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Research paper

Inter-observer variance and the need for standardization in the morphological classification of myelodysplastic syndrome☆

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ABSTRACT

In this era of genome medicine, the sub-classification of myeloid neoplasms, including myelodysplastic syndrome (MDS), is now supported by genetic testing in selected cases. However, as the initial suspicion and primary diagnosis of the disease still largely relies on morphological features and numbers of hematopoietic cells, the establishment of a uniform diagnostic basis, especially for cell morphology, is essential. In this study, we collected nearly 100,000 hematopoietic cell images from 499 peripheral blood smear specimens from patients with MDS and used these to evaluate the standardization of morphological classification by medical technologists. The observers in this study ranged between two to eleven for each image, and the images were classified according to MDS criteria through a web-based system. We found considerable inter-observer variance in the assessment of dysplastic features. Observers did not recognize cytoplasmic hypo–granularity unless almost all granules in neutrophils were absent. Pseudo Pelger–Huët anomalies were also often overlooked, except for cells with a very typical "pince-nez" appearance. Taken together, this study suggests a requirement for further standardization in terms of morphological cell classification, and a need for the development of automatic cell classification–supporting devices for the accurate diagnosis of MDS.

1. Introduction

Myelodysplastic syndrome (MDS) is a hematopoietic malignancy characterized by morphological atypia (dysplasia) of hematopoietic cells, ineffective hematopoiesis, peripheral cytopenia and progression to acute myeloid leukemia (AML). The disease is often found in the elderly: The median age of disease diagnosis is 76 years, with around 30 out of a 100,000 people aged 70 years or older diagnosed with MDS each year, whereas the frequency is 3–4 among people less than 70 years of age [1]. Recently, it was found that the disease becomes obvious when hematopoietic stem cells sequentially acquire several to ten somatic and/or germline gene mutations, and that gene mutations are frequently associated with disease phenotype or prognosis [2]. Based on these recent advances in the understanding of the molecular pathogenesis of the disease, the 2016 version of the WHO classification for myeloid neoplasms widely adopted gene mutation patterns for disease classification [3].

However, needless to say, the initial diagnosis of MDS is still, in principle, based on cell morphology and numbers, in which the disease is diagnosed by the following criteria: the ratio of myeloblasts is less than 5% in the bone marrow and less than 1% in the peripheral blood; and cytopenia or dysplasia is seen in at least one lineage [3], although the patterns and degree of dysplasia in peripheral blood cells, *per se*, seem not to be associated with an MDS subtype [4]. Nevertheless, morphological discrimination between normal and MDS cells is often very difficult, and thus requires substantial experience. For example, though dysplasia of MDS is defined as having a morphological abnormality in 10% or more of hematopoietic cells in a smear sample [3], other diseases or reactive conditions, such as various infections by pathogens or the administration of drugs, including anticancer agents,

☆ A commentary will follow in the upcoming issue for this article.

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sometimes display dysplastic features resembling MDS [5,6]. Hematological disorders, including aplastic anemia and primary myelofibrosis, also occasionally give rise to cells morphologically close to MDS. Furthermore, a certain number of healthy people exhibit dysplasia in hematopoietic cells [7]. Therefore, subtle changes in cell morphology may dissuade clinicians and medical technologists from a confident judgement [8], possibly giving rise to the variance observed in MDS diagnoses among observers and hospitals [9,10].

In this setting, the board for the standardization of cell morphology in the Japanese Society for Laboratory Hematology (JSLH) is working to standardize the definition of dysplasia. In Japan, Matsuda et al. proposed classifying dysplasia into two categories: (groups A and B), in which group A includes dysplastic features highly characteristic of MDS, including a pseudo Pelger–Huët anomaly, cytoplasmic hypo–-granularity (a decrease of cytoplasmic granules by more than 80% compared to normal granular cells), micromegakaryocytes and ring sideroblasts; while group B includes other dysplastic features less specific to MDS [11]. This categorization was also adopted by the study group for idiopathic hematopoietic disorders in the Japanese Ministry of Health, Labour and Welfare (MHLW). Likewise, the International Working Group on the Morphology of MDS (IWGM–MDS) proposes that the presence of a pseudo Pelger–Huët anomaly, cytoplasmic hypo–-granularity (decrease of granules by more than two-thirds), abnormal chromatin clumping and macrocytes in granulocytes should be taken into account for dysplasia in MDS [12].

Of course, the quality of smear samples impacts on the judgement of dysplasia. Factors that determine the quality of samples include the application of peripheral blood or bone marrow specimens to glass slides, including their drying and staining, and the camera settings for taking cell images (when stored as digital files and observed through a display monitor). As for sample staining, according to our experience, the Wright–Giemsa staining method tends to stain cytoplasmic granules of neutrophils weakly compared to May–Grünward–Giemsa staining. Thus the former staining may lead to overlooking a decrease in the number of cytoplasmic granules. Collectively, it is very important to standardize the procedure for the preparation of smear samples among institutes/hospitals. The systematic education of clinicians and medical technologists is also crucial so that they are able to evaluate the dysplasia of MDS appropriately. In Japan, medical technologists for hematology are certified by the JSLH, with 1234 medical technologists have been certified as of 2017. However, certified technologists are scarce, especially in regional cities; thus, clinicians and medical technologists who lack relevant experience often have no choice but to evaluate the dysplasia of MDS without input from more experienced personnel.

Under these circumstances, we have developed a cell classification system that can assist in the identification of MDS cells present in peripheral blood. This project is ultimately aimed at the implementation of software that distinguishes subtle dysplasia present in MDS cells utilizing a machine learning concept. Although the automatic identification of dysplastic cells from blood specimens using an automated hematopoietic cell counting system has been attempted previously [13,14], as far as we know, a system that identifies such cells using machine learning is lacking. To this end, we first obtained a total 97,924 cell images from 499 peripheral blood smear samples from patients with MDS (note, however, that the images unavoidably included non-malignant myeloid and non-myeloid cells because they were automatically taken by an imaging device, as described in Section Materials and methods). Each image was subsequently evaluated by at least two medical technologists (2–11, mean 5.67) who majored in hematology, and the data used as a “guide” for machine learning. However, we had previously found variation among observers for about 40% of the images. In this manuscript, we first disclose the evaluations entered by observers and discuss the issues that need to be overcome to realize an automatic diagnostic decision support system.

2. Materials and methods

2.1. Sample preparation and obtaining digital images of peripheral blood cells

A total of 499 peripheral blood smear samples from patients with MDS, which had been prepared for clinical usage at each hospital that participated in this study, were anonymized and collected at the Department of Laboratory Medicine in Kumamoto University Hospital. MDS diagnoses were made at each hospital. In this study, we put our focus on the evaluation of dysplasia in individual hematopoietic cells. Therefore, we collected samples regardless of the MDS subtype, so that we could collect as many samples as possible. Two hundred cell images for each sample, which included all types of nucleated cell lineages, were automatically taken using a CellaVision DM96 digital morphology imaging system (CellaVision AB, Lund, Sweden). The images were stored in JPEG format (360 × 363 pixels, 72 ppi), without having the device apply an automatic classification. Since smear samples that were covered with coverslips could not be screened by CellaVision, the cell images of such samples were manually captured under a light microscope. After the manual exclusion of images that were obviously not suitable for being classified, a final total of 97,924 cell images were initially used for the study.

The use of peripheral blood smear samples for this purpose was approved by the ethics committee at Kumamoto University, and the study was performed in accordance with the Declaration of Helsinki.

2.2. Classification of cells by medical technologists

The entering of data on cell classifications was executed through an original web-based system. A total 71 medical technologists, who mainly belonged to the Kyushu regional department of the JSLH, were recruited as observers in this study; the participants are listed in the Supplemental Table. They were individually issued with an user account to securely access websites to which cell images were uploaded, and were randomly assigned 1–76 (mean 42.5, median 52) smear samples (200 cells in each sample) and blinded to clinical data. The numbers of observers for the 499 MDS samples are shown in Supplemental Fig. S1 (mean observers/cell image is 5.67). The observers were asked to select a cell category from choices as shown in the Table 1. In addition, they were given an opportunity to check and correct their classification for 200 images again before final submission of their decision on each images of a smear specimen. Therefore, they had a chance to compare morphological features in a cell with other cells in the same specimen. Since we used peripheral blood smear samples in this study, it was expected that dysplastic cells seen in the samples were highly biased toward granulocytes (neutrophils and monocytes). Therefore, we put the choices of pseudo Pelger–Huët anomaly and cytoplasmic hypo–granularity in category A, and of micro- or giant-neutrophils, hyper-segmented neutrophils, pseudo Chédiak–Higashi granules and Auer bodies in category B of the classifications proposed by the Japanese MHLW study group for idiopathic hematopoietic disorders [11].

The categorizations entered by observers were statistically analyzed using R 2.8.1 (https://www.r-project.org/) and modified R commander software (http://www.rcommander.com/). Siegel’s kappa coefficient test was applied for the statistical analysis of inter-observer variance.

3. Results

3.1. Overall classification of cell images

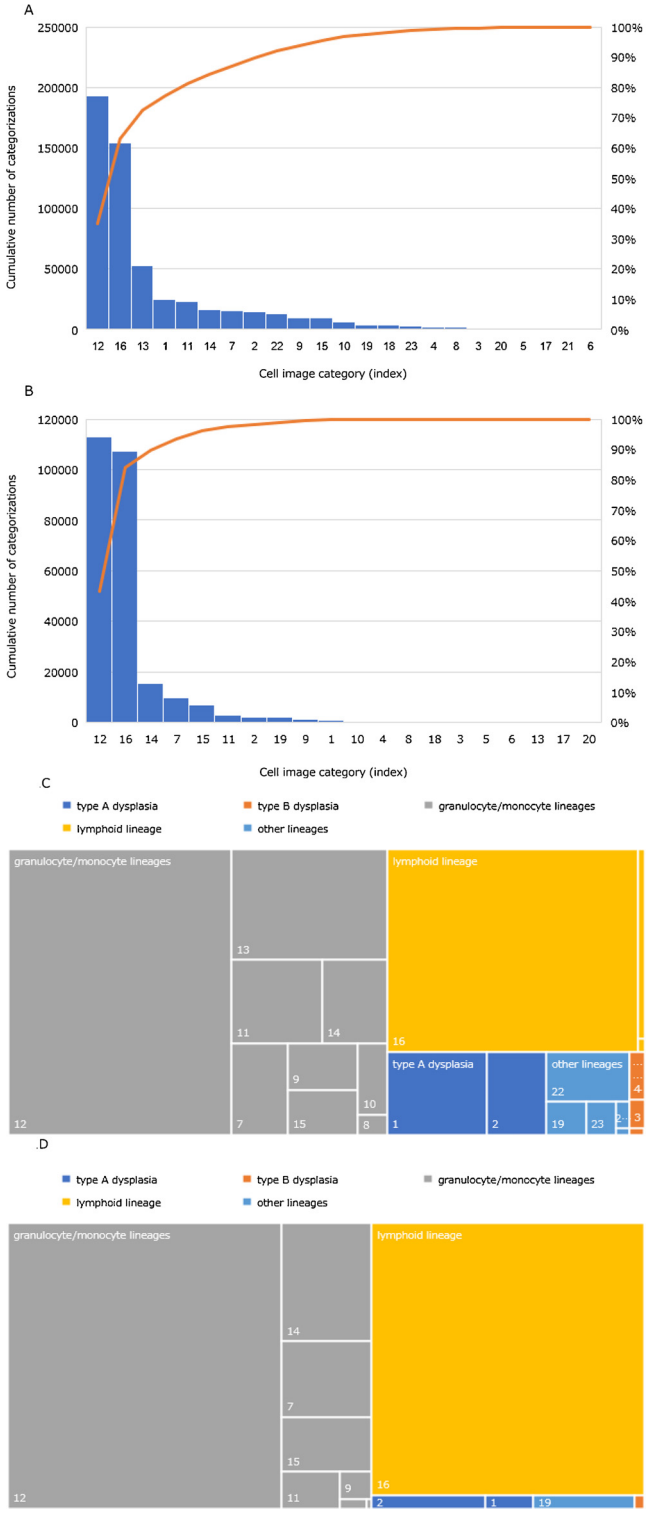
Among the 97,924 cell images independently classified by at least two observers, 880 images were classified as “platelet”, “no cell” or “non-typable” by more than 80% of observers. Although we omitted such images before providing them to observers, inappropriate images

Table 1
Cell classification categories used for this study. Types A and B dysplasias are morphological categories highly and less specific to myelodysplastic syndrome, respectively. For more information refer to the text and Ref. [11].

Class	Subclass	Index
type A dysplasia	cytoplasmic hypo-granularity	1
	pseudo Pelger-Huët anomaly	2
type B dysplasia	micro- or giant-neutrophil	3
	hypersegmental neutrophil	4
	pseudo Chédiak-Higashi granules	5
	auer body	6
granulocyte/monocyte lineages	myeloblast	7
	promyelocyte	8
	myelocyte	9
	metamyelocyte	10
	band neutrophil	11
	segmental neutrophil	12
	monocyte	13
	eosinophil	14
	basophil	15
lymphoid lineage	lymphocyte	16
	plasma cell	17
	atypical lymphocyte	18
other lineages	erythroblast	19
	megakaryocyte	20
	platelet	21
	non-typable	22
	no cell	23

still remained because we initially manually assembled the series of cell images. After excluding images not suitable for this study, we finally chose 97,044 images for consideration.

We first explored the distribution of classifications entered by the observers. As for the 97,044 cell images, Siegel's kappa coefficient was 0.752, with 53,915 images (55.6%) completely matched among observers. The distribution of all 97,044 images, and that of fully matched 53,915 images are shown in Fig. 1A and B, respectively. Among the 53,915 fully matched images, 20,418 (37.9%) and 19,661 (36.5%) cells were classified as "segmental neutrophil" (index 12 in the Table 1) and "lymphocyte" (index 16), respectively, by all observers, while only 246 (0.46%) and 386 (0.72%) of cells were classified as "cytoplasmic hypo–granularity" (index 1) and "pseudo Pelger–Huët anomaly" (index 2), respectively. Fig. 1C and D reveals that the rate of dysplastic features categorized into groups A and B decreased for fully matched images. However, according to the coefficient value for all 97,044 images, the classification was said to be basically reproducible among observers. When images tagged as "monocyte", "erythroblast", "lymphocyte", "atypical lymphocyte", "plasma cell" and "megakaryocyte" by more than 80% of observers were further excluded from the calculation, that is, only possible granulocytic lineage that consisted of 64,130 images were considered, the Siegel's kappa coefficient decreased to 0.605. Although the coefficient value was still relatively high, the data suggests that the observers found the classification of granulocytic lineage more difficult than for other cell types. Possibly, this is because of the existence of many choices in this lineage, and of the difficulty in the recognition of subtle nuances of morphological atypia in MDS cells.



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3.2. Detailed analysis of cytoplasmic hypo–granularity

Next, we assessed how cytoplasmic hypo–granularity was recognized by observers. Of 8050 cell images that were classified by between 8 and 11 observers, 2229 images were tagged as cytoplasmic hypo–granularity by at least one observer. Cumulative numbers for the classification of these 2229 images are shown in Fig. 2A; we found the consistency among observers was low in terms of the cell categorization of cytoplasmic hypo–granularity (Fig. 2B). The cell images tended to be classified more as neutrophils (segmental or band neutrophil) than cytoplasmic hypo–granularity (Fig. 2A and B), thus leading to Siegel's kappa coefficient being as low as 0.399 for the 2229 images. Therefore, it was likely that the observers hesitated in judging these images as morphologically abnormal unless the finding was obvious. We then analyzed cytoplasmic hypo–granularity in more detail. Fig. 2C and Supplemental Fig. S2A–K are a series of images of granulocytes taken from the same smear sample. In these figures, the cells ranged from normal cytoplasmic granules to cytoplasmic hypo–granularity; the relative levels of granules are shown under the images. While cytoplasmic

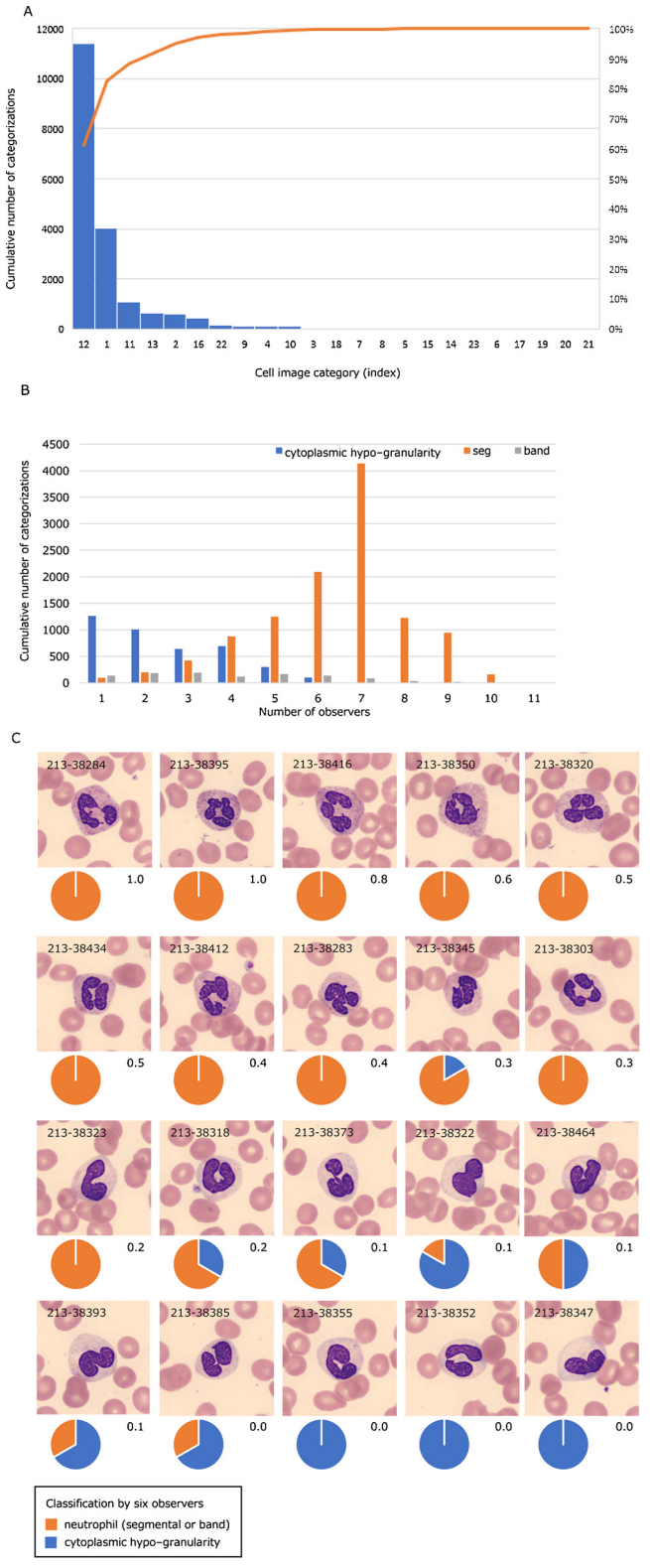

Fig. 1. Distribution of cell image classifications by observers. (A) A Pareto chart covering all cell images, but excluding 880 images that were classified as "platelet", "no cell" or "non-typable" (thus, 97,044 images in total were used). A total of 550,408 inputs were made for these 97,044 images (mean: 5.67 inputs per image). The indexes on the x-axis correspond to the cell classification as listed in the Table 1; classifications are in descending order. The left and right vertical axes denote cumulative inputs made for each image, and the cumulative percentage of the total number of cell classifications, respectively. (B) A Pareto chart covering the 53,915 images that were completely matched among observers. A total of 294,670 inputs were made for these 53,915 images. The x- and y-axes are the same as in (A). As mentioned in the text, most of the fully matched images were categorized as "segmental neutrophil" and "lymphocyte". (C) A tree-map chart for the 97,044 images as in (A). (D) A tree-map chart for the 53,915 images as in (B). As compared with (C), the rate of images classified to groups A and B dysplasia, designated as areas of blue and orange rectangles, respectively, are greatly decreased.

hypo–granularity was defined as a decrease in granules by more than 80% according to Japanese criteria, and by more than two-thirds according to IWGM–MDS criteria, the observers in this study did not decide to judge a cell as cytoplasmic hypo–granularity unless granules were almost completely absent.

3.3. Detailed analysis of pseudo Pelger–Huët anomaly

We subsequently evaluated how a pseudo Pelger–Huët anomaly was classified by observers. A total of 749 out of 8050 cell images selected by the criteria used as mentioned above were tagged as having a pseudo Pelger–Huët anomaly by at least one observer (Fig. 3A). Since Siegel's kappa coefficient for these 749 images was 0.371, the consistency for this anomaly among the observers was as low as that for cytoplasmic hypo–granularity. In particular, judging un-lobulated cells as having the anomaly fluctuated among the observers. Indeed, un-lobulated cells exhibiting a pseudo Pelger–Huët anomaly was not included in the group, for which all judgements matched among eight or more observers. Nevertheless, Fig. 3B reveals that the pseudo Pelger–Huët anomaly rather than cytoplasmic hypo–granularity tended to be tagged by multiple observers. This may be because this kind of change in nuclear morphology is more eye-catching than a cytoplasmic abnormality. Representative images for the abnormal finding are shown in Fig. 3C, in which the first 17 images are those consistently tagged as pseudo Pelger–Huët anomalies by all observers, while the rest of the images were those tagged as other categories by at least one observer. Note that cells such as 26-3895 and 212-38139 also harbored a feature of cytoplasmic hypo–granularity, but no one selected this classification. This further suggests that the pseudo Pelger–Huët anomaly is easier to recognize than cytoplasmic hypo–granularity.

In terms of pseudo Chédiak–Higashi granules and Auer bodies, these were tagged in only a small number of images (231 and 14 images were tagged as the former and latter categories by at least one observer, respectively), and such images were not consistently judged by all observers. Thus, a detailed analysis was not executed for these abnormal findings.

4. Discussion

MDS is a hematological malignancy that develops based mainly on the age-related accumulation of somatic gene mutations in hematopoietic stem cells. In recent years, genetic pathways that determine MDS development have been revealed owing to the rapid development of high-throughput parallel sequencing technology. A hematopoietic stem cell that initially acquired a mutation in the genes encoding epigenome regulators gives rise to a status of clonal hematopoiesis of an indeterminate potential, whereby a limited number of stem cells produce all the hematopoietic cells of the body [15]. Thereafter, the cells evolve into malignant clones by acquiring additional genetic mutations in a stepwise manner. In addition, a strong association between somatic mutation and disease phenotype is also evident [2]. A typical example is the *SF3B1* mutation in MDS with ring sideroblasts (MDS–RS), where nearly 80% of patients with MDS–RS harbor the mutation [16]. Furthermore, it has recently been shown that germline mutations in a variety of genes, including *DDX41*, *ETV6*, *SAMD9L* and *SAMD9*, are involved in MDS/AML development [17–22], leading to the increased

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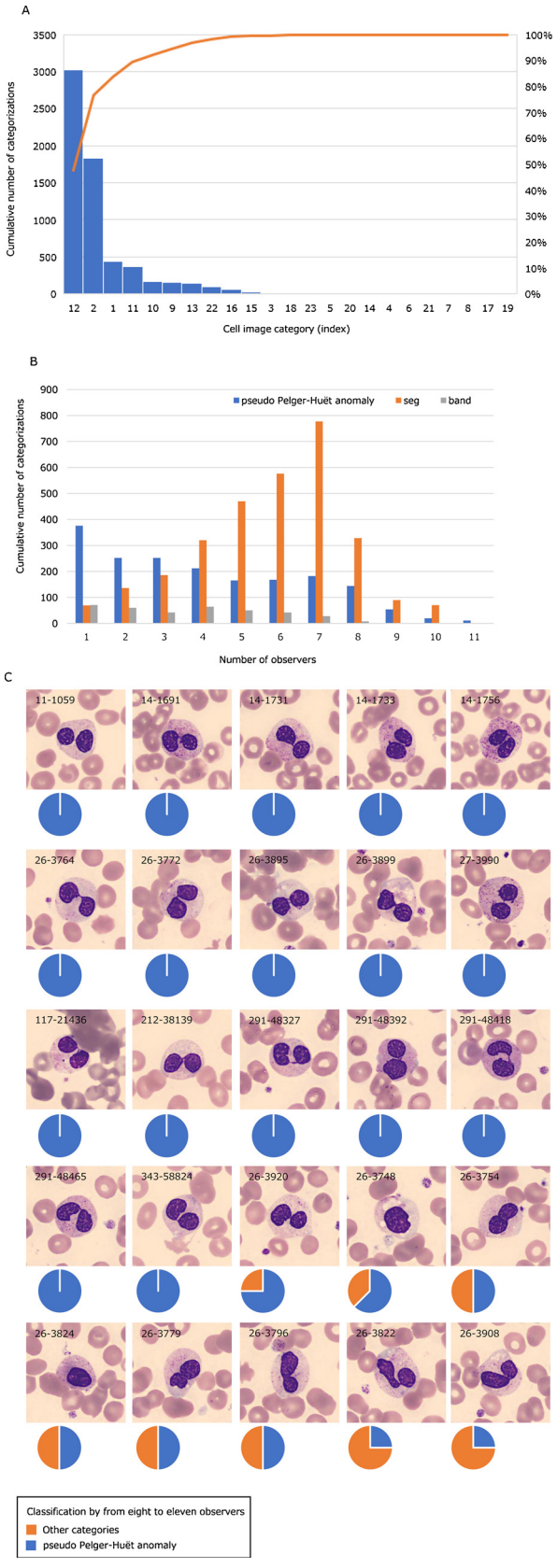

Fig. 2. Inconsistent classification of cytoplasmic hypo–granularity among observers. (A) A Pareto chart covering the 2229 cell images classified as cytoplasmic hypo–granularity by at least one out of from 8 to 11 observers. The cell classification is in descending order for the x-axis, for which cell names corresponding to indexes are listed in the Table 1. The left and right vertical axes indicate cumulative inputs made for each image, and the cumulative percentage of the total number of cell classifications, respectively. (B) The numbers of observers who classified a cell image as "cytoplasmic hypo–granularity", "segmental neutrophil" and "band neutrophil" are indicated as columns. The graph also used 2229 images classified as "cytoplasmic hypo–granularity" by at least one out of from 8 to 11 observers. The x- and y-axes indicate the number of observers and cumulative image numbers, respectively. The number of observers indicating the highest column in orange (segmental neutrophil) is seven, while that in blue (cytoplasmic hypo–granularity) is one; thus segmental neutrophils tended to be consistently judged among observers, while a judgement of cytoplasmic hypo–granularity was inconsistent among observers. (C) The association between the actual level of cytoplasmic granules in neutrophils and the classification made by observers. A series of neutrophils from sample ID 213, which was evaluated by six observers, is shown as a representative sample. The indexes under the images represent the level of cytoplasmic granules, with the Image 213–38,284 used as the reference level (1.0). The pie charts indicate the classifications made by observers for each image (pie wedge in orange: segmental or band neutrophil, blue: cytoplasmic hypo–granularity).

importance of taking a family history of hematological disorders when a patient is diagnosed with MDS/AML. According to these recent findings, the classification of myeloid malignancies based on gene mutations has been widely adopted in "The 2016 revision to the WHO classification of myeloid neoplasms and acute leukemia"; thus, the need for genetic testing has markedly increased [3].

Meanwhile, at present, MDS is still basically diagnosed and subclassified according to cell morphology and numbers, as well as blast counts [3,23]. In addition, as smear samples can usually be prepared within 30 min at hospital clinical laboratories, it goes without saying that cell morphology is the most important basis for the initial diagnosis of MDS. The detailed sub-classification of the disease and prediction of its prognosis can then be determined/estimated by additional testing, including flow cytometry, g-banding analysis and genetic testing. This is why standardization of the morphological judgement of hematopoietic cells is sought.

However, our present study revealed uneven classifications of cell morphology, even among observers with adequate experience in the diagnosis of hematological diseases. One reason for the uneven classification in this study may be because we allowed only one finding to be recorded for each cell. A pseudo Pelger–Huët anomaly and abnormal chromatin condensation, both of which are dysplasia observed in the nucleus, can be observed simultaneously within a single cell [12]. The images used in this study also included MDS cells with both a pseudo Pelger–Huët anomaly and cytoplasmic hypo–granularity; however, observers tended to select a pseudo Pelger–Huët anomaly over cytoplasmic hypo–granularity for such cells (Fig. 3B). At the time, we decided against accepting multiple findings for a single cell image. This was because we wanted to simplify entering a finding so that we could collect as many judgements as possible from observers for the development of a diagnostic decision support system for MDS utilizing a machine learning concept. The other reason for the uneven classification may be because observers were not familiar with the images obtained by CellaVision. In particular, determining the level of cytoplasmic granules and nuclear chromatin condensation may have been difficult since images taken by CellaVision are sharp and of high-contrast as compared with those observed under a light microscope, which are usually used for routine examinations.

Apart from the issues specific to this study, it has also been shown that pointing out subtle dysplasia in MDS cells is still challenging, and, therefore, standardization of the morphological classification of MDS has not yet been accomplished, at least in Japan. This would be partly because the criterion for dysplasia in MDS is still somewhat ambiguous.

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Cytoplasmic hypo–granularity is defined as a reduction of more than 80% of granules as compared with those of normal neutrophils according to Japanese criteria [11]. Similarly, IWGM–MDS describes the

Fig. 3. Inconsistent classification of pseudo Pelger–Huët anomalies among observers. (A) A Pareto chart covering the 749 cell images classified as a pseudo Pelger–Huët anomaly by at least one out of between 8 and 11 observers. The x- and y-axes are the same as in Figs. 1(A and B) and 2(A). (B) The number of observers who classified a cell image as a "pseudo Pelger–Huët anomaly", "segmental neutrophil" and "band neutrophil" is shown in the column graph. The graph also used the same 749 images as in (A). (C) Representative cell images of pseudo Pelger–Huët anomalies. The pie charts under the images indicate the classification of each image by observers (blue: pseudo Pelger–Huët anomaly, orange: other categories).

phenotype as a reduction of more than two-thirds of granules [12]. However, it is not clearly stated in both criteria which cells should be chosen as a normal reference. Actually, out of the 2229 cells tagged with cytoplasmic hypo–granularity by at least one observer, no cell was given an identical judgement by all observers. Most of the cells tagged with cytoplasmic hypo–granularity were simultaneously classified as neutrophils by other observers.

Moreover, this study also suggested difficulties in the identification of a pseudo Pelger–Huët anomaly. This dysplasia is characterized by hypo-lobulated nuclei of neutrophils that resemble those seen in a congenital Pelger–Huët anomaly [5]. It is known that the nucleus of a cell with the anomaly exhibits coarse chromatin condensation, and in the case of two lobes, these are connected by a fine or thin filament. Nonetheless, both of these are largely exposed to subjective judgement by observers, due largely to the ambiguity of the definition. According to the description of an anomaly by Colella et al. [24], the two nuclear lobes of the anomaly are often unsymmetrical, and the membrane outline is not as smooth as a congenital Pelger–Huët anomaly. It seems that observers waver between a segmented neutrophil and a pseudo Pelger–Huët anomaly when they find a hypo-lobulated neutrophil with one or both of the two lobes not round-shaped, but bent or twisted. Therefore, most of the cells tagged with a pseudo Pelger–Huët anomaly by an observer were also tagged as a neutrophil by the other observers.

Taken together, we detected a considerable inter-observer variance in cell classification among observers, using as many as nearly 100,000 cell images obtained from the peripheral blood smear samples of patients with MDS. Although we primarily planned to use the data for the development of a cell classification support system, we found that a standardized classification is still under development, at least in Japan, and that further data mining may be required for the development of a more efficient and accurate diagnostic decision support system. In addition, our data can also be used as material for the standardization of hematopoietic cell classification as well as for the improvement of classification skills in medical technologists.

Conflict of interest disclosure

The authors of this paper declare they do not have any competing financial interests to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.leukres.2018.04.003.

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