T cells were detectable in all three stem cell transplant recipients who received a conditioning regimen containing alemtuzumab in the present study. Previous studies showed auto-Abs specific to DRS-1 and moesin are frequently detected in PNH$^+$ patients (36, 37). It is thus possible that GPI-AP$^-$ T cells may be induced to proliferate by some auto-Abs specific to GPI-APs on T cells in PNH-T$^+$ patients. However, GPI-AP$^-$ T cells in alemtuzumab-treated patients showed a distinct phenotype pattern characterized by the expression of CD45RA, CCR7, and CD62L from that detectable in PNH-T$^+$ patients. There was no apparent T lymphocytopenia in PNH-T$^+$ patients which should occur in patients possessing auto-Abs specific to T cell antigens. It is therefore unlikely that CD48$^-$CD59$^-$ T cells were induced to proliferate by auto-Abs specific to GPI-APs.

The most likely explanation for the presence of PNH-T$^+$ patients is that humoral factors negatively regulating the proliferation of both HSCs and T-cell precursors via their interaction with GPI-APs are involved in the development of BMF in PNH-T$^+$ patients. Cytokine-mediated selection of *PIGA* mutant HSCs has been proposed as a mechanism for preferential proliferation of GPI-AP$^-$ cells (38), but no evidence supporting this mechanism has been shown. The present study demonstrated that GPI-AP$^-$ T cells show a decreased sensitivity to HVEM that transmit inhibitory signals through a GPI-AP receptor CD160 (27), as well as to TGF-β, a well-known inhibitor of haematopoiesis (39). Recent studies have demonstrated the presence of GPI-AP-type co-receptors for TGF-β (40). Although the T cells used in the current study were not T-cell precursors, memory T cells in the PB T cells may behave like HSCs in terms of their dormancy and activation in response to appropriate stimulation. HSCs may be rendered to express some GPI-APs capable of transmitting inhibitory signals upon activation as memory T cells express CD160 and as a result, both HSCs and T-cell precursors or memory T cells may become invulnerable to some inhibitory cytokines, such as TGF-β, because of the lack of GPI-AP type-receptors. Further analyses of T cells may therefore be useful for identifying GPI-AP type TGF-β receptors which permit the preferential proliferation of HSCs with *PIGA* mutation in patients with BMF.

Acknowledgements

The authors thank Ms. Rie Ohmi, Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, for the excellent technical assistance. We also thank all of the institutions in Japan who contributed patients to this study.

Disclosure of conflicts of interest

All authors have no financial or personal relationships with other people or organizations that could inappropriately influence this study. The authors declare no competing financial interest.

References

1. Schubert J, Vogt HG, Zielinska-Skowronek M, Freund M, Kaltwasser JP, Hoelzer D, Schmidt RE. Development of the glycosylphosphatitylinositol-anchoring defect characteristic for paroxysmal nocturnal hemoglobinuria in patients with aplastic anemia. *Blood* 1994;**83**:2323–8.
2. Schrezenmeier H, Hertenstein B, Wagner B, Raghavachar A, Heimpel H. A pathogenetic link between aplastic anemia and paroxysmal nocturnal hemoglobinuria is suggested by a high frequency of aplastic anemia patients with a deficiency of phosphatidylinositol glycan anchored proteins. *Exp Hematol* 1995;**23**:81–7.
3. Griscelli-Bennaceur A, Gluckman E, Scrobohaci ML, Jonveaux P, Vu T, Bazarbachi A, Carosella ED, Sigaux F, Socie G. Aplastic anemia and paroxysmal nocturnal hemoglobinuria: search for a pathogenetic link. *Blood* 1995;**85**:1354–63.
4. Dunn DE, Tanawattanacharoen P, Boccuni P, Nagakura S, Green SW, Kirby MR, Kumar MS, Rosenfeld S, Young NS. Paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure syndromes. *Ann Intern Med* 1999;**131**:401–8.
5. Wang H, Chuhjo T, Yasue S, Omine M, Nakao S. Clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria-type cells in bone marrow failure syndrome. *Blood* 2002;**100**:3897–902.
6. Sugimori C, Chuhjo T, Feng X, Yamazaki H, Takami A, Teramura M, Mizoguchi H, Omine M, Nakao S. Minor population of CD55-CD59- blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia. *Blood* 2006;**107**:1308–14.
7. Sugimori C, Mochizuki K, Qi Z, Sugimori N, Ishiyama K, Kondo Y, Yamazaki H, Takami A, Okumura H, Nakao S. Origin and fate of blood cells deficient in glycosylphosphatidylinositol-anchored protein among patients with bone marrow failure. *Br J Haematol* 2009;**147**:102–12.
8. Maciejewski JP, Follmann D, Nakamura R, Saunthararajah Y, Rivera CE, Simonis T, Brown KE, Barrett JA, Young NS. Increased frequency of HLA-DR2 in patients with paroxysmal nocturnal hemoglobinuria and the PNH/aplastic anemia syndrome. *Blood* 2001;**98**:3513–9.
9. Ishiyama K, Chuhjo T, Wang H, Yachie A, Omine M, Nakao S. Polyclonal hematopoiesis maintained in patients with bone marrow failure harboring a minor population of paroxysmal nocturnal hemoglobinuria-type cells. *Blood* 2003;**102**:1211–6.

10. Ishikawa T, Tohyama K, Nakao S, *et al.* A prospective study of cyclosporine A treatment of patients with low-risk myelodysplastic syndrome: presence of CD55 (-)CD59(-) blood cells predicts platelet response. *Int J Hematol* 2007;**86**:150–7.
11. Nakao S, Takamatsu H, Yachie A, Itoh T, Yamaguchi M, Ueda M, Shiobara S, Matsuda T. Establishment of a CD4+ T cell clone recognizing autologous hematopoietic progenitor cells from a patient with immune-mediated aplastic anemia. *Exp Hematol* 1995;**23**:433–8.
12. Karadimitris A, Manavalan JS, Thaler HT, Notaro R, Araten DJ, Nafa K, Roberts IA, Weksler ME, Luzzatto L. Abnormal T-cell repertoire is consistent with immune process underlying the pathogenesis of paroxysmal nocturnal hemoglobinuria. *Blood* 2000;**96**:2613–20.
13. Young NS, Maciejewski JP. Genetic and environmental effects in paroxysmal nocturnal hemoglobinuria: this little PIG-A goes "Why? Why? Why?" *J Clin Invest* 2000;**106**:637–41.
14. Murakami Y, Kosaka H, Maeda Y, Nishimura J, Inoue N, Ohishi K, Okabe M, Takeda J, Kinoshita T. Inefficient response of T lymphocytes to glycosylphosphatidylinositol anchor-negative cells: implications for paroxysmal nocturnal hemoglobinuria. *Blood* 2002;**100**:4116–22.
15. Gargiulo L, Lastraioli S, Cerruti G, Serra M, Loiacono F, Zupo S, Luzzatto L, Notaro R. Highly homologous T-cell receptor beta sequences support a common target for autoreactive T cells in most patients with paroxysmal nocturnal hemoglobinuria. *Blood* 2007;**109**:5036–42.
16. Rotoli B, Luzzatto L. Paroxysmal nocturnal haemoglobinuria. *Baillieres Clin Haematol* 1989;**2**:113–38.
17. Young NS. The problem of clonality in aplastic anemia: Dr Dameshek's riddle, restated. *Blood* 1992;**79**:1385–92.
18. Nicholson-Weller A, Spicer DB, Austen KF. Deficiency of the complement regulatory protein, "decay-accelerating factor," on membranes of granulocytes, monocytes, and platelets in paroxysmal nocturnal hemoglobinuria. *N Engl J Med* 1985;**312**:1091–7.
19. van der Schoot CE, Huizinga TW, van 't Veer-Korthof ET, Wijmans R, Pinkster J, von dem Borne AE. Deficiency of glycosyl-phosphatidylinositol-linked membrane glycoproteins of leukocytes in paroxysmal nocturnal hemoglobinuria, description of a new diagnostic cytofluorometric assay. *Blood* 1990;**76**:1853–9.
20. Schubert J, Alvarado M, Uciechowski P, Zielinska-Skowronek M, Freund M, Vogt H, Schmidt RE. Diagnosis of paroxysmal nocturnal haemoglobinuria using immunophenotyping of peripheral blood cells. *Br J Haematol* 1991;**79**:487–92.
21. Hall SE, Rosse WF. The use of monoclonal antibodies and flow cytometry in the diagnosis of paroxysmal nocturnal hemoglobinuria. *Blood* 1996;**87**:5332–40.
22. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposals for the classification of the acute leukaemias. French–American–British (FAB) co-operative group. *Br J Haematol* 1976;**33**:451–8.
23. Araten DJ, Nafa K, Pakdeesuwan K, Luzzatto L. Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals. *Proc Natl Acad Sci USA* 1999;**96**:5209–14.
24. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; **401**:708–12.
25. Chalasani G, Dai Z, Konieczny BT, Baddoura FK, Lakkis FG. Recall and propagation of allospecific memory T cells independent of secondary lymphoid organs. *Proc Natl Acad Sci USA* 2002;**99**:6175–80.
26. Mochizuki K, Sugimori C, Qi Z, Lu X, Takami A, Ishiyama K, Kondo Y, Yamazaki H, Okumura H, Nakao S. Expansion of donor-derived hematopoietic stem cells with PIGA mutation associated with late graft failure after allogeneic stem cell transplantation. *Blood* 2008;**112**:2160–2.
27. Cai G, Anumanthan A, Brown JA, Greenfield EA, Zhu B, Freeman GJ. CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. *Nat Immunol* 2008;**9**:176–85.
28. Camitta BM. What is the definition of cure for aplastic anemia? *Acta Haematol* 2000;**103**:16–8.
29. Chen G, Kirby M, Zeng W, Young NS, Maciejewski JP. Superior growth of glycophosphatidy linositol-anchored protein-deficient progenitor cells *in vitro* is due to the higher apoptotic rate of progenitors with normal phenotype *in vivo*. *Exp Hematol* 2002;**30**:774–82.
30. Ismail MM, Tooze JA, Flynn JA, Gordon-Smith EC, Gibson FM, Rutherford TR, Elebute MO. Differential apoptosis and Fas expression on GPI-negative and GPI-positive stem cells: a mechanism for the evolution of paroxysmal nocturnal haemoglobinuria. *Br J Haematol* 2003;**123**:545–51.
31. Classen S, Zander T, Eggle D, *et al.* Human resting CD4+ T cells are constitutively inhibited by TGF beta under steady-state conditions. *J Immunol* 2007;**178**:6931–40.
32. Das L, Levine AD. TGF-beta inhibits IL-2 production and promotes cell cycle arrest in TCR-activated effector/memory T cells in the presence of sustained TCR signal transduction. *J Immunol* 2008;**180**:1490–8.
33. Nakakuma H, Nagakura S, Kawaguchi T, Iwamoto N, Hidaka M, Horikawa K, Kagimoto T, Tsuruzaki R, Takatsuki K. Persistence of affected T lymphocytes in long-term clinical remission in paroxysmal nocturnal hemoglobinuria. *Blood* 1994;**84**:3925–8.
34. Rawstron AC, Rollinson SJ, Richards S, Short MA, English A, Morgan GJ, Hale G, Hillmen P. The PNH phenotype cells that emerge in most patients after CAMPATH-1H therapy are present prior to treatment. *Br J Haematol* 1999;**107**:148–53.
35. Garland RJ, Groves SJ, Diamanti P, *et al.* Early emergence of PNH-like T cells after allogeneic stem cell transplants utilising CAMPATH-1H for T cell depletion. *Bone Marrow Transplant* 2005;**36**:237–44.

36. Feng X, Chuhjo T, Sugimori C, Kotani T, Lu X, Takami A, Takamatsu H, Yamazaki H, Nakao S. Diazepam-binding inhibitor-related protein 1: a candidate autoantigen in acquired aplastic anemia patients harboring a minor population of paroxysmal nocturnal hemoglobinuria-type cells. *Blood* 2004;**104**:2425–31.
37. Takamatsu H, Feng X, Chuhjo T, Lu X, Sugimori C, Okawa K, Yamamoto M, Iseki S, Nakao S. Specific antibodies to moesin, a membrane-cytoskeleton linker protein, are frequently detected in patients with acquired aplastic anemia. *Blood* 2007;**109**:2514–20.
38. Parker CJ. The pathophysiology of paroxysmal nocturnal hemoglobinuria. *Exp Hematol* 2007;**35**:523–33.
39. Yamazaki S, Iwama A, Takayanagi S, Eto K, Ema H, Nakauchi H. TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. *Blood* 2009;**113**:1250–6.
40. Finnson KW, Tam BY, Liu K, Marcoux A, Lepage P, Roy S, Bizet AA, Philip A. Identification of CD109 as part of the TGF-beta receptor system in human keratinocytes. *FASEB J* 2006;**20**:1525–7.

Int J Hematol (2010) 92:708–712
DOI 10.1007/s12185-010-0719-1

ORIGINAL ARTICLE

Estimation of cardiac left ventricular ejection fraction in transfusional cardiac iron overload by R2* magnetic resonance

Juri Sakuta · Yoshikazu Ito · Yukihiko Kimura · Jinho Park · Koichi Tokuuye · Kazuma Ohyashiki

Received: 14 June 2010 / Revised: 18 October 2010 / Accepted: 27 October 2010 / Published online: 25 November 2010

Abstract Cardiac dysfunction due to transfusional iron overload is one of the most critical complications for patients with transfusion-dependent hematological disorders. Clinical parameters such as total red blood cell (RBC) transfusion units and serum ferritin level are usually considered as indicators for initiation of iron chelation therapy. We used MRI-T2*, MRI-R2* values, and left ventricular ejection fraction in 19 adult patients with blood transfusion-dependent hematological disorders without consecutive oral iron chelation therapy, and propose possible formulae of cardiac function using known parameters, such as total RBC transfusion units and serum ferritin levels. We found a positive correlation in all patients between both R2* values (reciprocal values of T2*) and serum ferritin levels ($r = 0.81$) and also total RBC transfusion volume ($r = 0.90$), but not when we analyzed subgroups of patients whose T2* values were over 30 ms (0.52). From the formulae of the R2*, we concluded that approximately 50 Japanese units or 2,900 pmol/L ferritin might be the cutoff value indicating possible future cardiac dysfunction.

Keywords Iron overload · Transfusion · Myocardial iron · Magnetic resonance · Iron chelation

J. Sakuta · Y. Ito (✉) · Y. Kimura · K. Ohyashiki
First Department of Internal Medicine (Division of Hematology), Tokyo Medical University,
6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
e-mail: yito@tokyo-med.ac.jp

J. Park · K. Tokuuye
Department of Radiology,
Tokyo Medical University, Tokyo, Japan

1 Introduction

Cardiac failure due to excess of myocardial iron accumulation is known to be a fatal complication of red blood cell (RBC) transfusion-dependent patients [1], although it is also reflected by chronic anemia itself. The recent development of oral iron chelation therapy is expected to improve their prognosis and has been reported to improve iron-induced organ dysfunction and to lower serum ferritin levels [2]. On the other hand, once cardiac failure progresses, the survival is usually poor due to rapid deterioration of cardiomyopathy despite intensive chelation [3]. Therefore, appropriate initiation of iron chelation therapy could improve the survival of patients with transfusion-dependent bone marrow failure (BMF) syndromes. Some corroboration may be expected to use these parameters from the viewpoint of cardiac iron overload.

Myocardial biopsy is sometimes difficult for patients with BMF syndromes, and is also difficult to evaluate myocardial iron concentration (MIC) adequately, because of nonhomogeneous myocardial deposition and small specimens [4]. That is why a simpler and easier way to quantify MIC is needed. Magnetic resonance imaging (MRI) can provide noninvasive assessment, yet has some disadvantages including increased cost of the examination and the relatively limited availability of systems. The T2* value and its reciprocal relaxation rate R2* value with cardiac MRI is known to correlate to tissue iron concentration [5, 6]. T2* values less than 20 ms are commonly used as the cutoff to evaluate severe cardiac iron overload [7]. While cardiac iron overload shortens T2* values having a negative non-linear regression to iron tissue concentration, the R2* values (reciprocals of T2*) are linearly proportional to iron overload [7]. Using R2* values would make it easier to evaluate the relationship between

iron deposit and cardiac function. We tried to measure T2* and R2* values with MRI in transfusion-dependent patients in our institution and discussed the clinical implications of some parameters based on retrospective data.

2 Materials and methods

We selected 19 transfusion-dependent patients (12 men and 7 women) without consecutive oral chelation therapy, who underwent cardiac MRI examination between 2004 and 2010 (Table 1). In this study, we recruited 7 patients who were also included in a previous study [8, 9] using this different technique to analyze them. Patients treated with anticancer drugs were excluded, since they can cause organ dysfunction. The 19 cases consisted of 7 patients with myelodysplastic syndromes (MDS) (4 with refractory anemia, 1 with refractory cytopenia with multi-lineage dysplasia, 2 with refractory anemia with excess blasts 1), 7 with aplastic anemia (AA) (6 with idiopathic AA, 1 with AA-paroxysmal nocturnal hemoglobinuria syndromes), 1 with pure red cell aplasia, 4 with myelofibrosis (2 with primary myelofibrosis and 2 with secondary myelofibrosis). Their ages ranged from 35 to 88 (median 67 years). None of the patients in this study had previously received consecutive oral iron chelation therapy, while 10 of them had received intermittent intravenous deferoxamine (9 of them) or transient use of deferasirox (1 of them).

We used a Magnetom Avanto 1.5 T scanner (Siemens AG, Erlangen, Germany) using a gradient echo (GRE) T2* MRI technique. GRE T2* weighted image (WI) was used to obtain a single mid-ventricular short-axis slice of the left cardiac ventricle at seven echo times (5, 7, 10, 13, 15, 17 and 20 ms). The repeat time between each radiofrequency pulse was 170 ms. Phased array coils, electrocardiogram (ECG) gating, and breath holding were also utilized. The region of interest (ROI) was set at the left ventricular wall. Measured signal intensity was fitted to an exponential curve and we obtained the T2* value of the myocardium, and then calculated the reciprocal R2* value. We also investigated serum ferritin levels, brain natriuretic peptide (BNP) and left ventricular ejection fraction (LVEF) measured by ultrasonography, which were all examined within 7 days before or after MRI examination. Total RBC transfusion units (using Japanese units, 1 unit is the value of that obtained from 200 mL of blood) given until the day of MRI examination were also counted. In some patients, liver iron overload was also tried to be measured using the method by Gandon et al. [10]. Pairs of 2 parameters were selected according to the purpose, and the simple correlation coefficient of each pair was calculated using Excel Statistics ver. 6 software (Esumi Co. Ltd., Tokyo, Japan).

Table 1 Characteristics of 19 patients

Underlying disease	
Myelodysplastic syndromes	7
Aplastic anemia	7
Myelofibrosis	4
Pure red cell aplasia	1
Ages (years)	35–88 (median 67)
Gender	
Male	12
Female	7
Previous iron chelation therapy	
None	9
Intermittent deferoxamine infusion	9
Intermittent oral chelation therapy	1

3 Results

The median serum ferritin level in the 19 patients was 6,809 pmol/L, ranging from 1,289 to 39,428 pmol/L, while the median total RBC transfusion volume was that obtained from 95 Japanese units, ranging from 10 to 488 units. The T2* value ranged from 11.55 to 94.34 ms with a median of 37.3 ms. Median LVEF was 57.8% ranging from 26.5 to 78.2%. One patient having previous history of myocardial infarction was excluded in the analysis on LVEF to avoid the influence of her ischemic heart disease. Twelve patients had an LVEF of less than 60% among 18 patients.

We calculated a simple correlation coefficient to clarify the relationship of each parameter (Table 2). It has been reported that total RBC transfusion volume correlates with iron accumulation in organs. In our study, a very strong correlation was noted between R2* and total RBC transfusion volume ($r = 0.90$, Fig. 1a). A strong correlation was also noted between R2* and total RBC transfusion volume ($r = 0.70$) when we analyzed in patients without previous iron chelation therapy (Fig. 1a). A distinct linear correlation was seen in all analyses (Table 2). There was also a correlation between serum ferritin level and total

Table 2 Simple correlation coefficients between pairs of parameters

	LVEF	RBC transfusion	Serum ferritin	BNP	Age (years)
R2* (s^{-1})	−0.71	0.90	0.81	−0.38	−0.50
LVEF (%)	–	−0.51	−0.46	0.49	0.58
RBC transfusion (unit)		–	0.79	0.03	−0.32
Serum ferritin (pmol/L)			–	0.29	−0.33
BNP (pg/mL)				–	0.86

LVEF left ventricular ejection fraction, *RBC transfusion* total red blood cell transfusion volume, *BNP* brain natriuretic peptide

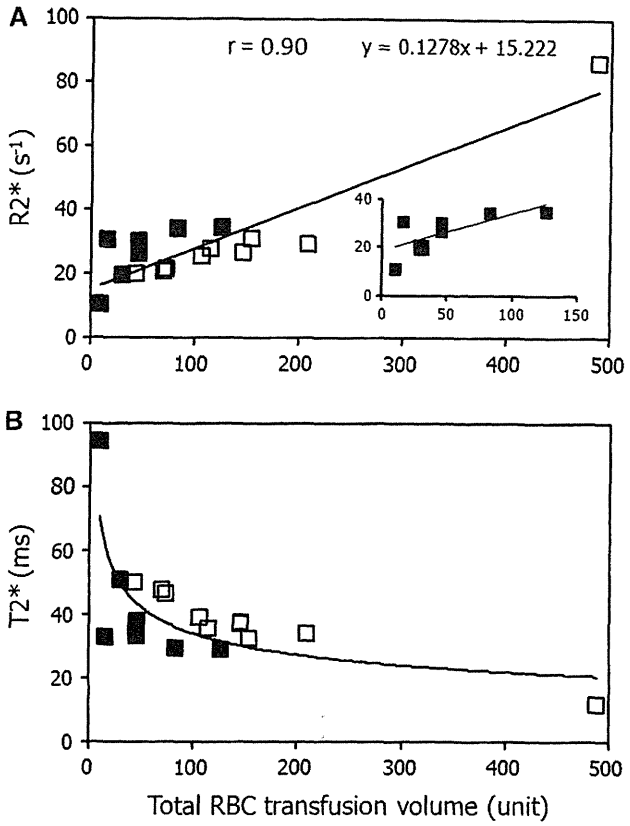

Fig. 1 Correlation between total RBC transfusion volume (Japanese units) and R2* values (**a**) and T2* values (**b**). The approximation formula was R2* (s^{-1}) = 0.1278 × [total RBC volume (Japanese unit)] + 15.222. *r* values are also given in the figures. Patients who received previous intermittent iron chelation therapy (*empty squares*) and those who had no previous iron chelation therapy (*solid squares*) were included. There was no significant difference between them. Correlation was also analyzed in patients without previous iron chelation therapy (small inset in **a**). A strong correlation was similarly noted between R2* and total RBC transfusion volume ($r = 0.70$)

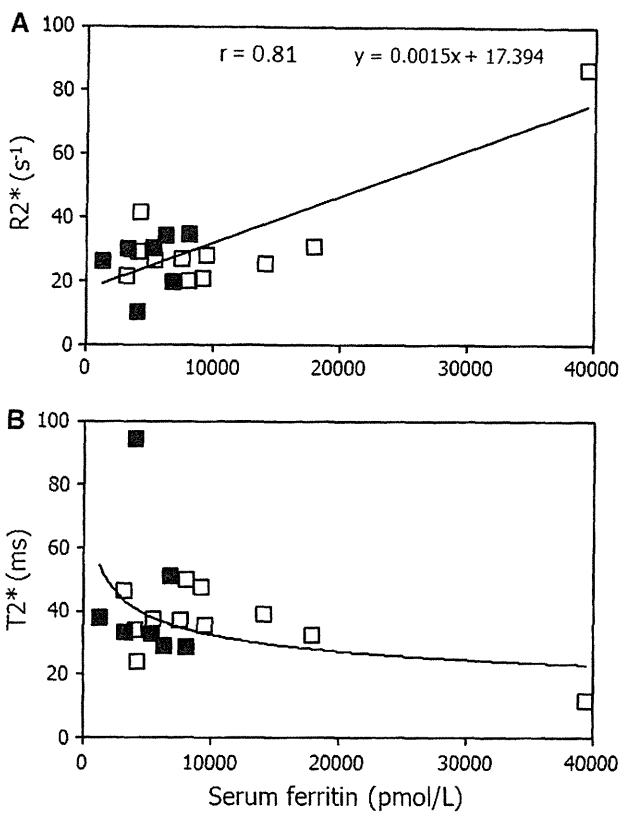

Fig. 2 Correlation between total serum ferritin levels and R2* values (**a**) and T2* values (**b**). The approximation formula was R2* (s^{-1}) = 0.0015 × [serum ferritin (pmol/L)] + 17.394. *r* values are also given in the figures. Patients who received previous intermittent iron chelation therapy (*empty squares*) and those who had no previous iron chelation therapy (*solid squares*) were included. There was no significant difference between them

RBC transfusion volume, which are major parameters frequently used in iron chelation therapy ($r = 0.79$, Table 2), although it was relatively lower than that between R2* and total RBC transfusion volume ($r = 0.90$). Serum ferritin levels and R2* values significantly correlated in all patients ($r = 0.81$, Fig. 2a). We then analyzed predictive parameters of cardiac dysfunction. There was no direct correlation between serum ferritin levels and LVEF ($r = -0.46$). No deterioration of LVEF was found in patients with serum ferritin levels below 20,000 pmol/L. We found no direct correlation between total RBC transfusion volume and LVEF ($r = 0.51$). To evaluate these results, we analyzed the correlation between R2* value and LVEF ($r = -0.71$, Fig. 3a). R2* correlates with total RBC transfusion volume or serum ferritin levels when all patients were analyzed together (Figs. 1a, 2a). In contrast, there was no correlation between them in the subgroup of patients with a T2* over 30 ms, who were examined to evaluate these values of the early stage of iron overload still sustaining normal cardiac function ($r = 0.52$). There was no correlation between R2* and BNP, a known indicator of cardiac failure ($r = -0.38$).

4 Discussion

We set out to verify whether evaluation by MRI could corroborate the current consensus of initiating iron chelation therapy for patients with transfusional iron overload. As already known, humans lack a mechanism to excrete excess iron, which is usually trapped by macrophages in the reticulo-endothelial system and stored as ferritin or hemosiderin, mainly in the liver. Serum iron generally exists as transferrin-bound iron which is not harmful. As the iron-binding capacity of transferrin becomes saturated due to transfusional iron overload, free iron appears in serum as toxic non-transferrin-bound iron (NTBI). When overloaded iron starts to be deposited in several organs including the heart, the cardiac T2* value begins to decrease [11]. At that stage, the cardiac ejection fraction

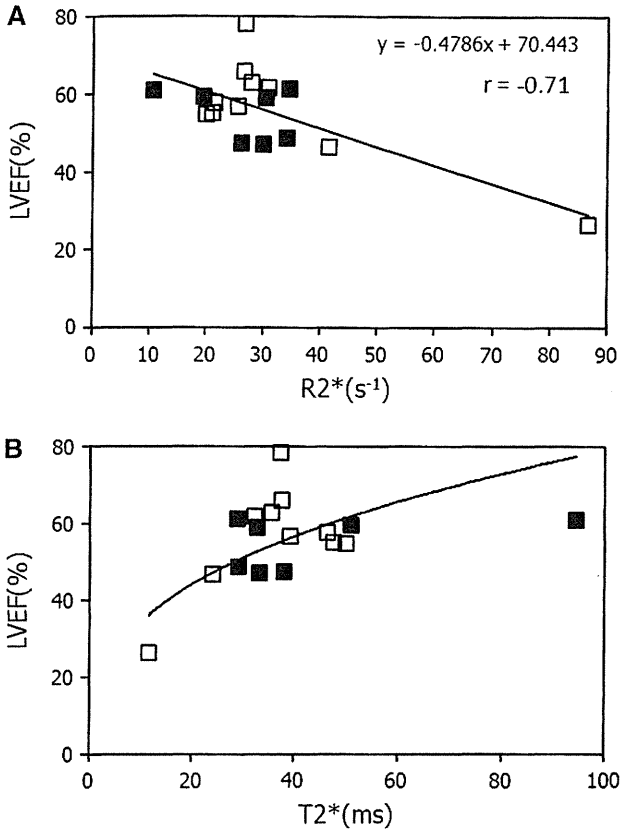

Fig. 3 Relationships between left ventricular ejection fraction (LVEF) and R2* (**a**) and T2* (**b**) were demonstrated. The approximation formula was LVEF (%) = −0.4786 × [R2* (s^{-1})] + 70.443. *r* values are also given in the figures. Patients who received previous intermittent iron chelation therapy (*empty squares*) and those who had no previous iron chelation therapy (*solid squares*) were included. There was no significant difference between them

remains normal [5], while polysaccharides, proteins, nucleic acids and lipids are subject to peroxidative damage, resulting in myocardial impairment, which is manifested clinically as cardiac failure [12].

Continuous iron chelation therapy is necessary to excrete overloaded iron and to improve dysfunction of liver, heart and other organs. In contrast to the liver, cardiac dysfunction is believed irreversible once iron overload reaches a critical level, despite intensive intravenous iron chelation [3]. This discrepancy is partially because iron is eradicated more slowly from the heart than the liver [5]. Effective removal of excess iron in the heart may require early initiation of chelation therapy before cardiac dysfunction appears. Prediction of the effects of iron chelation based on serum ferritin levels alone is still difficult [8]. According to the guidelines for iron chelation in Japan which was based mainly on the analysis of liver iron overload, serum ferritin levels are higher than 2,247 pmol/L (1,000 ng/mL) in 90% of patients with liver dysfunction. Thus, the Japanese guideline sets a serum ferritin level of 1,000 ng/mL (2,247 pmol/L) as the initiation criterion for iron chelation. However, Anderson et al. [5] suggested that there is no correlation between heart and liver iron concentration. This indicates that evaluation of heart function should also be studied from the viewpoint of cardiac iron overload.

Prediction of cardiac function deterioration is a major problem. We found no direct correlation between serum ferritin and LVEF, thus measurement of serum ferritin levels was not proved useful in predicting cardiac function deterioration. There was also no direct correlation between total RBC transfusion volume and LVEF. It was suggested that impairment of cardiac function became marked when the T2* value became below 20 ms (Fig. 3b). This result is compatible with the report by Anderson et al. [5] that decrease in LVEF appeared in the late phase of iron overload. By contrast, there was an obvious decrease in LVEF correlated with elevation of R2* values; therefore, the correlation coefficient between R2* and LVEF also indicated that measuring R2* value might be helpful to predict cardiac siderosis.

Using the approximation formula between the R2* value and LVEF (Fig. 3a), we calculated the possible cutoff of R2* value measuring to determine cardiac dysfunction. If we hypothesize 60% LVEF as the borderline of sufficient cardiac function, an R2* value of 21.8 s^{-1} (T2* value of 45.9 ms) becomes the critical point according to the calculation using the formula (Fig. 3a). We demonstrated that the correlation value of R2* and serum ferritin was less than that of R2* and the total volume of RBC transfusion. We, therefore, assumed that the total RBC transfusion volume may be the current best indicator of cardiac iron overload. When we substitute an R2* value of 21.8 s^{-1} in the formula between R2* and total RBC transfusion volume (Fig. 1a), an RBC transfusion volume of 51.4 units (approximately 50 units) may be the cutoff value for future cardiac dysfunction. If we substitute an R2* value of 21.8 s^{-1} in the formula for R2* and serum ferritin (Fig. 2a), a serum ferritin level of 2,937 pmol/L (approximately 1,300 ng/mL) becomes the cutoff value. The mathematical calculation values of our results agree with the Japanese guidelines recommending that patients receiving over 40 units of RBC transfusion should be given iron chelating therapy [13]. By meditation of R2* value, we can obtain simulated LVEF when total RBC transfusion volume or serum ferritin level is available.

Then, we carefully dissect Figs. 1a and 2a. We also realize the necessity to count disease heterogeneity. The early phase of iron overload may be overlooked when only using serum ferritin or total RBC transfusion volume as parameters. Jensen et al. [14] indicated that heart iron accumulation became clear after a serum ferritin level of 1,800 ng/mL (4,045 pmol/L) or more. The correlation between R2* and RBC transfusion volume disappeared in

the subgroup of patients at the early stage of iron overload. When we focus on the slope of regression curve at the early stage of iron overload (Figs. 1, 2), the T2* seem steeper than the R2*. To corroborate the disadvantage in detecting the early stage of iron accumulation by R2*, the T2* value may be sensitive and appropriate. On the other hand, the slope of R2* is steeper than the T2* at the late stage. Although the number of patients in this study is too small to conclude, we propose here that T2* value may be more useful at the early stage of iron overload while R2* value could be used in the late stage. Using the right parameter at the right stage may earn more precise evaluation of iron overload. To start iron chelation therapy at an appropriate time, based on the detailed and precise evaluation, is essential to contribute to survival of patients with iron overload.

Acknowledgments The authors are indebted to Prof. J. Patrick Barron of the Department of International Medical Communications of Tokyo Medical University for his review of this manuscript.

Conflict of interest None.

References

1. Takatoku M, Uchiyama T, Okamoto S, Kanakura Y, Sawada K, Tomonaga M, et al. Japanese National Research Group on Idiopathic Bone Marrow Failure Syndromes. Retrospective nationwide survey of Japanese patients with transfusion-dependent MDS and aplastic anemia highlights the negative impact of iron overload on morbidity/mortality. Eur J Haematol. 2007;78:487–94.
2. Shashaty G, Frankewich R, Chakraborti T, Choudary J, Al-Fayoumi S, Kacuba A, et al. Deferasirox for the treatment of chronic iron overload in transfusional hemosiderosis. Oncology. 2006;20:1799–806.
3. Felker GM, Thompson RE, Hare JM, Hruban RH, Clemetson DE, Howard DL, et al. Underlying causes and long-term survival in patients with initially unexplained cardiomyopathy. N Engl J Med. 2000;342:1077–84.
4. Buja LM, Roberts WC. Iron in the heart. Etiology and clinical significance. Am J Med. 1971;51:209–21.
5. Anderson LJ, Holden S, Davis B, Prescott E, Charrier CC, Bunce NH, et al. Cardiovascular T2-star (T2*) magnetic resonance for the early diagnosis of myocardial iron overload. Eur Heart J. 2001;22:2171–9.
6. Alexopoulou E, Stripeli F, Baras P, Seimenis I, Kattamis A, Ladis V, et al. R2 relaxometry with MRI for the quantification of tissue iron overload in β-thalassemic patients. J Magn Reson Imaging. 2006;23:163–70.
7. Wood JC, Otto-Duessel M, Aguilar M, Nick H, Nelson MD, Coates TD, et al. Cardiac iron determines cardiac T2*, T2, and T1 in the gerbil model of iron cardiomyopathy. Circulation. 2005;112:535–43.
8. Park J, Ohyashiki K, Akata S, Takara K, Uno R, Kakizaki D, et al. Evaluation of cardiac iron overload in transfusion-dependent adult marrow failure patients by magnetic resonance imaging. Leuk Res. 2009;33:756–8.
9. Kiguchi T, Ito Y, Kimura Y, Ohyashiki K. Restoration of cardiac function by an iron chelator, deferasirox, in a patient with aplastic anemia and cardiac iron overload. Int J Hematol. 2009;89:546–8.
10. Gandon Y, Olivié D, Guyader D, Aubé C, Oberti F, Sebille V, et al. Non-invasive assessment of hepatic iron stores by MRI. Lancet. 2004;363:357–62.
11. Wood JC, Otto-Duessel M, Aquilar M, Nick H, Nelson MD, Coates TD, et al. Cardiac iron determines cardiac T2*, T2, and T1 in the gerbil model of iron cardiomyopathy. Circulation. 2005;112:535–43.
12. Hershko C, Link G, Cabantchik I. Pathophysiology of iron overload. Ann N Y Acad Sci. 1998;850:191–201.
13. Suzuki T, Tomonaga M, Miyazaki Y, Nakao S, Ohyashiki K, Matsumura I, et al. Japanese epidemiological survey with consensus statement on Japanese guidelines for treatment of iron overload in bone marrow failure syndromes. Int J Hematol. 2008;88:30–5.
14. Jensen PD, Jensen FT, Christensen T, Eiskjaer H, Baandrup U, Nielsen JL. Evaluation of myocardial iron by magnetic resonance imaging during iron chelation therapy with deferrioxamine: indication of close relation between myocardial iron content and chelatable iron pool. Blood. 2003;101:4632–9.

Leukemia Research 34 (2010) 1151–1157

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Multicenter phase II trial of vitamin K_2 monotherapy and vitamin K_2 plus 1α-hydroxyvitamin D_3 combination therapy for low-risk myelodysplastic syndromes

Nobu Akiyama[a,g], Keisuke Miyazawa[b], Yoshinobu Kanda[c,g], Kaoru Tohyama[d,h], Mitsuhiro Omine[e,i], Kinuko Mitani[f,g,h], Kazuma Ohyashiki[b,g,h,*]

[a] Department of Internal Medicine, Teikyo University, Tokyo, Japan
[b] First Department of Internal Medicine, Tokyo 160-0023, Japan
[c] Department of Internal Medicine, Ohmiya Medical Center, Jichi Medical University, Saitama, Japan
[d] Department of Clinical Laboratory Medicine, Kawasaki Medical University, Okayama, Japan
[e] Department of Hematology, Showa University, Kanagawa School of Medicine, Japan
[f] Department of Hematology, Dokkyo Medical University School of Medicine, Tochigi, Japan
[g] Japan Hematology-Oncology Clinical Study Group (J-HOCS), Japan
[h] Japanese Research Committee for Research on Epoch-making Therapy for Myelodysplastic Syndrome, Japan
[i] Japanese Research Committee for Measures for Intractable Diseases, Japan

ARTICLE INFO

Article history:
Received 29 August 2009
Received in revised form 13 February 2010
Accepted 9 April 2010
Available online 1 June 2010

Keywords:
Myelodysplastic syndromes
Vitamin K_2
Vitamin D_3

ABSTRACT

We performed an open-labeled single-arm prospective phase II clinical trial of vitamin K_2 (menatetrenone: VK2) monotherapy and VK2 plus 1α-hydroxyvitamin D_3 (alfacalcidol: VD3) combination therapy for myelodysplastic syndromes (MDS) with refractory anemia and refractory cytopenia with multilineage dysplasia, having either low or intermediate-1 risks of the IPSS. The overall response rate to VK2 monotherapy (45 mg/day) after 16 weeks was 13% (5/38) including 4 cases with improvement of both anemia and thrombocytopenia and 1 case with thrombocytopenia. We then enrolled and evaluated 20 out of 33 VK2-monotherapy non-responders for VK2 plus VD3 (0.75 μg/day) combination therapy. The overall response rate at 16 weeks after initiation of VK2 plus VD3 was 30% (6/20). HI for hemoglobin (Hb) was observed in 6 out of 11 patients (55%) and for thrombocytopenia in 3 out of 11 patients (27%), respectively. No HI was observed for neutropenia in VK2 monotherapy and VK2 plus VD3 combination therapy. It was suggested that IPSS scores and absolute neutrophil counts positively correlated, and Hb levels inversely correlated with the response to VK2 plus VD3 combination therapy. Our study demonstrated that VK2 plus VD3 combination therapy appears to be promising for improvement of anemia and thrombocytopenia with low/intermediate-1 MDS.

1. Introduction

Myelodysplastic syndromes (MDS) are clonal stem cell disorders characterized by dysplastic features of hematopoietic and blood cells, cytopenias caused by ineffective hematopoiesis, and a variable risk of progression to acute myeloid leukemia (AML) due to accumulation of genetic abnormalities [1]. The treatment options available to patients with MDS are largely based upon the patient's age and prognosis as determined by the International Prognostic Scoring System (IPSS) [2]. For patients in the low to intermediate-1 by IPSS score categorized as "low-risk MDS", the goal of treatment is to improve ineffective hematopoiesis while providing the appropriate supportive care. The US National Comprehensive Cancer Network MDS recommendations are that therapies for the patients with low-risk with clinically significant cytopenias should be stratified into several groups, for example lenalidomide for del(5q), erythropoietin (Epo) for patients with low serum Epo [3]. DNA-hypomethylating agents as azacitidine or decitabine are also recommended for non-responders to these treatments. However, these therapeutic effects are not satisfactory for every patient and other options for low-risk MDS, especially elderly MDS patients, are still required [4].

Vitamin Ks are known to act as cofactors for γ-carboxylation of vitamin K-dependent coagulation factors. Menatetrenone, a vitamin K_2 analog, is approved as an active agent for osteoporosis in Japan [5,6]. As a coenzyme of γ-carboxylase, it promotes osteogen-

* Corresponding author at: First Department of Internal Medicine, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan.
Tel.: +81 3 3342 6111x5895; fax: +81 3 5381 6651.
E-mail address: ohyashik@rr.iij4u.or.jp (K. Ohyashiki).

0145-2126/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.leukres.2010.04.006

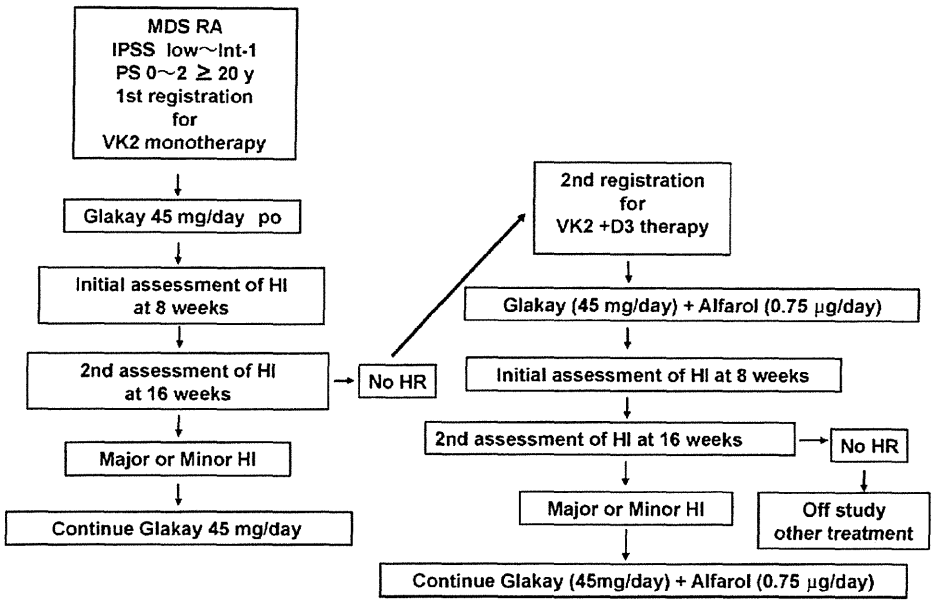

Fig. 1. Treatment strategy for VK2 monotherapy or VK2 plus VD3 combination therapy.

esis through γ-carboxylation of glutamate residues in osteocalcin. The safety of long term administration of menatetrenone has been well established [5,6]. The efficacy of oral menatetrenone therapy in RA and other types of MDS has been reported in Japan [7–10]. Regarding with the effect for improvement of cytopenias in clinical trials including pilot studies, the response varies 20–75%, and toxicity is tolerable [8–10]. The underlying mechanism of improvement of cytopenias by vitamin K_2 (VK2) remains to be cleared. However, VK2 has been reported to induce apoptosis and differentiation in some leukemic cell lines *in vitro* [11–13]. VK2 was also reported to improve hematopoietic supportive functions of the stromal cell lines established from MDS patients [14].

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 inhibits the proliferation and induces the differentiation of normal and leukemic myeloid cells *in vitro* [15]. There are reports demonstrating that vitamin D_3 analogs such as alfacalcidol (1α-hydroxyvitamin D_3), calcifediol (25-hydroxyvitamin D) and calcitriol (1α,25-dihydroxyvitamin D_3) have some therapeutic effects in patients with MDS [15–19]. Mellibovsky et al. reported that treatment with calcifediol showed some hematological improvements in 10 out of 14 MDS patients with low/intermediate risk [18]. Motomura et al. reported that alfacalcidol prevents the progression of MDS to overt leukemia under the effect of differentiation capacity from blasts to monocytes [19]. However, these therapeutic effects of VD3 analogs in MDS were controversial [20,21]. The therapeutic serum concentrations of these VD3 analogs based on *in vitro* studies were supposed to be practically difficult to achieve because of hypercalcaemia *in vivo*, a well known dose-limiting toxicity of vitamin D_3 (VD3) [21]. It is noteworthy that combination of VK2 plus either 22-oxa-1,25-dihydroxyvitamin D_3

Table 1
Characteristics of evaluable patients for VK2 monotherapy and VK2 plus VD3 combination therapy.

	VK2 monotherapy ($n=38$)			VK2 + VD3 combination therapy ($n=20$)		
Age	Median: 65 years, range: 23–84			Median: 65 years, range: 27–81		
Sex	Male	Female		Male	Female	
	20	18		10	10	
WHO classification	RA	RCMD		RA	RCMD	
	27	11		16	4	
IPSS	0	0.5	1	0	0.5	1
	13	19	5	7	8	5
Hb	Average: 8.99 ± 3.03 g/dl			Average: 9.47 ± 3.16 g/dl		
	<10 g/dl	10 g/dl≦		<10 g/dl	10 g/dl≦	
	24	14		11	9	
PLT	Average: 118,900 ± 121,000/μl			Average: 133,500 ± 149,400/μl		
	<100,000/μl	100,000/μl≦		<100,000/μl	100,000/μl≦	
	24	14		11	9	
ANC	Average: 1,426 ± 1,013.5/μl			Average: 1082.5 ± 746.8/μl		
	<1500/μl	1500/μl≦		<1500/μl	1500/μl≦	
	25	13		14	6	
Transfusion dependency	RBC	PLT		RBC	PLT	
	10	6		2	3	
Cytogenetics	Normal	Abnormal	Unknown	Normal	Abnormal	Unknown
	26	11	1	13	7	0

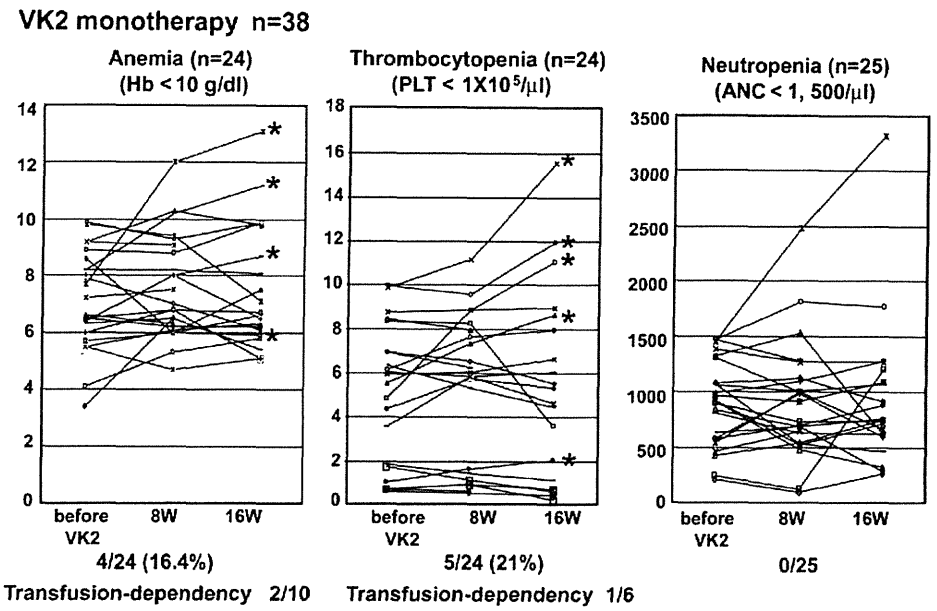

Fig. 2. Hematological response to VK2 monotherapy.*Indicates the responder in VK2 monotherapy.

or 1,25-dihydroxyvitamin D_3 synergistically enhances the induction of cellular differentiation in HL-60 cells along with inhibition of VK2-induced apoptosis *in vitro* [22,23]. The optimal concentration of VD3 analogs for differentiation induction could be reduced less than one tenth by combining with VK2. This may provide a therapeutic benefit for combining VK2 plus VD3 by preventing hypercalcemia as well as enhancement of cellular differentiation induction.

Although the precise therapeutic mechanism still remains to be clarified, all these data suggest that VK2 and VD3 may improve cytopenias in MDS by either differentiation induction, eradication of MDS clone by apoptosis, or improvement of hematopoietic microenvironment. Based on the previous clinical reports and *in vitro* studies, we conducted an open-labeled single-arm prospective phase II clinical trial to clarify the effectiveness of VK2 monotherapy for improving cytopenias in low-risk MDS, as a primary endpoint. Furthermore, the study was designed to investigate the therapeutic effects of VK2 plus VD3 (alfacalcidol) combination therapies for non-responders to VK2 monotherapy as a secondary endpoint.

2. Materials and methods

2.1. Patients

The protocol was approved by all local medical ethical committees, and the patients were required to provide written informed consent. From January 2003 to August 2005, 46 patients were enrolled in this clinical trial from 10 institutes involved in 2 Japanese clinical study groups; the Japan Hematology-Oncology Clinical Study Group (J-HOCS) and Japanese Research Committee for Research on Epoch-making Therapy for Myelodysplastic Syndrome. After obtaining informed consent, consecutive MDS patients who met all of the following eligibility criteria were registered in the study.

The eligibility criteria: (1) WHO classification (2001), RA or RA with multilineage dysplasia (RCMD) [24]; (2) IPSS, low or intermediate-1 risk [2]; (3) performance status (Zubrod criteria) of 0–2 [22]; (4) age 20 or more; (5) adequate functions in the liver, kidney, lung and heart; (6) no previous treatment for MDS within 4 weeks before administration of VK2, except for transfusions. The patients could not be taking warfarin potassium. Other criteria for exclusion were malabsorption of fat-soluble vitamin, other active malignancy, serious infection, uncontrolled diabetes mellitus, liver cirrhosis, serious psychiatric disorder, pregnancy, lactation, and seropositivity for the human immunodeficiency virus. The diagnosis of MDS RA or RCMD was made by hematological experts in each institute, but no central slide review was performed.

2.2. Treatment design

The regimen consisted of two steps. In the first step, a Glakay™ capsule containing 15 mg of menatetrenone was orally administered 3 times a day (45 mg/day) for 16 weeks. According to the International Working Group (IWG-2000) response criteria, hematologic response has to last for no less than 2 months. Therefore, the response was assessed at 8 and 16 weeks after the initiation of the therapy [25]. When hematological improvement was observed at 16 weeks, 45 mg/day of menatetrenone was continued for a year at least (Fig. 1). For non-responders to VK2 monotherapy, an Alfarol™ capsule containing 0.75 μg of alfacalcidol was orally administered in addition to 45 mg of menatetrenone for other 16 weeks as the

Table 2
Characteristics of responders in VK2 monotherapy and VK2 plus VD3 combination therapy.

Therapy	MDS type	Sex	Age	IPSS	Hb (g/dl)	PLT (/μl)	ANC (10^3/μl)	Transfusion		Karyotype	Hematological improvement		
								RBC	PLT		RBC	PLT	Neut
VK2	RCMD	M	71	0.5	4.1	48,000	1.45	+	–	del(20)(q11)	Minor	Maior	Absent
	RA	M	71	0.5	8.2	55,000	0.96	–	–	del(20)(q11), del(16)(q23)	Major	Minor	Absent
	RA	M	78	0.5	8.9	35,000	1.64	–	–	Normal	Minor	Minor	Absent
	RA	M	69	0.5	7.7	98,000	2.13	–	–	Normal	Major	Minor	Absent
	RA	F	52	0.5	6.4	10,000	1.79	–	+	Normal	Absent	Major	Absent
VK2+VD3	RCMD	F	46	1	7	46,000	1.73	–	–	8	Major	Absent	Absent
	RA	M	61	1	6.2	179,000	0.32	–	–	+l,der(l;7)(ql0;pl0)	Minor	Absent	Absent
	RA	M	79	1	6.6	176,000	2.22	–	–	add(17)(ql2)	Minor	Absent	Absent
	RA	M	81	1	6.7	5,000	1.09	+	+	Normal	Major	Major	Absent
	RA	M	74	0.5	5.5	34,000	2.90	+	–	Normal	Absent	Minor	Absent
	RA	F	55	0.5	9.8	66.000	2.15	–	–	Normal	Major	Minor	Absent

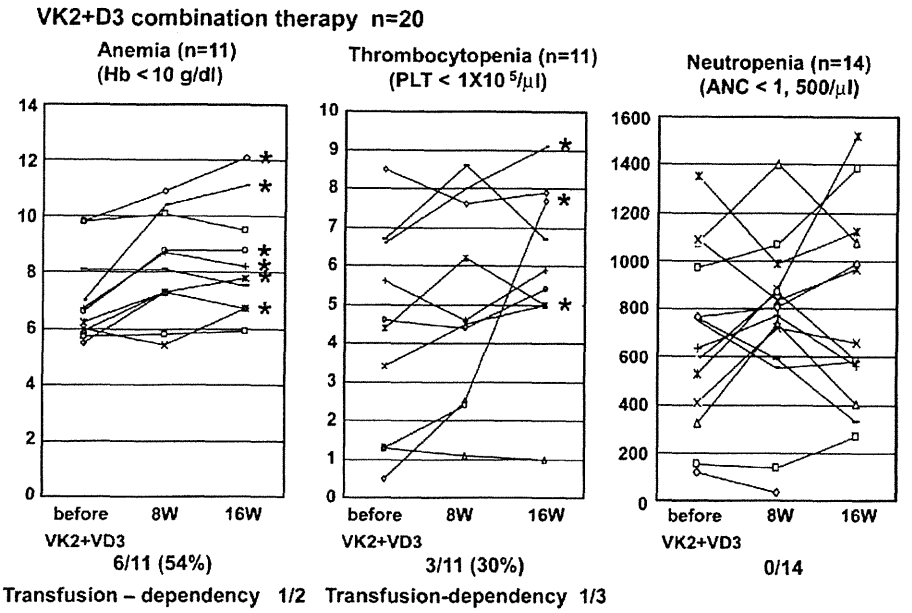

Fig. 3. Hematological response to VK2 plus VD3 combination therapy. *Indicates the responder in VK2 plus VD3 combination therapy.

second step after re-registration (Fig. 1). Response was re-assessed at 8 and 16 weeks after the initiation of the second steps of the regimes. When hematological improvement was achieved, both 45 mg/day of menatetrenone and 0.75 μg/day of alfacalcidol were further continued for a year at least. Since VK2 and VD3 are fat-soluble vitamins and they require biliary for efficient intestinal absorption, caution was paid for taking these vitamins either after meals or with a cup of milk. Glakay and Alfarol were kindly provided by Eisai Co., Ltd. (Tokyo, Japan) and Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively.

Concomitant use of medicines including erythropoietin, G-CSF, M-CSF, anabolic steroids, corticosteroids, cyclosporine A, antilymphocyte/antithymocyte globulin, antineoplastics, ascorbic acid, lithium carbonate, cepharanthine, amifostine, and thalidomide was prohibited within trial period. Transfusions of platelet and red blood cell were unrestrained. The amount of transfusions was taken into as count when response was assessed.

2.3. Response criteria

Eight and 16 weeks after the initiation of each treatment step, hematological improvement (HI) was assessed according to the standardize response criteria for MDS by IWG-2000 [25], and was determined when the improvement continued for 8 weeks or more. Previous reports demonstrated that the any hematological responses to VK2 cannot be observed within 8 weeks. Therefore, only patients who have received VK2 for more than 8 weeks should be included for evaluation [7–10]. The patients who have stopped the treatment within 8 weeks should be excluded for response evaluation but should be included for the assessment of any adverse events in this study. Achievement of either major or minor hematological improvement (HI) in one or more hematopoietic lineages was defined as "effective" in this study. However, if hemoglobin (Hb) concentration fell over 2 g/dl from the maximum concentration within a month after the treatment, or the counts of neutrophils and platelets dropped by more than 50% of the maximum counts within a month after the treatment, these conditions were denoted as "ineffective" despite achievement of any HI.

2.4. Endpoints and statistical analyses

The primary and secondary endpoints were the rate of HI at 16 weeks after the initiation of either VK2 alone or VK2 plus VD3, respectively. Differences in response rate were analyzed by Fisher's exact test. Comparison of characteristics between responders and non-responders was analyzed by Student's *t*-test, Fisher's exact test, and the *U*-test, according to variants.

3. Results

3.1. Patient characteristics

Thirty-eight out of 46 enrolled patients were evaluable. The enrolled cases who received menatetrenone for more than 8 weeks were all included for evaluation. Characteristics of evaluable patients are summarized in Table 1. The median age was 65 years. These were 27 and 11 patients with RA and RCMD, respectively. According to IPSS, 13 patients were at low-risk, 24 had intermediate-1, and 1 case was not assessable because of lack of cytogenetic data. The numbers of patients with a Hb level less than 10 g/dl, platelet count less than 100,000/μl, and absolute neutrophil count (ANC) less than 1.5×10^3/μl were 24, 24, and 25 cases, respectively. Ten cases were red blood cells (RBC) transfusions-dependent and 6 were dependent on platelets transfusions, respectively.

3.2. VK2 monotherapy

Overall response rate of the VK2 monotherapy was 13% (5/38) in all of the evaluable cases. Anemia improved in 4 out of 24 (17%) patients with a Hb level lower than 10 g/dl. Thrombocytopenia improved in 5 out of 24 (21%) patients with a platelet count less than 100,000/μl (Fig. 2). In 4 cases, anemia and thrombocytopenia ameliorated concurrently (Table 2). The efficacy tended to be better in patients with moderate to severe cytopenias. However, the differences between mild and moderate to severe cytopenias were not statistically significant by Fisher's exact test ($P = 0.38$ for anemia, $P = 0.08$ for thrombocytopenia). VK2 had no effects on neutropenia in any cases. In transfusion dependency, which was defined as requirement of 2 units or more red-cell concentrates or 10 units or more platelets concentrates for a month, it was relieved in 1 out of 6 patients with platelet transfusion dependency. None of 10 patients with RBC transfusion dependency became free from transfusions. Two of 5 responders had abnormal karyotypes in the bone marrow cells: one had del(20)(q11), and the other had del(20)(q11) and del(16)(q23) (Table 2).

3.3. VK2 and VD3 combination therapy

Twenty-three out of 33 patients receiving VK2 monotherapy did not show any hematologic improvement in 16 weeks. Twenty-three non-responders in VK2 monotherapy were re-registered after obtaining informed consent and transferred VK2 plus VD3 combination therapy, and 20 cases were evaluable (Table 1). The median age was 65 years (range: 27–81). The numbers of cases with RA

Table 3
Comparison of clinical characteristics between responders and non-responders, (): %.

Variables	VK2 monotherapy ($n=38$)				VK2+VD3 combination therapy ($n=20$)			
	Responders ($n=5$)	Non-responders ($n=33$)	*P*-value	Test	Responders ($n=6$)	Non-responders ($n=14$)	*P*-value	Test
Age (years) mean ± SD	68.2 ± 9.7	61.3 ± 16.6	0.372	(1)	66.0 ± 14.2	59.3 ± 15.5	0.375	(1)
Sex								
Male	4 (80.0)	16 (48.5)	0.344	(1)	4 (66.7)	6 (42.9)	0.628	(1)
Female	1 (20.0)	17 (51.5)			2 (33.3)	8 (57.1)		
MDS type								
RA	4 (80.0)	22 (68.6)	1	(1)	5 (83.3)	11 (78.6)	1	(1)
RCMD	1 (20.0)	10 (31.3)			1 (16.7)	3 (21.4)		
Karyotype								
Normal	2 (50.0)	23 (71.9)	0.57	(1)	3 (50.0)	10 (71.4)	0.613	(1)
Abnormal	2 (50.0)	9 (28.1)			3 (50.0)	4 (28.6)		
IPSS score								
0	1 (20.0)	12 (37.5)	0.864	(3)	0 (0.00)	7 (50.0)	0.028	(3)
0.5	4 (80.0)	15 (46.9)			3 (50.0)	5 (35.7)		
1	0 (0.00)	5 (15.6)			3 (50.0)	2 (14.3)		
Time from diagnosis to therapy (month)								
<6	5 (100)	29 (87.9)	0.441	(3)	6 (100)	12 (85.7)	0.384	(3)
6–12	0 (0.00)	1 (3.0)			0 (0.00)	1 (7.1)		
12<	0 (0.00)	3 (9.1)			0 (0.00)	1 (7.1)		
Prior therapy								
Absent	5 (100)	31 (93.9)	1	(1)	6 (100)	13 (92.9)	1	(1)
Present	0 (0.00)	2 (6.1)			0 (0.00)	1 (7.1)		
Performance status								
0	3 (60.0)	24 (72.7)	0.623	(3)	4 (66.7)	11 (78.6)	0.621	(3)
1	2 (40.0)	8 (24.2)			2 (33.3)	3 (21.4)		
2	0 (0.00)	1 (3.0)			0 (0.00)	0 (0.00)		
Complete blood counts								
Hb (g/dl)	7.06 ± 1.89	9.29 ± 3.08	0.126	(2)	7.07 ± 1.47	10.48 ± 3.15	0.021	(2)
PLT (/μl)	49,200 ± 32,200	129,500 ± 126,200	0.17	(2)	84,800 ± 74,600	154,600 ± 170,000	0.348	(2)
ANC (/μl)	1602.4 ± 421	1399.2 ± 1077	0.682	(2)	1735.2	802.7 ± 460.7	0.007	(2)
Transfusion dependency								
RBC								
Dependent	1 (20.0)	9 (47.4)	0.283	(1)	2 (33.3)	3 (21.4)	0.483	(1)
Independent	4 (80.0)	10 (52.6)			4 (66.7)	11 (78.6)		
PLT								
Dependent	1 (20.0)	5 (26.3)	0.633	(1)	1 (20.0)	2 (13.3)	0.601	(1)
Independent	4 (80.0)	14 (73.7)			4 (80.0)	13 (86.7)		
Bone marrow cellularity								
Hypocellular	0 (0.00)	12 (38.7)	0.552	(3)	2 (33.3)	7 (50.0)	0.59	(3)
Normocellular	5 (100)	12 (38.7)			3 (50.0)	5 (35.7)		
Hypercellular	0 (0.00)	7 (22.6)			1 (16.7)	2 (14.3)		
Blast in bone marrow (%) mean ± SD	1.26 ± 1.56	1.72 ± 1.31	0.479	(2)	0.97 ± 0.61	2.04 ± 1.87	0.193	(2)

The number of the column of "Test" means: (1) Fisher's exact test, (2) *t*-test, and (3) *U*-test.

Table 4
Summary of Japanese clinical trials of VK2 therapy for MDS RA/RCMD.

Reporters	No. of cases	Hematological Improvement (%)	Type of study	No of cases with HI	Ref
Miyazawa et al.	15	20 (3/15)	Retrospective	HI-E major: 2 HI-N minor: 1	[8]
Tsukamoto et al.	4	75 (3/4)	Prospective		[38]
Abe et al.	13	46 (6/13)	Prospective	HI-E major: 3, minor: 3	[9]
Takami et al.	9	56 (5/9)	Prospective	HI-E major: 2, minor: 1 HI-N major: 3 HI-P major: 2	[10]
Imamura et al.	35	29 (10/35)	Retrospective		[39]
Yoshinaga et al.	13	23 (3/13)	Retrospective		[40]
Tsurumi et al.	8	75 (6/8)	Prospective		[41]

and RCMD were 16 and 4, respectively. Overall response rate was 30% (6/20) in all of the evaluable cases. Forty-five % (5/11) of patients with Hb lower than 10 g/dl showed improvement of anemia. Thrombocytopenia was improved in 3 out of 10 patients (30%) (Fig. 3). In two cases, anemia and thrombocytopenia were concurrently ameliorated (Table 2). As well as monotherapy, the combination therapy did not show any effect for neutropenia in all of the cases. Five and 3 patients were dependent on RBC and platelet transfusion, respectively. One patient with both RBC and platelet transfusion dependency became free from transfusion. Three out of 6 responders had abnormal karyotypes in the bone marrow cells. One had +8, one had der(1;7)(q10;p10), and another had add(17)(p12).

3.4. Clinical characteristics of the responders

Differences in clinical characteristics between the responders and the non-responders were analyzed (Table 3). In the VK2 monotherapy, anemia and thrombocytopenia tended to be severer in the responders, but without statistic significance. There were no clinical characteristics for predicting good or poor responses to VK2. In the VK2 plus VD3 combination therapy, the IPSS score was higher, and anemia was severer in the responders with statistical significance. The ANC were higher in the responders. However, as enrolled case number for combination therapy was small, these data only appear to suggest that the hematological improvement by combination therapy can be observed even in the sever cases. The response rates did not differ between patients with or without transfusion dependency. There was no difference in efficacy between the cases with and without chromosomal abnormalities.

3.5. Adverse events and withdrawal cases

Adverse events were assessed in all 46 cases enrolled in this study [26]. Adverse events for VK2 and VD3 were minimal: 1 case with grade I nausea, 1 with grade I lower abdominal pain, and 1 with grade I skin rash in VK2 monotherapy; 1 with grade I skin rash in VK2 plus VD3 combination therapy. Respectively, 8 and 3 patients withdrew from the VK2 monotherapy and the VK2 plus VD3 combination therapy, respectively. The causes of withdrawal in VK2 monotherapy were as follows: 2 cases showed progression to RAEB or AML within 6 weeks after the initiation of VK2; 1 for gastric discomfort; 1 for lower abdominal pain; 1 case for receiving corticosteroid for fever of unknown origin; 3 cases stopped visiting hospital on their own decision. In VK2 plus VD3 combination therapy, the causes of withdrawal were; 1 case was due to progression of pancytopenia within 4 weeks after the initiation of VK2 and VD3; 1 halted taking medicine on his own decision within 8 weeks; 1 discontinued of hospital visit due to removal to another location.

4. Discussion

The response rates in hematological improvement of VK2 monotherapy were largely different in previous reports (Table 4). Three retrospective studies demonstrated that the response rates ranged from 20 to 29%, whereas 4 prospective studies revealed that it ranged from 46 to 75%. Several factors appears to contribute to this variation such as the small number of patients enrolled, concomitant use of other medications including VD3 and anabolic steroid, difference of background of recruited patients, and difference of response criteria. In these prospective studies, the response rates might be overestimated because of these biases. In this study, the response rate of 13% (5/38) for VK2 monotherapy appears to indicate minimal therapeutic benefit for low-risk MDS. In addition, we were unable to identify the predictive factors for VK2 responders (Table 3). However, we cannot definitively exclude the possibility of underestimation of the response in VK2 therapy, because assessment at 16 weeks may not reflect the full response to VK2. We had set the assessment of HI at 16 weeks because most previous reports showed hematological response within 16 weeks after initiation of VK2 [8–10].

Addition of alfacalcidol (VD3) to VK2 appears to improve anemia and thrombocytopenia in about one third of patients who did not respond to VK2 monotherapy by 16 weeks. It is still unclear whether this effect was completely due to the combination of VK2 and VD3 or not. In addition, one may question the mechanism of improvement of cytopenias by combining both vitamins. There appear be several possibilities to explain this effect, such as selective elimination of MDS clone via apoptosis, differentiation induction, and improvement of the hematopoietic microenvironment [13,14,27]. It was demonstrated that combination of VK2 and VD3 synergistically enhanced differentiation in addition to suppression of VK2-inducing apoptosis in HL-60 cells *in vitro* [22,23]. This may suggest some transcriptional interaction between VK2 and VD3, and also suggests that enhanced cellular differentiation induction may occur *in vivo* in the MDS clone as well as in leukemic cells. Regarding with cell-differentiation therapy in MDS using VD analogs, Mellibovsky et al. reported that VD3 monotherapy using calcifediol (25-hydroxyvitamin D) was effective in 11 out of 19 cases whereas Takahashi et al. reported that alfacalcidol was effective in 5 of 12 cases with MDS, respectively [18,28]. In contrast, Yoshida et al. reported that a randomized study of alfacalcidol (6 μg/day) versus supportive therapy in 23 evaluable patients with refractory MDS, alfacalcidol resulted in no therapeutic benefit [21]. To date, most previous reports of cell-differentiation therapy with either vitamin D_3 analogs or retinoids yielded only disappointing results [29]. However, VK2 has recently been reported to induce autophagy in various types of cell lines [30–32]. VD3 has also been reported to induce autophagy in leukemia cells [33]. Recent reports demonstrated that autophagy plays an important role in the turnover of organelles including, mitochondria and ribosomes, as well as cytosolic protein degradation [34]. Using conditional knockout mice, it has been demonstrated that deficiency of autophagy resulted in accumulation of damaged mitochondria and mis-folded proteins that may subsequently cause production of reactive oxygen species (ROS) by mitochondria and ER stress in ribosomes, respectively. Thus autophagy is involved in clearance of impaired mitochondria [35]. In bone marrow erythroblasts from RA and RARS patients, an increased formation of autophagosomes including iron-deposited mitochondria has been reported [36]: unfortunately, we could not enroll any RARS patients in this study. Autophagy mat function as cytoprotection via clearance of the damaged mitochondria from which apoptotic signals initiate [37]. Therefore, further induction of autophagy in response to VK2 and VD3 may play a role in protection from apoptosis in MDS clone resulting in improvement of cytopenias along with cellular differentiation induction. This concept may be supported by previous observations in MDS patients, showing that dysplastic features and cytogenetic abnormalities still can be detectable after improvement of cytopenias [7,8].

In VK2 plus VD3 combination therapy, IPSS score, Hb level and ANC were significantly different between responders and non-responders. The response rates were 0%, 28.5% and 66.7% for IPSS score 0, 0.5 and 1, respectively (Table 3). Since the percentage of bone marrow blasts does not contribute to the score in the low and int-1 risk groups, the scores are mainly related to cytopenia and cytogenetic abnormalities. In the responders, lower Hb level and higher ANC were statistically significance. Anemia was improved even in cases with high-risk abnormalities involving chromosome 7 or >2 chromosome abnormalities (Table 2). This suggests cyto-

genetic abnormalities may not affect the response in VK2 plusVD3 combination therapy.

This study demonstrated that VK2 mono and VK2 plus VD3 therapies showed minimal adverse effects with no toxicity of grade 2 or more. In addition, 0.75 μg-a-day of alfacalcidol did not provoke hypercalcaemia in our series. Based on the response rate and safety in our study, VK2 plus VD3 combination therapy appears to be promising for improvement of anemia and thrombocytopenia in elderly patients with low-risk MDS. Further studies are required to clarify the duration of efficacy in response to combination therapy.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgements

We thank Esai Co., Ltd. (Tokyo, Japan) for kindly providing Glakay (menatetrenone) and also Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan) for kindly providing for Alfarol (alfacalcidol) for this clinical study. This work was supported in part by a grant-in-aid from the Ministry of Health, Welfare and Labor of Japan (Japanese Research Committee for Research on Epoch-making Therapy for Myelodysplastic Syndrome; Chief researcher, Prof. Kinuko Mitani, Dokkyo Medical University School of Medicine).

References

[1] Steensma DP, Bennett JM. The myelodysplastic syndromes: diagnosis and treatment. Mayo Clin Proc 2006;81:104–30.
[2] Greenberg PL, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997;89:2079–88.
[3] National comprehensive cancer network NCCN clinical practice guidelines in oncology. Myelodysplastic syndromes V.2.2010; 2010. Available at (http://www.nccn.org/professionals/physician_gls/PDF/mds.pdf).
[4] Kasner MT, Luger SM. Update on the therapy for myelodysplastic syndrome. Am J Hematol 2009;84:177–86.
[5] Orimo H, Shiraki M, Tomita A, Morii H, Fujita T, Ohata M, et al. Effect of menatetrenone on the bone and calcium metabolism in osteoporosis. a double-blind placebo-controlled study. J Bone Miner Metab 1998;16:106–12.
[6] Sasaki N, Kusano H, Takahashi H, Ando Y, Yano K, Tsuda E, et al. Vitamin K2 (menatetrenone) effectively prevents fractures and sustains number bone mineral density in osteoprotegerin (OPG). J Bone Miner Metab 2005;23:41–7.
[7] Takami A, Nakao S, Ontachi Y, Yamauchi H, Matsuda T. Successful therapy of myelodysplastic syndrome with menatetrenone, a vitamin K2 analog. Int J Hematol 1999;69:24–6.
[8] Miyazawa K, Nishimaki J, Ohyashiki K, Enomoto S, Kuriya S, Fukuda R, et al. Vitamin K2 therapy for myelodysplastic syndromes (MDS) and post-MDS acute myeloid leukemia: information through a questionnaire survey of multi-center pilot studies in Japan. Leukemia 2000;14:1156–7.
[9] Abe Y, Muta K, Hirase N, Choi I, Matsushima T, Hara K, et al. Vitamin K2 therapy for myelodysplastic syndrome. Rinsho Ketsueki 2002;43:117–21 [Japanese].
[10] Takami A, Asakura H, Nakao S, Menatetrenone. A vitamin K2 analog, ameliorates cytopenia in patients with refractory anemia of myelodysplastic syndrome. Ann Hematol 2002;81:16–9.
[11] Yaguchi M, Miyazawa K, Katagiri T, Nishimaki J, Kizaki M, Tohyama K, et al. Vitamin K2 and its derivatives induce apoptosis in leukemia cells and enhance the effect of all-trans retinoic acid. Leukemia 1997;11:779–87.
[12] Nishimaki J, Miyazawa K, Yaguchi M, Katagiri T, Kawanishi Y, Toyama K, et al. Vitamin K2 induces apoptosis of a novel cell lines established from a patient with myelodysplastic syndrome in blastic transformation. Leukemia 1999;13:1399–405.
[13] Miyazawa K, Yaguchi M, Funato K, Gotoh A, Kawanishi Y, Nishizawa Y, et al. Apoptosis/differentiation-inducing effects of vitamin K2 on HL-60 cells: dichotomous nature of vitamin K2 in leukemia cells. Leukemia 2001;15:1111–7.
[14] Miyazawa K, Aizawa S. Vitamin K2 improves the hematopoietic supportive functions of bone marrow stromal cells in vitro: a possible mechanism of improvement of cytopenia for refractory anemia in response to vitamin K2 therapy. Stem Cells Dev 2004;13:449–51.
[15] Abe E, Miyamura C, Sakamaki H, Takeda M, Konno K, Yanazaki T, et al. Differentaiton of mouse myeloid leukemia cells induced by 1α,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA 1981;78:4990–5.
[16] Koefler HP, Hirji K, Itru L. 1,25-dihydroxyvitamin D3, in vivo and in vitro effects on human preluekemic and leukemic cells. Cancer Treat Rep 1985;69:1389–407.
[17] Mehta AB, Kumaran TO, Marsh GW. Treatment of advanced myelodysplastic syndrome with alfacalcidol. Lancet 1984;ii:761.
[18] Mellibovsky L, Díez A, Pérez-Vila E, Serrano S, Nacher M, Aubía J, et al. Vitamin D treatment in myelodysplastic syndromes. Br J Haematol 1998;100:516–20.
[19] Motomura S, Kanamori H, Maruta A, Kodama F, Ohkubo T. The effect of 1-hydroxyvitamin D3 for prolongation of leukemic transformation-free survival in myelodysplastic syndromes. Am J Hematol 1991;38:67–8.
[20] Rchird C, Mazo E, Cuadrado MA, Iriondo A, Bello C, Gandarrillas MA, et al. Treatment with myelodysplastic syndrome with 1,25-hydoxy-vitamin D3. Am J Hematol 1986;23:175–8.
[21] Yoshida Y, Oguma S, Uchino H, Maekawa T, Nomura T. A randomized study of alfacaldicol in refractory myelodysplastic anaemas. A Japanese cooperative study. Int J Clin Pharm Res 1993;13:21–7.
[22] Funato K, Miyazawa K, Yaguchi M, Gotoh A, Ohyashiki K. Combination of 22-oxa-1,25-dihyroxyvitamin D3, a vitamin D3 derivative, with vitamin K2 (VK2) synergistically enhances cell differentiation but suppresses VK2-inducing apoptosis in HL-60 cells. Leukemia 2002;16:1519–27.
[23] Iguchi T, Miyazawa K, Asada M, Gotoh A, Mizutani S, Ohyashiki K. Combined treatment of leukemia cells with vitamin K_2 and 1α,25-dihydroxy vitamin D_3 enhances monocytic differentiation along with becoming resistant to apoptosis by induction of cytoplasmic $p21^{CIP1}$. Int J Oncol 2005;27:893–900.
[24] Brunning RD, Bennet JM, Flandrin G, Matutes E, Head D, Vardiman JW, et al. Myelodysplastic syndromes. In: Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. WHO classification of tumors. pathology and genetics of tumors of haematopoietic and lymphoid tissues. Lyon: IARC Press; 2001. p. 61–74.
[25] Cheson BD, Bennet JM, Kantarjian H, Pinto A, Schiffer CA, Nimer SD, et al. Report of an international working group to standardize response criteria for myelodysplastic syndromes. Blood 2000;96:3671–4.
[26] Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol 1982;5:649–55.
[27] Yaguchi M, Miyazawa K, Otawa M, Katagiri T, Nishimaki J, Uchida Y, et al. Vitamin K2 selectively induces apoptosis of blastic cells in myelodysplastic syndrome: flow cytometric detection of apoptotic cells using APO2.7 monoclonal antibody. Leukemia 1998;12:1392–7.
[28] Takahashi T, Ichiba S, Okuno Y, Sugiyama H, Sakai Y, Imura H, et al. Therapeutic effectiveness of vitamin D3 in patients with myelodysplastic syndromes, leukemia and myeloproliferative disorders. Rinsho Ketsueki 1989;30:1–10.
[29] Kizaki M, Koeffler HP. Differentiation-inducing agents in the treatment of myelodysplastic syndromes. Semin Oncol 1992;19:95–105.
[30] Yokoyama T, Miyazawa K, Naito M, Toyotake J, Tauchi T, Itoh M, et al. Vitamin K2 induces autophagy and apoptosis simultaneously in leukemia cells. Autophagy 2008;4:629–40.
[31] Enomoto M, Tsuchida A, Miyazawa K, Yokoyama T, Kawakita H, Tokita H, et al. Vitamin K2-induced cell growth inhibition via autophagy formation in cholangiocellular carcinoma cell lines. Int J Mol Med 2007;20:801–8.
[32] Kawakita H, Tsuchida A, Miyazawa K, Naito M, Shigoka M, Kyo B, et al. Growth inhibitory effects of vitamin K2 on colon cancer cell lines via different types of cell death including autophagy and apoptosis. Int J Mol Med 2009;23:709–16.
[33] Wang J, Lian H, Zhao Y, Kauss MA, Spindel S. Vitamin D3 induces autophagy of human myeloid leukemia cells. J Biol Chem 2008;283:25596–605.
[34] Kirkin V, McEwan DG, Novak I, Dikic I. A role for ubiquitin in selective autophagy. Mol Cell 2009;34:259–69.
[35] Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008;132:27–42.
[36] Houwerzijl EJ, Pol HW, Blom NR, van der Want JJ, de Wolf JT, et al. Erythroid precursors from patients with low-risk myelodysplasia demonstrate ultrastructural features of enhanced autophagy of mitochondria. Leukemia 2009;23:886–91.
[37] Yu L, Strandberg L, Lenardo MJ. The selectivity of autophagy and its role in cell death and survival. Autophagy 2008;4:567–73.
[38] Tsukamoto N, Mastushima T, Nojima M, Karasawa M, Murakami H, Sakuraya M, et al. Therapeutic result of vitamin K2 therapy in myelodysplastic syndrome. J Jpn Society Int Med 2001;90:280 (abst. Japanese).
[39] Imamura T, Ostuka E, Ogata M, Ogata Y, Kouno K, Uno N, et al. Clinical trial: Effectiveness of vitamin K2 for the treatment of MDS patients. Hematology Frontier 2005;15:1858–63 (Japanese).
[40] Yoshinaga K, Teramura M, Hida M, Masuda M, Motoji T, Mizoguchi H. Therapeutic outcome of vitamin K2therapy in myelodysplastic syndromes. Rinsho Ketsueki 2002;44:878 (abst. Japanese).
[41] Tsurumi A, Kasahara C, Hara T, Yamada T, Sawada M, Ohyama M, et al. Combination therapy consisted of vitamin B6, vitamin D3 and vitamin K2 for myelodysplastic syndromes. Rinsho Ketsueki 2004;46:765 (abst. Japanese).

PNH-phenotype cells in patients with idiopathic cytopenia of undetermined significance (ICUS) with megakaryocytic hypoplasia and thrombocytopenia

The recent World Health Organization classification has identified a condition marked by having fewer than 10% of dysplastic cells and fewer than 5% of blasts in the bone marrow (BM) as idiopathic cytopenia of undetermined significance (ICUS) (Wimazal *et al*, 2007; Brunning *et al*, 2008). We recently reported that ICUS patients may be heterogeneous (Ando *et al*, 2010), and the pathophysiology of some ICUS patients may be different from that of myelodysplastic syndromes (MDS). During the last decade, the immunological background of MDS patients, especially in those with low-grade dysplasia, has been postulated as significant, and immunosuppressive therapy is widely proposed in the consensus guidelines for MDS treatment (Bowen *et al*, 2003). Candidates for immunosuppressive therapy (IST) are those with hypocellular bone marrow or the presence of DRB1*1501. The correlation between the presence of paroxysmal nocturnal haemoglobinuria (PNH)-phenotype cells (a minor PNH clone: <1% of CD55- and CD59-deficient cells) and a good response to IST has also been reported. It has been reported that patients with aplastic anaemia (AA) or MDS displaying >0·003% of PNH-phenotype cells showed significantly favourable responses to IST (Wang *et al*, 2002; Nakao *et al*, 2006). However, the prevalence of increased PNH-type cells in patients with ICUS remained unclear.

We analysed a total of 29 patients, including 11 patients with morphologically identified ICUS, nine with refractory cytopenia with unilineage dysplasia (RCUD), and nine patients with refractory cytopenia with multilineage dysplasia (RCMD): the haematological and clinical data of some patients were reported previously (Ando *et al*, 2010). Patients with fewer than 25 analysable megakaryocytes were defined as undetermined, even if the percentage of dysplastic megakaryocytes was more than 10%. MDS was diagnosed according to morphological criteria defined by the French-American-British classification and International Working Group on Morphology of myelodysplastic syndromes (Valent *et al*, 2007; Mufti *et al*, 2008). To detect a minor PNH clone, peripheral blood obtained from patients was transferred to Kanazawa University (transported overnight at 4°C), and analysed using a highly sensitive flow cytometric assay, as reported previously (Sugimori *et al*, 2006). We considered a patient to be positive for PNH-phenotype cells when the presence of CD55- and CD-59 deficient granulocytes were >0·003%, and when CD55- and CD59-deficient erythrocytes were >0·005%.

Of the 11 ICUS patients, two showed an increase in the percentage of PNH-type cells, whereas none of 9 RCUD or 9 RCMD patients did. Of the two patients with ICUS showing increased PNH-type cells, one showed clonal monosomy 8 and the other had a normal karyotype. Both patients who were positive for PNH–type cells had markedly low platelet counts, although there was no significant difference in the platelet count ($P = 0{\cdot}2125$ by unpaired *t*-test) (Fig 1) between the ICUS patients and those negative for PNH-type cells. The BM of the two patients (unique patient numbers 54 and 09–01) showed markedly megakaryocytic hypoplasia and was therefore difficult to assess for the morphology of megakaryocytes (Table SI).

It has been reported that approximately 20% of MDS-refractory anaemia and RCMD cases, and 50% of AA patients showed an increase in the percentage of PNH-type cells, and a common immunological background was postulated between the low-risk MDS and AA patient groups (Nakao *et al*, 2006; Sugimori *et al*, 2006). In the present study, PNH–type cells were detectable in only two patients with ICUS, both of whom showed prominent thrombocytopenia and megakaryocytic hypoplasia in the BM. This indicates that some ICUS patients with predominant thrombocytopenia may share immune pathophysiology characterized by the presence of increased PNH-type cells with patients with AA.

Recent research by Wang *et al* (2009) demonstrated that nine refractory anaemia (RA) patients had a minor population of PNH-phenotype granulocytes (<1% of granulocytes), and six of them showed neither ≥10% dysgranulopoiesis nor ≥10% dysmegakaryopoiesis; five of them lacked clonal cytogenetic abnormalities, suggesting the possibility that these patients could be given diagnoses of ICUS. Wang *et al* (2002) reported that 21 RA patients with increased PNH-type cells showed significantly lower percentages of abnormal karyotypes and pseudo-Pelger neutrophil anomalies, and significant lower platelet counts than RA patients negative for increased PNH-type cells. These findings, in combination with our results, indicate that an increase in the percentage of PNH-type cells may be characteristic of BM failure with few signs of dysplasia. Our findings further suggest that ICUS to be heterogeneous; some patients present with predominant neutropenia (Ando

doi:10.1111/j.1365-2141.2010.08257.x

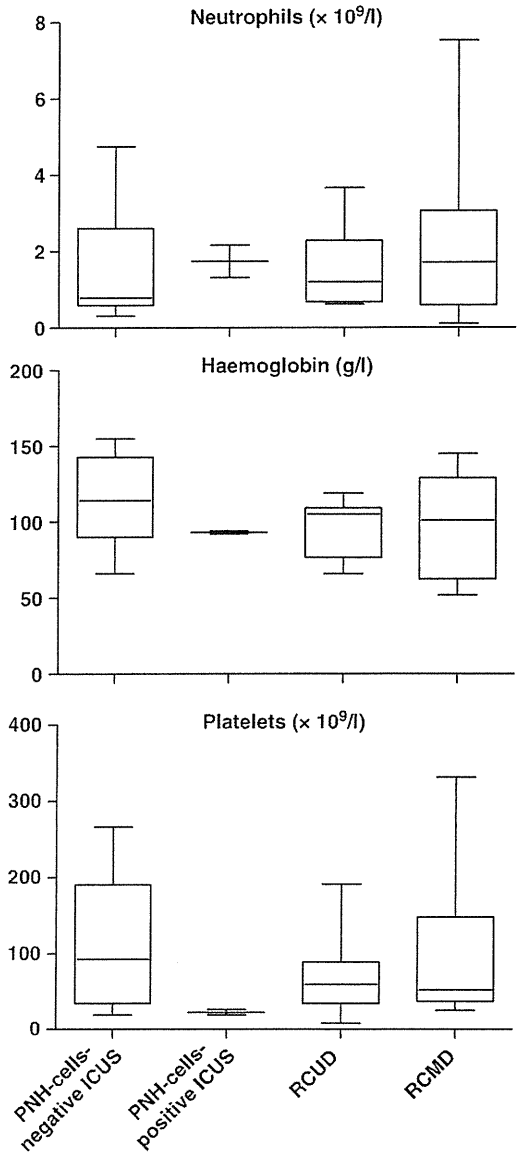

Fig 1. Haematological parameters in patients with idiopathic cytopenia of undetermined significance (ICUS) with or without a minor PNH clone, refractory cytopenia with unilineage dysplasia (RCUD), or refractory cytopenia with multilineage dysplasia (RCMD). Patients with ICUS with a minor PNH clone (n = 2) showed prominent thrombocytopenia. Boxes show 95 percentile confidence intervals; lines indicate the range of haematological parameters.

et al, 2010) and others show predominant thrombocytopenia with increased PNH-phenotype cells.

Another important issue is the limitation of diagnosing ICUS with the current criteria. Patients given diagnoses of ICUS in this study did not show hypoplastic BM suggestive of AA. However, BM sites other than the one examined by biopsy may have been hypocellular (Brunning *et al*, 2008), and the diagnosis of non-severe AA could not be completely excluded, although the degree of their cytopenias did not meet the criteria for AA. Morphological assessment of megakaryocytes is important to diagnose low-grade-dysplasia in MDS patients, although some ICUS patients, for example those with increased PNH-type cells in the present study, do not have enough megakaryocytes to be assessed. These findings indicate that further discussion from both pathophysiological and morphological approaches is necessary to understand overlapping categories of idiopathic cytopenia.

Acknowledgements

Thanks are due to Roderick J. Turner and Professor J. Patrick Barron of the Department of International Medical Communications Centre of Tokyo Medical University, for their review of this manuscript. Thanks are also due to Ms A. Hirota for her assistance. This work was supported in part by a grant-in-aid from the Ministry of Health, Welfare and Labour of Japan (Japanese Research Committee for Intractable Haematopoietic Diseases).

Conflict of interest

No potential conflicts of interest were disclosed.

Keiko Ando[1]
Yuko Tanaka[1]
Yuko Hashimoto[1]
Junko H. Ohyashiki[2]
Naomi Sugimori[3]
Shinji Nakao[3]
Kazuma Ohyashiki[1]

[1]*First Department of Internal Medicine, Haematology Division,* [2]*Intractable Disease Research Centre, Tokyo Medical University, Tokyo, and* [3]*Cellular Transplantation Biology Division, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan.*
E-mail: ohyashik@rr.iij4u.or.jp

References

Ando, K., Kodama, A., Iwabuchi, T., Ohyashiki, J.H. & Ohyashiki, K. (2010) Idiopathic neutropenia with fewer than 5% dysplasia may be a distinct entity of idiopathic cytopenia of undetermined significance. *Annals of Hematology*, doi:10.1007/s00277-009-0845-0

Bowen, D., Culligan, D., Jowitt, S., Kelsey, S., Mufti, G., Oscier, D. & Parker, J.; UK MDS Guidelines Group. (2003) UK MDS Guidelines Group Guidelines for the diagnosis and therapy of adult myelodysplastic syndromes. *British Journal of Haematology*, **120**: 187–200.

Brunning, R.D., Orazi, A., Germing, U., Le Beau, M.M., Porwit, A., Baumann, I., Vardiman, J.W. & Hellström-Lindberg, E. (2008) Myelodysplastic syndromes. In: *WHO Classification of Tumours of Hematopoietic and Lymphoid Tissues* (ed. by S.H. Swerdlow, E. Campo, N.L. Harris, E.S. Jaffe, S.A. Pileri, H. Stein, J. Thiele & J.W. Vardiman), pp. 90–107. IARC Press, Lyon.

Mufti, G., Bennett, J.M., Goasguen, J., Bain, B.J., Baumann, I., Brunning, R., Cazzola, M., Fenaux, P., Germing, U., Hellström-Lindberg,

E., Jinnai, I., Manabe, A., Matsuda, A., Niemeyer, C.M., Sanz, G., Tomonaga, M., Vallespi, T. & Yoshimi, A.; International Working Group on Morphology of Myelodysplastic Syndrome. (2008) Diagnosis and classification of myelodysplastic syndrome: International Working Group on Morphology of myelodysplastic syndrome (IWGM-MDS) consensus proposals for the definition and enumeration of myeloblasts and ring sideroblasts. *Haematologica*, **93,** 1712–1727.

Nakao, S., Sugimori, C. & Yamazaki, H. (2006) Clinical significance of a small population of paroxysmal nocturnal hemoglobinuria-type cells in the management of bone marrow failure. *International Journal of Hematology*, **84,** 118–122.

Sugimori, C., Chuhjo, T., Feng, X., Yamazaki, H., Takami, A., Teramura, M., Mizoguchi, H., Omine, M. & Nakao, S. (2006) Minor population of CD55- CD59- blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia. *Blood*, **107,** 1308–1314.

Valent, P., Horney, H.-P., Bennett, J.M., Fonatsch, C., Germing, U., Greenberg, P., Haferlach, T., Haase, D., Kolb, H.J., Krieger, O., Loken, M., van de Loosdrecht, A., Ogata, K., Orfao, A., Pfeilstöcker, M., Rüter, B., Sperr, W.R., Stauder, R. & Wells, D.A. (2007) Definitions and standards in the diagnosis and treatment of the myelodysplastic syndromes: consensus statements and report from a working conference. *Leukemia Research*, **31,** 727–736.

Wang, H., Chuhjo, T., Yasue, S., Omine, M. & Nakao, S. (2002) Clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria-type cells in bone marrow failure syndrome. *Blood*, **100,** 3897–3902.

Wang, S.A., Pozdnyakova, O., Jorgensen, J.L., Medeiros, L.J., Stachurski, D., Anderson, M., Raza, A. & Woda, B.A. (2009) Detection of paroxysmal nocturnal hemoglobinuria clones in patients with myelodysplastic syndromes and related bone marrow diseases, with emphasis on diagnostic pitfalls and caveats. *Haematologica*, **94,** 29–37.

Wimazal, F., Fonatsch, C., Thalhammer, R., Schwarzinger, I., Müllauer, L., Sperr, W.R., Bennett, J.M. & Valent, P. (2007) Idiopathic cytopenia of undetermined significance (ICUS) versus low risk MDS: the diagnostic interface. *Leukemia Research*, **31,** 1461–1468.

Keywords: idiopathic cytopenia of undetermined significance, PNH-phenotype, thrombocytopenia.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table SI. Frequency of PNH clone in myelodysplastic syndrome with marrow blasts fewer than 5% or idiopathic cytopenia of undetermined significance.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

ARTICLE

doi:10.1038/nature10496

Frequent pathway mutations of splicing machinery in myelodysplasia

Kenichi Yoshida[1]*, Masashi Sanada[1]*, Yuichi Shiraishi[2]*, Daniel Nowak[3]*, Yasunobu Nagata[1]*, Ryo Yamamoto[4], Yusuke Sato[1], Aiko Sato-Otsubo[1], Ayana Kon[1], Masao Nagasaki[5], George Chalkidis[6], Yutaka Suzuki[7], Masashi Shiosaka[1], Ryoichiro Kawahata[1], Tomoyuki Yamaguchi[8], Makoto Otsu[4], Naoshi Obara[9], Mamiko Sakata-Yanagimoto[9], Ken Ishiyama[10], Hiraku Mori[11], Florian Nolte[3], Wolf-Karsten Hofmann[3], Shuichi Miyawaki[10], Sumio Sugano[7], Claudia Haferlach[12], H. Phillip Koeffler[13,14], Lee-Yung Shih[15], Torsten Haferlach[12], Shigeru Chiba[9], Hiromitsu Nakauchi[4,8], Satoru Miyano[2,6] & Seishi Ogawa[1]

Myelodysplastic syndromes and related disorders (myelodysplasia) are a heterogeneous group of myeloid neoplasms showing deregulated blood cell production with evidence of myeloid dysplasia and a predisposition to acute myeloid leukaemia, whose pathogenesis is only incompletely understood. Here we report whole-exome sequencing of 29 myelodysplasia specimens, which unexpectedly revealed novel pathway mutations involving multiple components of the RNA splicing machinery, including *U2AF35*, *ZRSR2*, *SRSF2* and *SF3B1*. In a large series analysis, these splicing pathway mutations were frequent (~45 to ~85%) in, and highly specific to, myeloid neoplasms showing features of myelodysplasia. Conspicuously, most of the mutations, which occurred in a mutually exclusive manner, affected genes involved in the 3′-splice site recognition during pre-mRNA processing, inducing abnormal RNA splicing and compromised haematopoiesis. Our results provide the first evidence indicating that genetic alterations of the major splicing components could be involved in human pathogenesis, also implicating a novel therapeutic possibility for myelodysplasia.

Myelodysplastic syndromes (MDS) and related disorders (myelodysplasia) comprise a group of myeloid neoplasms characterized by deregulated, dysplastic blood cell production and a predisposition to acute myeloid leukaemia (AML)[1]. Although the prevalence of MDS has not been determined precisely, more than 10,000 people are estimated to develop myelodysplasia annually in the United States[2]. Their indolent clinical course before leukaemic transformation and ineffective haematopoiesis with evidence of myeloid dysplasia indicate a pathogenesis distinct from that involved in *de novo* AML. Currently, a number of gene mutations and cytogenetic changes have been implicated in the pathogenesis of MDS, including mutations of *RAS*, *TP53* and *RUNX1*, and more recently *ASXL1*, *c-CBL*, *DNMT3A*, *IDH1/2*, *TET2* and *EZH2* (ref. 3). Nevertheless, mutations of this set of genes do not fully explain the pathogenesis of MDS because they are also commonly found in other myeloid malignancies and roughly 20% of MDS cases have no known genetic changes (ref. 4 and unpublished data). In particular, the genetic alterations responsible for the dysplastic phenotypes and ineffective haematopoiesis of myelodysplasia are poorly understood. Meanwhile, the recent development of massively parallel sequencing technologies has provided an expanded opportunity to discover genetic changes across the entire genomes or protein-coding sequences in human cancers at a single-nucleotide level[5–10], which could be successfully applied to the genetic analysis of myelodysplasia to obtain a better understanding of its pathogenesis.

Overview of genetic alterations

In this study, we performed whole-exome sequencing of paired tumour/control DNA from 29 patients with myelodysplasia (Supplementary Table 1). Although incapable of detecting non-coding mutations and gene rearrangements, the whole-exome approach is a well-established strategy for obtaining comprehensive registries of protein-coding mutations at low cost and high performance. With a mean coverage of 133.8, 80.4% of the target sequences were analysed at more than ×20 depth on average (Supplementary Fig. 1). All the candidates for somatic mutations ($N = 497$) generated through our data analysis pipeline were subjected to validation using Sanger sequencing (Supplementary Methods I and Supplementary Fig. 2). Finally, 268 non-synonymous somatic mutations were confirmed with an overall true positive rate of 53.9% (Supplementary Fig. 3), including 206 missense, 25 nonsense, and 10 splice site mutations, and 27 frameshift-causing insertions/deletions (indels) (Supplementary Fig. 4). The mutation rate of 9.2 (0–21) per sample was significantly lower than that in solid tumours (16.2–302)[7,11,12] and multiple myeloma (32.4)[6], but was comparable to that in AML (7.3–13)[13–15] and chronic lymphocytic leukaemia (11.5)[16]. Combined with the genomic copy number profile obtained by single nucleotide polymorphism (SNP) array karyotyping, this array of somatic mutations provided a landscape of myelodysplasia genomes (Supplementary Fig. 5)[17,18].

[1]Cancer Genomics Project, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. [2]Laboratory of DNA Information Analysis, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. [3]Department of Hematology and Oncology, Medical Faculty Manheim of the University of Heidelberg, 1–3 Theodor-Kutzer-Ufer, Mannheim 68167, Germany. [4]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. [5]Laboratory of Functional Genomics, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. [6]Laboratory of Sequence Data Analysis, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. [7]Division of Systems Biomedical Technology, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. [8]Nakauchi Stem Cell and Organ Regeneration Project, Exploratory Research for Advanced Technology, Japan Science and Technology Agency, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. [9]Department of Hematology, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-shi, Ibaraki, 305-8571, Japan. [10]Division of Hematology, Tokyo Metropolitan Ohtsuka Hospital, 2-8-1 Minami-Ohtsuka, Toshima-ku, Tokyo 170-0005, Japan. [11]Division of Hematology, Internal Medicine, Showa University Fujigaoka Hospital, 1-30 Fujigaoka, Aoba-ku, Yokohama, Kanagawa 227-8501, Japan. [12]Munich Leukemia Laboratory, Max-Lebsche-Platz 31, Munich 81377, Germany. [13]Hematology/Oncology, Cedars-Sinai Medical Center, 8700 Beverly Blvd, Los Angeles, California 90048, USA. [14]National University of Singapore, Cancer Science Institute of Singapore, 28 Medical Drive, Singapore 117456, Singapore. [15]Division of Hematology-Oncology, Department of Internal Medicine, Chang Gung Memorial Hospital, Chang Gung University, 199 Tung Hwa North Rd, Taipei 105, Taiwan.
*These authors contributed equally to this work.

Novel gene targets in myelodysplasia

The list of the somatic mutations (Supplementary Table 2) included most of the known gene targets in myelodysplasia with similar mutation frequencies to those previously reported, indicating an acceptable sensitivity of the current study. The mutations of the known gene targets, however, accounted for only 12.3% of all detected mutations ($N = 33$), and the remaining 235 mutations involved previously unreported genes. Among these, recurrently mutated genes in multiple cases are candidate targets of particular interest, for which high mutation rates are expected in general populations. In fact, 8 of the 12 recurrently mutated genes were among the well-described gene targets in myelodysplasia (Supplementary Table 3). However, what immediately drew our attention were the recurrent mutations involving *U2AF35* (also known as *U2AF1*), *ZRSR2* and *SRSF2* (*SC35*), because they belong to the common pathway known as RNA splicing. Including an additional three genes mutated in single cases (*SF3A1*, *SF3B1* and *PRPF40B*), six components of the splicing machinery were mutated in 16 out of the 29 cases (55.2%) in a mutually exclusive manner (Fig. 1, Supplementary Fig. 6 and Supplementary Table 2).

Frequent mutations in splicing machinery

RNA splicing is accomplished by a well-ordered recruitment, rearrangement and/or disengagement of a set of small nuclear ribonucleoprotein (snRNP) complexes (U1, U2, and either U4/5/6 or U11/12), as well as many other protein components onto the pre-mRNAs. Notably, the mutated components of the spliceosome were all engaged in the initial steps of RNA splicing, except for PRPF40B, whose functions in RNA splicing are poorly defined. Making physical interactions with SF1 and a serine/arginine-rich (SR) protein, such as SRSF1 or SRSF2, the U2 auxiliary factor (U2AF) that consists of the U2AF65 (U2AF2)–U2AF35 heterodimer, is involved in the recognition of the 3′ splice site (3′SS) and its nearby polypyrimidine tract, which is thought to be required for the subsequent recruitment of the U2 snRNP, containing SF3A1 as well as SF3B1, to establish the splicing A complex (Fig. 1)[19]. ZRSR2 (or Urp), is another essential component of the splicing machinery. Showing a close structural similarity to U2AF35, ZRSR2 physically interacts with U2AF65, as well as SRSF1 and SRSF2, with a distinct function from its homologue, U2AF35 (ref. 20).

To confirm and extend the initial findings in the whole-exome sequencing, we studied mutations of the above six genes together with three additional spliceosome-related genes, including *U2AF65*, *SF1* and *SRSF1*, in a large series of myeloid neoplasms ($N = 582$) using a high-throughput mutation screen of pooled DNA followed by confirmation/identification of candidate mutations (refs 21 and 22 and Supplementary Methods II).

In total, 219 mutations were identified in 209 out of the 582 specimens of myeloid neoplasms through validating 313 provisional positive events in the pooled DNA screen (Supplementary Tables 4 and 5). The mutations among four genes, *U2AF35* ($N = 37$), *SRSF2* ($N = 56$), *ZRSR2* ($N = 23$) and *SF3B1* ($N = 79$), explained most of the mutations with much lower mutational rates for *SF3A1* ($N = 8$), *PRPF40B* ($N = 7$), *U2AF65* ($N = 4$) and *SF1* ($N = 5$) (Fig. 2). Mutations of the splicing machinery were highly specific to diseases showing myelodysplastic features, including MDS either with (84.9%) or without (43.9%) increased ring sideroblasts, chronic myelomonocytic leukaemia (CMML) (54.5%), and therapy-related AML or AML with myelodysplasia-related changes (25.8%), but were rare in *de novo* AML (6.6%) and myeloproliferative neoplasms (MPN) (9.4%) (Fig. 3a). The mutually exclusive pattern of the mutations in these splicing pathway genes was confirmed in this large case series, suggesting a common impact of these mutations on RNA splicing and the pathogenesis of myelodysplasia (Fig. 3b). The frequencies of mutations showed significant differences across disease types. Surprisingly, *SF3B1* mutations were found in the majority of the cases with MDS characterized by increased ring sideroblasts, that is, refractory anaemia with ring sideroblasts (RARS) (19/23 or 82.6%) and refractory cytopenia with multilineage dysplasia with ≥ 15% ring sideroblasts (RCMD-RS) (38/50 or 76%) with much lower mutation frequencies in other myeloid neoplasms. RARS and RCMD-RS account

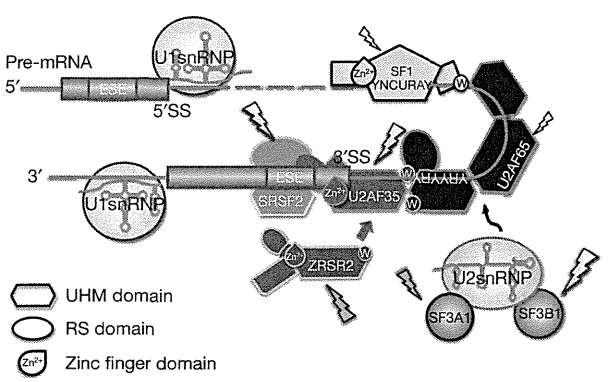

Figure 1 | Components of the splicing E/A complex mutated in myelodysplasia. RNA splicing is initiated by the recruitment of U1 snRNP to the 5′SS. SF1 and the larger subunit of the U2 auxiliary factor (U2AF), U2AF65, bind the branch point sequence (BPS) and its downstream polypyrimidine tract, respectively. The smaller subunit of U2AF (U2AF35) binds to the AG dinucleotide of the 3′SS, interacting with both U2AF65 and a SR protein, such as SRSF2, through its UHM and RS domain, comprising the earliest splicing complex (E complex). ZRSR2 also interacts with U2AF and SR proteins to perform essential functions in RNA splicing. After the recognition of the 3′SS, U2 snRNP, together with SF3A1 and SF3B1, is recruited to the 3′SS to generate the splicing complex A. The mutated components in myelodysplasia are indicated by arrows.

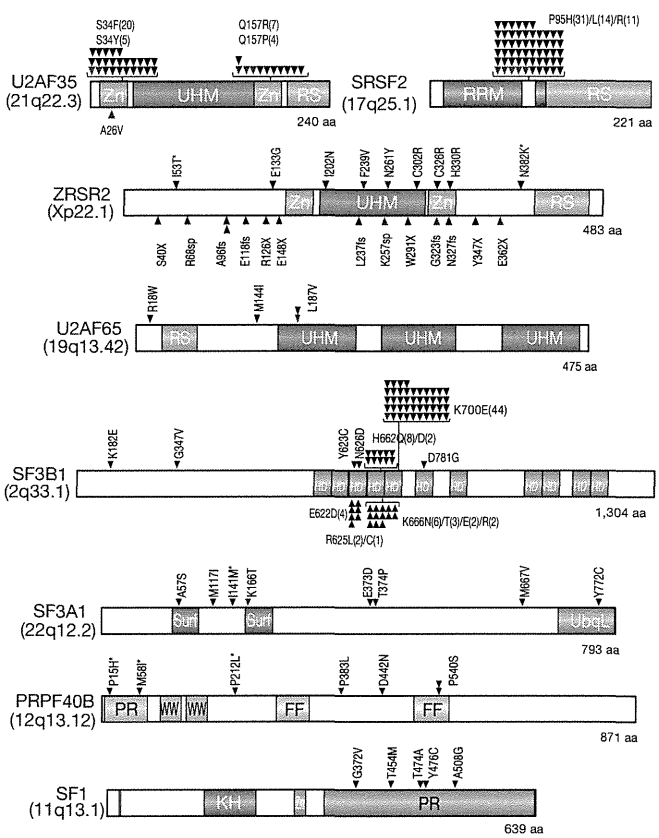

Figure 2 | Mutations of multiple components of the splicing machinery. Each mutation in the eight spliceosome components is shown with an arrowhead. Confirmed somatic mutations are discriminated by red arrows. Known domain structures are shown in coloured boxes as indicated. Mutations predicted as SNPs by MutationTaster (http://www.mutationtaster.org/) are indicated by asterisks. The number of each mutation is indicated in parenthesis. ZRSR2 mutations in females are shown in blue.