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Original Article

Utility of a Fluorescence Microscopy Imaging System for Analyzing the DNA Ploidy of Pathological Megakaryocytes Including 5q- Syndrome

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To investigate megakaryocyte (MK) DNA ploidy in various hematological diseases, fluorescence microscopy imaging system (FMI) can be used to analyze DNA ploidy with cell morphology at the single-cell level by using specialized image-processing software. Here we compared DNA ploidy obtained by FMI measured with that obtained flow cytometry (FCM). With FMI, we could evaluate the DNA ploidy in long-term preserved bone marrow smear samples after staining. We next analyzed the MK DNA ploidy in 42 bone marrow smear samples including 26 myeloid neoplasm cases, and we compared the DNA ploidy and platelet counts in the patients' peripheral blood; the production of platelets was significantly high compared to DNA ploidy in the myeloproliferative neoplasms group. The FMI method revealed that the patients with 5q- syndrome exhibited relatively low DNA ploidy despite high platelet counts, and this result suggested that increased DNA ploidy is not indispensable to abundant platelet production. The FMI method for DNA ploidy will be a useful tool to clarify the relationship between DNA ploidy and platelet production by MKs.

Key words: fluorescence microscopy image analysis, DNA ploidy, megakaryocytes, MDS with isolated del(5q), 5q- syndrome

Mature megakaryocytes (MKs) are large multinucleated cells and produce many of platelets. Their growth is accompanied by a distinct pattern of nuclear division (endomitosis) [1-5]. Mitosis is regulated at the molecular level in normal dividing cells. DNA replication in the absence of cytokinesis causes MKs to grow into large, multinucleated cells, some of which have DNA ploidy beyond 128N. However, the molecular mechanisms governing how DNA ploidy increases and how nuclear lobation occurs remain to be elucidated. One general feature of myeloproliferative neoplasms (MPN) is increased MK counts, but characteristics such as cell size, ploidy changes, lobation patterns, and platelet production capacity differ among the MPN subtypes. Very few studies have been performed to examine the associations between MK DNA content and hematological disease type [6].

Techniques for analyzing DNA ploidy include laser-scanning cytometry (LSC) and flow cytometry (FCM), and the better technique to use depends on the aim of the analysis [7-13]. LSC is used to quantify and evaluate DNA content and protein localization in cells, and to compare these data with morphological observa-

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tions. Few devices are available that are specialized for LSC; moreover, the technology is complex and difficult to operate, and — in the case of dense cell populations — it cannot analyze DNA content in individual cells. In contrast, FCM is simple to operate and makes it easy to measure the DNA content of individual cells in a short time. However, it is hard to record the morphology and the DNA content of a given cell at the same time. Another technique for analyzing DNA ploidy is imaging flow cytometry (IFC), a newer technology by which users can observe images of individual cells in combination with FCM [14, 15].

In the present study, we attempted to conduct a DNA ploidy analysis using fluorescence microscopy imaging (FMI), a technique that combines fluorescent microscopy with specialized image-processing software. FMI is simple to operate and, unlike FCM and LSC, FMI can quantify the DNA ploidy of individual cells. Using this technique, a user can take new images after each round of staining to analyze DNA content at a later date, and the FMI method is unaffected by specimen fading. With the use of an analysis system consisting of fluorescent microscopy and specialized image-processing software, we evaluated the DNA ploidy of MKs in long-term preserved bone marrow smear samples, and we compared the results obtained with the data of cytological imaging findings to search for features of MK ploidy in a variety of hematological diseases.

Materials and Methods

Cell lines and patient samples. A human myeloid cell line, MDS-L, was derived from a patient with myelodysplastic syndromes (MDS). MDS-L cells were cultured in RPMI1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin-G/streptomycin.

The patient samples consisted of 42 bone marrow smears collected at Kawasaki Medical School Hospital (n = 40) and the Hiroshima University Department of Hematology (n = 2) from 2004 to 2013. All bone marrow smears were stained by May-Grünwald-Giemsa (MGG) solution and long-term preserved at room temperature. The sample smears from patients: (a) who achieved complete remission following treatment, or (b) in whom no abnormal cell infiltration was observed in a bone marrow examination for malignant lymphoma staging were assigned to the normal control group. The sample smears from patients with blood diseases were assigned to the disease group (n = 26). An overview of patients and their diseases is provided in Table 1. Hematological diseases were categorized according to the WHO classification scheme. Platelet count values were taken from the test results for the blood sampled at the time of the preparation of bone marrow smear samples (Table 1).

This study was conducted with the approval of the ethics committee of Kawasaki Medical School (No. 517, 1478).

DNA ploidy analysis by FCM. MDS-L cells were washed in phosphate-buffered saline (PBS) (94222-61: Nacalai Tesque, Kyoto, Japan) followed by fixation with 70% methanol for 20 min at 4°C. The cells were then washed twice with PBS and resuspended in staining solution (10% FBS). After being washed twice with PBS, the cells were incubated with 0.1 mg/mL ribonuclease A (Sigma, St Louis, MO, USA) for 30 min at 37°C, followed by DNA staining with 0.05 mg/mL propidium iodide (PI, Sigma) for 20 min at room temperature. The cells were measured with a FACS Calibur flow cytometer (BD Bioscience, Mansfield, MA, USA). The PI fluorescence was measured at the FL2 channel. Up to 10,000 events for each sample were measured by CellQuest software (BD Bioscience), and DNA ploidy was analyzed with ModFit LT™ software (Verity Software House, Topsham, ME, USA).

Preparation of MDS-L cells and bone marrow smear samples for DNA ploidy analysis by FMI. Cytospin preparations of MDS-L cells were fixed in 4% formalin/50% acetone for 15 sec at room temperature,

Table 1 Patient Information

Diagnosis	n	Age median (range)	Plt ($\times 10^3/\mu L$) median (range)
Control	16	57.5 (13–74)	200 (38–1049)
MDS with del(5q)	4	82 (69–83)	318 (206–499)
MDS without del(5q)	10	75.5 (30–88)	78 (21–203)
CML	5	63 (48–76)	351 (202–1111)
MPN (ET + PV)	7	70 (29–91)	867 (405–2240)

Platelet count data was only available for 3 of the MDS patients with 5q- syndrome. The MPN group consisted of 3 ET and 4 PV patients; the 5 CML patients were not included in this group.
Plt, platelet counts; MDS, myelodysplastic syndromes; CML, chronic myeloid leukemia; MPN, myeloproliferative neoplasms; ET, essential thrombocythemia; PV, polycythemia vera.

and washed with Tris-buffered saline (TBS) (S3001; Dako, Japan). Nuclear staining was performed with either 0.05 mg/mL PI (Sigma) or 0.01 mg/mL 4', 6-diamidino-2-phenylindole (DAPI) (Dojindo Laboratories, Mashiki, Japan). Ribonuclease A solution (final concentration 0.1 mg/mL) was added to the samples only prior to nuclear staining with PI.

Bone marrow smear samples were stained with MGG solution. After MK image data (≥ 30 cells per sample) were saved in the storage system of a fluorescence microscope (BZ-8100; Keyence, Osaka, Japan) with the *x-y* coordinate mapping information, the specimens were soaked in destaining solution (acetate/methanol) and DAPI nuclear staining was performed. Each MK after nuclear staining could be identified in the same visual fields as the MGG-stained image data using the *x-y* coordinate mapping information.

DNA ploidy analysis by FMI. We used BZ-II Analyzer analysis software (Keyence) to analyze the FMI data. In brief, the cells were counted in the MDS-L spot samples in 20 visual fields (the average cell count per field was 330 cells). The nuclear DNA content is expressed as the luminescence integrated value (LSum), which was estimated by multiplying the area of the PI- or DAPI-stained image and the luminescence intensity (Fig. 1B).

The DNA content of the FMI analysis was visualized by plotting the LSum values of individual cells as histograms. Each representative histogram indicates the DNA ploidy distributions from MDS-L cells measured by FCM (Fig. 1A) and FMI (Fig. 1C, D).

Statistical analysis. Statistical analysis was carried out using Stat Flex ver. 6 software. All statistical tests were performed using two-sided, unpaired Student's *t*-test or the Mann-Whitney *U*-test. Significance was defined as a p value of less than 0.05.

Results

The DNA ploidy pattern of the cell lines by the FCM analysis is comparable to that of the FMI analysis. Aiming at the development of the DNA ploidy evaluation of blood smear samples, we compared the DNA ploidy pattern analyzed by FCM with that analyzed by FMI of cytospin preparations in the proliferating MDS-L cell line. The MDS cell line has rarely undergone abnormal karyomitosis, and as a consequence is allohexaploid. A DNA ploidy distribution can be identified by defining ploidy classes based on the sequential doubling of DNA content from the 2N peak set on total nucleated cells. For the FMI (PI), we considered the value of an LSum between 32,000 and 42,000 as diploid, 67,000-77,000 as tetraploid, and 102,000-112, 000 as hexaploid (Fig. 1C). For the FMI (DAPI), we considered the LSum value 11,000-15,000 diploid, 23,000-27,000 tetraploid, and 37,000-41,000 hexaploid (Fig. 1D). MDS-L cells were considered aneuploid if clear aneuploid peaks (4N, 6N) were present.

For the FCM data, the mean distribution ratios for DNA ploidy (2N: 4N: 6N) of the MDS-L cells were $35.6 \pm 1.0\%$: $12.3 \pm 0.7\%$: $0.3 \pm 0.1\%$ (Fig. 1A). For the FMI (PI) data, the mean distribution ratios for DNA ploidy (2N: 4N: 6N) of the MDS-L cells were $37.6 \pm 4.3\%$: $11.5 \pm 2.0\%$: $0.3 \pm 0.3\%$ (Fig. 1C); and for FMI (DAPI) data, the corresponding data were $37.3 \pm 3.3\%$: $11.6 \pm 1.5\%$: $0.4 \pm 0.3\%$ (Fig. 1D). There were no significant differences in the DNA ploidy distribution ratios (2N: 4N: 6N) by nuclear stain type (PI vs. DAPI) or by measurement technique (FCM vs. FMI (PI)) (Fig. 1E).

The variations in the exposure time and the variation in the RBG element did not result in significant differences in DNA ploidy data in the FMI (PI) or FMI (DAPI) staining specimens (data not shown).

Evaluation of DNA ploidy of MKs in myeloid neoplasms by FMI. We measured the MK DNA ploidy in bone marrow smear specimens prepared from 42 patients. The disease group was further classified into myelodysplastic syndromes (MDS), chronic myelogenous leukemia (CML), and myeloproliferative neoplasms (MPN). The MDS samples were further divided into MDS with isolated del(5q) or 5q- syndrome and the other MDS cases without del(5q). The MPN samples were further divided by disease subtype: essential thrombocythemia (ET: $n = 3$) and polycythemia vera (PV: $n = 4$).

The standard reference LSum value was determined by a selection of mature neutrophils whose ploidy was assumed to be 2N. We calculated reference LSum values corresponding to DNA ploidy of 2N separately for each specimen by taking the mean LSum of 5-10 mature neutrophils. We then determined the DNA ploidy for each MK by dividing each cell's LSum by the 2N reference value and by doubling the value (Fig. 2A).

Representative examples of MKs in bone marrow and their DNA content are shown for various hemato-

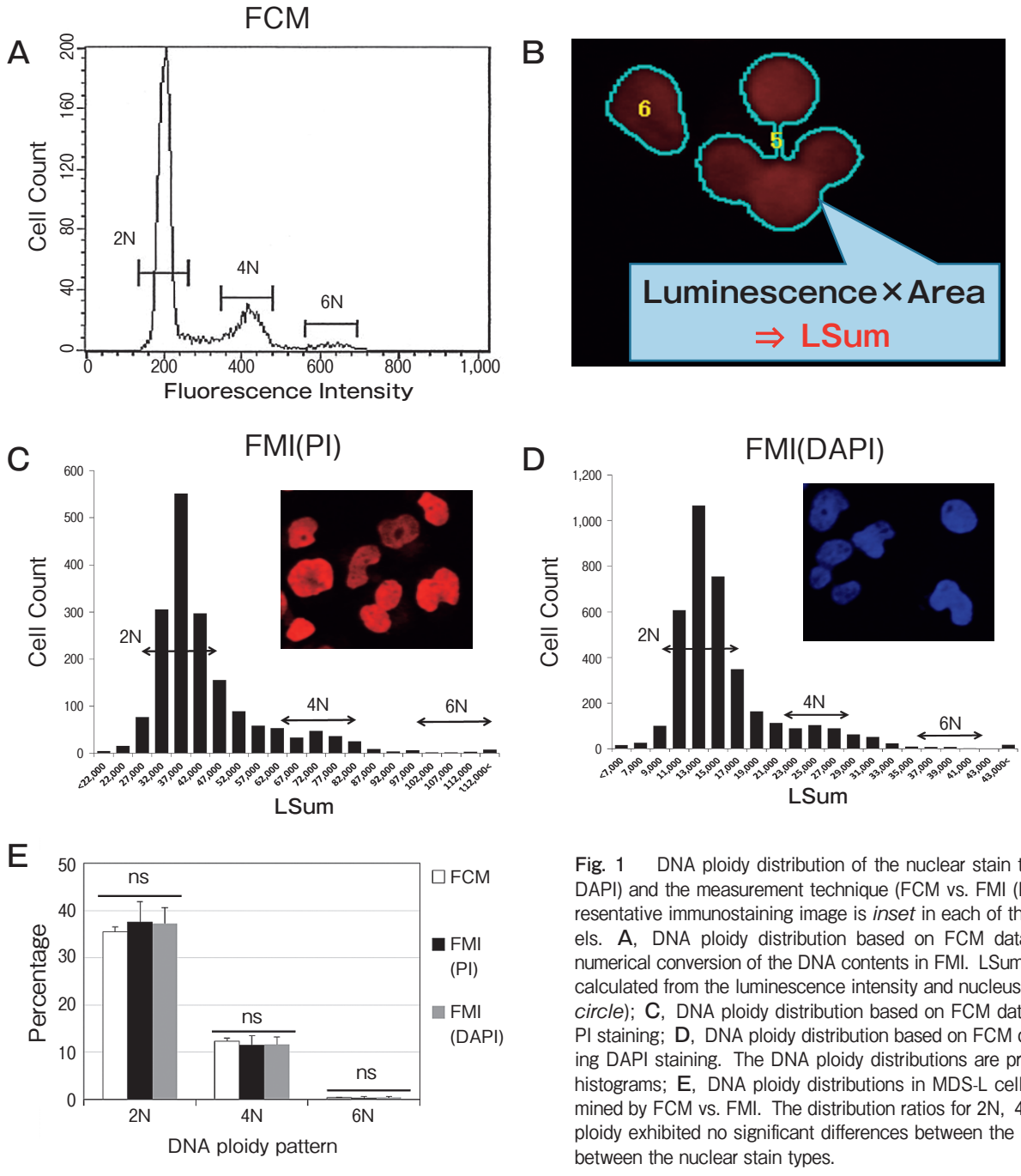

Fig. 1 DNA ploidy distribution of the nuclear stain type (PI vs. DAPI) and the measurement technique (FCM vs. FMI (PI)). A representative immunostaining image is *inset* in each of the FMI panels. **A**, DNA ploidy distribution based on FCM data; **B**, The numerical conversion of the DNA contents in FMI. LSum values are calculated from the luminescence intensity and nucleus area (*blue circle*); **C**, DNA ploidy distribution based on FCM data following PI staining; **D**, DNA ploidy distribution based on FCM data following DAPI staining. The DNA ploidy distributions are presented as histograms; **E**, DNA ploidy distributions in MDS-L cells as determined by FCM vs. FMI. The distribution ratios for 2N, 4N, and 6N ploidy exhibited no significant differences between the methods or between the nuclear stain types.

logical diseases in Fig. 2B. The number inset in each image is the DNA content of the individual MK. The % MK according to DNA ploidy in each disease is summarized in Table 2 and Fig. 3A. The MPN group showed a tendency to have higher DNA contents than the CML group, but the difference was not significant. In contrast, the MDS group showed a tendency to have lower MK DNA ploidy than the normal control group (Fig. 3A).

The platelet counts of each disease group are indicated in Fig. 3B and showed significant differences between the normal control group and the MDS with-

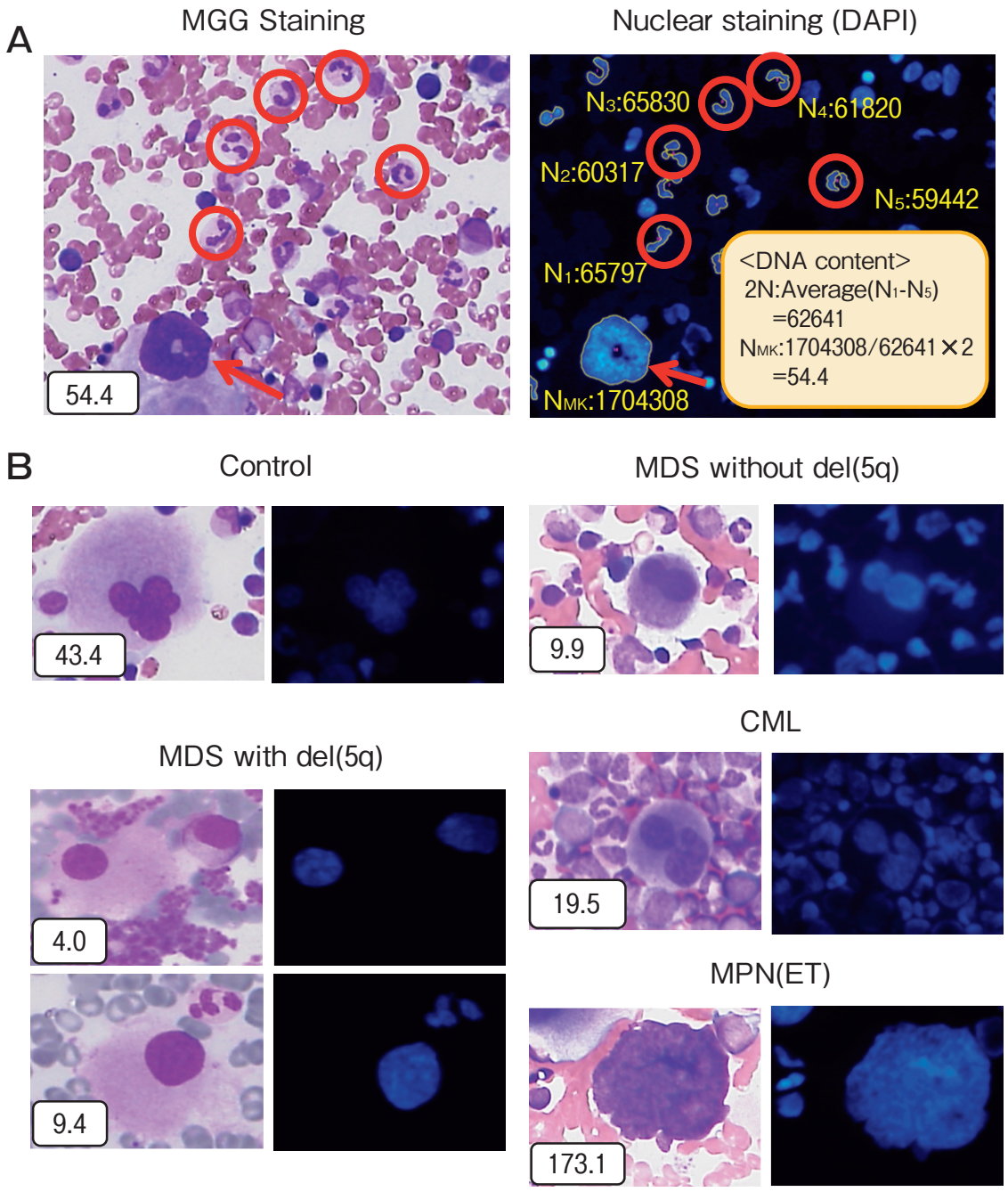

Fig. 2 Morphological features and DNA contents of MKs in hematological diseases. *Left-side* images are May-Grünwald-Giemsa (MGG) stains; *right-side* images are DAPI nuclear stains. **A**, *Circles* denote mature neutrophils for the 2N reference value for integrated luminescence (LSum); an *arrow* indicates a megakaryocyte. The LSum (shown as a number) is the product of the nuclear area and the fluorescence intensity, and is calculated separately for each cell; **B**, Bone marrow MKs and their DNA ploidy are shown for various hematological diseases. *Lower-left numbers* denote DNA content (N).

out del(5q) group and between the normal control group and the MPN group (Fig. 3B).

To clarify the relation between the platelet counts and the DNA content by the disease, the ratio of DNA content and the platelet counts (DNA/Plt ratio) or the product of DNA content and the platelet counts (DNA×Plt products) was calculated. DNA/Plt ratio showed statistical difference between the normal con-

Table 2 DNA ploidy distribution of Megakaryocyte of hematological diseases

Diagnosis	% MK according to DNA ploidy							DNA contents
	＜4N	4–8N	8–16N	16–32N	32–64N	64–128N	128N＜	AVE
Control	0 ± 0.8	2 ± 3.4	15 ± 10.9	42 ± 13.3	33 ± 14.3	7 ± 6.0	2 ± 4.2	33.5 ± 10.0
MDS with del(5q)	6 ± 6.8	9 ± 7.1	29 ± 14.8	43 ± 19.2	14 ± 9.9	0	0	19.6 ± 5.3
MDS without del(5q)	4 ± 6.7	5 ± 8.2	22 ± 18.9	36 ± 13.6	27 ± 15.9	5 ± 6.3	0 ± 0.9	27.0 ± 10.7
CML	1 ± 1.3	6 ± 7.0	20 ± 16.3	42 ± 17.3	26 ± 25.4	5 ± 10.6	1 ± 1.5	28.1 ± 15.0
MPN	2 ± 3.5	6 ± 8.0	17 ± 13.3	35 ± 15.0	27 ± 13.1	9 ± 8.4	3 ± 4.7	35.4 ± 16.2

Average values of DNA contents are shown. Values are presented as mean ±SD.

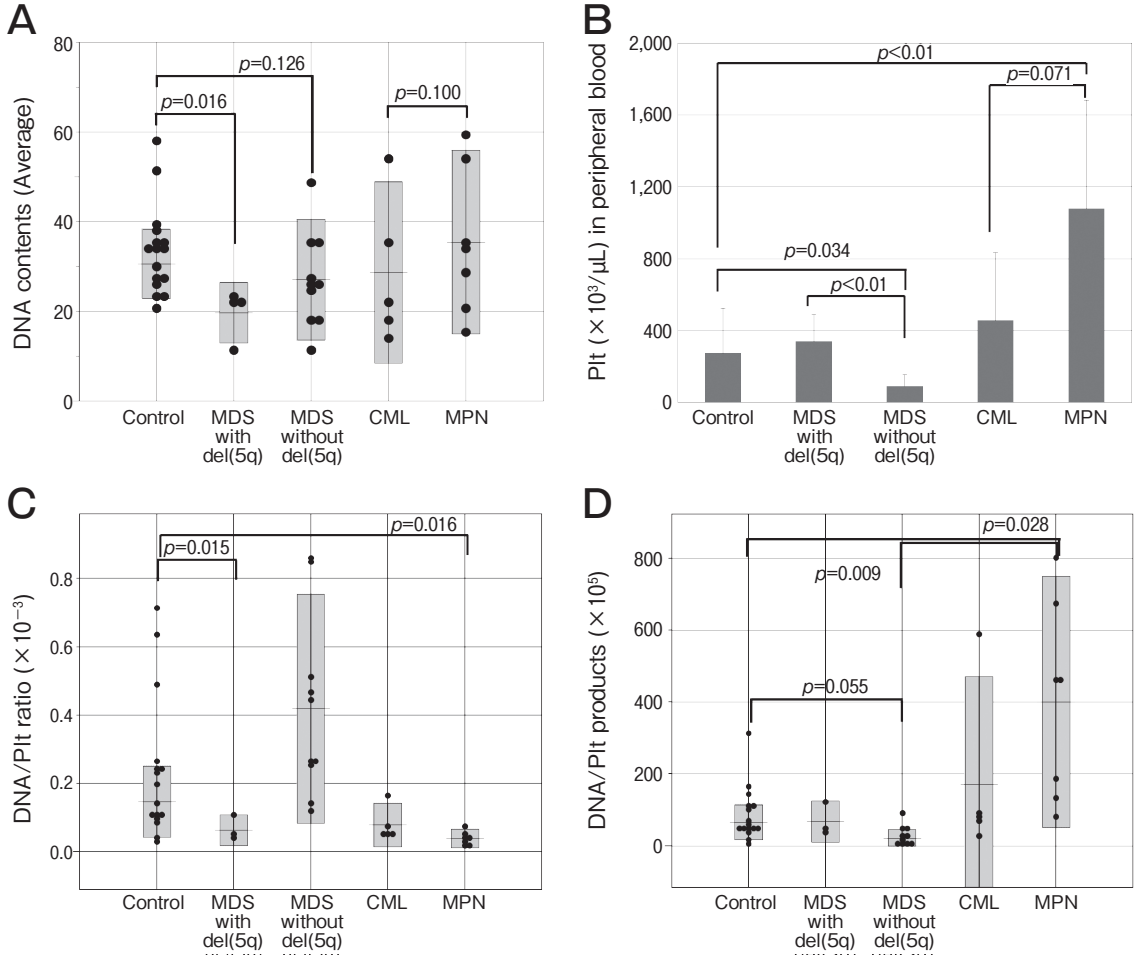

Fig. 3 Comparison of the DNA contents and platelet counts of hematological diseases. **A**, The average DNA contents of bone marrow MKs by hematological diseases; **B**, Platelet counts in peripheral blood were compared between the diseases examined. Values are mean ± SD; **C**, The ratio of DNA content and the platelet counts (DNA/Plt ratio); **D**, The product of the DNA content and the platelet counts (DNA×Plt products).

trol group and MDS with del(5q), the normal control group and MPN group (Fig. 3C). In contrast, the DNA × Plt products showed a significant difference between the normal control group and the MDS without del(5q) group and between the normal control group and the MPN group (Fig. 3D).

In the present study, we focused on the ploidy of hypolobated MKs detected exclusively in the patients with MDS with isolated del(5q) or 5q- syndrome. Although only 4 cases of MDS with del(5q) were examined for DNA ploidy analysis, the ploidy distribution was found to deviate significantly to lower the range from that of the normal control group (Fig. 3A). In contrast, the platelet count in the MDS with del(5q) group was comparable to that of the normal control group (Fig. 3B).

Discussion

We established a system for analyzing DNA ploidy by comparing cytological images with their ploidy data. This FMI system consists of a fluorescent microscope and specialized image-processing software, and the specimens used in this study were preserved bone marrow smears after MGG staining. Compared to the conventional techniques LSC and FCM, the FMI system can be easily operated and also yields favorable analyses, and it enabled us to successfully quantify DNA content and thus the DNA ploidy of normal to abnormal MKs, especially from the long-term preserved bone marrow smears of patients with a variety of hematological diseases.

In general, mature MKs have more DNA content with nuclear multilobation and release platelets when their ploidy reaches 8N or more. The degree of nuclear lobation and DNA ploidy thus seem to correlate with each other [1, 14, 16]. The morphological features of MKs in MPN are somewhat different between the subtypes. For instance, the MKs of patients with CML are known to be characterized by their relatively small size and hypolobation, whereas the MKs of patients with ET are large, polyploid, and markedly hyperlobated, and the MKs of patients with PV exhibit a phenotype intermediate to those observed in CML and ET. As these findings were not significant in the present study, an analysis with a larger sample size is necessary to confirm these features of MK in MPN subtypes. Our present findings also support the previous reports that MKs in MDS patients show lower DNA ploidy compared to those in normal controls [17-21], although a larger sample size study is necessary to examine this result.

MDS with isolated del(5q) or 5q- syndrome is discriminated into the distinct MDS subtypes characterized by macrocytic anemia, absent or mild leukopenia, normal or elevated platelet counts, and abnormal hypolobated MK with eccentric nuclei [22]. It is important to identify the DNA ploidy of hypolobated MKs. In Japan, 5q- syndrome is quite rare [23] and we were able to collect only 4 samples for the present study, but as shown in Fig. 2B and Fig. 3A, the ploidy distribution of such MKs deviated significantly to a range that was lower than that of the normal control group. This result might be expected from the observation of the bone marrow smear of the patients with 5q- syndrome, but to the best of our knowledge, this study is the first to actually demonstrate the DNA hypoploidy of hypolobated MKs. Considering the features of 5q- syndrome-specific MKs that produce a large amount of platelets regardless of DNA hypoploidy, it could be presumed that increased ploidy and nuclear lobation are not indispensable to abundant platelet production.

There are various mechanisms in the polyploidy formation of MKs [24-27], including a report that the activation and localization of aurora B by survivin and RhoA promote the cleavage furrow formation [28]. However, most of the mechanisms are still unknown. Further investigation of these mechanisms in conjunction with the interrelation of nuclear lobation, DNA ploidy, and platelet production is required.

Our clinical sample findings also indicate that the DAPI staining of cells from normal or pathological tissues will be the best application for FMI.

We intend to use FMI to further measure the DNA ploidy of several hematopoietic cells and clarify the relationship between the DNA ploidy and cell dysplasia in bone marrow disorders.

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