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RESEARCH

Over-Expression of Cancerous Inhibitor of PP2A (CIP2A) in Bone Marrow Cells from Patients with a Group of High-Risk Myelodysplastic Syndromes

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Abstract Cancerous inhibitor of PP2A (protein phosphatase 2A) (CIP2A) is an inhibitor of PP2A, a phosphatase and tumor suppressor that regulates cell proliferation, differentiation, and survival. The aim of this study was to investigate whether CIP2A plays a role in the progression of myelodysplastic syndromes (MDS). Immunohistochemical analysis revealed that a fraction patients having refractory anemia with excess blasts (RAEB)-1 (4 out of 12) and RAEB-2 (10 out of 14) exhibited significant expression of CIP2A in bone marrow hematopoietic cells, while all patients with refractory cytopenia with unilineage or multilineage dysplasia (RCUD/RCMD) (0 out of 18) and the control group (0 out of 17) were negative. CIP2A was mainly expressed by the MPO-positive myeloid series of cells and partly by the CD34-positive cells in association with the expression of phosphorylated c-MYC (p-c-MYC) protein and the cell cycle-related proteins Ki-67, MCM2, and geminin. The percentage of p-c-MYC-positive cells in the bone marrow of CIP2A-positive MDS cases was significantly higher than that in CIP2A-negative MDS cases ($P<0.01$). The expression levels of mRNA for *CIP2A* and *PP2A* exhibited positive correlation in MDS/control bone marrow. These results suggest that up-regulated expression of CIP2A might play a role in the proliferation of blasts in the MDS bone marrow and in disease progression in at least some cases.

Keywords CIP2A · c-MYC · Myelodysplastic syndromes · Bone marrow · Immunohistochemistry

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Introduction

Myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal disorders involving hematopoietic progenitor cells, in which the predominant symptoms are ineffective hematopoiesis and peripheral cytopenias [1, 2]. Although the genetic and molecular aberrations in MDS cell clones have been determined, their survival, proliferation, and apoptosis are influenced by multiple factors in the bone marrow microenvironment [3–8]. MDS bone marrow cells possess a highly proliferative character [9], while several cytokines, including TNF-α, Fas-ligand, and IFN-γ, are known to contribute to their apoptosis [6, 10–12], causing cytopenias [11, 13]. By contrast, bone marrow hematopoietic cells transformed from MDS to overt leukemia (OL) tend to be immortal, possibly due to accumulated chromosomal/genetic abnormalities. Thus, the mechanisms regulating cellular proliferation/apoptosis in MDS bone marrow hematopoietic cells seem to be altered during disease progression.

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a human oncoprotein over-expressed in various malignancies, such as head and neck squamous cell carcinoma, and colonic adenocarcinoma [14–16]. Although not necessarily in the cases with hematological neoplasms, CIP2A interacts with protein phosphatase 2A (PP2A) and prevents PP2A-mediated dephosphorylation of the oncogene *c-MYC* in

human malignancies [15]. CIP2A is a marker of reduced overall survival in certain subgroups of gastric cancer [17] as well as serous ovarian cancer [18], and its expression is associated with high grade and lymph node metastasis in breast cancer [19]. Recently, Lucas et al. reported that CIP2A is a critical determinant of disease progression in chronic myeloid leukemia (CML) [20]. The authors showed that CIP2A played a key role in inhibiting PP2A in CML, and observed higher CIP2A levels in patients who later progressed to acute leukemia. In acute myeloid leukemia (AML) cases, suppression of PP2A phosphatase activity was a recurrent event [21]. However, the role of CIP2A in the pathogenesis of MDS and its progression is unknown. To address the role of CIP2A in MDS, we analyzed the expression of CIP2A protein in bone marrow cells by using immunohistochemistry, and investigated the association of the expression of CIP2A protein with clinico-pathological variables and the distribution of phosphorylated c-MYC-expressing cells in bone marrow.

Materials and Methods

Patients

For clinical samples, bone marrow clots were obtained by aspiration during routine diagnostic procedures. Formalin-fixed paraffin-embedded (FFPE) bone marrow aspiration samples from 17 control individuals (male:female, 10:7; median age, 55 [range, 33–77 years]) and 44 patients with MDS (male:female, 30:14; median age, 71 [range, 28–89 years]) were analyzed using immunohistochemistry. In addition, 9 cases with overt leukemia (OL) transformed from MDS (MDS/AML) were also used for the immunohistochemical analysis. These FFPE samples were collected at the National Hospital Organization Kumamoto Medical Center and the Tokyo Medical and Dental University Hospital.

In addition, the expression of mRNA was analyzed in bone marrow specimens from newly diagnosed patients at the National Hospital Organization Kumamoto Medical Center between 2006 and 2010. Fresh frozen bone marrow samples from 15 MDS patients (male: female, 9:6; median age, 68 [range, 31–80 years]) were analyzed using the polymerase chain reaction. Samples from 6 age-matched individuals with no hematological diseases or morphological abnormalities were included as controls (male:female, 3:3; median age, 60 [range, 44–70 years]). The patients were not infected with specific viruses, including human T cell leukemia virus type 1 (HTLV-1), and had not been treated with therapeutic drugs prior to the study.

All procedures followed standards established by the ethical committees of The National Hospital Organization Kumamoto Medical Center and The Tokyo Medical and Dental University, and the experimental schedule was approved by the ethical committees of both institutions.

Immunohistochemical Staining for CIP2A, Phosphorylated c-MYC, Cell Cycle-Related Proteins, and Cell Markers

To examine the occurrence of CIP2A-expressing cells in the bone marrow of control and MDS cases, rabbit monoclonal antibody against CIP2A (Novus Biologicals, Littleton, CO, USA) was applied to 4-μm-thick FFPE tissue sections after treatment with 0.3 % hydrogen peroxide in methanol (to eliminate endogenous peroxidase activity) and 10 % normal horse serum (to block non-specific binding of the antibody). Sections were then treated with primary antibodies (dilution 1:250) overnight at 4 °C. Detection was performed by the streptavidin-biotin-peroxidase complex technique (ABC Kit; Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (DAB) as chromogen. For CIP2A, the nuclei of more than 300 cells from randomly selected fields of the whole section were counted for each case. The percentage of brown-staining nuclei (CIP2A +ve) was calculated. Cases with more than 50 % of nucleated cells CIP2A positive were evaluated as ++, 10 % to 50 % as +, and less than 10 % as -.

To determine the distribution of phospho-c-MYC (p-c-MYC) protein in the bone marrow of MDS cases expressing CIP2A, adjacent sections were stained for p-c-MYC by using mouse monoclonal antibody (Abcam, Cambridge, MA, USA). The staining procedure and evaluation process for p-c-MYC positivity were similar to those for CIP2A.

To clarify the cell cycle status and cell type of CIP2A-expressing bone marrow MDS cells, double immunostaining was performed for CIP2A and CIP2A cell-cycle-related proteins and cell markers. FFPE bone marrow sections of thickness 4 μm were used. After de-paraffinization, heat-based antigen retrieval, endogenous peroxidase inactivation with 3 % hydrogen peroxide, and blocking were performed. The primary antibodies were as follows: CIP2A, rabbit monoclonal, 1:250 (Novus Biologicals, Littleton, CO, USA); Ki-67, mouse monoclonal, ready to use (Nichirei Bioscience, Tokyo, Japan); minichromosome maintenance 2 (MCM2), mouse monoclonal, 1:2000 (clone 46; BD Transduction Laboratories, Lexington, KY, USA); geminin, mouse monoclonal, 1:100 (NOVOCASTRA, Newcastle Upon Tyne, UK); CD71, mouse monoclonal, ready to use (Invitrogen, Carlsbad, CA, USA); myeloperoxidase (MPO), rabbit monoclonal, 1:4000 (NOVOCASTRA); CD61, mouse polyclonal, 1:50 (NOVOCASTRA); CD34, mouse monoclonal, 1:100 (NOVOCASTRA). Primary antibody incubation was performed overnight at 4 °C. Antigen retrieval was performed using a microwave for CD61, CD71, MPO and CD34 staining, and an autoclave for CIP2A, MCM2, Ki-

67, and geminin staining. Detection was performed with the following reagents: ABC Kit (Vector Laboratories) with diaminobenzidine (DAB, Nichirei Bioscience) or Ni-diaminobenzidine (Ni-DAB, Nichirei Bioscience) as the chromogen; or HISTOFINE Simple Stain AP Series (Nichirei Bioscience) with Vector Blue (Vector Laboratories) or Warp Red Chromogen Kit (Biocare Medical, Concord, CA, USA) as the chromogen.

Preparation of mRNA and Quantitative Assay for CIP2A and PP2A using Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from fresh-frozen bone marrow specimens of patients with MDS and control subjects, and RT-PCR was performed as described previously [22]. For quantitative RT-PCR, specific primers were used with the Lightcycler Sybr Green Master Mix (Roche, Basel, Switzerland). The sequences of the primers were as follows: for *CIP2A*, GAACAGATAAGAAAAGAGTTGAGCATT and CGACCTTCTAATTGTGCCTTT; for *PP2A*, ACTGGTGCCATGACCGGAATGT and TCGCCTCTACGAGGTGCTGGG; and for *β-actin*, AGCACAGAGCCTCGCCTTT and CACGATGGAGGGGAAGAC. PCR products were detected using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Data on the quantity of RNA (ng) for *CIP2A* and *PP2A* were normalized by the 2-ΔΔCT method using the data for *β-actin* for each sample, [23].

Statistical Analysis

Significant differences were demonstrated in the immunohistochemical positivity of CIP2A using a chi-square test and StatView statistical software. Non-parametric tests were used, including Mann-Whitney's *U*-test and the Kruskal-Wallis test, to compare the fraction of p-c-MYC positive bone marrow cells in control and MDS cases. Data from correlations of *CIP2A* with *PP2A* expression levels were compared with the collated data by using Spearman's rank correlation coefficient. The level of significance was set at 0.05 for all tests.

Results

Immunohistochemistry for CIP2A and Phosphorylated c-MYC

To determine the CIP2A expression in bone marrow cells, immunostaining was performed using FFPE samples from 44 MDS patients and 17 control subjects (Fig. 1a–d). As shown

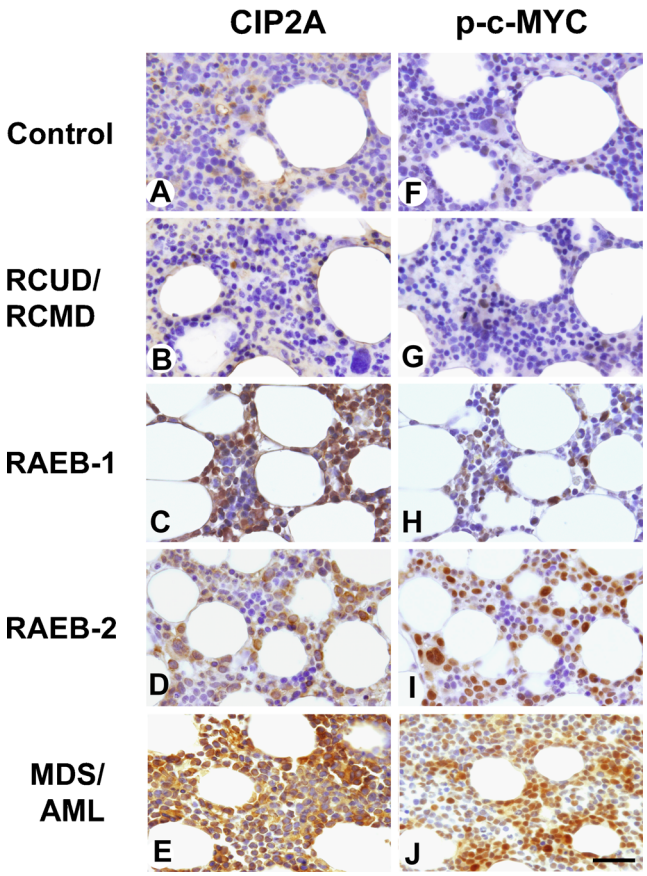

Fig. 1 Immunohistochemical localization of CIP2A (**a**–**e**) and p-c-MYC (**f**–**j**) in bone marrow cells from control (**a** and **f**), RCUD/RCMD (**b** and **g**), RAEB-1 (**c** and **h**), RAEB-2 (**d** and **i**), and MDS/AML (**e** and **j**) cases. Bar indicates 50 μm. Note the cytoplasmic localization of CIP2A in RAEB-1 (**c**), RAEB-2 (**d**) and MDS/AML (**e**) cases. By contrast, control (**a**) and RCUD/RCMD (**b**) cases did not stain for CIP2A. p-c-MYC was localized to the nucleus of the bone marrow cells. Control (**f**) and RCUD/RCMD (**g**) cases showed a few positive signals, whereas RAEB-1 (**h**), RAEB-2 (**i**) and MDS/AML (**j**) cases exhibited frequent signals for p-c-MYC. CIP2A and p-c-MYC showed similar patterns of distribution in the bone marrow of RAEB-1, RAEB-2 and MDS/AML cases

in Fig. 1c and d, CIP2A was mainly expressed in the cytoplasm of the hematopoietic cells in some MDS cases, in contrast to a negative reaction in the control and RCUD/RCMD bone marrow (Fig. 1a and b). Although many MDS cases did not stain significantly, 14 of 44 cases showed distinct staining for CIP2A (the upper column of Table 1). Notably, CIP2A-positive cases were classified as RAEB-1 and RAEB-2, while all RCUD/RCMD cases were CIP2A-negative by immunohistochemical staining.

In addition to control and MDS cases, we determined the expression of CIP2A in the bone marrow samples from OL cases transformed from MDS (MDS/AML) (the lower column of Table 1). These cases were also analyzed as the cases with MDS before transformation (one case of RCMD, 4 cases of RAEB-1 and 4 cases of RAEB-2 in the upper column of

Table 1 Immunohistochemical localization of CIP2A in bone marrow from control and MDS cases (number of cases)

Subtypes	CIP2A		
	–	+	++
Control	17	0	0
RCUD/RCMD	18	0	0
REAB-1	8	4	0
RAEB-2	4	5	5
MDS/AML	2	3	4

Differences were significant between the proportion of CIP2A-positive cases (+ and ++) in the control and RAEB (RAEB-1 and RAEB-2), and between RCUD/RCMD and RAEB cases ($P<0.001$ by chi-square test, respectively). The samples of MDS were taken at the time of initial diagnosis. Results from MDS/AML cases were shown in the lower column of the table. MDS/AML cases developed overt leukemia from one case of RCMD, four cases of RAEB-1 and four cases of RAEB-2. MDS/AML samples were taken after the evolution of overt leukemia from the same patients of MDS that were tabulated in the upper column

Table 1), thus we could examine the chronological changes of CIP2A expression in the same patients during the course of transformation. As shown here, 7 out of 9 cases of MDS/AML exhibited positive staining for CIP2A. The comparison of CIP2A expression in MDS and MDS/AML revealed that two cases with CIP2A-negative bone marrow at the time of initial diagnosis showed negative staining for CIP2A even when OL developed. Another two MDS cases with CIP2A-negative bone marrow turned to CIP2A-positive after transformation to OL. By contrast, five MDS cases with CIP2A-positive bone marrow were also positive for CIP2A expression after OL development, although 2 among 5 cases exhibited stronger staining pattern when OL developed. These results suggested that CIP2A never showed reduced expression during the course of transformation to OL from MDS. Thus, higher CIP2A expression in MDS bone marrow could be associated with the blastic proliferation as well as the development of OL at least in a part of MDS cases.

As the over-expression of CIP2A is known to up-regulate phosphorylation of c-MYC via inhibition of the PP2A phosphatase, immunohistochemical staining was performed to visualize phosphorylated c-MYC (p-c-MYC) in the bone marrow cells of MDS cases and control subjects. As shown in Fig. 1f–j, p-c-MYC was localized to the nucleus of bone marrow cells. The localization of p-c-MYC-positive cells was similar to that of CIP2A-positive cells in MDS cases, although p-c-MYC-positive cells were more frequent. Furthermore, all control subjects and CIP2A-negtive MDS cases exhibited a positive reaction for p-c-Myc in some bone marrow cells (Fig. 1f and g), in contrast to the absence of staining for CIP2A (Fig. 1a and b). The p-c-MYC positive cell percentage in bone marrow was higher in MDS cases than in control cases (Fig. 2a). Differences were significant between

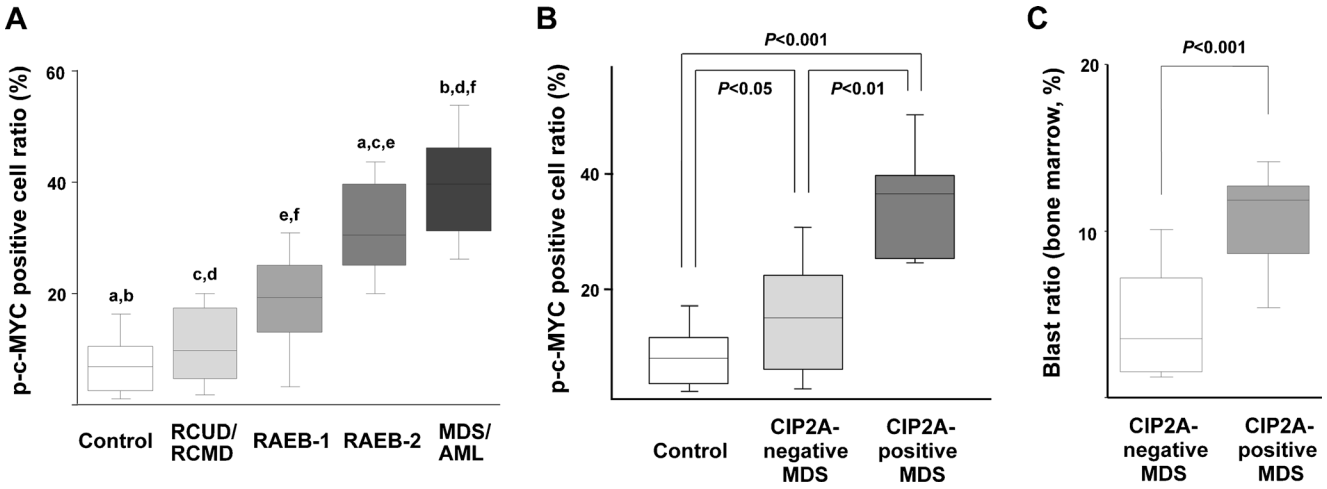

Fig. 2 **a** Box plots comparing the proportions of p-c-MYC positive cells in the bone marrow of control, MDS and MDS/AML cases. Bars indicate 90 and 10 percentiles, and boxes indicate 75 to 25 percentiles with median values shown as a line inside the boxes. Differences were significant between control and RAEB-2 cases (**a**), control and MDS/AML cases (**b**), RCUD/RCMD and RAEB-2 cases (**c**), RCUD/RCMD and MDS/AML cases (**d**), RAEB-1 and RAEB-2 cases (**e**), and RAEB-1 and MDS/AML cases (**f**) (**a** and **b**, $P\leq0.0001$; **c** and **d**, $P\leq0.01$; **e** and **f**, $P\leq0.05$). *P* values were calculated using Mann-Whitney's *U*-test and Kruskal-Wallis analysis. **b** Box plots comparing the proportions of p-c-MYC positive cells in the bone marrow of CIP2A-negative and -positive MDS cases. Differences were significant between control and CIP2A-negative MDS cases, control and CIP2A-positive MDS cases, and CIP2A-negative MDS and CIP2A-positive MDS cases ($P<0.05$, $P<0.001$, and $P<0.01$, respectively) by Mann-Whitney's *U*-test. **C**: Box plots comparing the bone marrow blast ratio of CIP2A-negative and -positive MDS cases. Differences were significant between CIP2A-negative MDS and CIP2A-positive MDS cases ($P\leq0.001$) by Mann-Whitney's *U*-test

Table 2 Clinico-pathological parameters in control, CIP2A-negative, and CIP2A-positive MDS cases [median, (range)]

Groups	Age (years)	RBC ($\times 10^{10}$/L)	WBC ($\times 10^{6}$/L)	Plt ($\times 10^{10}$/L)	Blast (%, BM)
Control	55 (33–77)	435 [a,b] (228–509)	5.0 (2.9–13)	21 [c,d] (12–50)	N.D.
CIP2A- negative MDS	72.5 (28–89)	241 [a] (161–413)	3.8 (0.8–14)	6.2 [c] (1.3–47)	3.5 [e] (0.8–11.8)
CIP2A- positive MDS	70 (55–86)	210 [b] (150–375)	4.3 (1.6–9.8)	9.8 [d] (3.1–34)	11.9 [e] (4.1–17.8)

N.D. not demonstrated

BM bone marrow

[a–e] Differences were significant ($P<0.01$ by Mann-Whitney's U-test) between the RBC and Plt counts in control and MDS cases (a–d), and between the blast ratio of CIP2A-negative and -positive cases (e)

control and RAEB-1 cases ($P<0.05$), control and RAEB-2 cases ($P<0.0001$), RCUD/RCMD and RAEB-2 cases ($P<0.01$), and RAEB-1 and RAEB-2 cases ($P<0.05$). Furthermore, the percentage in CIP2A-positive MDS cases was significantly higher than that in CIP2A-negative MDS cases ($P<0.01$) (Fig. 2b).

We compared clinico-pathological parameters, including peripheral blood cell counts and bone marrow blast percentage, between control, CIP2A-negative, and CIP2A-positive cases. As shown in Table 2, MDS cases exhibited significantly lower red blood cell (RBC) and platelet (Plt) counts compared with control cases. Notably, the fraction of blasts in the bone marrow exhibited significant differences between CIP2A-negative and CIP2A-positive MDS cases (Table 2 and Fig. 2c, $P\leq0.001$), while the RBC, white blood cell (WBC) and Plt counts were not significantly different. These results suggest that over-expression of CIP2A in bone marrow cells is possibly associated with the proliferation of blasts, but does not contribute to the cytopenic condition of patients caused by ineffective hematopoiesis.

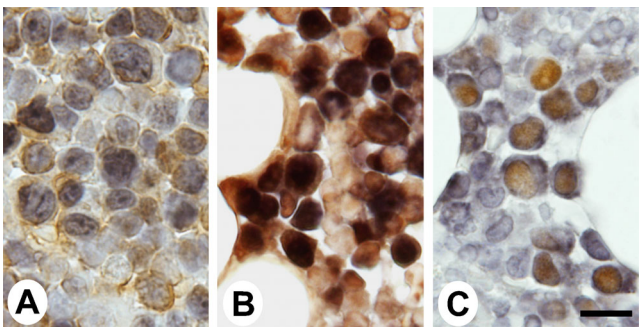

Fig. 3 Expression of Ki-67 (**a**), MCM2 (**b**), and geminin (**c**) in bone marrow cells of CIP2A-positive MDS cases. Most of the CIP2A-positive cells (cytoplasm, *brown*) were positively stained for Ki-67 (nucleus, *black*) (**a**) and MCM2 (nucleus, *black*) (**b**). Note that the majority of geminin-positive cells (nucleus, *brown*) were positive for CIP2A (cytoplasm, *black*) (**c**). Bar indicates 20 μm

Expression of CIP2A and Cell Cycle Markers

To determine the cell cycle status of bone marrow cells from CIP2A-positive MDS cases, double immunostaining was performed for CIP2A and Ki-67 (Fig. 3a). The majority of CIP2A-positive cells (brown) expressed Ki-67 (black) (median, 73.3 %; range, 63.2 % to 86.8 %), while the percentage expressing Ki-67 was lower in CIP2A-negative cells (median, 43.8 %; range, 32.9 % to 75.0 %). To further differentiate the G1 and S/G2/M status of bone marrow cells, MCM2 and geminin were stained immunohistochemically. As shown in Fig. 3b and c, most of the CIP2A-positive cells bone marrow cells exhibited MCM2+ (black) geminin+ (black) phenotype, whereas bone marrow cells with a CIP2A-negative phenotype were $MCM2^{+}$ $geminin^{-}$. The percentage of CIP2A-positive cells (brown) that were MCM2-positive (black) was higher (median, 75.6 %; range, 59.4 % to 83.7 %) than that of CIP2A-negative cells that were MCM2-positive (median, 29.9 %; range, 20.1 % to 45.5 %). Furthermore, the geminin-positive cells (brown) were concentrated in CIP2A-positive cells (black) (median, 40.1 %; range, 34.4 % to 58.7 %), in contrast with the very low proportion of geminin-positive cells in CIP2A-negative cells (median, 5.6 %; range, 3.4 % to 13.4 %).

Immunohistochemistry for CIP2A and Cell Markers

To determine the cell types of CIP2A-expressing bone marrow cells from MDS cases, double immunostaining was performed for erythroid cell marker, CD71 and CIP2A, myeloid cell marker, MPO and CIP2A, megakaryocyte marker, CD61 and CIP2A and blast/stem cell marker, CD34 and CIP2A. As shown in Fig. 4a and c, CD71-positive cells (blue) and CD61-positive cells (brown) did not express CIP2A (brown in Fig. 4a and black in c), i.e., the majority of CD71- and CD61-positive cells were single positive. In

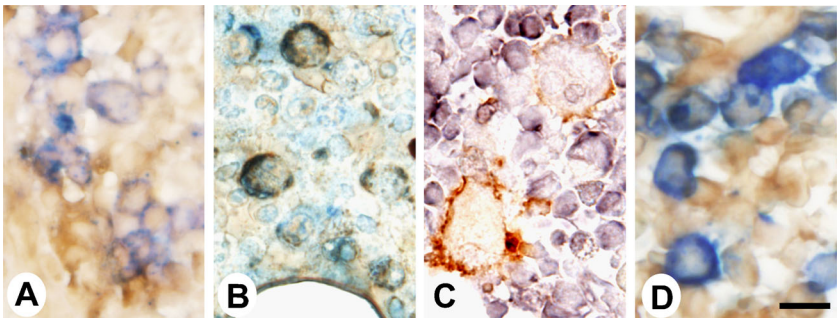

Fig. 4 Double immunostaining for CIP2A and cell markers in the bone marrow of CIP2A-positive MDS cases. The erythroid cell marker, CD71 (cell surface, *blue*) and CIP2A (cytoplasm, *brown*) (**a**), the myeloid cell marker, MPO (cytoplasm, *blue*) and CIP2A (cytoplasm, *brown*) (**b**), the megakaryocyte marker, CD61 (cell surface, *brown*) and CIP2A (cytoplasm, *black*) (**c**) and the blast/stem cell marker, CD34 (cell surface, *blue*) and CIP2A (cytoplasm, *brown*) were identified immunohistochemically. Note the CIP2A positive reaction in the MPO-positive cells (**b**) and CD34-positive cells (**d**) but not in the CD71- (**a**) or the CD61-positive cells (**c**). Bar indicates 20 μm

contrast, CIP2A-positive cells (brown) were also positive for MPO (blue), exhibiting double positive staining (Fig. 4b). The CD34-positive cells (blue) were also positive for CIP2A (brown) exhibiting double positive signals, although a part of CIP2A-positive cells were CD34-negative. These results suggested that CIP2A was mainly expressed in myeloid cells including blastic cells in the bone marrow of MDS patients.

Correlation of the Expression Levels of mRNA for *CIP2A* and *PP2A* Determined by Real-Time PCR

We investigated the relationship between the expression levels of *CIP2A* and *PP2A* in MDS patients. We found a positive correlation between the expression levels of *CIP2A* and *PP2A* ($P<0.02$ by Spearman's rank coefficient, two-tail test) (Fig. 5a). The relationship in all samples, including MDS and control subjects, also exhibited a significant correlation ($P<0.01$) (Fig. 5b). These results suggest that over-expression of CIP2A possibly inhibits PP2A function and could therefore induce the production of PP2A in the bone marrow of MDS patients as well as in control cases.

Discussion

Current evidence clearly indicates that malignant transformation of tumors requires PP2A inhibition [14, 24, 25]. Therefore, CIP2A, a protein that inhibits PP2A in human malignancies and has transforming activity, is important in cancer progression. CIP2A inhibits PP2A-mediated c-MYC dephosphorylation and proteolytic degradation [15]. c-MYC is stabilized by phosphorylation, firstly by ERK at serine 62 (S62), and then by GSK-3β at threonine 58 (T58). After the conformational change in doubly phosphorylated c-MYC, S62 becomes a substrate for PP2A, leading to dephosphorylation of that residue. The resulting T58 mono-phosphorylated c-MYC is a target of ubiquitin ligase complex, leading to proteosomal degradation of c-MYC. Therefore, inhibition of PP2A by CIP2A protects c-MYC from

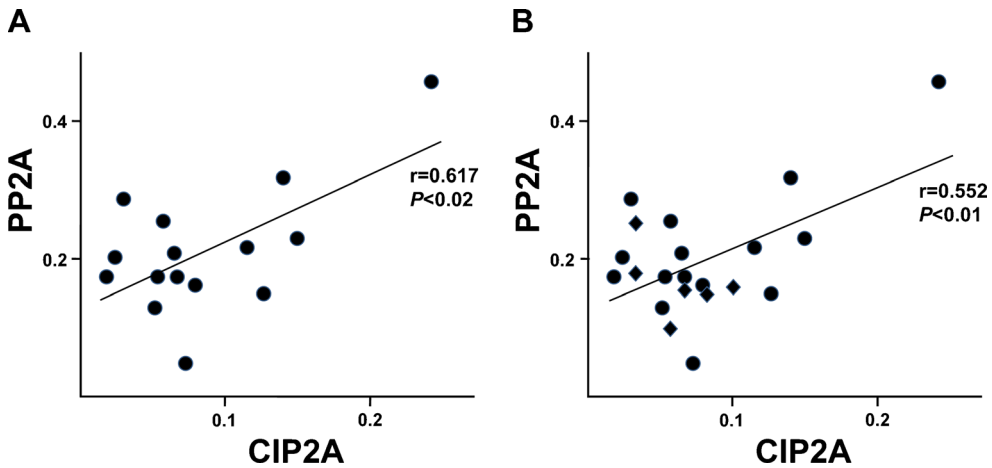

Fig. 5 Correlation between mRNA expression levels for *CIP2A* and *PP2A* determined by RT-PCR in MDS (**a**) and MDS + control (**b**) cases. Significance was demonstrated using the Spearman's rank correlation coefficient ($P<0.02$ and $P<0.01$, respectively)

degradation. c-MYC status is directly related to oncogenic activity [26] as well as the clinical outcome of breast cancers [27]. Furthermore, CIP2A directly binds to c-MYC and shield it from PP2A-mediated dephosphorylation [15]. Although not in the cases with MDS, stabilization of c-MYC has been observed in various tumor cells, including hematological malignancies, promoting cell transformation and tumor progression [14, 28, 29]. Inhibition of CIP2A by bortezomib in hepatocellular carcinoma caused activation of PP2A and decreased phospho-Akt, resulting in induction of apoptosis in the tumor cells [30].

On the other hand, down-regulation of PP2A activity also induced apoptotic responses to tumor cells in several situations. Lenalidomide, which has broad biological effects, including inhibition of angiogenesis and enhancement of tumor-specific immunity, is effective in suppressing clones of malignant del(5q) MDS [31]. This effect was shown to be associated with the inhibition of phosphatases such as Cdc25C and PP2A that are encoded within the common deleted region (CDR) of del(5q) MDS. Using animal model systems, we have recently shown that inhibition of PP2A by cytoplasmic MCM2 induces hyper-phosphorylation of DNA-PK to activate p53, resulting in the enhanced induction of apoptosis in the hematopoietic cells under DNA-damage [32].

Here we demonstrated an association of CIP2A and p-c-MYC expression mainly in the myeloid series of hematopoietic cells and blasts of MDS cases. Stabilization of c-MYC was observed in some RAEB-1 and RAEB-2 cases and was associated with over-expression of CIP2A. These cells appeared to have higher proliferative activity, suggesting that CIP2A expression is related to disease progression in MDS cases. c-MYC amplification has been reported previously in a case of progression from MDS to OL [33]. Thus, the role of c-MYC could be important in the progression of MDS. On the other hand, the clinical parameters for cytopenias in CIP2A-positive MDS cases did not show significant differences as compared with those in CIP2A-negative MDS cases. These results suggest that the suppression of PP2A was not correlated with the apoptotic enhancement of hematopoietic cells in MDS. However, over-expression of CIP2A was detected only in a fraction of high-risk MDS (RAEB-1 and RAEB-2, total 14 of 26). To confirm the CIP2A expression pattern in MDS bone marrow, we searched for the data-base showing the expression profile of CIP2A (GEO, http://www.ncbi.nlm.nih.gov/gds; GSE4619). The data-base revealed that the majority of MDS cases exhibited the similar levels of CIP2A expression with those of control subjects. However, a minor fraction of MDS cases showed the remarkably high levels of CIP2A expression. The results were compatible with our findings in the present study. Thus, certainly the cases with CIP2A over-expression formed a minor but a significant group in MDS cases. The present study showed that these MDS cases were usually belonging to the high-risk type that would frequently transform to OL. Therefore we could speculate that the biological significance would exist in the over-expression status of CIP2A in the MDS bone marrow cells.

Down-regulation of PP2A by CIP2A in bone marrow cells should correlate with shortening of the G1 phase in the cell cycle, and accelerated cell proliferation [34]. Although flow cytometric analysis might provide more reliable evidences for cell cycle analysis, in vivo studies could demonstrate significant clinical implications of the expression for CIP2A as well as cell cycle-related proteins [35, 36]. The present study demonstrated expression of Ki-67, MCM2 and geminin in CIP2A-positive bone marrow cells of MDS cases. As is well known, Ki-67 is expressed in cells in late G1 to M phase, MCM2 in early G1 to M phase, and geminin in S/G2/M phase [37]. Therefore, CIP2A-positive cells of MDS cases, especially the myeloid series of cells, might have high proliferative activity. The majority of CIP2A-positive cells were believed to be in S/G2/M phase (Ki-67$^+$, MCM2$^+$, geminin$^+$), although abnormal expression patterns of MCM2 as well as geminin could occur in the bone marrow cells of patients with myeloid disorders/neoplasms [38, 39].

Although PP2A mRNA measured by RT-PCR appeared to be highly expressed in CIP2A-expressing cases, p-c-MYC expression was also detected. These results suggest that the function of PP2A might be suppressed in CIP2A-positive MDS cases, resulting in the complementary production of PP2A mRNA. Further study should clarify the molecular mechanisms regulating the correlation of the CIP2A-PP2A-c-MYC axis.

In conclusion, immunohistochemical detection of CIP2A in the bone marrow of patients with MDS possibly indicates up-regulated activity in hematopoietic cell proliferation, resulting in disease progression. The mechanisms of CIP2A over-expression and the clinical outcomes of CIP2A-positive MDS cases need to be clarified in future studies.

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