Table 5 Serial changes in laboratory data and blood pressure after the change in the route of CsA administration

	Mean (minimum–maximum)			
	Serum creatinine (mg per 100 ml)	*ALT (IU/l)*	*Total bilirubin (mg per 100 ml)*	*Blood pressure level (mm Hg)*
Day 0	0.87 (0.60–1.43)	64.4 (16–182)	0.63 (0.24–1.06)	Systolic 130 (114–173) Diastolic 82 (63–103)
Day 3	0.86 (0.32–1.63)	50.1 (10–106)	0.62 (0.27–1.47)	Systolic 124 (109–150) Diastolic 79 (51–103)
Day 7	0.92 (0.69–1.31)	44.6 (10–103)	0.61 (0.30–1.17)	Systolic 122 (109–132) Diastolic 80 (51–103)
Day 14	0.83 (0.67–1.29)	65.8 (10–300)	0.64 (0.27–0.96)	Systolic 121 (113–135) Diastolic 76 (68–89)

Abbreviation: ALT = alanine aminotransferase.

weeks after the conversion. The AUC of CsA was rather lower after conversion, and thus CsA was not considered to be the causative agent of liver dysfunction. Otherwise, no notable changes in laboratory and clinical data were observed (Table 5).

Four patients had developed grade I acute GVHD of the skin before the change in the route of CsA administration. During the 2 weeks after the switch, 3 of the 4 patients had persistent grade I skin GVHD, whereas GVHD was improved in 1 patient. Among the eight patients who did not have acute GVHD at the switch, one patient developed grade I acute GVHD of the skin, which was well controlled by topical steroid, and the other seven patients did not develop acute GVHD during the observation period. No clinically significant changes in vital or biological parameters occurred in the study patients. One patient (No. 9) developed nausea soon after conversion. An excessive increase in the CsA concentration was considered to be the cause of nausea and this symptom was improved after the dose of Neoral was reduced.

Discussion

Neoral is a microemulsion formulation of CsA that has improved bioavailability and reduced variability in pharmacokinetic parameters within and between patients compared with a conventional CsA formulation (Sandimmun).[4] Its bioavailability has been reported to be 0.38 (38%) in healthy volunteers.[13] However, allogeneic HSCT patients have complications that could influence the CsA pharmacokinetics, such as damaged gastrointestinal mucosa and multiple drug interactions. The results of this study showed that the median value of the bioavailability of Neoral was 0.685 (range, 0.45–1.04). Detailed analyses revealed that the oral administration of VRCZ strongly affected the bioavailability of Neoral (0.87 vs 0.54). Therefore, although the switch from intravenous to oral administration of CsA at a ratio of 1:2 seemed to be appropriate in most patients, a lower conversion ratio such as 1:1.1 or 1:1.2 may be better in patients taking oral VRCZ.

The drug interactions between CsA and azole antifungal agents including FLCZ, ITCZ, and VRCZ have been well recognized.[14] Azole antifungal agents are metabolized through the cytochrome P450-3A (CYP3A4) enzyme system, interfere with the metabolism of CsA, and thereby increase the exposure to CsA. Therefore, careful monitoring of the blood CsA concentration is recommended when these agents are added during CsA administration. On the other hand, there are considerable differences among azole antifungals with regard to their ability to inhibit CYP3A4.[14] Interestingly, the concomitant use of oral VRCZ significantly increased the bioavailability of Neoral. We confirmed that VRCZ was started at least 7 days before the switch from intravenous to oral administration of CsA and was continued at the same dose after the switch. Therefore, the drug interaction between CsA and VRCZ seemed to be stronger during oral administration than during the intravenous infusion of CsA. We hypothesized that this stronger interaction can be explained by the presence of the P450 enzyme system in the gastrointestinal mucosa. The CYP3A4 isoenzymes are the most abundant isoforms of CYP and it has been postulated that CsA is also metabolized in the intestine by gut CYP3A4 isoenzymes.[15] The administration of VRCZ might have inhibited the gut metabolism of CsA and increased the bioavailability of CsA. However, a prospective controlled study is required to confirm this hypothesis.

ITCZ, another strong inhibitor of CYP3A4, did not increase the bioavailability of Neoral. As the ratio of $AUC_{IV}/DOSE_{IV}$ was higher not only in patients taking VRCZ but also in patients taking ITCZ compared with other patients (median 47.5, 55, and 41), ITCZ might have inhibited liver CYP3A4 similar to VRCZ, but inhibited gut CYP3A4 less strongly than VRCZ. This might have been affected by the different bioavailable dose of these agents, as the bioavailability of ITCZ is lower than that of VRCZ, in addition to the fact that the dose of ITCZ was lower than that of VRCZ (200 vs 400 mg/day).

With regard to the route of VRCZ, it was exclusively administered orally in this study. Therefore, we could not conclude whether the intravenous administration of VRCZ would similarly affect the bioavailability of CsA. In earlier reports, the extent of drug interaction between CsA and azole antifungals varied according to the route of administration and the dose or kind of antifungal agent. Numerous reports have shown a significant interaction (>84%) between oral FLCZ with a dose of 200 mg/day or greater and oral CsA.[16,17] On the other hand, Osowski *et al*.[18] evaluated the drug interaction between intravenous FLCZ at 400 mg/day and intravenous CsA in HSCT recipients and there was a statistically significant but smaller increase (21%) in the serum CsA concentration.

Mihara *et al.*[19] reported that the mean steady-state whole-blood level of CsA significantly increased after the route of FLCZ administration was switched from intravenous to oral. These data suggest that the drug interaction between CsA and FLCZ was stronger when FLCZ was administered orally. With regard to other azole antifungal agents, not only oral but also intravenous administration of ITCZ significantly affected the blood concentration of CsA.[20–22] Concerning the interaction between VRCZ and CsA, Mori *et al.*[23] reported that the administration of VRCZ to patients receiving CsA resulted in a significant increase in the concentration/dose ratio of CsA, but the route of VRCZ administration did not affect the changes in the concentration/dose ratio. If we consider these findings together, it may be reasonable to suggest that the interaction between azole antifungal agents and CsA is stronger when the antifungals are given orally, but the difference becomes unclear with ITCZ and VRCZ, as the interactions of these agents are stronger than that of FLCZ and can be detected even when they are given intravenously. Therefore, when we interpret pharmacokinetic data of CsA, we must be cautious not only about concomitantly used agents but also the route of administration of both CsA and the other drugs. For example, Parquet *et al.* reported that a ratio of 1:2 in the switch from intravenous to oral administration was appropriate,[5] whereas a 1:1 ratio seemed to be appropriate in the study by McGuire *et al.*[6] In the former study, oral FLCZ was used concomitantly and thus their conclusion was consistent with our data. In the latter study, information on the use of antifungal agents was not described, and thus the data were difficult to interpret.

When we switch the route of CsA administration from continuous infusion to twice-daily oral administration, the target blood concentration should also be changed. Nakamura *et al.*[12] reported that the CsA blood concentration during continuous infusion was estimated to be 2.55 times the trough level during twice-daily oral administration of Neoral to obtain an equal AUC of CsA in kidney transplant patients. In this study, we concluded that the CsA concentration during continuous infusion should be doubled compared with the trough concentration during twice-daily oral administration in allogeneic HSCT recipients. Although the calculation method was different, the conclusion was consistent (mean 2.01) when we applied their methods. Although the reason for the difference between these studies remains unclear, it may have been due to the differences in the use of concomitant drugs or the status of the gastrointestinal tract.

In conclusion, when switching CsA from continuous infusion to oral administration, concomitant medications that could affect the bioavailability of CsA, especially azole antifungal agents, should be taken into account. Although a 1:2 ratio on switching may be appropriate in most patients, a lower conversion ratio is recommended in patients taking oral VRCZ.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Ruutu T, Niederwieser D, Gratwohl A, Apperley JF. A survey of the prophylaxis and treatment of acute GVHD in Europe: a report of the European Group for Blood and Marrow, Transplantation (EBMT). Chronic Leukaemia Working Party of the EBMT. *Bone Marrow Transplant* 1997; **19**: 759–764.
2 Kanda Y, Hyo R, Yamashita T, Fujimaki K, Oshima K, Onoda M *et al.* Effect of blood cyclosporine concentration on the outcome of hematopoietic stem cell transplantation from an HLA-matched sibling donor. *Am J Hematol* 2006; **81**: 838–844.
3 Oshima K, Kanda Y, Nakasone H, Arai S, Nishimoto N, Sato H *et al.* Decreased incidence of acute graft-versus-host disease by continuous infusion of cyclosporine with a higher target blood level. *Am J Hematol* 2008; **83**: 226–232.
4 Holt DW, Mueller EA, Kovarik JM, van Bree JB, Kutz K. The pharmacokinetics of Sandimmun Neoral: a new oral formulation of cyclosporine. *Transplant Proc* 1994; **26**: 2935–2939.
5 Parquet N, Reigneau O, Humbert H, Guignard M, Ribaud P, Socié G *et al.* New oral formulation of cyclosporin A (Neoral) pharmacokinetics in allogeneic bone marrow transplant recipients. *Bone Marrow Transplant* 2000; **25**: 965–968.
6 McGuire TR, Honaker M, Lynch JC, Tarantolo SR, Bishop MR, Ketcham MA *et al.* Renal dysfunction associated with cyclosporine (CSA) prophylaxis in HLA matched sibling peripheral blood stem cell transplantation (AlloBSCT): conversion from intravenous CSA to a new oral formulation (Neoral). *Blood* 1999; **94**(Suppl 1): 334A abstr 1492.
7 Okuda S, Terasako K, Oshima K, Sato M, Nakasone H, Kako S *et al.* Fludarabine, cyclophosphamide, anti-thymocyteglobulin, and low-dose total body irradiation conditioning enables 1-HLA-locus-mismatched hematopoietic stem cell transplantation for very severe aplastic anemia without affecting ovarian function. *Am J Hematol* 2009; **84**: 167–169.
8 Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J *et al.* 1994 Consensus conference on acute GVHD grading. *Bone Marrow Transplant* 1995; **15**: 825–828.
9 Asano-Mori Y, Kanda Y, Oshima K, Kako S, Shinohara A, Nakasone H *et al.* Long-term ultra-low-dose acyclovir against varicella-zoster virus reactivation after allogeneic hematopoietic stem cell transplantation. *Am J Hematol* 2008; **83**: 472–476.
10 Kanda Y, Mineishi S, Saito T, Seo S, Saito A, Suenaga K *et al.* Pre-emptive therapy against cytomegalovirus (CMV) disease guided by CMV antigenemia assay after allogeneic hematopoietic stem cell transplantation: a single-center experience in Japan. *Bone Marrow Transplant* 2001; **27**: 437–444.
11 Wolf BA, Daft MC, Koenig JW, Flye MW, Turk JW, Scott MG. Measurement of cyclosporine concentrations in whole blood: HPLC and radioimmunoassay with a specific monoclonal antibody and 3H- or 125I-labeled ligand compared. *Clin Chem* 1989; **35**: 120–124.
12 Nakamura Y, Takeuchi H, Okuyama K, Akashi T, Jojima Y, Konno O *et al.* Evaluation of appropriate blood level in continuous intravenous infusion from trough concentrations after oral administration based on area under trough level in tacrolimus and cyclosporine therapy. *Transplant Proc* 2005; **37**: 1725–1727.
13 Ku YM, Min DI, Flanigan M. Effect of grapefruit juice on the pharmacokinetics of microemulsion cyclosporine and its

metabolite in healthy volunteers: does the formulation difference matter? *J Clin Pharmacol* 1998; **38**: 959–965.
14 Leather HL. Drug interactions in the hematopoietic stem cell transplant (HSCT) recipient: what every transplanter needs to know. *Bone Marrow Transplant* 2004; **33**: 137–152.
15 Dresser GK, Spence JD, Bailey DG. Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clin Pharmacokinet* 2000; **38**: 41–57.
16 Canafax DM, Graves NM, Hilligoss DM, Carleton BC, Gardner MJ, Matas AJ. Interaction between cyclosporine and fluconazole in renal allograft recipients. *Transplantation* 1991; **51**: 1014–1018.
17 Lopez-Gil JA. Fluconazole-cyclosporine interaction: a dose-dependent effect? *Ann Pharmacother* 1993; **27**: 427–430.
18 Osowski CL, Dix SP, Lin LS, Mullins RE, Geller RB, Wingard JR. Evaluation of the drug interaction between intravenous high-dose fluconazole and cyclosporine or tacrolimus in bone marrow transplant patients. *Transplantation* 1996; **61**: 1268–1272.
19 Mihara A, Mori T, Aisa Y, Yamazaki R, Iketani O, Tanigawara Y *et al*. Greater impact of oral fluconazole on drug interaction with intravenous calcineurin inhibitors as compared with intravenous fluconazole. *Eur J Clin Pharmacol* 2008; **64**: 89–91.
20 Kramer MR, Merin G, Rudis E, Bar I, Nesher T, Bublil M *et al*. Dose adjustment and cost of itraconazole prophylaxis in lung transplant recipients receiving cyclosporine and tacrolimus (FK 506). *Transplant Proc* 1997; **29**: 2657–2659.
21 Florea NR, Capitano B, Nightingale CH, Hull D, Leitz GJ, Nicolau DP. Beneficial pharmacokinetic interaction between cyclosporine and itraconazole in renal transplant recipients. *Transplant Proc* 2003; **35**: 2873–2877.
22 Leather H, Boyette RM, Tian L, Wingard JR. Pharmacokinetic evaluation of the drug interaction between intravenous itraconazole and intravenous tacrolimus or intravenous cyclosporin A in allogeneic hematopoietic stem cell transplant recipients. *Biol Blood Marrow Transplant* 2006; **12**: 325–334.
23 Mori T, Aisa Y, Kato J, Nakamura Y, Ikeda Y, Okamoto S. Drug interaction between voriconazole and calcineurin inhibitors in allogeneic hematopoietic stem cell transplant recipients. *Bone Marrow Transplant* 2009; **44**: 371–374.

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LETTER TO THE EDITOR

Target blood concentrations of CYA and tacrolimus in randomized controlled trials for the prevention of acute GVHD after hematopoietic SCT

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In a recent issue of *Bone Marrow Transplant*, Ram *et al.*[1] reported a systematic review and meta-analysis of prophylaxis regimens for GVHD. They combined three randomized controlled trials that compared the combination of CYA and MTX (CYA–MTX) with the combination of tacrolimus and MTX (TAC–MTX), and concluded that although TAC–MTX was superior to CYA–MTX in terms of acute GVHD reduction, the incidence of all-cause mortality was similar.[1–4] A large retrospective study in Japan also revealed that the incidence of grades II–IV acute GVHD was significantly lower in patients who received TAC than in those who received CYA in matched unrelated donor transplantation, whereas no such difference was observed in matched sibling donor transplantation.[5] Although it is difficult to directly compare the incidence of GVHD among these studies because of the difference in the study population, these findings suggest that although TAC is more effective than CYA for preventing acute GVHD, this benefit does not confer a survival benefit, probably because of increased toxicities.

However, before making such a conclusion, we have to consider whether CYA and TAC were administered at appropriate doses in these studies. Table 1 summarizes the design and results of the three randomized controlled trials. CYA was continuously infused with a target blood concentration between 150 and 450 ng/ml. This target concentration might have been too low, as the target concentration was equivalent to the target trough concentration that is widely accepted in European centers when CYA was administered twice daily.[6] The target steady-state concentration in the continuous infusion of CYA should be higher than the trough concentration in twice-daily administration to provide an equal area under the concentration-time curve.[7] In fact, in a retrospective study, the incidence of acute GVHD was significantly higher in patients who received a continuous infusion of CYA with a target concentration between 250 and 400 ng/ml than in those who received a twice-daily infusion of CYA with a target trough concentration between 150 and 300 ng/ml.[8] In contrast, the target concentration of TAC was between 10 and 30 ng/ml in the two randomized trials in the United States and between 20 and 25 ng/ml in the Japanese trial. These target concentrations were apparently higher than that in current transplantation practice. Couriel *et al.*[9] recommended a blood concentration of TAC between 8 and 12 ng per 100 ml based on their retrospective and prospective studies.

It has been shown that the blood concentrations of CYA and TAC affect the incidences of acute GVHD and adverse events.[10] In addition, an increase in the target blood concentration from 300 to 500 ng/ml in continuously infused-CYA significantly decreased the incidence of acute GVHD.[11] This difference was more prominent in transplantation from an unrelated donor, similar to the fact that the difference in the incidence of acute GVHD between patients who received CYA and those who received TAC was observed only in unrelated donor transplantation[5,11] (Table 2). Therefore, continuous infusion of CYA with a target concentration at 500 ng/ml may be as effective as TAC with a target concentration used in our daily practice. In contrast, although the relationship between the blood concentration of TAC and the incidence of acute GVHD was not clear, an increase in the blood concentration was associated with greater renal dysfunction.[10] Therefore, renal toxicity associated with TAC could be reduced by decreasing the target blood concentration to a range

Table 1 Summary of the randomized controlled trials of CYA and tacrolimus (TAC)

Study	*Group*	*Initial dose*	*Target concentration*	*Grades II–IV acute GHVD (%)*	*2-year survival (%)*
Hiraoka *et al.*[2]	CYA	Not fixed[a]	Not fixed[a]	48	65
	TAC	0.05 mg/kg per day continuous i.v.[b]	20–25 ng/ml	18 ($P<0.0001$)	63 ($P=0.93$)
Ratanatharathorn *et al.*[4]	CYA	3 mg/kg continuous i.v.	150–450 ng/ml	44	57
	TAC	0.03 mg/kg continuous i.v.	10–30 ng/ml	32 ($P=0.01$)	47 ($P=0.02$)
Nash *et al.*[3]	CYA	3 mg/kg continuous i.v.	150–450 ng/ml	74	50
	TAC	0.03 mg/kg continuous i.v.	10–30 ng/ml	56 ($P=0.0002$)	54 ($P=0.46$)

[a]Determined by each institution.
[b]0.15 mg/day orally was allowed.

Table 2 Retrospective comparison of CYA and tacrolimus (TAC)

Study	*Donor*	*Group*	*Target concentration*	*Grades II–IV acute GVHD (%)*
Yanada *et al.*[5]	HLA-matched sibling	CYA	Not fixed[a]	38
		TAC	Not fixed[a]	33
	HLA-matched unrelated donor	CYA	Not fixed[a]	58
		TAC	Not fixed[a]	36
Oshima *et al.*[11]	HLA-matched sibling	CYA	300 ng/ml	44
		CYA	500 ng/ml	33
	HLA-matched unrelated donor	CYA	300 ng/ml	59
		CYA	500 ng/ml	24

[a]Determined by each institution.

between 10 and 20 ng/ml, without increasing the incidence of acute GVHD. If we consider all of these points, neither CYA nor TAC was administered at an appropriate dose in the earlier three randomized controlled trials of CYA–MTX and TAC–MTX. To clarify this problem, a randomized controlled trial of CYA–MTX and TAC–MTX with target blood concentrations at 500 and 15 ng/ml, respectively, is being performed in the Kanto Study Group for Cell Therapy.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Ram R, Gafter-Gvili A, Yeshurun M, Paul M, Raanani P, Shpilberg O. Prophylaxis regimens for GVHD: systematic review and meta-analysis. *Bone Marrow Transplant* 2009; **43**: 643–653.
2 Hiraoka A, Ohashi Y, Okamoto S, Moriyama Y, Nagao T, Kodera Y *et al.* Phase III study comparing tacrolimus (FK506) with cyclosporine for graft-versus-host disease prophylaxis after allogeneic bone marrow transplantation. *Bone Marrow Transplant* 2001; **28**: 181–185.
3 Nash RA, Antin JH, Karanes C, Fay JW, Avalos BR, Yeager AM *et al.* Phase 3 study comparing methotrexate and tacrolimus with methotrexate and cyclosporine for prophylaxis of acute graft-versus-host disease after marrow transplantation from unrelated donors. *Blood* 2000; **96**: 2062–2068.
4 Ratanatharathorn V, Nash RA, Przepiorka D, Devine SM, Klein JL, Weisdorf D *et al.* Phase III study comparing methotrexate and tacrolimus (prograf, FK506) with methotrexate and cyclosporine for graft-versus-host disease prophylaxis after HLA-identical sibling bone marrow transplantation. *Blood* 1998; **92**: 2303–2314.
5 Yanada M, Emi N, Naoe T, Sakamaki H, Takahashi S, Hirabayashi N *et al.* Tacrolimus instead of cyclosporine used for prophylaxis against graft-versus-host disease improves outcome after hematopoietic stem cell transplantation from unrelated donors, but not from HLA-identical sibling donors: a nationwide survey conducted in Japan. *Bone Marrow Transplant* 2004; **34**: 331–337.
6 Ruutu T, Niederwieser D, Gratwohl A, Apperley JF. A survey of the prophylaxis and treatment of acute GVHD in Europe: a report of the European Group for Blood and Marrow, Transplantation (EBMT). Chronic Leukaemia Working Party of the EBMT. *Bone Marrow Transplant* 1997; **19**: 759–764.
7 Nakamura Y, Takeuchi H, Okuyama K, Akashi T, Jojima Y, Konno O *et al.* Evaluation of appropriate blood level in continuous intravenous infusion from trough concentrations after oral administration based on area under trough level in tacrolimus and cyclosporine therapy. *Transplant Proc* 2005; **37**: 1725–1727.
8 Ogawa N, Kanda Y, Matsubara M, Asano Y, Nakagawa M, Sakata-Yanagimoto M *et al.* Increased incidence of acute graft-versus-host disease with the continuous infusion of cyclosporine A compared to twice-daily infusion. *Bone Marrow Transplant* 2004; **33**: 549–552.
9 Couriel DR, Thall P, Mickler K, De Lima M, Giralt S, Qazilbash MH *et al.* Phase II/III randomized study comparing two different tacrolimus blood levels for the prevention of graft-versus-host disease (GVHD). *Blood* 2005; **106**: 45a (abstract 142).
10 Wingard JR, Nash RA, Przepiorka D, Klein JL, Weisdorf DJ, Fay JW *et al.* Relationship of tacrolimus (FK506) whole blood concentrations and efficacy and safety after HLA-identical sibling bone marrow transplantation. *Biol Blood Marrow Transplant* 1998; **4**: 157–163.
11 Oshima K, Kanda Y, Nakasone H, Arai S, Nishimoto N, Sato H *et al.* Decreased incidence of acute graft-versus-host disease by continuous infusion of cyclosporine with a higher target blood level. *Am J Hematol* 2008; **83**: 226–232.

Reverse seroconversion of hepatitis B virus after allogeneic hematopoietic stem cell transplantation in the absence of chronic graft-versus-host disease

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The appearance of hepatitis B surface antigen (HBsAg) in patients previously positive for antibody to this antigen (HBsAb) is called reverse seroconversion, a rare complication after hematopoietic stem cell transplantation (HSCT), which occurs almost exclusively after HSCT from an HBsAb-negative donor and the development of chronic graft-versus-host disease (CGVHD). However, we experienced a patient who developed reverse seroconversion 23 months after unrelated HSCT even in the absence of immunosuppressants use or CGVHD. Serum immunoglobulin level was persistently normal. Therefore, all HBsAb-positive recipients should be considered to be at risk for HBV reactivation, even in patients without any risk factors.

Keywords: Hematopoietic stem cell transplantation, hepatitis B virus, reverse seroconversion, graft-versus-host disease

Introduction

The reactivation of hepatitis B virus (HBV) may cause fulminant hepatitis after hematopoietic stem cell transplantation (HSCT) in patients positive for hepatitis B virus surface antigen (HBsAg). The prophylactic use of antiviral agents such as lamivudine or entecavir has reduced the risk of severe hepatitis, although the emergence of resistant mutations was observed during an extended treatment with lamivudine.[1,2] Recently, several papers have reported the reactivation of HBV even in HSCT recipients previously positive for antibody to HBsAg (HBsAb).[3–7] This is called reverse seroconversion (RS) and the risk factors for RS included HSCT from an HBsAb-negative donor, the development of chronic graft-versus-host disease (GVHD), and the use of immunosuppressants. However, we experienced a patient who developed RS 23 months after unrelated HSCT for acute lymphoblastic leukemia even in the absence of the use of immunosuppressants or the development of chronic GVHD. This experience suggested that close monitoring of HBV markers is required even for patients without any risk factors.

Case report

A 42-year-old woman was diagnosed as acute lymphoid leukemia with t(4;11)(q21;q23) translocation in June 2005. She was positive for HBsAb and antibody to HBV core antigen (HBcAb) and negative for HBsAg at the onset of leukemia, suggesting the resolution of prior HBV infection. She achieved complete remission with a single course of induction chemotherapy. After three cycles of post-remission chemotherapy, she underwent bone marrow transplantation in November 2005, from an HLA-matched unrelated donor who was negative for

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HBsAg, HBsAb, and HBcAb. The conditioning regimen was cyclophosphamide and total body irradiation. HBV markers just before the conditioning regimen had not changed from pre-transplant levels (Table 1). Prophylaxis against GVHD was performed with cyclosporine and short-term methotrexate. Neutrophil engraftment was achieved on day 16 after HSCT. She developed grade I acute GVHD of the skin, which improved with topical steroid. She did not develop chronic GVHD and cyclosporine was stopped 10 months after HSCT. Although cellular immune recovery was not evaluated in detail, total lymphocyte count became greater than 1000/μl after the discontinuation of cyclosporine. Serum immunoglobulin G level was persistently higher than 1200 mg/dl after HSCT. The clinical course was uneventful. HBV monitoring was performed at six-month intervals and she was persistently positive for HBsAb and negative for HBsAg until 17 months after HSCT.

Twenty-three months after HSCT, however, routine blood test showed an elevation of serum aspartate aminotransferase (AST, 52 U/l) and alanine aminotransferase (ALT, 62 U/l) without any other abnormalities. We suspected reactivation of HBV and evaluated HBV markers. RS of HBV was diagnosed by the serological data including positive HBsAg, positive HBeAg, negative HBsAb, and negative HBeAb (Table 1). Quantitative polymerase chain reaction (PCR) revealed 6·7 log copies/ml of HBV-DNA in the serum.

Entecavir at 0·5 mg/day was immediately started and the dose was elevated to 1 mg/day one month later due to the lack of clinical response. The serum ALT and total bilirubin level increased up to 1450 U/l and 16·28 mg/dl, respectively, two months after the onset of RS, but thereafter gradually decreased and were normalized one more month later (Fig. 1). Twenty-eight months after HSCT, HBV became undetectable by PCR and she became positive for HBsAb and negative for HBsAg.

Table 1 Hepatitis B virus markers of the donor and recipient

	Donor	Recipient before HSCT	Recipient at RS
HBsAg	–	–	>2000 COI
HBsAb	–	484 U/l	(–)
HBeAg	NT	–	>1600 COI
HBeAb	NT	66 U/l	(–)
HBcAb	–	97 INH%	96 INH%
HBV-DNA	NT	<2·6 log copies/ml	6·7 log copies/ml

NT=not tested.

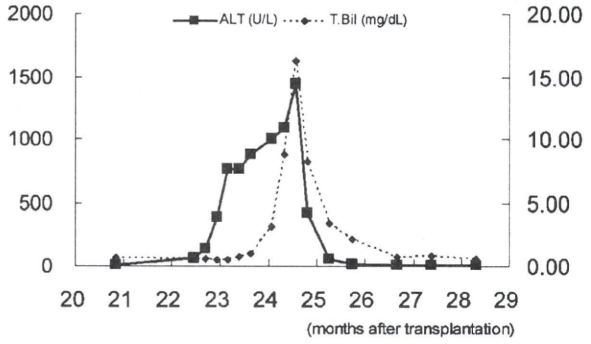

Figure 1 Clinical course of the patient: ALT=alanine aminotransferase; T.Bil=total bilirubin

Retrospective evaluation of the HBsAb titer revealed gradual decrease in HBsAb titer within two years after HSCT (Table 2).

Discussion

Previously, the seroconversion of HBsAg to HBsAb was considered to indicate an eradication of HBV. However, recent data has shown that HBV-DNA remains latent in the liver and in the peripheral blood mononuclear cells after seroconversion.[8,9] The latent virus can be reactivated in severely immunosuppressed patients, such as human immunodeficiency virus-positive patients, patients receiving rituximab, solid organ transplantation recipients, and allogeneic HSCT recipients.[10–12] Autologous HSCT recipients receiving steroid also caused this complication.[3]

Onozawa *et al.* retrospectively analyzed the clinical course of 14 allogeneic HSCT recipients who were HBsAb-positive before HSCT.[5] In 12 patients, HBsAb became undetectable at a median of 13 (range 10–38) months after HSCT. Among these, RS was observed in seven patients at a median of 20 (range 12–50) months after HSCT, while RS did not occur in the remaining five patients even after the loss of HBsAb. All seven patients with RS had chronic GVHD. Kempinska *et al.* reviewed 12 RS patients whose clinical information was available in the literature.[4] They revealed that RS occurred almost exclusively in patients who underwent HSCT form an

Table 2 Retrospective evaluation of the HBsAg and HBsAb titers

	HBsAg	HbsAb (U/l)
Before transplantation	–	484
2 months after transplantation	–	329
6 months after transplantation	–	347
17 months after transplantation	–	202
23 months after transplantation	>2000 COI	–
25 months after transplantation	128 COI	61
28 months after transplantation	–	64

HBsAb-negative donor and have developed chronic GVHD. To the best of our knowledge, the present patient is the first patient who developed RS with clinical hepatitis in the absence of chronic GVHD and the use of immunosuppressants. Furthermore, peripheral blood lymphocyte count and serum immunoglobulin level had already recovered to normal range. Therefore, all HBsAb-positive recipients should be considered to be at risk for HBV reactivation, even in patients without any risk factors. Also, we were monitoring HBV markers every six months, but it was insufficient to detect the disappearance of HBsAb before RS. Therefore, more frequent monitoring may be mandatory.

Prophylactic administration of antiviral agents is a possible strategy to prevent RS, but an emergence of resistant mutation is a serious problem.[2] Vaccination of the donor may reduce the risk of RS, but RS was observed in a patient who underwent HSCT from a donor who was actively vaccinated against HBV (HBsAb titre 14 642 U/l).[6] Considering these findings together, close monitoring of the HBV markers in all HBsAb-positive recipients and vaccination of the recipients with declining HBsAb titer may be the best strategy to prevent RS. However, prospective studies are required to address the efficacy of recipient vaccination.

References

1 Ohnishi M, Kanda Y, Takeuchi T, Won Kim S, Hori A, Niiya H, Chizuka A, Nakai K, Saito T, Makimoto A *et al.* Limited efficacy of lamivudine against hepatitis B virus infection in allogeneic hematopoietic stem cell transplant recipients. *Transplantation* 2002; **73**: 812–815.

2 Hsiao LT, Chiou TJ, Liu JH, Chu CJ, Lin YC, Chao TC, Wang WS, Yen CC, Yang MH, Tzeng CH *et al.* Extended lamivudine therapy against hepatitis B virus infection in hematopoietic stem cell transplant recipients. *Biol Blood Marrow Transplant* 2006; **12**: 84–94.

3 Goyama S, Kanda Y, Nannya Y, Kawazu M, Takeshita M, Niino M, Komeno Y, Nakamoto T, Kurokawa M, Tsujino S *et al.* Reverse seroconversion of hepatitis B virus after hematopoietic stem cell transplantation. *Leuk Lymphoma* 2002; **43**: 2159–2163.

4 Kempinska A, Kwak EJ, Angel JB. Reactivation of hepatitis B infection following allogeneic bone marrow transplantation in a hepatitis B-immune patient: case report and review of the literature. *Clin Infect Dis* 2005; **41**: 1277–1282.

5 Onozawa M, Hashino S, Izumiyama K, Kahata K, Chuma M, Mori A, Kondo T, Toyoshima N, Ota S, Kobayashi S *et al.* Progressive disappearance of anti-hepatitis B surface antigen antibody and reverse seroconversion after allogeneic hematopoietic stem cell transplantation in patients with previous hepatitis B virus infection. *Transplantation* 2005; **79**: 616–619.

6 Knoll A, Boehm S, Hahn J, Holler E, Jilg W. Long-term surveillance of haematopoietic stem cell recipients with resolved hepatitis B: high risk of viral reactivation even in a recipient with a vaccinated donor. *J Viral Hepat* 2007; **14**: 478–483.

7 Dhedin N, Douvin C, Kuentz M, Saint Marc MF, Reman O, Rieux C, Bernaudin F, Norol F, Cordonnier C, Bobin D *et al.* Reverse seroconversion of hepatitis B after allogeneic bone marrow transplantation: a retrospective study of 37 patients with pre-transplant anti-HBs and anti-HBc. *Transplantation* 1998; **66**: 616–619.

8 Michalak TI, Pasquinelli C, Guilhot S, Chisari FV. Hepatitis B virus persistence after recovery from acute viral hepatitis. *J Clin Invest* 1994; **93**: 230–239.

9 Rehermann B, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nat Med* 1996; **2**: 1104–1108.

10 Rouphael NG, Talati NJ, Rimland D. Hepatitis B reverse seroconversion in HIV-positive patients: case series and review of the literature. *AIDS* 2007; **21**: 771–774.

11 Sarrecchia C, Cappelli A, Aiello P. HBV reactivation with fatal fulminating hepatitis during rituximab treatment in a subject negative for HBsAg and positive for HBsAb and HBcAb. *J Infect Chemother* 2005; **11**: 189–191.

12 Blanpain C, Knoop C, Delforge ML, Antoine M, Peny MO, Liesnard C, Vereerstraeten P, Cogan E, Adler M, Abramowicz D. Reactivation of hepatitis B after transplantation in patients with pre-existing anti-hepatitis B surface antigen antibodies: report on three cases and review of the literature. *Transplantation* 1998; **66**: 883–886.

Diabetes mellitus is associated with high early-mortality and poor prognosis in patients with autoimmune hemolytic anemia

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The incidence of autoimmune hemolytic anemia (AIHA) is highest among the elderly, and thus it is frequently associated with co-morbidities such as diabetes mellitus (DM). However, there have been few reports on the impact of these co-morbidities on survival in patients with AIHA. Therefore, we retrospectively reviewed the records of 53 consecutive AIHA patients and assessed the impact of DM on survival. Eighteen of the 53 patients had DM. The estimated 4-year overall survival (4y-OS) for all patients was 84·9%. Infection was the most frequent cause of death, and fatal infections were exclusively observed in patients with DM. The deaths in DM patients occurred frequently within 1 year, to give significantly poor survival (4y-OS; 69·3% versus 93·6%, P=0·0064). The presence of DM was identified as the only significant risk factor for survival. A large prospective investigation is warranted to assess the impact of co-morbidities on survival in patients with AIHA.

Keywords: Diabetes, autoimmune hemolytic anemia, prognostic markers

Introduction

Autoimmune hemolytic anemia (AIHA) is an uncommon disorder with an incidence of approximately 1–3 cases per 100,000 per year. AIHA is either idiopathic or secondary to malignancy, infection, connective tissue disease (CTD) or drug administration.[1] AIHA is characterized by the production of auto-antibodies and the destruction of red blood cells. The immunological dysfunction is derived from the dysregulation of not only auto-reactive B cells but also auto-reactive T cells.[2,3] Although long-term follow-up studies have shown a mortality rate of 20%,[4] few reports have directly identified the risk factors for death in AIHA. The incidence of AIHA is highest in patients aged 60–70 years.[1] Therefore, patients with AIHA may have some co-morbidities such as diabetes mellitus (DM). DM has been identified as a risk factor for variable disease-onset or disease-outcome.[5] To the best of our knowledge, however, the impact of DM on survival in patients with AIHA has not yet been assessed. Therefore, the current study was performed to clarify the impact of DM on the clinical outcome of AIHA.

Patients and methods

Patients

We retrospectively reviewed the records of consecutive AIHA patients who were referred to Saitama

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Medical Center of Jichi Medical University (SMC) between August 1991 and August 2007 and Kanto Medical Center NTT EC (KMC) between January 1994 and April 2008. AIHA was diagnosed by the presence of anemia, laboratory evidence of hemolysis (such as elevated reticulocytes, elevated lactate dehydrogenase and reduced haptoglobin), and a positive direct anti-globulin test or a direct detection of anti-erythrocyte antibodies.

Statistical analysis

The following data were collected from the clinical records; sex, age at diagnosis, the presence of immunological disease, malignancy and DM, treatment, complications, survival and cause of death. The presence of DM was defined according to the standard criteria,[6] the presence of hemoglobin A1c >6·3% and/or routine administration of anti-diabetic agents. Patients who developed steroid-induced DM were considered to have glucose intolerance before the administration of steroid and were categorized into the DM group.

The patient characteristics were compared using Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous variables. Overall survival (OS) was calculated using the Kaplan-Meier method and log rank test. Risk factors for complications were also assessed by a logistic regression model. Variables with a P value <0·10 were entered in a multivariable analysis using a backward stepwise proportional-hazard model for survival and a logistic regression model for complications. Finally, statistical significance was defined as a two-sided P value of <0·05.

Results

A total of 53 patients were included in this study (Table 1). The survivors were observed for a median of 4·0 years (range: 0·16–16·6). The median age at diagnosis was 65 years (range: 28–88). Forty-three patients received steroids or other immunosuppressive agents including chemotherapy, while 10 did not receive such treatments. Eighteen of the 53 patients

Table 1 Clinical characteristics of the AIHA patients

	Total	AIHA with DM	AIHA without DM	*P* value
Total	53	18	35	
Sex				
Male	30	12	18	
Female	23	6	17	0·38
Median age	65 years old (range: 28–88)	67·5 years old (range: 54–88)	61·5 years old (range: 28–88)	0·15
Associated disease at Dx				
Immune disease	7	2	5	
ITP	4	1	3	
SLE	2	0	2	
adult Still's	1	1	0	
SjS	1	0	1	0·62
Malignancy	10	2	8	
Indolent NHL	3	1	2	
HL	2	1	1	
RCC	2	0	2	
Pros Ca	1	0	1	
MDS/MPD	2	0	2	0·65
Treatment				
none	10	1	9	
IST	32	13	19	
IST with CT	5	3	2	
IST with S	6	1	5	0·14
Death	10	7	3	*0·022
Death cause				
Infection	6	6	0	
CHF	1	0	1	
malignancy	1	0	1	
PE	1	0	1	
unknown cause	1	1	0	*0·04

Dx, diagnosis; ITP, immune thrombocytopenic purpura; SjS, Sjogren syndrome; NHL, non-Hodgkin lymphoma; HL, Hodgkin lymphoma; RCC, renal cell carcinoma; Pros Ca, prostate carcinoma; MDS, myelodysplastic syndrome; MPD, myeloproliferative disease; IST, immunosuppressive therapy; CT, chemotherapy; S, splenectomy; DM, diabetes mellitus; CHF, congestive heart failure; PE, pulmonary thromboembolism; AIHA, autoimmune hemolytic anemia. Statistical significance was defined as a two-sided *P* value of <0·05.

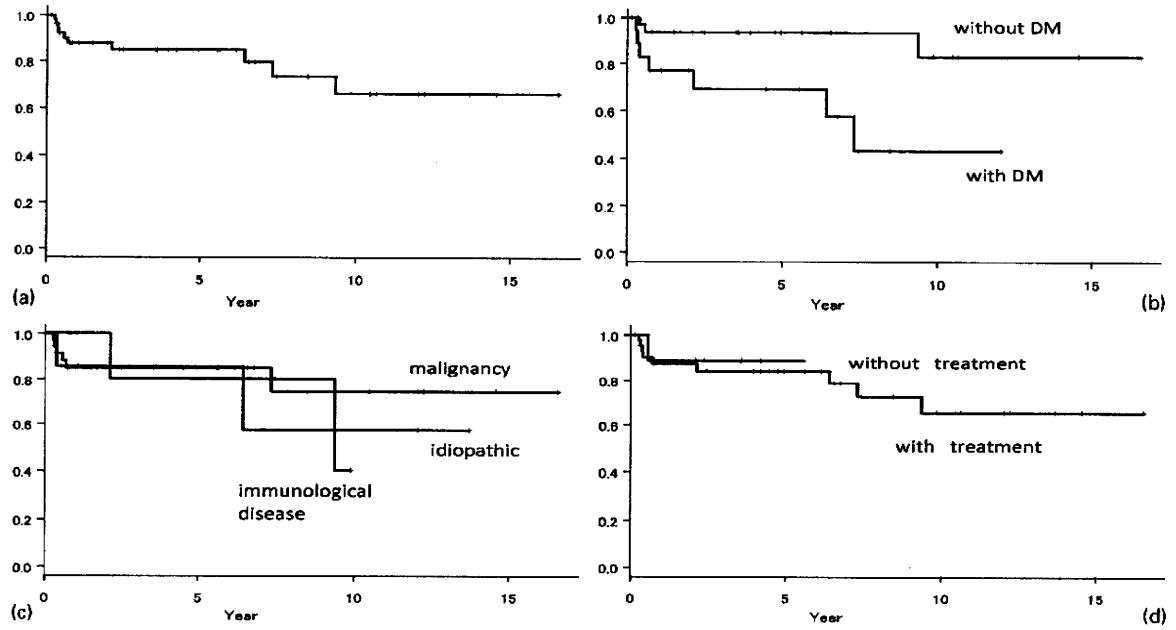

Figure 1 Overall survival *a* of all patients with AIHA, *b* in patients with and without DM, *c* in patients with idiopathic and secondary AIHA and *d* in patients with and without treatment. Statistical significance was defined as a two-sided *P* value of <0·05

had DM, including three patients who developed steroid-induced DM after treatment. Although patients with DM tended to be older, these differences were not statistically significant. Ten patients died during observation (Table 1). The estimated 4-year OS (4y-OS) for all patients was 84·9±5·4% (±standard error) (Fig. 1*a*). Six of the patients died due to infections (2 methicillin-resistant *Staphylococcus aureus*, 1 *Pseudomonas aeruginosa*, 1 aspergillosis, 1 miliary tuberculosis, 1 not identified), and all of them had DM. These six patients were receiving prednisolone (PSL, 5–30 mg/day) at death. The estimated 4y-OS in DM patients was significantly lower than that in patients without DM (69·3±11·7% versus 93·6±4·4%, P=0·0064) (Fig. 1*b*). Patients with idiopathic AIHA, those with AIHA secondary to immunological diseases and those with AIHA secondary to malignancy showed similar 4y-OS values (85·1±6·2%, 85·7±13·2% and 80±17·9%, respectively, P=0·89) (Fig. 1*c*). There was no difference in 4y-OS between patients who received treatment for AIHA and those who did not (84·1±6·1% and 88·9±10·5%, P=0·73) (Fig. 1*d*). The presence of DM was the only significant risk factor for survival in the multivariable analysis (relative risk [RR] 0·239 [95% confidence interval (CI); 0·058–0·932], P=0·039). Four of the seven deaths in the DM group occurred within one year from the diagnosis of AIHA, importantly. The infection incidence was the only significant difference between death and alive among DM patients.

The cumulative incidence of recurrence was similar in those with and without DM (P=0·35, Fig. 2).

With regard to major complications of AIHA, infections, congestive heart failure (CHF) and PE occurred in 15, 8 and 2 patients, respectively. The presence of DM was identified as a risk factor for the incidence of CHF in a multivariable analysis [HR 8·20 (95% CI: 1·24–54·4), P=0·029]. Older age was associated with CHF, with borderline significance [HR 1·09 (95% CI: 0·999–1·20), P=0·054].

Discussion

We retrospectively reviewed consecutive patients with AIHA and identified the presence of DM as an only significant risk factor for survival. In patients with DM, infection occurs more frequently than in the general population.[7] Therefore, the co-existence of DM in an immunocompromised host who receive steroid may further worsen immunological protection against infection. However, after heart transplantation, where recipients received administrations of steroid long time, there were no differences in the incidence of infection and survival between DM and non-DM recipients.[8,9] With regard to other autoimmune disease, there have been very few reports about the association between the prognosis and DM in CTD, such as systemic lupus erythematosus (SLE)

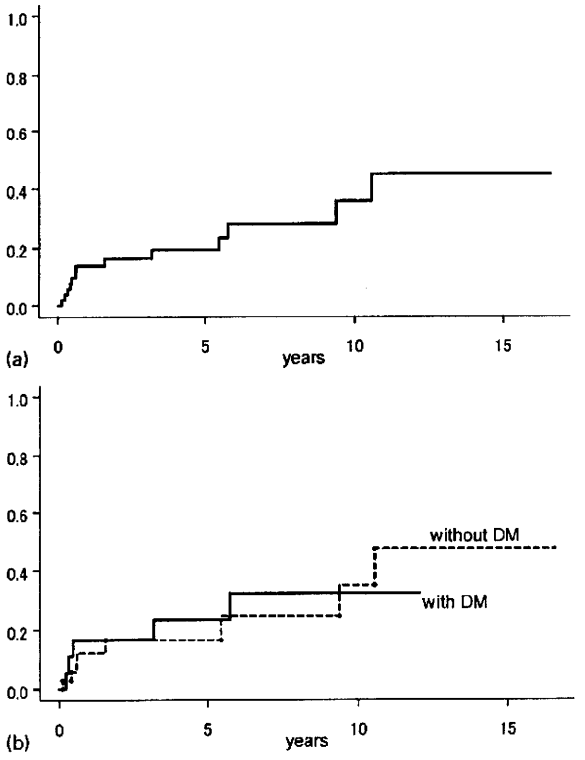

Figure 2 Cumulative incidence of recurrence *a* of all patients and *b* between patients with and without diabetes mellitus

or rheumatoid arthritis (RA), where patients received administrations of steroid long time, too. In patients with SLE, who are thought to be younger than patients with AIHA, DM was associated with a high mortality, although this difference was not significant.[10] In patients with RA, whom are thought to be similar in age to those with AIHA, DM was associated with increased cardiovascular death,[11] while it has not been established whether the presence of DM would increase the frequency of infection.[12] In addition, deaths in CTD seemed to occur exclusively later than in our study population with AIHA.[13,14] Interestingly, the infection-related mortality was increased in SLE patients who had hemolytic anemia.[14] If we take all of these findings into consideration, the high incidence of early fatal infections in the presence of DM in this study might be supposed to be exclusively specific to patients with AIHA. Leukocyte functions such as adherence, chemotaxis, phagocytosis and neutrophil degranulation are thought to be impaired in DM patients.[15–17] In addition, DM induces endothelial activation and dysfunction, which would alter the migration kinetics of T cells, and regulation of the immune reaction.[18–21] Additionally, since von Willebrand factor is known to be increased in AIHA,[22] other adhesion molecules, such as E-selectin, ICAM-1 and VCAM-1, might be increased and activate endothelial cells just as in other intravascular hemolytic anemia.[23] If so, the activation and dysfunction of endothelial cells could strongly worsen the protection against infection in AIHA with DM, although further basic research is needed to verify this hypothesis.

With regard to major complications, the presence of DM and older age was associated with the incidence of CHF. These findings are consistent with data in the general population, where DM and age were risk factors for cardiovascular events. However, a fatal cardiovascular event was observed in only one patient during the observation period in this study (Table 1). Other aspects of endothelial dysfunction in AIHA with DM might also be associated with the cardiovascular events.

In conclusion, DM was shown to be associated with high early-mortality within one year from diagnosis due to fatal infections in this analysis of consecutive patients with AIHA. Therefore, prophylactic antibiotics might be beneficial during treatment in AIHA patients with DM, and this should be evaluated in a prospective trial. Although we focused only on DM in this analysis, a co-morbidity index that included cardiac disease, cerebrovascular disease, obesity, and so on was shown to significantly affect the prognosis of hematopoietic stem cell transplant recipients and elderly patients undergoing chemotherapy for acute myeloid leukemia.[24,25] Therefore, further prospective investigations will be warranted to assess the impact of co-morbidities other than DM on survival in AIHA.

References

1 Hendrick AM. Auto-immune haemolytic anaemia – a high-risk disorder for thromboembolism? *Hematology* 2003; **8**(1): 53–56.
2 Barker RN, Hall AM, Standen GR, Jones J, Elson CJ. Identification of T-cell epitopes on the Rhesus polypeptides in autoimmune hemolytic anemia. *Blood* 1997; **90**(7): 2701–2715.
3 Ward FJ, Hall AM, Cairns LS *et al.* Clonal regulatory T cells specific for a red blood cell autoantigen in human autoimmune hemolytic anemia. *Blood* 2008; **111**(2): 680–687.
4 Allgood JW, Chaplin H, Jr. Idiopathic acquired autoimmune hemolytic anemia. A review of forty-seven cases treated from 1955 through 1965. *Am J Med* 1967; **43**(2): 254–273.
5 Standards of medical care in diabetes – 2008. *Diabetes Care* 2008; **31**(Suppl 1): S12–54.
6 Genuth S, Alberti KG, Bennett P *et al.* Follow-up report on the diagnosis of diabetes mellitus. *Diabetes Care* 2003; **26**(11): 3160–3167.
7 Muller LM, Gorter KJ, Hak E *et al.* Increased risk of common infections in patients with type 1 and type 2 diabetes mellitus. *Clin Infect Dis* 2005; **41**(3): 281–288.
8 Lang CC, Beniaminovitz A, Edwards N, Mancini DM. Morbidity and mortality in diabetic patients following cardiac transplantation. *J Heart Lung Transplant* 2003; **22**(3): 244–249.

9 Livi U, Caforio AL, Grassi G *et al.* Mid-term results of heart transplantation in diabetic patients. *J Cardiovasc Surg (Torino)* 1994; **35**(6 Suppl 1): 115–118.

10 Molad Y, Levin-Iaina N, Vaturi M, Sulkes J, Sagie A. Heart valve calcification in young patients with systemic lupus erythematosus: a window to premature atherosclerotic vascular morbidity and a risk factor for all-cause mortality. *Atherosclerosis* 2006; **185**(2): 406–412.

11 Maradit-Kremers H, Nicola PJ, Crowson CS, Ballman KV, Gabriel SE. Cardiovascular death in rheumatoid arthritis: a population-based study. *Arthritis Rheum* 2005; **52**(3): 722–732.

12 Doran MF, Crowson CS, Pond GR, O'Fallon WM, Gabriel SE. Frequency of infection in patients with rheumatoid arthritis compared with controls: a population-based study. *Arthritis Rheum* 2002; **46**(9): 2287–2293.

13 Blanco FJ, Gomez-Reino JJ, de la Mata J *et al.* Survival analysis of 306 European Spanish patients with systemic lupus erythematosus. *Lupus* 1998; **7**(3): 159–163.

14 Jacobsen S, Petersen J, Ullman S *et al.* A multicentre study of 513 Danish patients with systemic lupus erythematosus. II. Disease mortality and clinical factors of prognostic value. *Clin Rheumatol* 1998; **17**(6): 478–484.

15 Delamaire M, Maugendre D, Moreno M, Le Goff MC, Allannic H, Genetet B. Impaired leucocyte functions in diabetic patients. *Diabet Med* 1997; **14**(1): 29–34.

16 Joshi N, Caputo GM, Weitekamp MR, Karchmer AW. Infections in patients with diabetes mellitus. *N Engl J Med* 1999; **341**(25): 1906–1912.

17 Stegenga ME, van der Crabben SN, Blumer RM *et al.* Hyperglycemia enhances coagulation and reduces neutrophil degranulation, whereas hyperinsulinemia inhibits fibrinolysis during human endotoxemia. *Blood* 2008; **112**(1): 82–89.

18 Leon B, Ardavin C. Monocyte migration to inflamed skin and lymph nodes is differentially controlled by L-selectin and PSGL-1. *Blood* 2008; **111**(6): 3126–3130.

19 McGettrick HM, Hunter K, Moss PA, Buckley CD, Rainger GE, Nash GB. Direct observations of the kinetics of migrating T cells suggest active retention by endothelial cells with continual bidirectional migration. *J Leukoc Biol* 2009; **85**: 98–107.

20 Ou R, Zhang M, Huang L, Flavell RA, Koni PA, Moskophidis D. Regulation of immune response and inflammatory reactions against viral infection by VCAM-1. *J Virol* 2008; **82**(6): 2952–2965.

21 Tepper OM, Galiano RD, Capla JM *et al.* Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 2002; **106**(22): 2781–2786.

22 Nilsson TK, Sondell KM, Norberg BO, Back O. Plasma levels of endothelial-derived haemostatic factors in autoimmune thrombocytopenia and haemolytic anaemia. *J Intern Med* 1990; **228**(3): 249–252.

23 Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. *JAMA* 2005; **293**(13): 1653–1662.

24 Giles FJ, Borthakur G, Ravandi F *et al.* The haematopoietic cell transplantation comorbidity index score is predictive of early death and survival in patients over 60 years of age receiving induction therapy for acute myeloid leukaemia. *Br J Haematol* 2007; **136**(4): 624–627.

25 Sorror ML, Maris MB, Storb R *et al.* Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. *Blood* 2005; **106**(8): 2912–2919.

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Letter to the Editor

CD8$^+$ memory T cells predominate over naïve T cells in therapy-free CML patients with sustained major molecular response

To the Editor,

While imatinib mesylate (IM) is currently the gold standard for treating patients with chronic myeloid leukemia (CML) in the chronic phase (CP), the leukemia-initiating stem cells of CML are resistant to IM and the majority of patients who stop it after achieving a complete molecular response (CMR) are reported to relapse [1–3], suggesting that CML is unlikely to be cured by IM monotherapy. However, recent data indicate that CMR can be sustained after discontinuation of IM, particularly in patients with a history of prolonged interferon-alpha (IFN) therapy [4]. We analyzed peripheral blood lymphocyte subsets in nine CML patients who discontinued therapy after showing a major molecular response (MMR) for more than 2 years: four patients relapsed and the remaining five patients remain in MMR without treatment.

Table 1
Peripheral blood lymphocyte subsets in nine CML patients who discontinued therapy after showing MMR for more than 2 years.

	Lymphocyte (/μl)	CD3$^+$ cells (/μl)	CD4$^+$ cells (/μl)	CD8$^+$ cells (/μl)	CD4/CD8 ratio	CD56$^+$ cells (/μl)	RO$^+$/RA$^-$/CD8 cells (/μl)	RO$^-$/RA$^+$/CD8 cells (/μl)	RO$^+$/RA$^+$ (CD8) ratio
Sustained MMR (n=5)	1530 ± 578	797 ± 187	434 ± 100	296 ± 143	1.69 ± 0.763	321 ± 202	115 ± 55.7	124 ± 123	1.74 ± 1.16* (p=0.026)
Relapse (n=4)	1490 ± 538	848 ± 300	520 ± 199	257 ± 111	2.28 ± 1.22	243 ± 151	57.9 ± 33.9	144 ± 57.3	0.46 ± 0.27
Healthy volunteers (n=6)		1070 ± 90.6	636 ± 134	381 ± 103	1.86 ± 0.947	142 ± 160	66.6 ± 34.7	138 ± 41.9	0.49 ± 0.35

Each value is shown in mean ± standard deviation.
* Statistically significant difference between sustained CCyR cases and healthy volunteers.

The median age of the patients was 49 years (35–72) and seven were men. MMR has been sustained, respectively, for 108+, 54+, 24+, 14+, and 6+ months after stopping therapy in five patients including one in CMR. All five patients with sustained therapy-free MMR had received IFN before discontinuation of treatment and two of them had also received IM. Three patients had a low Sokal score and two had an intermediate score. Four other patients with a low Sokal score relapsed at, 1, 5, 5, and 36 months, respectively, after discontinuation of therapy (IM alone in two, IFN alone in one, and IM + IFN in one). All of the patients with relapse resumed IM and achieved MMR within 6 months. The reasons for discontinuation of therapy included adverse effects ($n=1$), poor compliance ($n=1$), patient's choice ($n=3$), and the possibility that IFN might be unnecessary after stable MMR had been achieved according to previous reports [5,6] ($n=4$).

Flow cytometric analysis of peripheral blood lymphocytes showed that the ratio of CD45RO$^+$/RA$^-$ (memory) to CD45RO$^-$/RA$^+$ (naïve) CD8$^+$ T cells was significantly higher in the patients with sustained therapy-free MMR (1.74 ± 1.16) than in normal volunteers ($n=6$; 0.49 ± 0.35; $p=0.026$). It was also higher than in relapsed patients (0.46 ± 0.27), but there was no significant difference (possibly due to the small sample size). CD8$^+$ memory T cell numbers were higher in sustained MMR patients (115 ± 55.7/μl) than in relapsed patients and normal volunteers (57.9 ± 33.9 and 66.6 ± 34.7/μl, respectively) without a significant difference, and there was little difference of CD8$^+$ naïve T cells among the three groups (124 ± 123, 144 ± 57.3, and 138 ± 41.9/μl, respectively). There were no significant differences of the lymphocyte count, CD3$^+$ cells, CD4$^+$ T cells, CD3$^-$CD56$^+$ cells, or CD4/CD8 ratio among the three groups (Table 1).

In the patients with sustained therapy-free MMR, CD8$^+$ memory T cells showed significant predominance over naïve T cells, suggesting that effector memory T cells specific for the Philadelphia chromosome (Ph)-positive clone may be involved in maintaining MMR. It is well known that Ph-positive leukemia cells express a number of tumor-associated antigens that provoke a cytotoxic T cell (CTL) response, including PR1, WT1, BCR-ABL, and PRAME. It would be interesting to examine the CTL response to these antigens in our patients. Similar to previous reports [4–6], all of the patients in this series who remain in therapy-free MMR had previously received IFN, implying that IFN has a role in the induction of the CTL response. Since IM induces MMR much more effectively than IFN and Carella [7] recently reported that molecular response could be maintained by IFN after discontinuation of IM, our findings suggest that a clinical trial is warranted to evaluate the efficacy of IFN maintenance therapy for CML-CP after IM-induced MMR.

Conflicts of interest

The authors indicated no potential conflicts of interest.

Acknowledgements

Contributions. Conception and design of the study, or acquisition of data: Kensuke Usuki, Kazuaki Yokoyama, Tokiko Nagamura-Inoue, Ayumu Ito, Michiko Kida, Koji Izutsu, Akio Urabe, and Arinobu Tojo. Drafting the article or revising it: Kensuke Usuki,

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References

[1] Cortes J, O'Brien S, Kantarjian H. Discontinuation of imatinib therapy after achieving a molecular response. Blood 2004;104:2204–5.
[2] Mauro MJ, Druker BJ, Maziarz RT. Divergent clinical outcome in two CML patients who discontinued imatinib therapy after achieving a molecular remission. Leukemia Res 2004;28(Suppl 1):S71–3.
[3] Usuki K, Iijima K, Iki S, Urabe A. CML cytogenetic relapse after cessation of imatinib therapy. Leukemia Res 2005;29:237–8.
[4] Rousselot P, Huguet F, Rea D, Legros L, Cayuela JM, Maarek O, et al. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. Blood 2007;109:58–60.
[5] Bonifazi F, de Vivo A, Rosti G, Guilhot F, Guilhot J, Trabacchi E, et al. Chronic myeloid leukemia and interferon-alpha: a study of complete cytogenetic responders. Blood 2001;98(10):3074–81.
[6] Mahon FX, Delbrel X, Cony-Makhoul P, Fabères C, Boiron JM, Barthe C, et al. Follow-up of complete cytogenetic remission in patients with chronic myeloid leukemia after cessation of interferon alfa. J Clin Oncol 2002;20:214–20.
[7] Carella AM. Interferon-α is able to maintain complete molecular remission induced by imatinib after its discontinuation. Leukemia 2008;22:1090–1.

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Gain-of-function mutations and copy number increases of Notch2 in diffuse large B-cell lymphoma

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Signaling through the Notch1 receptor has a pivotal role in early thymocyte development. Gain of Notch1 function results in the development of T-cell acute lymphoblastic leukemia in a number of mouse experimental models, and activating *Notch1* mutations deregulate Notch1 signaling in the majority of human T-cell acute lymphoblastic leukemias. *Notch2*, another member of the *Notch* gene family, is preferentially expressed in mature B cells and is essential for marginal zone B-cell generation. Here, we report that 5 of 63 (~8%) diffuse large B-cell lymphomas, a subtype of mature B-cell lymphomas, have *Notch2* mutations. These mutations lead to partial or complete deletion of the proline-, glutamic acid-, serine- and threonine-rich (PEST) domain, or a single amino acid substitution at the C-terminus of Notch2 protein. Furthermore, high-density oligonucleotide microarray analysis revealed that some diffuse large B-cell lymphoma cases also have increased copies of the mutated *Notch2* allele. In the Notch activation-sensitive luciferase reporter assay *in vitro*, mutant Notch2 receptors show increased activity compared with wild-type Notch2. These findings implicate *Notch2* gain-of-function mutations in the pathogenesis of a subset of B-cell lymphomas, and suggest broader roles for *Notch* gene mutations in human cancers. (*Cancer Sci* 2009; 100: 920–926)

Signaling through the Notch receptor, triggered by the binding of ligands expressed on neighboring cells, has a key role in determining cell fate in a variety of cell lineages, including lymphocytes.[1,2] In mammals, there are four *Notch* genes that encode structurally similar single-pass and heterodimeric transmembrane receptors. Ligand binding initiates a series of intramolecular cleavages, which eventually liberates the intracellular domain of the transmembrane subunit of the intracellular Notch receptor (ICN). The ICN is then translocated to the nucleus and creates a transcriptional activating complex with RBP-Jκ, a constitutive DNA binding protein. During these processes, Notch proteins are intricately regulated by glycosylation, endocytosis, recycling, phosphorylation, and ubiquitylation before and after ICN liberation. Many of these regulatory processes appear to modify the biologic activity of Notch.[3] Notably, polyubiquitylation-based degradation is dependent on the proline-, glutamic acid-, serine- and threonine-rich (PEST) domain, located at the C-terminus of the Notch protein.

The physiologic roles of *Notch1* and *Notch2* have been clarified in mouse models, particularly in the lymphoid system. *Notch1* is preferentially expressed in immature T cells and is essential for specification of early hematopoietic progenitors toward the T cell fate and for early T cell development in the thymus.[4] In contrast, *Notch2* is preferentially expressed in mature B cells and is required for the generation of a mature B-cell subset, known as splenic marginal zone B (MZB) cells in mice.[5] *Notch1* was originally identified as a transforming gene in human T-cell acute lymphoblastic leukemia (T-ALL) cells harboring the t(7;9)(q34;q34) chromosomal translocation.[6] The N-terminal truncated form of Notch1 expressed in this type of T-ALL cell can induce the development of T-ALL when expressed in bone marrow cells that are then transplanted into recipient mice.[7] Importantly, more than 50% of childhood and 30–40% of adult human T-ALL cases carry *Notch1* mutations that lead to deregulated activation of Notch signaling,[8–11] indicating that accelerated Notch signaling contributes to the development of human neoplasms.

Two regions of the *Notch1* gene are major targets of oncogenic mutations in T-ALL. Missense, insertion, and deletion mutations in the heterodimerization domains are thought to decrease the stability of the dimer, consisting of the extracellular and transmembrane subunits, which results in the progression of Notch1 cleavage without ligand stimulation.[8,12] The other series of mutations accumulate in the PEST domain and its N-terminally flanking transactivation domain. All of these mutations cause partial or complete deletion of the PEST domain, considered to result in the prolonged half-life of Notch1 ICN, because the PEST domain is responsible for polyubiquitylation-based degradation of ICN.[13]

These lines of information about *Notch* genes led us to examine the possibility that deregulation of Notch2 signaling is involved in the development of mature B-cell lymphomas. We screened *Notch2* gene mutations at the heterodimerization and PEST domains in 109 B-cell lymphoma samples, and found mutations in five samples, all of which were diffuse large B-cell lymphomas (DLBCL). Interestingly, two of the five samples had an increased copy number of the mutated *Notch2* allele, and in another sample of the five, the total copy number of the *Notch2* allele was increased. Furthermore, we confirmed that the mutation-carrying Notch2 receptors had increased activity when stimulated by a ligand *in vitro*. We postulate that gain-of-function mutations of *Notch2* are involved in the pathogenesis of a subset of DLBCL.

Materials and Methods

Patient materials and genomic DNA preparation. Patients ($n = 109$) with various B-cell lymphomas were enrolled in the study after informed consent was obtained. The study design was approved by the ethics committees of the University of Tokyo (Tokyo, Japan) and Aichi Cancer Center (Nagoya, Japan). Genomic DNA

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was extracted from cryopreserved samples using a commercial kit (Puregene; Gentra Systems, Minneapolis, MN, USA).

Polymerase chain reaction–single-stranded conformational polymorphism (PCR-SSCP). Based on the information of *Notch1* mutations in T-ALL and the high similarity between *Notch1* and *Notch2* genes, we confined our mutation analysis to exons 26, 27, and 34 of *Notch2* that correspond to the heterodimerization domains (exons 26 and 27) and the C-terminal region containing the transactivation and PEST domains (exon 34). Oligonucleotide primers designed to amplify whole exon 26 and exon 27, and five divided portions of exon 34 are listed in the Supporting Information (Table S1). The ^{32}P-labeled PCR product was subjected to SSCP analysis as described in published reports.[14] In brief, the PCR mixture was heated at 80°C and applied to 5% polyacrylamide gel containing 10% glycerol. After 2–4 h electrophoresis with cooling, the gel was dried on filter paper and exposed to X-ray film. The PCR products were directly sequenced or bands with aberrant migration were excised from the gel and subjected to direct sequencing when indicated.

High-density oligonucleotide microarray analysis. Genome-wide copy number detection analysis was carried out as described previously.[15] In brief, Affymetrix GeneChip Mapping 100K high-density oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA) were used and the data were analyzed using the CNAG algorithm (Version 2.0. Genome Laboratory, University of Tokyo Hospital, Tokyo, Japan).

Fluorescence *in situ* hybridization. Bacterial artificial chromosome (BAC) clones RP11–723d17 (*Notch2*) and RP11–80d6 (1q23.3) were used to evaluate the copy number of the *Notch2* gene. BACs were obtained from the BAC/PAC Resource Center (Children's Hospital, Oakland, CA, USA). Fluorescence *in situ* hybridization experiments on interphase nuclei were carried out as described previously.[16]

Quantitative real-time PCR for genomic DNA. For the copy number evaluation of the *Notch2* gene by quantitative real-time PCR, genomic DNA was extracted from: samples L8 and W121672; a stomach cancer cell line (MKN45) that had a copy number loss at the *Notch2* (1p13) locus (data from microarray analysis not shown); and normal peripheral blood mononuclear cells. The *Notch2* gene dosage was measured using the primers: forward, TTCCCCAAGTGAGAGACATTT; and reverse, CAGACACTT-CACAGAACAGAA, and normalized by the relative DNA quantities measured by real-time PCR using the control locus (2q35) primers: forward, TGGCTGATGAACTTTTGCAC; and reverse, AGCGGTTGAGGTCTGTGAAC. Student's *t*-test was used for the statistical analysis.

Immunohistochemistry. Tissue sections were mounted on silanated slides, deparaffinized with xylene, rehydrated with a series of graded ethanols, processed with an autoclave in 10 mmol/L citrate buffer for 5 min, pH 6.0, treated with horse serum albumin to block non-specific staining, and immunostained. The detection of antibody binding was visualized by the avidin–biotin complex method using diaminobenzidine as the chromogen. The sections were counterstained with hematoxylin.

Plasmid preparation. In the human full-length Notch2 cDNA (wtN2) (a gift from S. Artavanis-Tsakonas, Harvard University, Cambridge, MA, USA), the stop codon corresponding to the nonsense mutation (7454 C/T), the single-base deletion mutation corresponding to 7120Del, and the point mutation corresponding to 7614 G/A were introduced. Mutant primers were used for PCR and the resulting products were sequenced and used to replace the corresponding fragment of wtN2 cDNA to create Notch2 with the nonsense mutation and the R2453Q mutation (nsmN2, delstN2, and rqN2, respectively). These cDNAs were inserted in pTracerCMV (Invitrogen, Carlsbad, CA, USA).

Establishment of CHO(r) cells stably expressing wild-type and mutant human Notch2. CHO(r) cells were transfected with pTracerCMV/wtN2, pTracerCMV/nsmN2, pTracerCMV/delstN2,

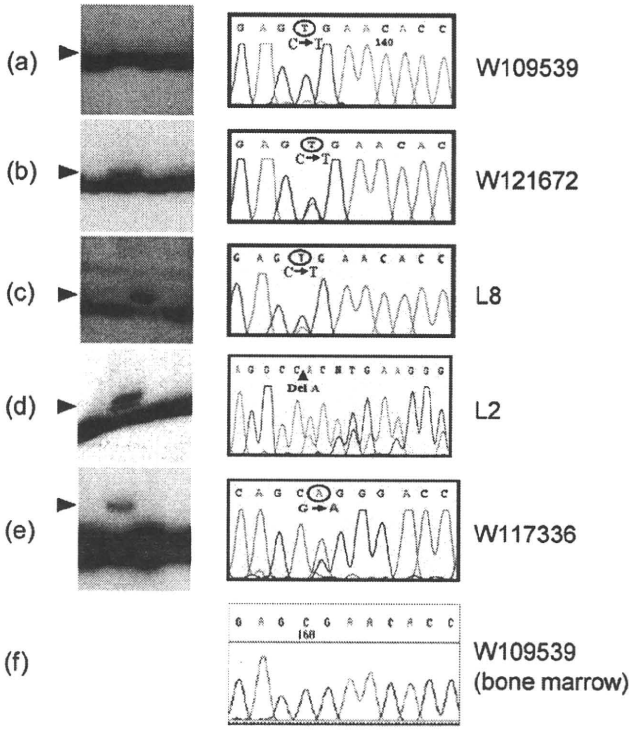

Fig. 1. Mutations of the *Notch2* gene in diffuse large B-cell lymphomas. Polymerase chain reaction–single-stranded conformational polymorphism and sequence analyses for samples having the nonsense mutation at 7454, C/T (W109539, W121672 and L8). Arrowheads indicate shifted bands. The shifted bands in (a) and (c) are obviously dominant against the normal band, suggesting the small amount of normal tissue contamination and unbalanced ratio of mutant and normal alleles. Those in (b) and (e) are minor compared with the normal band, suggesting the contamination of normal tissues, and those in (c) are comparable with the normal band. The shifted bands were excised from the gel and the extracted DNA was sequenced for samples W121672 and W117336. (f) Sequence of DNA prepared from the bone marrow cells obtained from the patient W109539.

and pTracerCMV/rqN2, and selected for zeocin (400 μg/mL) resistance. The resulting zeocin-resistant cells were single-cell sorted using the antihuman Notch2 monoclonal antibody (mAb). The antihuman Notch2 (MHN2-25, mouse IgG_{2a}) mAb was generated by immunizing BALB/c mice with human Notch2-Fc (the Fc portion of human IgG_1 was fused to the 22nd epidermal growth factor repeat of the extracellular region of human Notch2) and screening hybridomas producing mAbs specific for Notch2-Fc by enzyme-linked immunosorbent assay. MHN2-25 reacted with CHO(r) cells expressing human Notch2, as indicated by flow cytometry (Supporting Information Fig. S1).

Western blot analysis. Immunoblotting was carried out as described previously.[17] In brief, 1×10^6 wtN2/CHO(r), nsmN2/CHO(r), delstN2/CHO(r), and rqN2/CHO(r) cells were solubilized in 0.1 mL lysis buffer containing 1% NP-40, electrophoresed in 7.5% sodium dodecylsulfate polyacrylamide gel, transferred onto Immobilon-P membrane (Millipore, Billerica, MA, USA). It was then probed with a mAb recognizing the intracellular domain of human and murine Notch2 (C651.6DbHN; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) and an alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI, USA).

Transcriptional activation assay. The luciferase assay was carried out as described previously.[17] In brief, 2×10^5 CHO(r) cells expressing wtN2, Notch2 with truncatation at 2400 (nsmN2),

Table 1. *Notch2* mutational status in five patients with diffuse large B-cell lymphoma

Sample	Nucleic acid change	Amino acid change	Copy number	Immunohistochemistry		
				CD10	BCL6	MUM-1
W109539	7454 C/T	2400 Stop	Multiple	–	+	+
W121672	7454 C/T	2400 Stop	3	–	+	+
L8	7454 C/T	2400 Stop	2†	–	+	+
L2	7120 Del A	2288PLKGSTStop	NA	–	+	+
W117336	7614 G/A	2453 R/Q	2	–	+	+

†Uniparental disomy for the mutated *Notch2* allele is indicated. NA, information not available.

Table 2. Characteristics of five patients with diffuse large B-cell lymphoma who had *Notch2* mutations

Patient	Age/sex	CS/IPI	Treatment/Response	Survival	Others
W109539	64/M	IIIA/LI	R-CHOP/CRu/relapse	1.6 y (d1)	Acromegaly, DM, AAA (postoperation)
W121672	71/M	NA	NA	NA	–
L8	66/M	IVA/NA	NA	NA	–
L2	61/F	IV/NA	CHOP/CR	7 y (alive)	BCL2 rearrangement
W117336	83/F	IIIA/LI	RT, CHOP	0.3 y (d2)	–

AAA, abdominal aortic aneurysm; CHOP, cyclophosphamide, adriamycin, vincristine and predonisolon; CR, complete remission; CRu, complete remission uncertain; CS, clinical stage; d1, died of advanced lymphoma; d2, died of advanced lymphoma after first chemotherapy; DM, diabetes mellitus; F, female; IPI, international prognostic index; LI, low intermediate; M, male; NA, information not available; R-CHOP, rituximab plus CHOP, with 4-0-tetrahydropyranyl-adriamycin instead of adriamycin, four courses; RT, radiation therapy; y, years.

Notch2 with truncation after 6 amino acids insertion at 2288 (delstN2), and Notch2 with an R2453Q mutation (rqN2) were inoculoated in a 6-well dish and the next day transfected with the pGa981-6 luciferase reporter plasmid (2 μg) using the Superfect transfection reagent (Qiagen, Hilden, Germany). The β-galactosidase-expressing plasmid, pCMV/β-Gal (0.2 μg) was cotransfected when indicated. The cells were harvested after 3 h, suspended in 3 mL medium, and a 200 μL aliquot was replated in a 48-well dish coated with soluble human Delta1 (Delta1-Fc, a chimeric protein composed of the extracellular domain of human Delta1 and the Fc portion of human IgG,[18,19] a gift from S. Sakano, Asahi Kasei, Tokyo, Japan). After 24 h incubation, the cellular extracts were used to measure luciferase and, when applied, β-galactosidase activities. Two independent clones were used to compare the luciferase activity of each Notch2 protein and bulk transfectants were used to evaluate the effect of N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Calbiochem, San Diego, CA, US), a γ-secretase inhibitor.

Results

***Notch2* gene is mutated in a subset of DLBCL.** *Notch2* gene mutations were screened in 109 B-cell lymphoma samples, including 63 DLBCLs, 18 follicular lymphomas, and 28 MZB-cell lymphomas or mucosa-associated lymphoid tissue lymphomas. Exons 26 and 27, encoding the N- and C-terminal heterodimerization domains, and a portion of exon 34, encoding the PEST domain and its bilateral flanking regions, were amplified by PCR using genomic DNA with the primers listed in the Supporting Information (Table S1) and examined for mutations using the PCR-SSCP method.[20]

Five distinct nucleotide changes were detected in 11 of the 109 B-cell lymphoma samples, exclusively in exon 34. Whereas two of the five changes detected in 6 of the 11 samples were single nucleotide polymorphisms (SNP) without amino acid changes, the other three nucleotide changes detected in the remaining 5 samples (Fig. 1a–e) were thought to represent somatic mutations resulting in premature truncation or single amino acid substitution (Table 1). A nonsense mutation, C to T at nucleotide 7454 (based on the published human *Notch2* sequence, NM_024408), in three cases (Fig. 1a–c) and a single-base deletion at position 7120 in another case (Fig. 1d), led to premature truncation of the Notch2 protein (Table 1). These Notch2 proteins lacked a part or the entire region of the PEST domain. The other single nucleotide change, G to A at 7614, resulted in the replacement of arginine with glutamine on the C-terminal side of the PEST domain (Fig. 1e and Table 1). The G7614A change is not listed in the public SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/; as of October 23, 2007). In addition, the dose of the mutant A allele was unbalanced relative to the wild-type G allele (Fig. 1e), further decreasing the possibility of an SNP. Constitutive DNA was available in one case (W109539) and was confirmed to be the wild-type sequence (Fig. 1f), which definitely concluded that the mutation in the tumor was of somatic origin. Clinical information of the five patients is summarized in Table 2.

Mutation-carrying cases show same expression pattern of CD10, BCL6, and MUM-1. All five cases with *Notch2* mutations were diagnosed as DLBCL, and were uniformly immunohistochemically negative for CD10 and positive for BCL6 and MUM-1 (Fig. 2). We have reviewed 24 DLBCL subjects without *Notch2* mutations for expression of CD10, BCL6 and MUM-1. The immunohistochemistry study revealed that CD10, BCL6, and MUM-1 were positive in 4, 19, and 16 subjects, respectively. Among these, the CD10-negative, BCL6-positive, and MUM-1-positive staining pattern was seen in 10 (data not shown). Thus, this pattern was seen in five out of five *Notch2* mutation-carrying subjects and 10 out of 24 *Notch2* mutation-negative subjects, making the comparison statistically significant ($P = 0.042$; Fisher's exact test). This estimation is consistent with the previous report[21] and indicates that CD10-negative, BCL6-positive, and MUM-1-positive DLBCL might represent a fraction of non-germinal center B-cell-like (non-GCB)-DLBCL, according to the immunohistochemistry-based DLBCL subclassification.[21] DLBCL cases carrying the gain-of-function type *Notch2* mutations, thus, might constitute a discrete subset of non-GCB-DLBCL.

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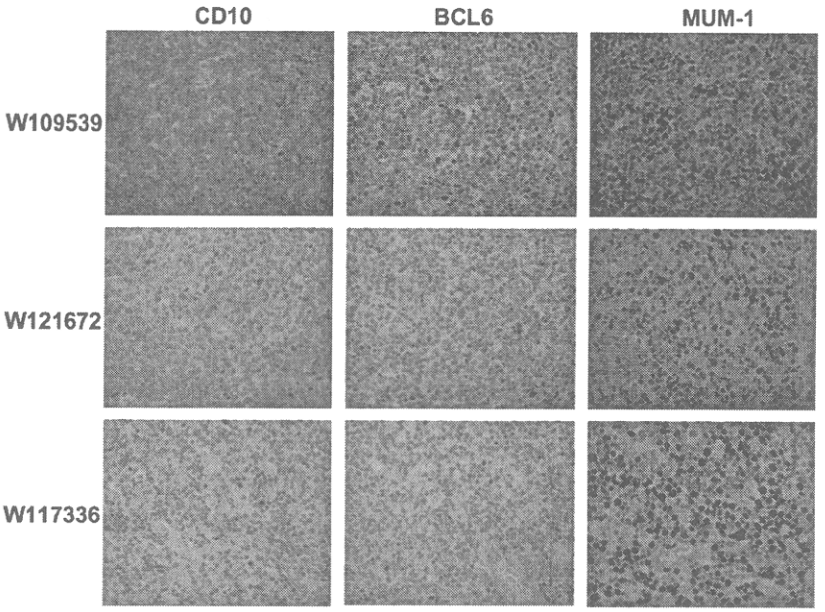

Fig. 2. Immunohistochemical staining of lymphoma specimens for CD10, BCL6, and MUM-1. Antibodies used were anti-CD10 monoclonal antibody (mAb) (56C6; Novocastra, Norwell, MA, USA), anti-BCL6 mAb (P1F6; Novocastra), and antihuman MUM-1 mAb (MUM1p; Dako, Glostrup, Denmark). The detection of antibody binding was visualized by the avidin–biotin complex method using diaminobenzidine as the chromogen. An Elipse 80i microscope was used (Nikon, Tokyo, Japan); original magnification, × 200. Camera, Dxm1200F (Nikon). Acquisition software, Act-1 (Nikon).

Some mutation-carrying samples have increased copy number of mutated Notch2 allele. Of particular interest is the fact that some oncogenic mutations are associated with increases in DNA copy number.[22,23] A high-density oligonucleotide microarray analysis[15] was carried out for 35 of 63 DLBCL samples in the current cohort to evaluate genome-wide copy number alterations. This analysis revealed an increased copy number of the *Notch2* allele in two samples, both of which carried the nonsense mutation. The other 33 samples did not show *Notch2* copy number alterations. In one sample (W109539), amplification of the *Notch2* locus in chromosome 1p was indicated by microarray (Fig. 3a, left panel) and fluorescence *in situ* hybridization (Fig. 3b) analyses. An allele-specific copy number detection analysis revealed an increase in the copy number of a single *Notch2* allele (Fig. 3a, left panel). This allele must correspond to the allele carrying the mutated *Notch2* gene because the mutated band was overwhelmingly dominant in the PCR-SSCP analysis (Fig. 1a). In the other sample (W121672) with a *Notch2* copy number increase, the genomic region encompassing the *Notch2* locus on chromosome 1p through the telomere of chromosome 1q had three copies, whereas most of the 1p region had only one copy (Fig. 3a, right panel). The *Notch2* copy number increase was confirmed by a quantitative real-time PCR analysis (Fig. 3c). We were unable to determine whether the third *Notch2* allele contained wild-type or mutant *Notch2* in this sample. In the third sample carrying the nonsense mutation (L8), a change in the *Notch2* copy number was not detected in the microarray analysis (data not shown) and quantitative PCR analysis revealed that the copy number was normal (Fig. 3c). Both *Notch2* alleles in this sample, however, were likely to have the nonsense mutation, thus representing uniparental disomy, losing the wild-type *Notch2*, because the mutant band was overwhelmingly dominant in the PCR-SSCP analysis (Fig. 1c). Taken together, these findings indicate that some DLBCL samples have *Notch2* mutations and an increased copy number of the mutated *Notch2* gene.

***Notch2* receptors with mutations have increased activity *in vitro*.** To investigate the function of the Notch2 receptors encoded by mRNA with the nonsense mutation (nsmN2), the single-base deletion mutation (delstN2), and missense mutation (rqN2), we established CHO(r) cell lines[17] expressing wild-type Notch2, nsmN2, delstN2, and rqN2 [wtN2/CHO(r), nsmN2/CHO(r), delstN2/CHO(r), and rqN2/CHO(r)] and obtained independent clones expressing each Notch2 protein at similar levels, using fluorescence-activated cell sorting with human Notch2-specific antibody (Fig. 4a; Supporting Information Fig. S1). A Western blot analysis showed that the expected sizes of the transmembrane subunit species were expressed at comparable levels (Fig. 4b). In a Notch-sensitive luciferase reporter assay,[24] the luciferase activity was significantly increased in nsmN2/CHO(r), delstN2/CHO(r), and rqN2/CHO(r) cells, compared with that in wtN2/CHO(r) cells when stimulated with Delta1-Fc. Basal luciferase activities with control IgG also tended to be higher in the three mutant Notch2-expressing CHO(r) cells lines than in wtN2/CHO(r) (Fig. 4c). These results indicated that all three kinds of mutation-carrying Notch2 had significantly increased levels of transcriptional activity compared with wtN2, irrespective of the strength of the Delta1 stimulation.

To evaluate the effect of γ-secretase inhibitor on wtN2 and nsmN2, we added graded concentrations of DAPT to the Delta1-Fc-stimulated bulk wtN2/CHO(r) and nsmN2/CHO(r). The elevated luciferase activity was reproducible with the bulk nsmN2/CHO(r), which was reduced by DAPT in a concentration-dependent manner (Fig. 4d). The luciferase levels of both wtN2/CHO(r) and nsmN2/CHO(r) at 3 μM DAPT in the presence of Delta1-Fc were below those in the presence of control IgG without DAPT, implying spontaneous Notch2 activity with only IgG in the culture system. The results also indicate that increased Notch2 activity by the PEST domain deletion is still dependent on γ-secretase activity.

Discussion

The results of the present study showed gain-of-function mutations of *Notch2* and increased copy numbers of the mutated *Notch2* allele in a subset of DLBCL. Both nonsense mutations and single-base deletion mutations that we found in *Notch2* cause partial or complete deletion of the Notch2 PEST domain. Given the marked structural similarities between Notch1 and Notch2, these mutations are thought to prolong the half-life of Notch2 ICN. In some T-ALL cell lines, both heterodimerization and PEST domain mutations lie in *cis* in the same *Notch1* allele. The reporter transcriptional activity of Notch1 with these double mutations was remarkably higher than that of wild-type Notch1 and Notch1 with a single mutation at either the heterodimerization

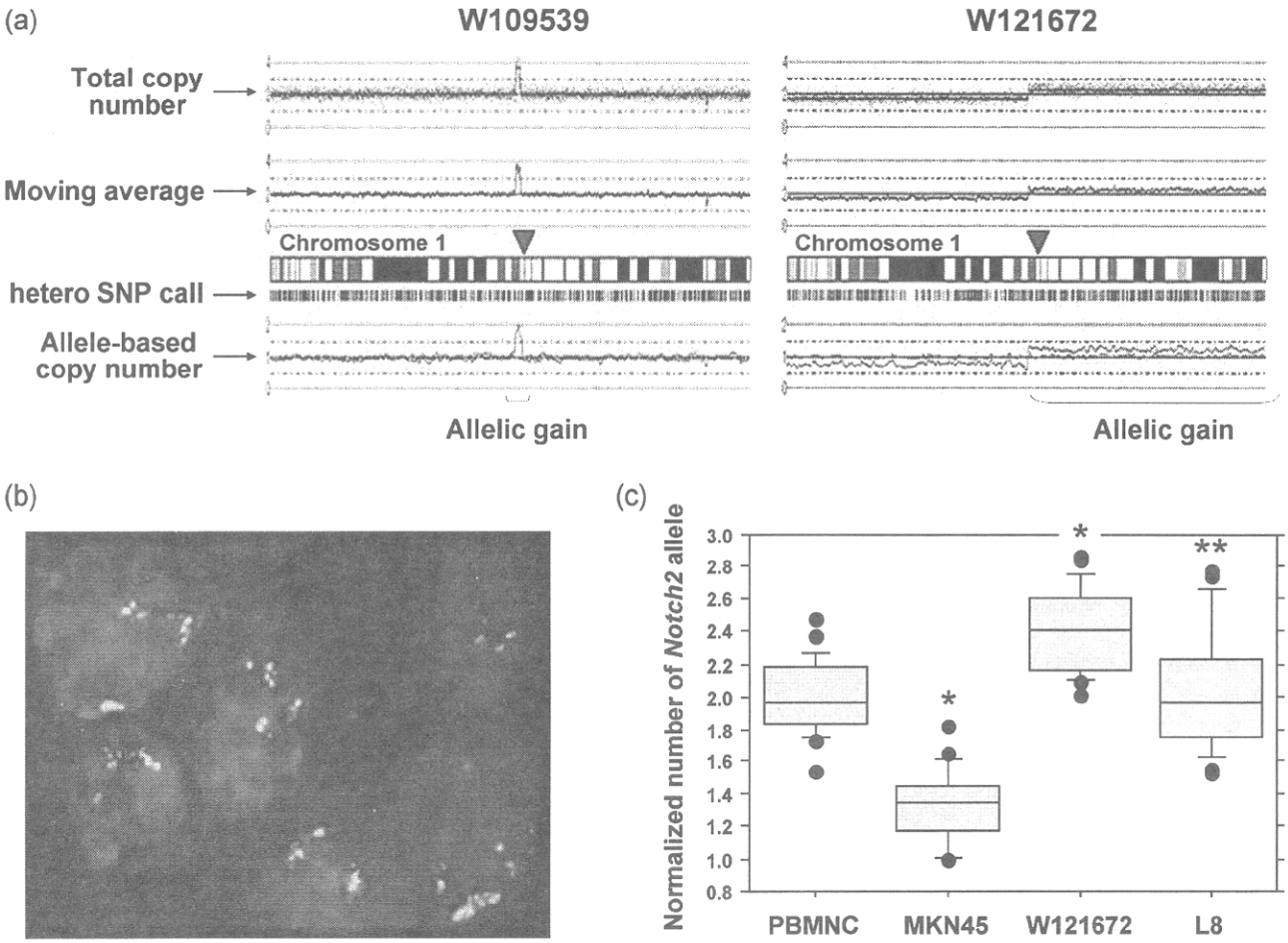

Fig. 3. Copy number increases of mutated Notch2 allele in diffuse large B-cell lymphomas. (a) High-density oligonucleotide microarray analysis using the CNAG program (CREST, Japan Science and Technology Agency, Tokyo, Japan) for samples W109539 and W121672. The copy number of the *Notch2*-encompassing allele is greatly increased in W109539 and mildly increased in W121672. Red arrow, centromere. hetero, heterozygous; SNP, single nucleotide polymorphism. (b) Fluorescence *in situ* hybridization analysis for sample W109539 using probes corresponding to *Notch2* (green signals) and a reference sequence on 1q23.3 (red signals). (c) Copy number evaluation of the *Notch2* gene by quantitative real-time polymerase chain reaction for samples L8 and W121672. The quantity of genomic DNA, extracted from samples L8 and W121672, MKN45 [a stomach cancer cell line having a copy number loss at the *Notch2* (1p13) locus], and normal peripheral blood mononuclear cells (PBMNC), was normalized by real-time reverse transcription–polymerase chain reaction for the control locus (2q35). Statistical analysis (Student's *t*-test) showed that the *Notch2* gene dose was unchanged in sample L8, and significantly increased in sample W121782, relative to the *Notch2* gene dose in the PBMNC, whose mean level was adjusted to two copies. The number of samples was 24 in each arm. $*P < 0.0001$; $**P = 0.79$.

or PEST domain in the absence of exogenous ligand stimulation. The activity of Notch1 with a PEST domain deletion mutation alone was only marginally higher than that of wild-type Notch1[8]. We did not detect mutations in either heterodimerization domain of Notch2 in the current cohort. It might be possible to identify those mutations if the number of samples is increased. With the PEST domain deletion alone, however, nsmN2 had a highly significant increase in activity compared with wtN2. Thus, there appears to be some disagreement between the effects of Notch1 PEST domain deletion and Notch2 PEST domain deletion, although difference in the experimental systems used in the two studies might cause such apparent disagreement. It remains to be determined whether similar mutations found in Notch1 and Notch2 have different biochemical and biologic significance.

The activity was also increased in rqN2, which has the 2453R/Q single amino acid substitution. This amino acid is located on the C-terminal side of the PEST domain, and it is not known whether this change affects the structure or function of the PEST domain. Nevertheless, as the arginine residue is often a target of protein modification such as methylation,[25,26] this amino acid change might convey a significant alteration in the protein function and be involved in lymphomagenesis.

There are other examples of copy number increases associated with oncogenic gene alterations, such as double Philadelphia chromosomes (*BCR/ABL* copy number increase) in the blastic crisis of chronic myelogenous leukemia[27] and homozygous *JAK2* mutations in polycythemia vera,[22,23] both of which represent clonal evolution and selection. In the present study, we showed that at least two (or possibly three) cases had increased copy numbers of the mutated *Notch2* allele due to gene amplification or mitotic recombination. This finding agrees with the recent understanding that the allelic copy number increase after an oncogenic mutation is a common mechanism of further transformation and selection of neoplastic cells.

Whether the presence of *Notch2* gain-of-function mutations has a prognostic indicator or further define a clinical entity within DLBCL is yet to be clarified. Although the number of cases is still small, our finding that all five cases with *Notch2*

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