CD34^{+}$ cells ≥1% of all nucleated cells ($CD34^{+}$ ≥1%); (3) Lack of CD13 expression in the myeloblasts; and (4) Aberrant expression of non-myeloid antigens, such as CD56, in at least 25% of myeloblasts.

Analysis of aberrant immunophenotypes in mature granulocytes: Mature granulocytes were selected by FCM ($CD45^{int}SSC^{int\text{-}bright}$). Dysplastic mature granulocytes can be identified by increased or decreased expression of antigens (CD45, CD13, CD33, CD11b, CD16, and CD64), lack of CD10 and CD13 in mature granulocytes, or aberrant expression of immature myeloid or

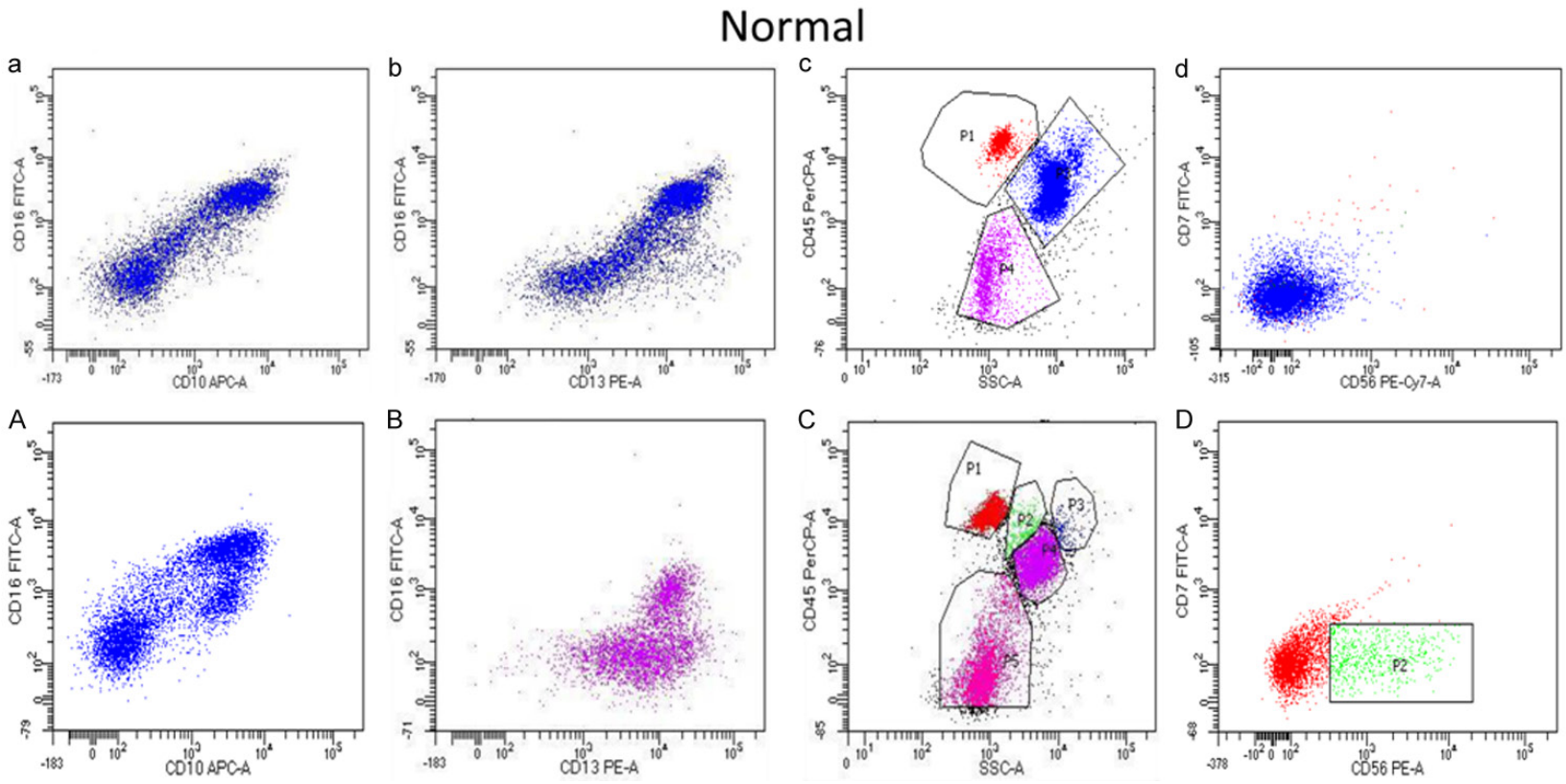

Figure 2. Immunophenotypic patterns in mature granulocytes of RCMD bone marrow samples compared with normal controls. Dysplastic mature granulocytes can be one or more aberrant expression by increased or decreased expression of antigens or an aberrant relationship among two or more antigens. (a) Expression pattern between CD16 and CD10 in normal controls. (b) Expression pattern between CD16 and CD13 in normal controls. (c) Mature granulocytes were selected by CD45 and SSC in normal controls. (d) CD56 was negative in the normal controls. (A) The aberrant expression pattern between CD16 and CD10. (B) The aberrant expression pattern between CD16 and CD13. (C) Compared with (c), the mature granulocytes compartment had an abnormally decreased SSC reflecting hypogranularity, a well-known phenomenon in MDS. (D) Compared with (d), CD56 was aberrant expression in mature granulocytes.

Table 2. Aberrant immunophenotypic differences between RCMD and AA (HRCMD and AA)

Aberrant expression parameters	RCMD N=168 (%)	HRCMD N=39 (%)	AA N=77 (%)	RCMD and AA (*P* value)	HRCMD and AA (*P* value)
Aberrant immunophenotypes of myeloblasts					
$CD34^+$ cells ≥1%	84 (50.0)	21 (53.8)	4 (5.2)	<0.05	<0.05
Myeloblasts ≥3%	71 (42.3)	13 (33.3)	1 (1.3)	<0.05	<0.05
Lack of CD13	32 (19.0)	6 (15.4)	2 (2.6)	<0.05	<0.05
CD56 aberrant expression	15 (8.9)	4 (10.3)	3 (3.9)	>0.05	>0.05
Aberrant immunophenotypes of mature granulocytes					
SSC low	54 (32.1)	20 (51.3)	17 (22.1)	>0.05	<0.05
Lack of CD10	33 (19.60)	6 (15.40	5 (6.5)	<0.05	>0.05
Lack of CD13	53 (31.5)	5 (12.8)	7 (9.1)	<0.05	>0.05
Decrease intensity of CD16	42 (25.0)	11 (28.2)	19 (24.7)	>0.05	>0.05
Increase intensity of CD64	14 (8.3)	2 (5.1)	3 (3.9)	>0.05	>0.05
Increase intensity of CD33	23 (13.7)	5 (12.8)	2 (2.6)	<0.05	<0.05
CD117 aberrant expression	9 (5.4)	2 (5.1)	0 (0.0)	<0.05	<0.05
CD56 aberrant expression	28 (16.7)	9 (23.1)	8 (10.4)	>0.05	>0.05

non-myeloid antigens, such as CD117 or CD56 [8, 15, 26, 27]. Expression patterns of CD16 versus CD10 (**Figure 2A**) and CD16 versus CD13 (**Figure 2B**) permit the identification of whether granulocytes are expressing a normal maturation sequence or not. Aberrant immunophenotypes in mature granulocytes were summarized as: (1) Low SSC by at least 150 mean fluorescence channels, **Figure 2C**; (2) Decreased expression intensity of CD16 (changes of mean fluorescence intensity >1/3 of a decade on a log scale); (3) Increased expression intensity of CD33 (changes of mean fluorescence intensity >1/3 of a decade on a log scale); (4) Increased expression intensity of CD64 (changes of mean fluorescence intensity >1/3 of a decade on a log scale); (5) Lack of CD10 expression in mature granulocytes; (6) Lack of CD13 expression in mature granulocytes; (7) Aberrant expression of immature myeloid antigens in at least 10% of mature granulocytes, such as CD117; and (8) Aberrant expression of non-myeloid antigens of CD56 in at least 20% of myeloblasts, **Figure 2D**.

Statistical analysis

Differences between the two groups of aberrant immunophenotypes were analyzed by Pearson's Chi-squared test. *P*-values were two-tailed, with p values <0.05 indicating statistical significance. To estimate the diagnostic efficiency in single parameter diagnostic tests, sensitivity, specificity, positive predictive value (+PV), positive likelihood ratio (+LR), and receiver operating characteristic curve (ROC) were performed. Aberrant immunophenotypes which were statistically different between RCMD (HRCMD) and AA were combined in the logistic regression models to estimate the diagnostic efficiency of these models by sensitivity, specificity, and area under ROC curve (AUC). Weights of each aberrant immunophenotype were given based on the diagnosis value from diagnostic tests to make a new scoring system of FCM for differential diagnosis between RCMD and AA. ROC curves were illustrated to determine diagnostic cut-offs and to estimate the diagnostic efficiency of the new scoring system. Statistical analysis was performed using Stata version 14 (StataCorp LP, Texas, USA).

Results

Aberrant immunophenotypes in myeloblasts and mature granulocytes

The present research mainly analyzed immunophenotypes in myeloblasts and mature granulocytes of bone marrow cells. Frequencies and percentages of aberrant immunophenotypes are listed in **Table 2**. As much as 50% of RCMD showed aberrant expression of $CD34^+$ cells ≥1% myeloblasts and 53.8% in HRCMD. There was no aberrant expression of CD117 observed in granulocytes of AA patients.

Differences in aberrant immunophenotypes between RCMD and AA were analyzed by Pearson's Chi-squared test (**Table 2**). Aberrant

Table 3. Diagnostic efficiency of single aberrant immunophenotype between RCMD and AA (HRCMD and AA)

	RCMD and AA					HRCMD and AA				
Aberrant expression parameters	Sen (%)	Spe (%)	+LR	+PV	AUC	Sen (%)	Spe (%)	+LR	+PV	AUC
Analysis of myeloblasts										
CD34$^+$ cells ≥1%	50.0	94.8	9.6	95.5	0.7240	53.8	94.8	10.3	84.0	0.7433
Myeloblasts ≥3%	42.3	98.7	32.5	98.6	0.7048	33.3	98.7	25.6	92.9	0.6602
Lack of CD13	19.0	97.4	7.3	94.1	0.5823	15.4	97.4	5.9	75.0	0.5639
CD56 aberrant expression	8.9	96.1	2.3	83.3	0.5252	10.3	96.1	2.6	57.1	0.5318
Analysis of granulocytes										
SSC low	32.1	77.9	1.5	76.1	0.5503	51.3	77.9	2.3	54.1	0.6460
Decrease intensity of CD16	25.0	75.3	1.0	68.9	0.5016	28.2	75.3	1.1	36.7	0.5176
Lack of CD10	19.6	93.5	3.0	86.8	0.5657	15.4	93.5	2.4	54.5	0.5445
Lack of CD13	31.5	90.9	3.5	88.3	0.6123	12.8	90.9	1.4	41.7	0.5186
Increase intensity of CD64	8.3	96.1	2.1	82.4	0.5222	5.1	96.1	1.3	40.0	0.5062
Increase intensity of CD33	13.7	97.4	5.3	92.0	0.5555	12.8	97.4	4.9	71.4	0.5511
CD117 aberrant expression	5.4	100.0	~*	100.0	0.5268	5.1	100.0	~*	100.0	0.5256
CD56 aberrant expression	16.7	89.6	1.6	77.8	0.5314	16.7	89.6	1.6	52.9	0.5314

*: Not count for the denominator was 0. Abbreviation: Sen: sensitivity, Spe: specificity, +PV: positive predictive value, +LR: positive likelihood ratio, AUC: area of the ROC curve.

immunophenotypes of CD34$^+$ cells ≥1%, myeloblasts ≥3%, and lack of CD13 expression in myeloblasts were statistically different between the two groups (P<0.05). However, aberrant expression of CD56 in myeloblasts showed no statistically significant differences between the two groups (P>0.05). Analysis of mature granulocytes demonstrated that aberrant immunophenotypes lacked CD10 expression, lacked CD13 expression, increased expression intensity of CD33, and aberrant expression of CD117, which were significantly different between the two groups (P<0.05). Aberrant expression of SSC, CD16, CD64, and CD56 on granulocytes showed no statistically significant differences between RCMD and AA (P>0.05).

Similarly, aberrant immunophenotypes of CD34$^+$ cells ≥1%, myeloblasts ≥3%, and lack of CD13 expression in myeloblasts were also statistically different between HRCMD and AA (P<0.05). According to analysis of mature granulocytes, only SSC low, CD33, and CD117 were aberrantly expressed and were significantly different (P<0.05) (**Table 2**).

Diagnostic efficiency in single parameter diagnostic tests

Between RCMD and AA, the diagnostic specificity of single aberrant immunophenotypes in myeloblasts and mature granulocytes ranged from 75.3% to 100%, but the sensitivity was very low, ranging from 5.4% to 50% (**Table 3**). The specificity of CD117 aberrant expression in granulocytes was as high as 100% but the sensitivity was only 5.4%. Only the parameters of CD34$^+$ ≥1% and myeloblasts ≥3% showed diagnostic significance, with an AUC of more than 0.7 (0.724, 95% CI: 0.68-0.77; 0.7048, 95% CI: 0.67-0.74). Myeloblasts ≥3% showed a positive likelihood ratio (+LR) of >10, identifying the disease well.

Between HRCMD and AA, the specificity of single aberrant immunophenotype diagnostic tests ranged from 75.3% to 100%, while sensitivity was very low, ranging from 5.1% to 53.8% (**Table 3**). Parameters of CD34$^+$ cells ≥1% and myeloblasts ≥3% could well distinguish HRCMD from AA, because the +LR was more than 10 (10.3, 25.6, respectively). Parameters of CD34$^+$ cells ≥1% had an AUC of more than 0.7 (0.7433, 95% CI: 0.66-0.83).

Diagnostic efficiency in multi-parameter diagnostic tests

For diagnostic tests of single aberrant immunophenotypes which had a high specificity with low sensitivity, multi-parameter diagnostic tests were conducted to improve diagnostic

Table 4. Diagnostic efficiency of multi-parameter diagnostic tests by logistic regression models

No. of combinations	Immunophenotypes combined	RCMD and AA			HRCMD and AA		
		Sen	Spe	AUC	Sen	Spe	AUC
2	$CD34^+$ cells ≥1%/Lack of CD13 in myeloblasts	59.5	92.2	0.765	59.0	94.8	0.753
3	$CD34^+$ cells ≥1%/myeloblasts ≥3%/CD117 aberrant in granulocytes	63.1	92.2	0.790	61.5	92.2	0.769
4	$CD34^+$ cells ≥1%/myeloblasts ≥3%/Lack of CD13 in myeloblasts/CD117 abnormal in granulocytes	63.7	92.2	0.793	61.5	92.2	0.769
5	$CD34^+$ cells ≥1%/myeloblasts ≥3%/Lack of CD13 in myeloblasts/CD117 abnormal in granulocytes/ Increase intensity of CD33 in granulocytes	64.9	89.6	0.796	61.5	89.6	0.777

Abbreviation: Sen: sensitivity, Spe: specificity, AUC: area of the ROC curve.

Table 5. Diagnostic efficiency of the new scoring system for differential diagnosis between RCMD and AA (HRCMD and AA)

Score	RCMD and AA				HRCMD and AA			
	Sen	Spe	+PV	+LR	Sen	Spe	+PV	+LR
≥0.5	0.827	0.688	85.3	2.7	0.795	0.688	56.4	2.6
≥1.0	0.696	0.857	91.4	4.9	0.641	0.857	69.4	4.5
≥1.5	0.649	0.935	95.6	10.0	0.615	0.922	80.0	7.9
≥2.0	0.548	0.974	97.9	21.1	0.538	0.935	80.8	8.3
≥2.5	0.488	0.974	97.6	18.8	0.462	0.974	90.0	17.8
≥3.0	0.435	0.987	98.6	33.5	0.410	0.974	88.9	15.8
≥3.5	0.298	0.987	98.0	22.9	0.250	0.987	90.9	19.7
≥4.0	0.202	1.000	100.0	~*	0.179	0.987	87.5	13.8
≥4.5	0.119	1.000	100.0	~*	0.103	1.000	100.0	~*
≥5.0	0.083	1.000	100.0	~*	0.077	1.000	100.0	~*
≥5.5	0.042	1.000	100.0	~*	0.051	1.000	100.0	~*
≥6.0	0.012	1.000	100.0	~*	/	/	/	/
≥7.5	0.006	1.000	100.0	~*	/	/	/	/

*: Not count for the denominator was 0. Abbreviation: Sen: sensitivity, Spe: specificity, +PV: positive predictive value, +LR: positive likelihood ratio.

efficiency. Aberrant immunophenotypes which demonstrated statistically significant differences between RCMD and AA or HRCMD and AA (**Table 2**) were chosen and combined to make logistic regression models. This study performed sensitivity, specificity, and AUC of ROC curves of each model to evaluate their diagnostic accuracy (**Table 4**).

Sensitivity was increased with the number of combined aberrant immunophenotypes, while specificity was decreased (**Table 4**). The combination of $CD34^+$ cells ≥1%, myeloblasts ≥3% in myeloblasts, and CD117 aberrant expression in granulocytes was considered to be the most ideal model with less parameters and with a better diagnostic value of sensitivity (63.1%), specificity (92.2%), and AUC (0.79), respectively, between RCMD and AA. However, in multi-parameter diagnostic tests by logistic regression models, only particular aberrant immunophenotypes were involved. It was difficult to determine the weight of the others, thus we tried to give a specific scoring system for each aberrant immunophenotype.

New scoring system of aberrant immunophenotypes in myeloblasts and mature granulocytes

A flow cytometric scoring system was devised as a means of condensing multiple flow cytometric abnormalities into numerical scores to distinguish RCMD from AA. Scores were calculated based on aberrant immunophenotypes in myeloblasts and mature granulocytes, which showed statistically significant differences between RCMD and AA or HRCMD and AA. Additional weights of each aberrant immunophenotype were given based on comprehensive analysis of the diagnostic value of a single aberrant immunophenotype, such as sensitivity, specificity, and AUC (**Table 3**). This was done by allowing 1.5 points for each parameter of $CD34^+$ cells ≥1%, myeloblasts ≥3%, and CD117 aberrant expression in the granulocytes, while 1 point was given for lack of CD13 expression in the myeloblasts and increased intensity of CD33 in the granulocytes. A total of 0.5 points was given for lack of CD10 expression in the granulocytes, lack of CD13 expression in the granulocytes, and SSC low in the mature granulocytes.

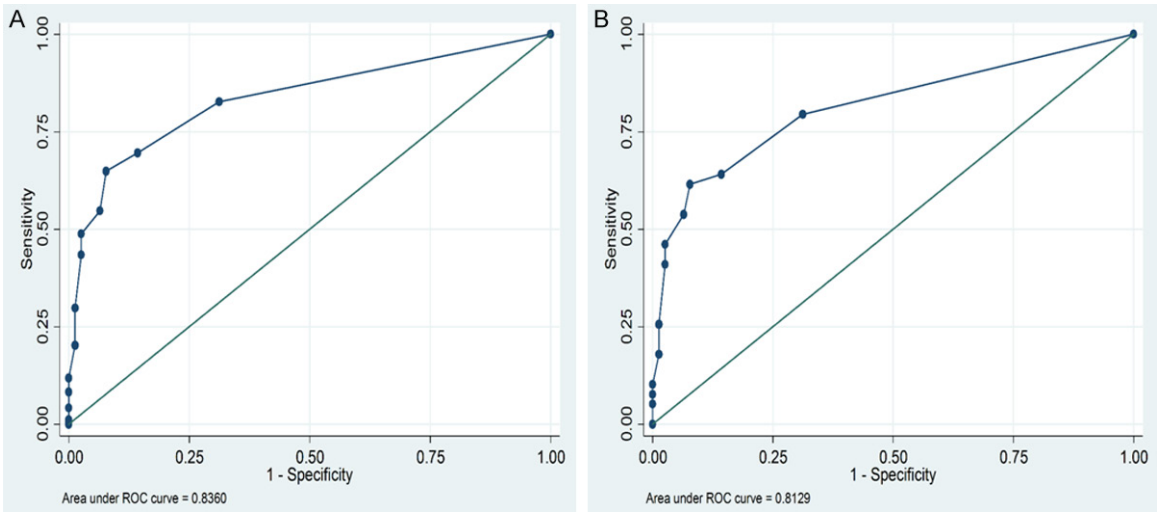

Figure 3. A. ROC curve of the new scoring system of aberrant immunophenotypes for differential diagnosis between RCMD and AA. The AUC was 0.836±0.02, 95% CI: 0.79-0.88. B. ROC curve of the new scoring system of aberrant immunophenotypes for differential diagnosis between HRCMD and AA. The AUC was 0.8129±0.03, 95% CI: 0.69-0.86.

The new scoring system of aberrant immunophenotypes was compared with a gold standard to evaluate its diagnostic efficiency. Each score had its corresponding diagnostic sensitivity and specificity values (**Table 5**). ROC curves for the scoring system are presented in **Figure 3.**

Between RCMD and AA, the AUC of the new scoring system was 0.836±0.02, 95% CI: 0.79-0.88 (**Figure 3A**). For differential diagnosis between HRCMD and AA, the AUC was also as high as 0.819±0.04, 95% CI: 0.73-0.89 (**Figure 3B**), and was more than 0.7, with a very good diagnostic value. The score of 1.5 points was determined as the best cut-off for diagnosis tests. This indicated that the scoring system of aberrant immunophenotypes with more than 1.5 points were diagnosed with RCMD, if not AA. Thus, the new scoring system of aberrant immunophenotypes in the myeloblasts and mature granulocytes was ideal in distinguishing RCMD from AA. It was applied equally for differential diagnosis between HRCMD and AA.

Discussion

Identification of hypoplastic myeloid neoplasms has been compounded by a lack of clear cut diagnostic criteria, which assists in diagnostic contradiction. According to the definition of a case, these both are in relation which truly is hypocellular as well as the separation between HMDS, hypoplastic AML, and aplastic anemia [5]. AA and hypocellular MDS have a number of overlapping features, such as the appearance of cytopenia, a clone of paroxysmal nocturnal hemoglobinuria (PNH) cells, or evidence of T-cell mediated myelosuppression, suggesting that they share a common pathophysiologic pathway [6]. Flow cytometric immunophenotyping is a reliable method in characterizing hematopoietic cells, acting as a potential diagnostic tool in the evaluation of MDS. This study prospectively analyzed FCM immunophenotypes in the bone marrows of 168 cases of RCMD and 77 cases of AA. Data was compared against the comprehensive diagnostic criteria (the gold standard) to estimate the value of differential diagnosis between RCMD and AA.

The present research mainly analyzed immunophenotypes on myeloblasts and mature granulocytes, but not erythrocytes and megakaryocytes. This might be because erythroid and megakaryocytic antigens are relatively scarce and not routinely detected. Panels which require complete immunophenotypic analysis of all 3 lineages are extensive as well as costly. Additionally, thrombocytic antigens CD41 and CD61 were expressed on the platelets and adhered to the surface of nucleated cells, which can lead to false positive results. Stetler-Stevenson et al. [28] analyzed granulocytic, erythrocytic, and megakaryocytic immunephe-

notypes, finding that the FCM of granulocytes were relatively more sensitive than morphological abnormalities. However, in the erythroid and megakaryocytic cells, the morphological abnormalities were more sensitive to FCM.

The present study analyzed aberrant immunophenotypes of RCMD compared with AA patients. Specificity ranged from 75%~100%, but the sensitivity was very low, ranging from 5.4% to 32.1%. The best parameter was $CD34^+$ cells ≥1% in the myeloblasts, with an AUC was 0.724±0.02, 95% CI: 0.67-0.77. All parameters showed diagnostic value to some degree. Myeloblasts ≥3% had a positive +LR >10, indicating that diagnostic tests could not distinguish RCMD from AA very well. To improve the sensitivity for performing multi-parameter diagnostic tests by logistic regression models and estimate the diagnostic efficiency of these models by sensitivity, specificity, and AUC, the present study chose parameters that were statistically different between RCMD and AA or between HRCMD and AA, in making logistic regression models. The AUC of the logistic regression models were all more than 0.7, with some diagnostic value. Eight aberrant immunophenotypes were chosen, which were significantly different between RCMD (HRCMD) and AA to perform the new scoring system. This confirmed that the accumulation of phenotypic abnormalities as quantified by the scoring system was an independent predictor of diagnosis, compared to single or parallel diagnostic tests of aberrant immunophenotypes.

To prove that the scoring system of FCM could distinguish HRCMD from AA, this study separated 39 HRCMD from RCMD patients that compared with AA. The AUC of scoring system of aberrant immunophenotypes was 0.836± 0.02, 95% CI: 0.79-0.88, which identified HRCMD from AA patients. However, a limitation of the present study was the limited cases of HRCMD patients available to evaluate the diagnostic power.

Conclusion

In summary, the present study estimated the diagnostic efficiency of aberrant immunophenotypes of MDS bone marrow by FCM, successfully generating a new scoring system of FCM as an independent predictor for differential diagnosis between RCMD and AA. The new scoring system also identified HRCMD from AA patients, showing several overlapping features. The new system was unlike the Ogata score and FCSS scoring system, which focus on the correlation of scoring systems and prognosis evaluation, analyzing all subtypes of MDS, while not mentioning HRCMD or differential diagnosis between HRCMD and AA.

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Disclosure of conflict of interest

None.

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