学位論文

Diagnostic Value of Flow Cytometry Standardized
Using the European LeukemiaNet for Myelodysplastic Syndrome

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ABSTRACT

Background: Myelodysplastic syndromes (MDS) and idiopathic cytopenia of undetermined significance (ICUS) are heterogeneous hematological disorders characterized by hematopoietic dysplasia and/or chromosomal aberrancy. Objectives: This study aimed to evaluate the diagnostic value of flow cytometry standardized using the European LeukemiaNet (ELN) for MDS and ICUS by analyzing samples obtained from patients with cytopenia based on morphological examination, cytogenetic analysis, and flow cytometry. Method: We retrospectively analyzed bone marrow samples aspirated from 253 consecutive patients (median age: 66 [range: 1–92] years) to identify the cause of cytopenia. Results: Sixty patients presented with MDS and 16 with ICUS. MDS subtypes were distributed as follows: MDS with single lineage dysplasia (10), MDS with multilineage dysplasia (10), MDS with ringed sideroblasts (4), MDS with excess blasts-1 (9), MDS with excess blasts-2 (13), MDS unclassified (5), 5q-syndrome (6), and MDS/myeloproliferative neoplasms (3). Four representative ELN indexes were used. Two or more ELN MDS indexes were in the abnormal range in 35 MDS cases (58.3%) and four ICUS cases (25.0%). Conclusions: Morphological examination remains the standard for MDS diagnosis. Considering the low incidence of genetically proven ICUS (20.2%–27.5%), the low sensitivity of ELN MDS indexes for ICUS is

considered to be a valuable alternative.

(199/200 words)

Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous disease characterized by dysplasia and clonal chromosomal aberration [1]. Therefore, the diagnosis of MDS is somewhat subjective and uncertain in some cases. Notably, in the absence of a characteristic morphology, the confirmative diagnosis of MDS is challenging. Idiopathic cytopenia with undetermined significance (ICUS) [1] is a conceptual and temporary diagnosis of unexplained cytopenia, which does not fulfill the minimal criteria for MDS. In some cases, cytopenia develops and results in MDS/acute myeloid leukemia (AML) [2]. Thus, ICUS is considered to have a pre-MDS/AML or potential status for underlying MDS. This clinical observation was recently associated with chromosomal/genetic factors. Kwok *et al.* have shown that 20% of patients with ICUS harbored clonal mutations [3], indicating that MDS-associated somatic mutations are common and can result in clonal evolution. This clonal pathogenetic change was termed clonal cytopenias of undetermined significance (CCUS) [3]. Accordingly, CCUS are more specific types of pre-MDS rather than ICUS. The authors have proven that CCUS is frequently associated with *TP53*, *TET2*, and *DNMT3A* mutations but not with *SF3B1* mutation. Therefore, a specific target gene sequence may be useful to validate the diagnosis or identify the predisposing risk factors for MDS [3].

However, molecular biological detection of clonal mutations is not completely practical with regard to a realistic clinical situation. Therefore, we investigated the efficacy of surface markers based on the MDS criteria from the European LeukemiaNet (ELN) as a more conventional tool for the diagnosis of MDS.

Material and Methods

1. Study population and sample preparation

We retrospectively assessed 253 consecutive patients who underwent bone marrow aspiration to examine the cause of cytopenia at our institution between 2012 and 2015. The aspirated bone marrow samples were immediately mixed with 1 mL of RPMI1640 and 10% fetal calf serum. The bone marrow cells were counted and suspended at $2.0 \times 10^4/\mu L$ in normal saline. For cell surface antigen staining, the cells were incubated with FITC-, PE-, or PC5-conjugated mouse anti-human mAbs for 30 min on ice at $1.0 \times 10^6/50$ μL. After staining, the cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 1.0 mL of PBS. We lysed the cells after staining by in-house conditioned 0.826% ammonium chloride (NH_4Cl) lysing buffer in individual tube. Staining with propidium iodide (Sigma–Aldrich, St. Louis, MO, USA) preceded all experiments to remove dead cells. At least 1 million cells were obtained from the procedure.

2. Flow cytometry

Data were acquired using the Beckman Coulter NAVIOS flow cytometer (6 colors, 2 lasers; Beckman Coulter, Tokyo, Cat# 775213) and analyzed using the FlowJo software version 8.8.2. (Tomy Digital Biology, Tokyo). Mean fluorescence intensity (MFI) was calculated. We used the following antibodies for conjugated fluorescence: FITC-CD2, 3, 7, 8, 14, 20, and 34 and PE-CD4, 7, 10, 11b, 13, 15, 19, 33, 56, and 117. We prepared 13 columns as follows: 1) FITC-mouse IgG1 isotype and PE-mouse IgG1, 2) FITC-HLA-DR and PE-CD13, 3) FITC-CD7 and PE-CD33, 4) FITC-CD2 and PE-CD7,

5) FITC-CD3 and PE-CD56, 6) FITC-CD8 and PE-CD4, 7) FITC-CD20 and PE-CD16, 8) FITC-CD34 and PE-CD117, 9) FITC-CD14 and PE-CD15, 10) FITC CD34 and PE-CD56, 11) FITC CD34 and PE-CD15, 12) FITC CD34 and PE-CD11b, and 13) FITC-CD34 and PE-CD10. Each column was stained with PC5-conjugated anti-CD45 antibody to perform the CD45 gating assay in all the analysis columns. To analyze ELN MDS indexes, we used CD45 and CD34 expression, and other antibodies were used to differentiate other causes of cytopenia.

3. Clinical and laboratory definition

Four representative ELN MDS indexes were conventionally selected from the working criteria [4,5] as follows: (1) a proportion of the myeloblast-related cluster in all nucleated cells ≥2.0%, (2) a proportion of the B-progenitor-related cluster in all $CD34^+$ cells ≤5.0%, (3) a ratio of CD45-positive cells MFI in lymphocytes compared with $CD34^+$ myeloblasts ≤4.0 or ≥7.4, and (4) a ratio of SCC peak value in $CD45^{high}$ SCC^{high} granulocytes and lymphocytes ≤6.0 [4,5] (Fig. 1). To analyze ELN MDS markers, which we adopted for the analysis, column #13 (FITC-CD34 and PE-CD10 gated by PC5-CD45) was sufficient. Columns #1–#12 were used for the differential diagnosis of cytopenia.

More than two hematologists/specialists determined the final diagnosis. Patients with MDS and ICUS were included in the study population. MDS was diagnosed according to the consensus criteria [6, 7]. Furthermore, ICUS was defined according to the same criteria [6, 8]. We identified cases that fulfilled the following requirements: bone marrow blast count of <5.0%, single or multi-lineage dysplasia of

<10%, and absence of the clonal chromosomal abnormality characteristic of MDS. Chromosomal analysis was conducted for the differential diagnosis of MDS and ICUS.

4. Statistical analysis

To calculate the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and positive likelihood ratio (PLR), the following equations were used. Sensitivity was defined as the proportion of patients with an ELN MDS index of ≥2with MDS or ICUS. Specificity was defined as the proportion of patients with an ELN MDS index <2 without MDS or ICUS. PPV was defined as the proportion of patients with MDS or ICUS with an ELN MDS index ≥2. NPV was defined as the proportion of patients without MDS or ICUS with an ELN MDS index <2. A crosstab was used to calculate statistics. PRL is calculated as sensitivity/(1 − specificity). This indicated the likelihood of having the disease if the test is positive.

Results

We analyzed the bone marrow samples aspirated from 253 patients (117 females and 136 males; median age, 66 [range: 1–92] years). In total, 60 patients had MDS (24 females; median age, 69 [range: 30–91] years) and 16 had ICUS (8 females; median age, 75 [range: 54–88] years). MDS subtypes were distributed according to the World Health Organization (WHO) classification (2017) as follows: MDS with single

lineage dysplasia (MDS-SLD, 10), MDS with multilineage dysplasia (MDS-MLD, 10), MDS with ringed sideroblasts (MDS-RS, 4), MDS with excess blasts-1 (MDS-EB-1, 9), MDS with excess blasts-2 (MDS-EB-2, 13), MDS unclassified (MDS-U, 5), 5q- syndrome (6), and MDS/myeloproliferative neoplasms (MPN, 3). The ELN MDS indexes were ≥2 in 35 patients with MDS (58.3%, 35/60) and ≥2 in four patients with ICUS (25.0%, 4/16).

The MDS subtypes of patients with ELN MDS indexes ≥2 were distributed as follows: MDS-SLD (40.0%, 4/10), MDS-MLD (50.0%, 5/10), MDS-RS (25.5%, 1/4), MDS-EB-1 (77.8%, 7/9), MDS-EB-2 (100.0%, 13/13), MDS-U (0.0%, 0/5), 5q- syndrome (33.3%, 2/6), and MDS/MPN (100.0%, 3/3). With the grade progression of the MDS subtypes, the proportion of patients with ELN MDS indexes ≥2 increased (Fig. 2).

With regard to patients with ICUS, four (25.0%, 4/16) were positive for ELN MDS indexes. The following diseases were observed in patients with ELN MDS indexes ≥2, except for MDS and ICUS: anemia of chronic disease (0.0%, 0/5), renal anemia (11.1%, 1/9), megaloblastic anemia (0%, 0/4), aplastic anemia (8.3%, 1/12), AML (42.9%, 3/7), chronic myeloid leukemia (10.5%, 2/19), MPN (75.0%, 6/8), idiopathic thrombocytopenic purpura (0%, 0/10), infectious diseases (0%, 0/9), collagen

disease (19.0%, 4/21), and other diseases (19.1%, 9/47). Except for individuals with hematological malignancies, approximately 16.9% (30/177) of patients had ELN MDS indexes ≥2 (Table 1). When a cutoff score such as ELN MDS indexes ≥2 was used, the sensitivity and specificity for the diagnosis of MDS were 58.3% (35/60) and 83.1% (147/177), respectively. Furthermore, PPV was 54.7% (35/64), and NPV was 85.0% (147/173; Table 2).

The positive likelihood ratio of MDS was 3.64 in ELN MDS index-positive patients. In Japan, according to the registry data of the Ministry of Health, Labor and Welfare, the prevalence of MDS in the general population is 0.62% (6.2 per 100,000 people). This indicates that the probability of MDS increases from 0.62% to 2.22% in patients with ELN MDS indexes ≥2. The diagnostic sensitivity for MDS and ICUS were 58.3% and 25.0%, respectively. Furthermore, the diagnostic specificity for MDS and ICUS were 83.1% and 83.1%, respectively. These data indicated that a lower diagnostic sensitivity for ICUS rather than MDS can be compensated by a higher specificity (83.1%) for both diseases. This high specificity contributes to the diagnostic value of ELN MDS indexes in patients suspected with MDS or ICUS. For instance, if the pre-test probability of MDS and ICUS was 25.0% for each patient referred for a consultation to a hematologist, the post-test probability increases up to 77.0% and

59.7%, respectively.

Discussions

Our study revealed that positive scores for the ELN MDS index increased corresponding to the disease status of MDS (such as from MDS-SLD, MDS-MLD, MDS-RS, and MDS-EB-1 to MDS-EB-2) [7]. However, the positive scores of the index were relatively low in individuals with ICUS and MDS-U. This finding was disappointing because the ELN MDS indexes did not exhibit a high sensitivity for ICUS and low-risk MDS, such as MDS-U. The possible reasons for this low sensitivity are as follows. First, ICUS supposedly includes a wide spectrum of disease characteristics preceding MDS, along with cytopenia of unknown etiology. Second, low-risk MDS is not a manifestation of a monoclonal disease. The hypothesis of disease progression is supported by the recent clonal expansion theory in MDS pathogenesis [2].

According to the ELN criteria modified by Ogata *et al.* [9], when using four and seven parameters of the ELN MDS indexes, the diagnostic sensitivity for MDS was 30.8% and 65.4%, respectively. According to other previous studies, the diagnostic sensitivity was 23.1%–50.0% using four parameters [4], and the sensitivity could be improved using seven parameters. Considering the differentiation of MDS from other

hematological diseases, including aplastic anemia, MDS/MPN, and ICUS, the low sensitivity and specificity are challenging with regard to clinical use. Among a subset of patients with non-MDS diseases, ELN MDS indexes were observed to be positive. This may indicate acquired bone marrow failure disorders, including aplastic anemia/MDS, hypoplastic MDS, and related diseases, that specifically are positive for ELN MDS indexes.

Previous studies have shown that the ELN MDS index is associated with the Revised International Prognostic Scoring System (IPSS-R) [10] and refined WHO classification-based Prognostic Scoring System (WPSS) [11] and is used as a risk model at the MD Anderson Cancer Center [12]. Our findings showed an increased positivity for the ELN MDS indexes according to the grade of the MDS subtype. This result may represent the clonal evolution of MDS [2]. Ogata *et al.* have described that MDS is a stem cell disease in which the myeloblast $CD34^+$ precursor expresses a pathological hallmark. Then, the total amount and proportion of $CD34^+$ precursors result in higher diagnostic sensitivity for MDS [5]. The grade of the MDS subtype progresses further when the dysregulation of the $CD34^+$ precursor becomes more frequent and severe. In previous studies, aberrant myeloid or B-cell lineage expressions on $CD34^+$ progenitor cells in the bone marrow, which is a component of the ELN MDS indexes, are the

diagnostic [12] and prognostic tools [11] for low-risk MDS. Further investigation may reveal the correlation between ELN MDS indexes and prognostic score for MDS, such as IPSS, WPSS, and other new prognostic tools [10]. In future studies, we aim to improve the diagnostic sensitivity and specificity using ELN MDS indexes and further evaluate their association with disease prognosis.

Targeted sequencing of 22 MDS-associated genes identified 27.5% (33/120 cases) of clonal abnormalities in patients with ICUS [3]. In this study, patients with less quantified dysplasia (<10%) were included as cases that did not fulfill the diagnostic criteria of MDS. When limited to patients with no evidence of dysplasia or an MDS-defining karyotype abnormality, 20.2% (20/99 cases) of patients were confirmed to have non-dysplastic idiopathic cytopenia by targeted sequencing in patients with CCUS. As shown in this study, genetically defined ICUS is the most confirmative evidence of clonal abnormality resulting in MDS. A 20%–30% prevalence of clonal evidence can be reasonable among patients with ICUS. Our study indicated that using ELN MDS indexes for the diagnosis of ICUS can be a useful alternative in patients with clinically probable ICUS. Similarly, clonal hematopoiesis of indeterminate potential, which is a novel concept, is defined as clonal expansion that can potentially result in clonal hematopoiesis but does not include patients with cytopenia [13].

True clonal cytopenia is observed among patients with ICUS, particularly those with ICUS that evolves to clonal hematopoietic malignancy [8, 13]. ICUS and CCUS are analogous diseases that partially mutually overlap. Because ICUS is a highly heterogeneous disease, its potential clonality must be screened using an objective and reproducible technique. In addition, clinically significant cytopenia is a critical aspect that must be addressed to improve disease prognosis, and if required, it can modestly improve the therapeutic course. In our study population, the conclusions were limited because of the absence of long-term follow-up.

Conclusions

This investigation was performed in a large series of patients, and it confirmed the low sensitivity of ELN MDS indexes for low-risk MDS and ICUS. However, the ELN MDS index can identify with high specificity a subset of patients with ICUS, thereby identifying the characteristics of MDS that can be useful for follow-up.

Appendix

Tables

Table 1 Positivity for ELN MDS indexes in individuals with MDS and non-hematological diseases

N = 253		MDS (N = 60)	ICUS (N = 16)	Non-hematological diseases (N = 177)
ELN MDS index	≥2	35	4	30
	<2	25	12	147

Abbreviations: ELN, European LeukemiaNet; ICUS, idiopathic cytopenia of undetermined significance; MDS, myelodysplastic syndrome

Table 2 Diagnostic values of ELN MDS indexes in individuals with MDS and ICUS

	Sensitivity	Specificity	PPV	NPV
MDS	58.3% (35/60)	83.1% (147/177)	54.7% (35/64)	85.0% (147/173)
ICUS	25.0% (4/16)	83.1% (147/177)	11.8% (4/34)	92.5% (147/159)

Abbreviations: ELN, European LeukemiaNet; MDS, myelodysplastic syndrome; ICUS, idiopathic cytopenia of undetermined significance; PPV, positive predictive value; NPV, negative predictive value

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Figure Legends

Fig. 1. Flow cytometry analysis of the ELN MDS indexes.

(a) Gating of all nucleated cells (ANC), SSC-FSC scattering, R1; relatively low SCC cell population. (b) Gating of R1, CD45-CD34 scattering, R2; diminished CD45-expressing $CD34^+$ fraction. (c) Gating of R2, CD45-SSC scattering, R3; $CD34^+$ B progenitor-related cluster, R4; $CD34^+$ myeloblast-related cluster. (d) Gating of ANC, CD45-SSC scattering, R5; granulocytes excluding myeloblasts, R6; lymphocytes. (e) Gating of R5, CD45 of lymphocyte in top panel, and gating of R6, CD45 of granulocyte in the bottom panel. (f) Gating of R5, SCC of lymphocyte in top panel, and gating of R6, SCC of granulocyte in the bottom panel.

Fig. 2. Number of patients with ELN MDS indexes ≥2.

The number and proportion of patients with ICUS and each MDS subtype with ELN MDS indexes ≥2.

Figure 1

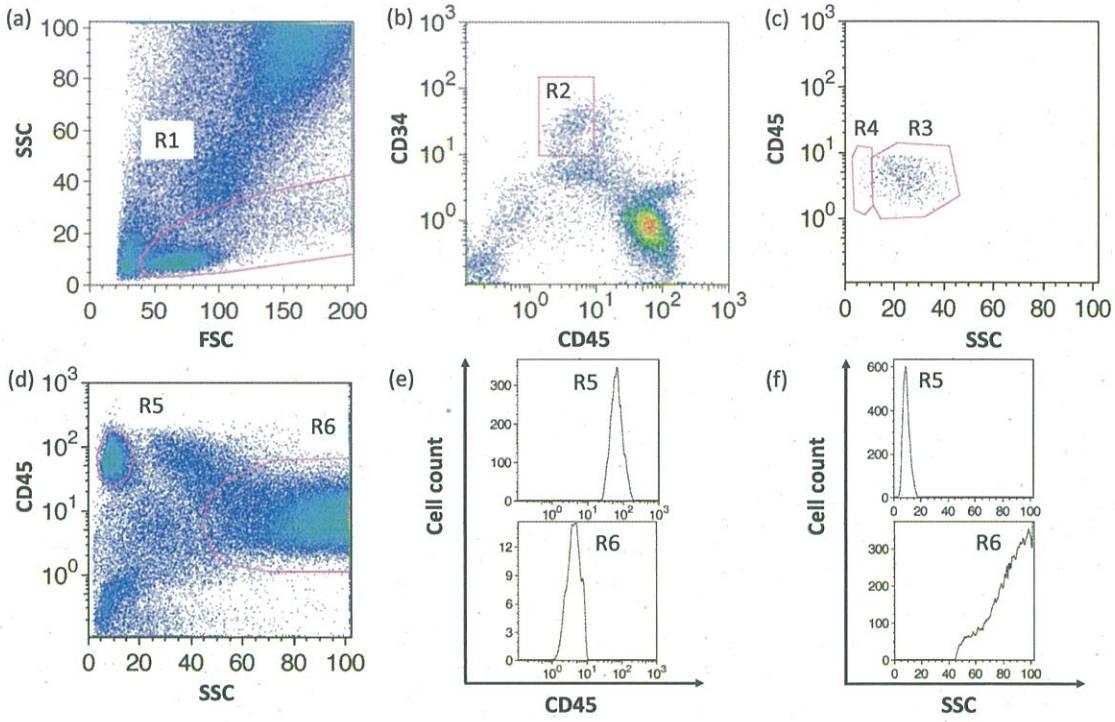
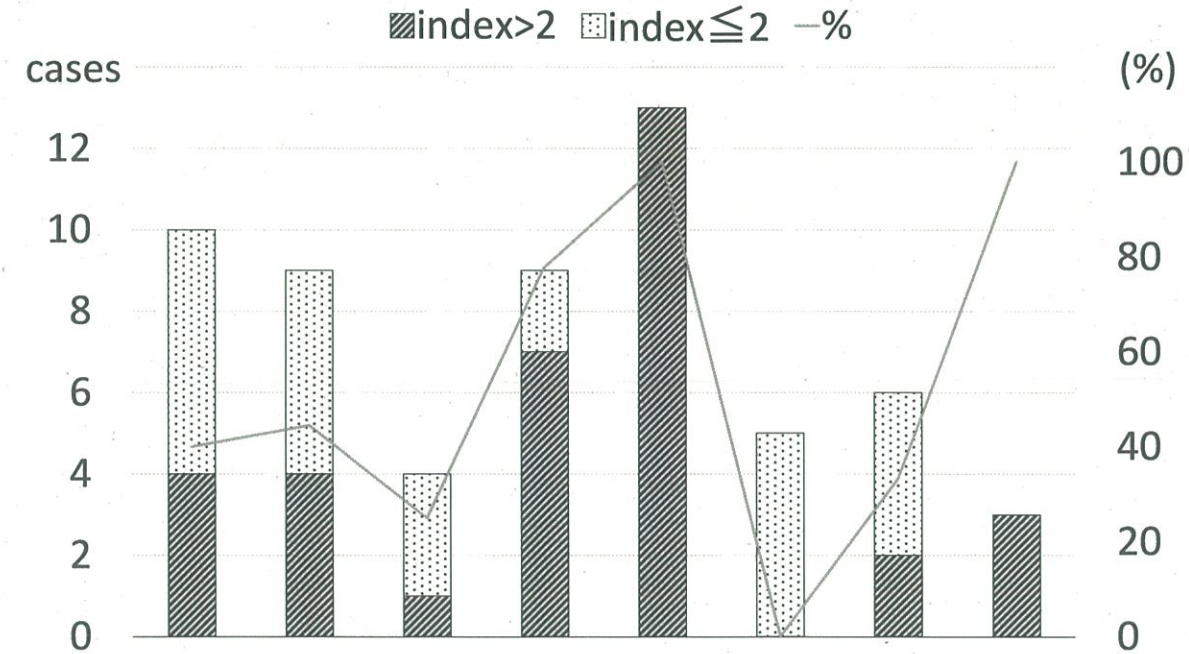


Figure 2



	MDS-SLD	MDS-MLD	MDS-RS	MDS-EB-1	MDS-EB-2	MDS-U	5q-	MDS/MPN
index>2	4	5	1	7	13	0	2	3
index≦2	6	5	3	2	0	5	4	0
%	40.0	50.0	25.0	77.8	100.0	0.0	33.3	100.0