

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/ $\mu$ l 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.

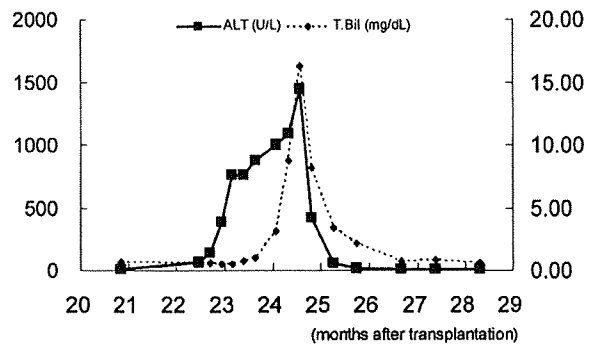
Twenty-three months after HSCT, however, routine blood test showed an elevation of serum aspartate aminotransferase (AST, 52 U/l) and alanine aminotransferase (ALT, 62 U/l) without any other abnormalities. We suspected reactivation of HBV and evaluated HBV markers. RS of HBV was diagnosed by the serological 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.<sup>8,9</sup> 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.<sup>10-12</sup> Autologous HSCT recipients receiving steroid also caused this complication.<sup>3</sup>

Onozawa *et al.* retrospectively analyzed the clinical course of 14 allogeneic HSCT recipients who were HBsAb-positive before HSCT.<sup>5</sup> 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.<sup>4</sup> They revealed that RS occurred almost exclusively in patients who underwent HSCT from 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.<sup>2</sup> 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).<sup>6</sup> 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.

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# 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.<sup>1</sup> 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.<sup>2,3</sup> Although long-term follow-up studies have shown a mortality rate of 20%,<sup>4</sup> few

reports have directly identified the risk factors for death in AIHA. The incidence of AIHA is highest in patients aged 60–70 years.<sup>1</sup> 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.<sup>5</sup> 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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© W. S. Maney & Son Ltd 2009  
Received 5 April 2009; accepted 15 June 2009  
DOI 10.1179/102453309X12473408860262

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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,<sup>6</sup> 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.

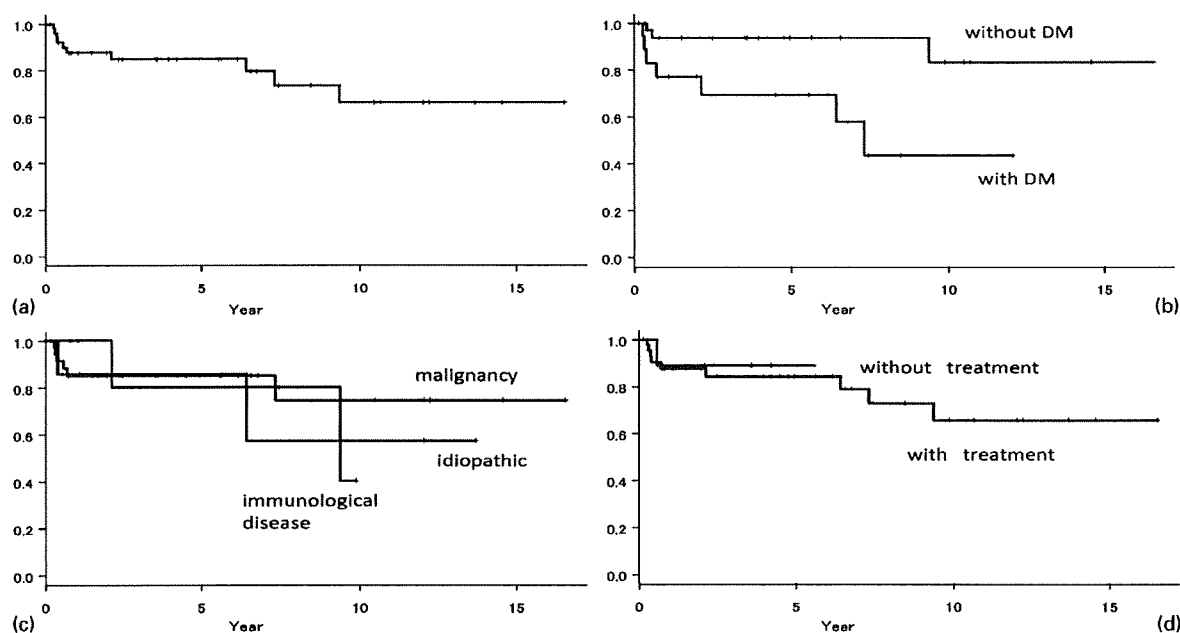
**Results**

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	<i>P</i> 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  $84.9 \pm 5.4\%$  ( $\pm$  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 ( $69.3 \pm 11.7\%$  versus  $93.6 \pm 4.4\%$ ,  $P=0.0064$ ) (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 ( $85.1 \pm 6.2\%$ ,  $85.7 \pm 13.2\%$  and  $80 \pm 17.9\%$ , 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 ( $84.1 \pm 6.1\%$  and  $88.9 \pm 10.5\%$ ,  $P=0.73$ ) (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

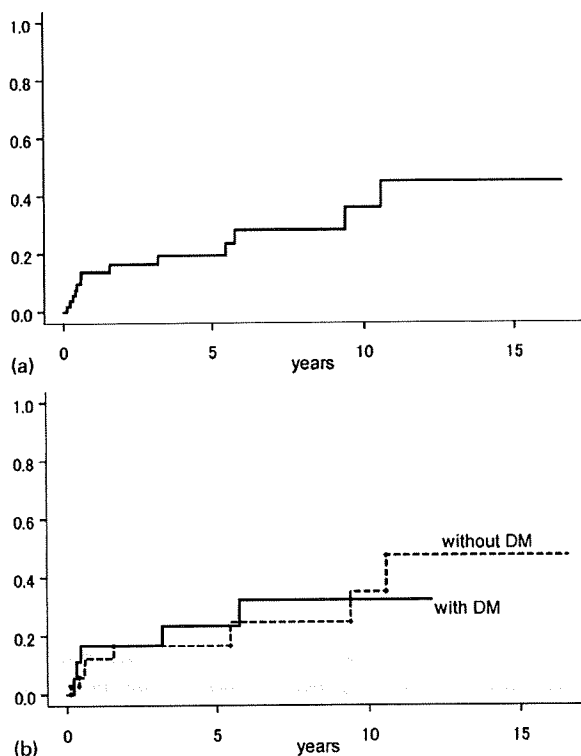
only significant difference between death and alive among DM patients.

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.<sup>7</sup> 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.<sup>8,9</sup> 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.<sup>10</sup> In patients with RA, whom are thought to be similar in age to those with AIHA, DM was associated with increased cardiovascular death,<sup>11</sup> while it has not been established whether the presence of DM would increase the frequency of infection.<sup>12</sup> In addition, deaths in CTD seemed to occur exclusively later than in our study population with AIHA.<sup>13,14</sup> Interestingly, the infection-related mortality was increased in SLE patients who had hemolytic anemia.<sup>14</sup> 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.<sup>15-17</sup> In addition, DM induces endothelial activation and dysfunction, which would alter the migration kinetics of T cells, and regulation of the immune reaction.<sup>18-21</sup> Additionally, since von Willebrand factor is known

to be increased in AIHA,<sup>22</sup> 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.<sup>23</sup> 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.<sup>24,25</sup> Therefore, further prospective investigations will be warranted to assess the impact of co-morbidities other than DM on survival in AIHA.

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## Letter to the Editor

**CD8<sup>+</sup> 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 <sup>+</sup> cells (/μl)	CD4 <sup>+</sup> cells (/μl)	CD8 <sup>+</sup> cells (/μl)	CD4/CD8 ratio	CD56 <sup>+</sup> cells (/μl)	RO <sup>+</sup> /RA <sup>-</sup> /CD8 cells (/μl)	RO <sup>-</sup> /RA <sup>+</sup> /CD8 cells (/μl)	RO <sup>+</sup> /RA <sup>+</sup> (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<sup>+</sup>/RA<sup>-</sup> (memory) to CD45RO<sup>-</sup>/RA<sup>+</sup> (naïve) CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells, CD4<sup>+</sup> T cells, CD3<sup>-</sup>CD56<sup>+</sup> cells, or CD4/CD8 ratio among the three groups (Table 1).

In the patients with sustained therapy-free MMR, CD8<sup>+</sup> 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,

Arinobu Tojo, final approval of the version to be submitted: Kensuke Usuki, Kazuaki Yokoyama, Tokiko Nagamura-Inoue, Ayumu Ito, Michiko Kida, Koji Izutsu, Akio Urabe, and Arinobu Tojo.

Role of the funding source: There is no study sponsor.

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13 March 2009  
Available online 16 April 2009

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

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(Received December 28, 2008/Revised January 22, 2009/Accepted January 22, 2009/Online publication March 25, 2009)

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.<sup>(1,2)</sup> 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-J $\kappa$ , 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.<sup>(3)</sup> 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.<sup>(4)</sup> 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.<sup>(5)</sup> *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.<sup>(6)</sup> 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.<sup>(7)</sup> 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,<sup>(8–11)</sup> 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.<sup>(8,12)</sup> 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.<sup>(13)</sup>

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* 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 <sup>32</sup>P-labeled PCR product was subjected to SSCP analysis as described in published reports.<sup>(14)</sup> 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.<sup>(15)</sup> 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).

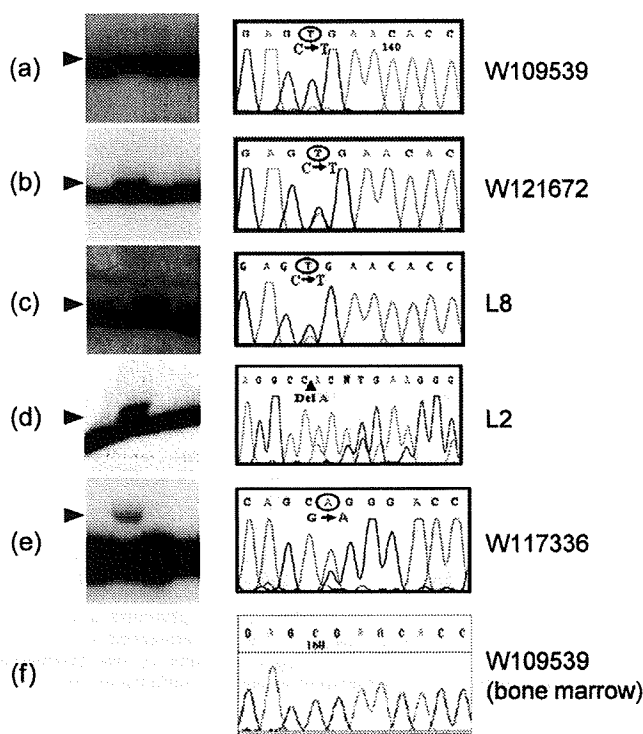
**Fluorescence *in situ* hybridization.** Bacterial artificial chromosome (BAC) clones RP11–723d17 (*Notch2*) and RP11–80d6 (1q23.3) were used to evaluate the copy number of the *Notch2* gene. BACs were obtained from the BAC/PAC Resource Center (Children's Hospital, Oakland, CA, USA). Fluorescence *in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

**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, TTCCCAAGTGAGAGACATTT; 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<sub>2a</sub>) mAb was generated by immunizing BALB/c mice with human *Notch2*-Fc (the Fc portion of human IgG<sub>1</sub> 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.<sup>(17)</sup> In brief,  $1 \times 10^6$  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.<sup>(17)</sup> In brief,  $2 \times 10^5$  CHO(r) cells expressing wtN2, *Notch2* with truncation 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 prednisolon; 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-tetrahydropyranil-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 inoculated 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,<sup>(18,19)</sup> 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.

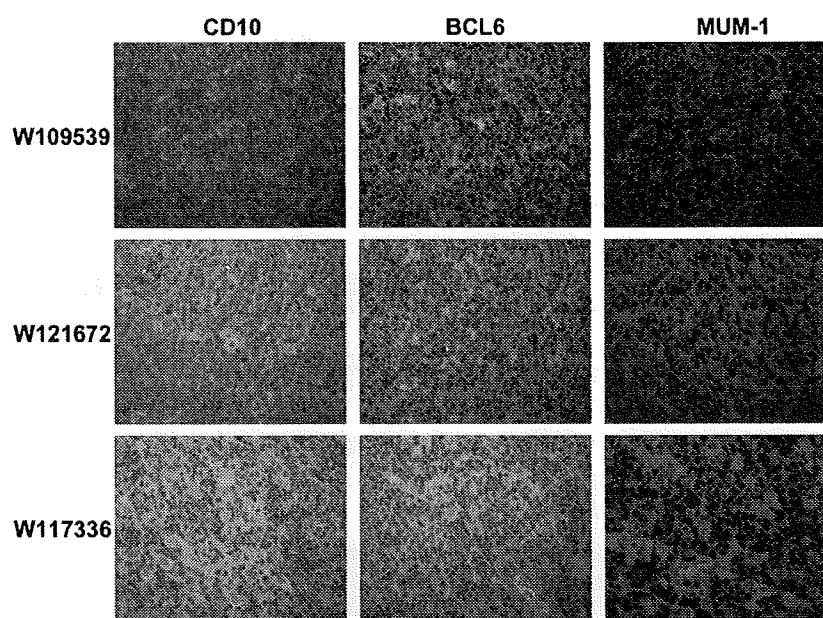
## Results

***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.<sup>(20)</sup>

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<sup>(21)</sup> 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.<sup>(21)</sup> DLBCL cases carrying the gain-of-function type *Notch2* mutations, thus, might constitute a discrete subset of non-GCB-DLBCL.



**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,  $\times 200$ . 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.<sup>(22,23)</sup> A high-density oligonucleotide microarray analysis<sup>(15)</sup> 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.

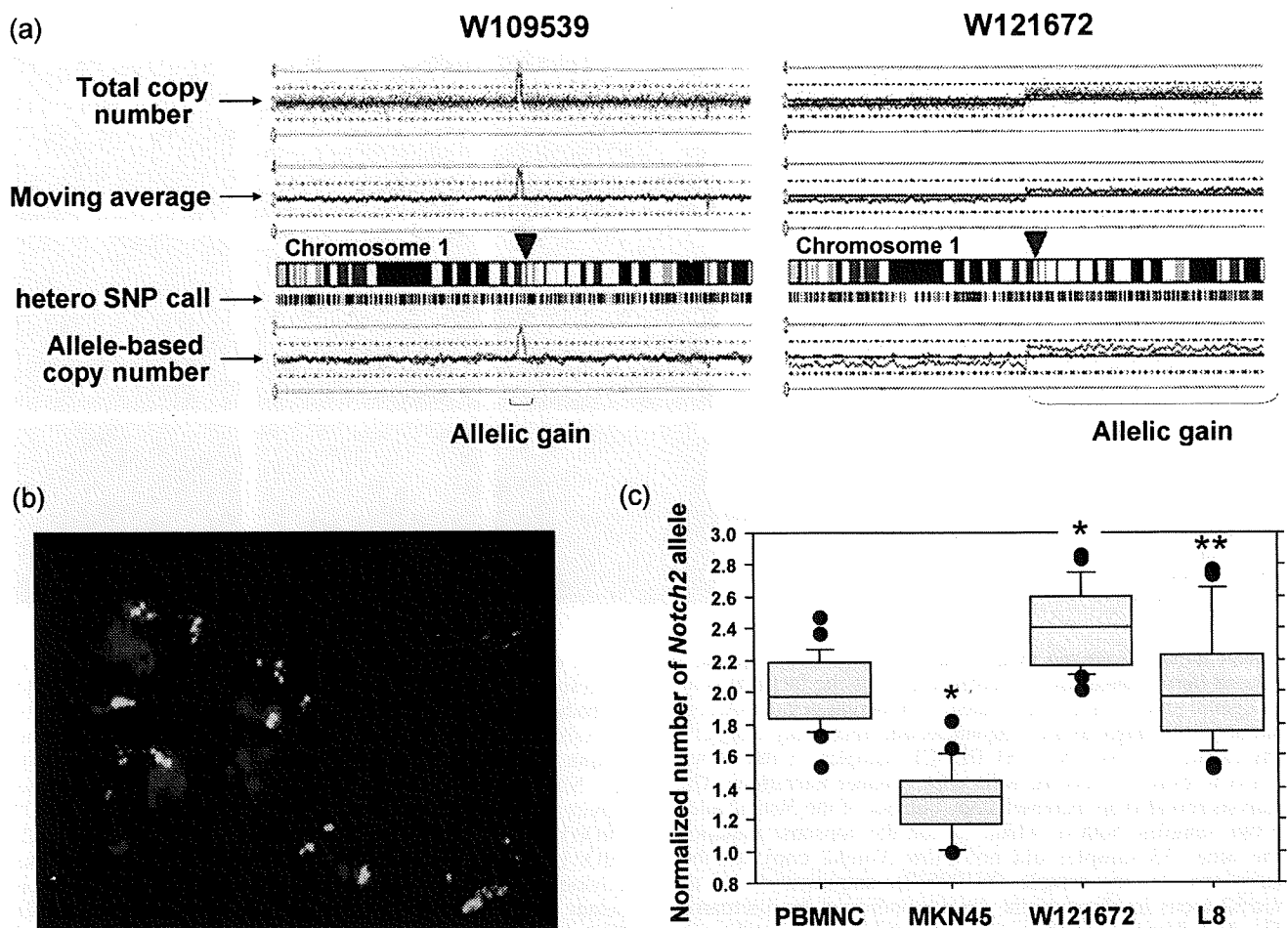
***Notch2* receptors with mutations have increased activity *in vitro*.** To investigate the function of the *Notch2* receptors encoded by mRNA with the nonsense mutation (nsmN2), the single-base deletion mutation (delstN2), and missense mutation (rqN2), we established CHO(r) cell lines<sup>(17)</sup> 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,<sup>(24)</sup> 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  $\gamma$ -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  $\mu$ M 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  $\gamma$ -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. \* $P < 0.0001$ ; \*\* $P = 0.79$ .

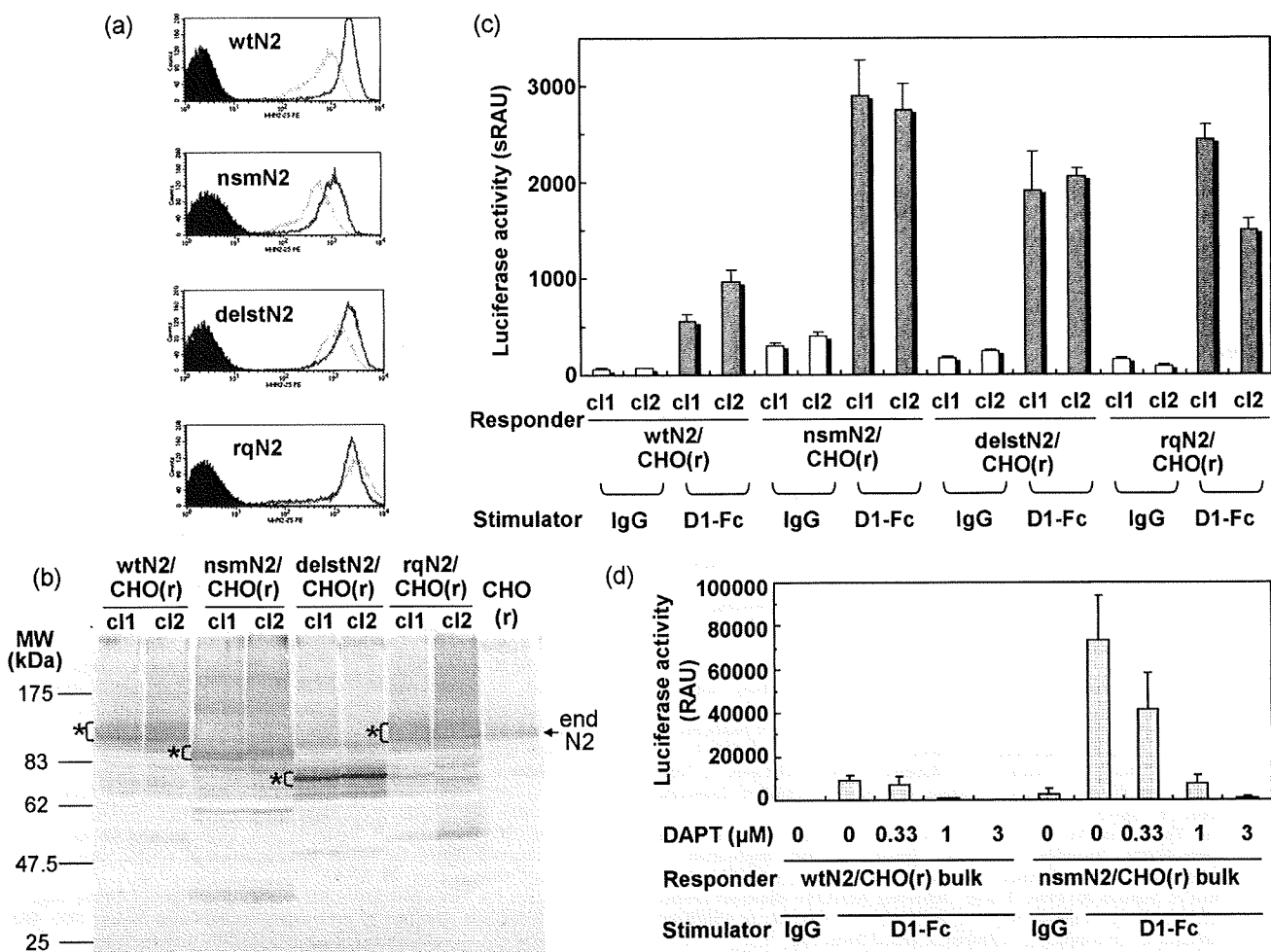
or PEST domain in the absence of exogenous ligand stimulation. The activity of Notch1 with a PEST domain deletion mutation alone was only marginally higher than that of wild-type Notch1<sup>(8)</sup>. We did not detect mutations in either heterodimerization domain of Notch2 in the current cohort. It 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,<sup>(25,26)</sup> 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<sup>(27)</sup> and homozygous *JAK2* mutations in polycythemia vera,<sup>(22,23)</sup> 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*



**Fig. 4.** Functional analysis of human full-length Notch2 cDNA (wtN2), and Notch2 with the nonsense mutation (nsmN2), single-base deletion mutation (delstN2), or R2453Q mutation (rqN2). (a) Flow cytometric analysis of CHO(r) clones expressing wtN2, nsmN2, delstN2, and rqN2 at similar expression levels. Each clone (c1 and c2 represented by green and red lines, respectively) of wtN2/CHO(r), nsmN2/CHO(r), delstN2/CHO(r), and rqN2/CHO(r) was analyzed by flow cytometry using the anti-human Notch2 antibody MHN2-25. Purple curves represent isotype control. (b) Western blot analysis of CHO(r) clones expressing wtN2, nsmN2, delstN2, and rqN2 using an antibody recognizing the intracellular domain of Notch2. Asterisks indicate the transmembrane species of each Notch2 protein. MW, molecular weight. (c) Reporter gene transactivation by wtN2, nsmN2, delstN2, and rqN2. Each clone (c1 and c2) was cultured in a dish coated with human Delta1-Fc (D1-Fc) or control IgG. Data are means of quadruplicate experiments. Error bars represent standard deviations. A representative experiment from repeated experiments is shown. sRAU, relative arbitrary units standardized by  $\beta$ -galactosidase activity. (d) Inhibition of luciferase activity by N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a  $\gamma$ -secretase inhibitor. Bulk CHO(r) cells transfected with wtN2 or nsmN2 were stimulated with D1-Fc or control IgG with graded concentrations of DAPT. RAU, relative arbitrary units.

mutations showed the same immunohistochemical staining pattern for CD10, BCL6 and MUM-1 might provide insight into this issue. DLBCL is highly heterogeneous clinically, morphologically, and genetically. The tissue microarray study based on immunostaining of the tissue samples identified three antigens (CD10, BCL6 and MUM-1) as useful markers to predict the results of mRNA expression array studies<sup>(28-30)</sup> and the staining pattern of these three antigens could be used to divide DLBCL cases into GCB and non-GCB groups.<sup>(21)</sup> Whereas all the five cases carrying *Notch2* mutations in our study belonged to the non-GCB group of DLBCL in this criterion, Troen *et al.* recently reported *Notch2* mutations in two cases of MZB-cell lymphomas.<sup>(31)</sup> Positions of these mutations are different from those that we found, and their effect on the Notch2 function is not shown. We did not find *Notch2* mutations in MZB-cell lymphomas in our cohort, yet the number of samples was not sufficient to draw conclusions. Although we were unable to find evidence that some or all the five cases carrying *Notch2* mutations in our

cohort are DLBCL transformed from MZB-cell lymphoma, this might be an interesting possibility.

Enhanced activation of Notch signaling by exogenous ligand stimulation or expression of constitutively active Notch proteins supports the growth of a variety of tumor cells, including chronic lymphocytic leukemia,<sup>(32)</sup> non-Hodgkin's lymphoma, and multiple myeloma<sup>(33)</sup> cells. Alternatively, inhibition of Notch signaling by  $\gamma$ -secretase inhibitors suppresses the growth of those tumor cells, in which enhanced Notch signaling might be involved in tumorigenesis.<sup>(34)</sup> In contrast, a study of mice with a *Notch1* deletion in keratinocytes revealed the tumor-suppressive feature of Notch signaling.<sup>(35)</sup> In a similar context, Notch2 activation induces growth suppression in a wide range of B-cell malignancies, raising the possibility that Notch2 functions as a tumor suppressor in B cells.<sup>(36)</sup> Thus, there appears to be a controversy regarding whether Notch signaling has an oncogenic or antioncogenic role in mature B-cell malignancies. It might be possible that Notch signaling can induce both growth suppression and tumor promo-

tion in the B-cell compartment, depending on the target window within the various developmental stages of B cells.

Although it will require additional studies, including development of animal models, to draw a definitive conclusion about the role of Notch2 mutations in lymphomagenesis, our observations in this study strongly indicate that deregulation of Notch2 signaling by somatic *Notch2* gene abnormalities contributes to the development of a subset of DLBCL, the most frequent type of non-Hodgkin's lymphoma. Developing inhibitors of individual Notch molecules might provide a new strategy for the treatment of different kinds of malignancies, including T-ALL and DLBCL.

## Acknowledgments

We thank Dr S. Sakano (Asahi Kasei) for the human Delta1 cDNA, Dr S. Shirahata (Kyushu University) for CHO(r), Dr S. Artavanis-Tsakonas (Harvard University) for the human full-length Notch2 cDNA, and Dr A. Harashima (Hayashibara Biomedical Institute) for the cell lines. We are grateful for the support provided by Dr M. Kato (University of Tokyo) and technical assistance by Y. Mori. Financial support was provided by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (KAKENHI #18013012 and #19390258), and grants from the Sagawa Foundation for the Promotion of Cancer Research and Osaka Cancer Foundation (SC).

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## Supporting Information

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

**Fig. S1.** Specific binding of the mouse antihuman Notch2 monoclonal antibody (MHN2-25). MHN2-25 was added to individual CHO(r) cells and analyzed by fluorescence-activated cell sorting. CHO(r), parental CHO(r); wtN1/CHO(r), CHO(r) cells stably transfected with pTracerCMV/wild-type human Notch1; wtN2/CHO(r), CHO(r) cells stably transfected with pTracerCMV/wild-type human Notch2. Broken lines, biotin-conjugated mouse IgG2a/k (isotype control); solid lines, biotin-conjugated MHN2-25.

**Table S1.** Primers for polymerase chain reaction–single-stranded conformational polymorphism

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## CASE REPORT

# Monobactam and aminoglycoside combination therapy against metallo- $\beta$ -lactamase-producing multidrug-resistant *Pseudomonas aeruginosa* screened using a 'break-point checkerboard plate'

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### Abstract

Metallo- $\beta$ -lactamase-producing multidrug-resistant *Pseudomonas aeruginosa* (MDR *P. aeruginosa*) is a cause of life-threatening infections. With parenteral colistin not available in Japan, we treated MDR *P. aeruginosa* sepsis with monobactam and aminoglycoside combination therapy, with screening using a 'break-point checkerboard plate'.

### Introduction

Multidrug-resistant *Pseudomonas aeruginosa* (MDR *P. aeruginosa*), defined as *P. aeruginosa* resistant to aminoglycosides, carbapenems, and fluoroquinolones, has emerged as an increasingly problematic cause of hospital-acquired infection. The outcome of MDR *P. aeruginosa* sepsis in severely immunocompromised patients is usually poor. With parenteral colistin not available in Japan, effective antimicrobial options are severely limited. Therefore, combination therapy involving available antimicrobial agents is expected. We treated MDR *P. aeruginosa* sepsis with monobactam and aminoglycoside combination therapy, with screening using a 'break-point checkerboard plate' [1].

### Case reports

#### *Patient 1*

A 58-y-old woman was admitted to Toranomon Hospital, Tokyo (890 beds) for the treatment of malignant lymphoma. She was treated with an unrelated donor bone marrow transplantation. The patient suffered from grade IV acute graft-

versus-host disease (GVHD). On day 74 of transplantation, peritonitis due to perforation was suspected. MDR *P. aeruginosa* was cultured from the blood, and treatment with intravenous aztreonam (1 g i.v. every 6 h) and amikacin (400 mg i.v. every 24 h) were started according to the break-point checkerboard plate results. The patient subsequently recovered from MDR *P. aeruginosa* sepsis.

#### *Patient 2*

A 54-y-old man was admitted to Toranomon Hospital for the treatment of malignant lymphoma. He received chemotherapy (R-HyperCVAD/MA) and pelvic radiation therapy. He became febrile 9 days after the most recent course of chemotherapy, with a neutrophil count of 176/ $\mu$ l. Treatment with meropenem and vancomycin was ineffective and the high fever persisted. MDR *P. aeruginosa* was isolated from blood culture, and combination therapy with aztreonam (2 g i.v. every 12 h) and amikacin (400 mg i.v. every 24 h) was selected for MDR *P. aeruginosa* according to the break-point checkerboard plate results. The patient recovered successfully from MDR *P. aeruginosa* sepsis.

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(Received 20 October 2009; accepted 22 October 2009)

ISSN 0036-5548 print/ISSN 1651-1980 online © 2009 Informa UK Ltd. (Informa Healthcare, Taylor & Francis AS)

DOI: 10.3109/00365540903443157

## Patient 3

A 63-y-old man was admitted to Toranomon Hospital for the treatment of acute myelogenous leukaemia (AML; World Health Organization classification M1). He was treated with cord blood transplantation. The patient developed redness and swelling in the right eyelid on day 1 of transplantation. He became febrile on day 3 of transplantation. On day 5 of transplantation, MDR *P. aeruginosa* was cultured from the eye discharge, and his neutrophil count was 0/ $\mu$ l. On day 8 of transplantation, MDR *P. aeruginosa* was cultured from the blood, and treatment with intravenous aztreonam (1 g i.v. every 6 h) and arbekacin (600 mg i.v. every 24 h) was started. The break-point checkerboard plate results suggested synergistic effects of amikacin in combination with aztreonam and piperacillin. The patient subsequently recovered from MDR *P. aeruginosa* sepsis.

The clinical characteristics of these 3 patients with MDR *P. aeruginosa* sepsis are shown in Table I.

## Discussion

We have reported cases for which monobactams and aminoglycosides were successfully used concomitantly for MDR *P. aeruginosa* infection. The production of metallo- $\beta$ -lactamase was demonstrated by the 3 MDR *P. aeruginosa* strains using the SMA disc method employing sodium mercaptoacetate (SMA), ceftazidime, and imipenem disks (EIKEN CHEMICAL). Pulsed-field gel electrophoresis was also conducted. The strains from patients 1 and 2 were closely related. The strain from patient 3 was different from those of patients 1 and 2 [2]. In Japan, where intravenous colistin cannot be used, combination antimicrobial therapy is expected to be effective for the treatment of MDR *P. aeruginosa*. A break-point checkerboard plate is used to evaluate the effect of combination therapy in reference to the breakpoint concentration established from the correlation with

clinical efficacy, allowing simultaneous evaluation of the effect of combination antimicrobial therapy using 8 clinically important agents (ceftazidime, piperacillin, imipenem, aztreonam, gentamicin, ciprofloxacin, polymyxin B, and rifampicin) on a single plate. In Japan, a break-point checkerboard plate is commercially available as a BC plate 'EIKEN' from EIKEN CHEMICAL, which includes amikacin, meropenem, and colistin instead of gentamicin, imipenem, and polymyxin B.

Most MDR *P. aeruginosa* patients remain in a carrier state. Usually, MDR *P. aeruginosa* seldom causes infection. Therefore, no treatment is recommended for carriers. However, MDR *P. aeruginosa* is associated with a very poor prognosis when it causes infection, particularly sepsis. Thus, when MDR *P. aeruginosa* is detected in monitoring cultures for immunocompromised patients, it is important to predict the effect of concomitant use on a break-point checkerboard plate to conduct appropriate early antimicrobial therapy for the infection.

In Japan, the production of metallo- $\beta$ -lactamase is often involved in the high-level resistance of *P. aeruginosa*. IMP encoded by the *bla<sub>IMP</sub>* gene on a plasmid has been reported [3,4]. Effective combinations of antibacterial drugs seem to vary with strains. Strains producing IMP-type metallo- $\beta$ -lactamase often remain susceptible to monobactams [5]. The concomitant use of monobactams and aminoglycosides seems to be promising. This regimen is considered to provide a promising second drug of choice for patients in whom intravenous colistin cannot be used.

Aminoglycosides for concomitant use with monobactams will be examined in the future. In Japan, a major drug resistance mechanism against aminoglycosides is inactivation of the antibacterial drugs via acetylation, phosphorylation, etc., by aminoglycoside-modifying enzymes produced by resistant bacteria [6,7]. Other known mechanisms include the methylation of 16S rRNA [8] and increased expression of drug-efflux pumps [9]. Arbekacin is characterized by

Table I. Clinical characteristics of patients with multidrug-resistant *Pseudomonas aeruginosa* sepsis.

Patient	Sex, age	Underlying disease	Predisposing conditions	Neutropenia	Site of infection	Combination selected by BC plate	Treatment	Clinical response
1	Female, 58 y	Malignant lymphoma	U-BMT, GVHD, diarrhoea	No	Blood, peritonitis	AZT/AMK, PIPC/AMK	AZT/AMK	Recovered
2	Male, 54 y	Malignant lymphoma	Chemotherapy, radiation	Yes (176/ $\mu$ l)	Blood, intestinal tract	AZT/AMK	AZT/AMK	Recovered
3	Male, 63 y	AML	CBT	Yes (0/ $\mu$ l)	Blood, eyelid cellulitis	AZT/AMK, PIPC/AMK	AZT/ABK	Recovered

AML, acute myelogenous leukaemia; U-BMT, bone marrow transplantation from an unrelated donor; GVHD, graft-versus-host disease; CBT, cord blood transplantation; BC plate, break-point checkerboard plate; AZT, aztreonam; AMK, amikacin; PIPC, piperacillin; ABK, arbekacin.

the effect against Gram-negative bacilli, including *P. aeruginosa*, as well as methicillin-resistant *Staphylococcus aureus* (MRSA) [8,10,11]. Like amikacin, arbekacin is regarded as a strong candidate for concomitant use with monobactams [12].

**Declaration of interest:** There is no conflict to declare.

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## 腸管 GVHD 患者に対する経口ベクロメタゾン投与時の 血中濃度に関する検討

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[*Jpn J Cancer Chemother* 37(2): 267-270, February, 2010]

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### Summary

In this study, we investigated the level of gut absorption following oral beclomethasone dipropionate (BDP) administration by measuring the blood concentration of its metabolites measured by LC-MS/MS using the HPLC method. Five patients who were administered BDP orally for gut GVHD were included. The blood concentrations of beclomethasone-17-monopropionate (17BMP), which is one of the active metabolites of BDP, were 618~1,749 pg/mL in 4 of the studied 5 patients, which was comparable to that after inhalation of BDP; however, it was relatively higher in one patient (2,439±161 pg/mL). As the blood concentration of 17BMP in this study patient was higher compared with healthy volunteers administered a single oral BDP 4 mg, GVHD patients might have a higher concentration than healthy volunteers.

Given that a higher grade of gut GVHD was associated with a higher blood level of 17BMP, BDP absorption might be associated with gut mucosal injury. Thus, the systemic adverse effect following oral BDP administration might not be negligible especially in gut GVHD patients. Key words: Beclomethasone dipropionate, Graft-versus-host disease, Stem cell transplantation (Received Jul. 6, 2009/Accepted Aug. 20, 2009)

**要旨** 腸管 GVHD に対してベクロメタゾン (BDP) を経口投与した患者を対象として、BDP およびその代謝物の血中濃度を高速液体クロマトグラフィを用いて LC-MS/MS で測定し、経口 BDP の消化管からの吸収について検討した。全対象症例 5 例より BDP の主要活性代謝物である 17BMP が検出された。5 例中 4 例における 17BMP の血中濃度は 618~1,749 pg/mL であり、吸入剤を投与した時と同程度あるいはそれ以下であった。1 例は 17BMP の血中濃度が 2,439±161 pg/mL を示し、吸入剤投与時以上に血中濃度が上昇した。本症例における 17BMP の血中濃度は、健康人に BDP 4 mg を単回経口投与した際の最高血中濃度と比較して高値を示したことから、GVHD 患者では健康人よりも血中濃度が上昇する症例が存在することが示唆された。腸管 GVHD の stage が高い症例に 17BMP の血中濃度が高値である症例が認められたことから、腸管粘膜障害と BDP 吸収の亢進との関連が示唆された。以上の成績から、腸管 GVHD に対する経口 BDP 投与は、必ずしも全身的副作用が無視できるものではない点に留意すべきと考えられた。

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