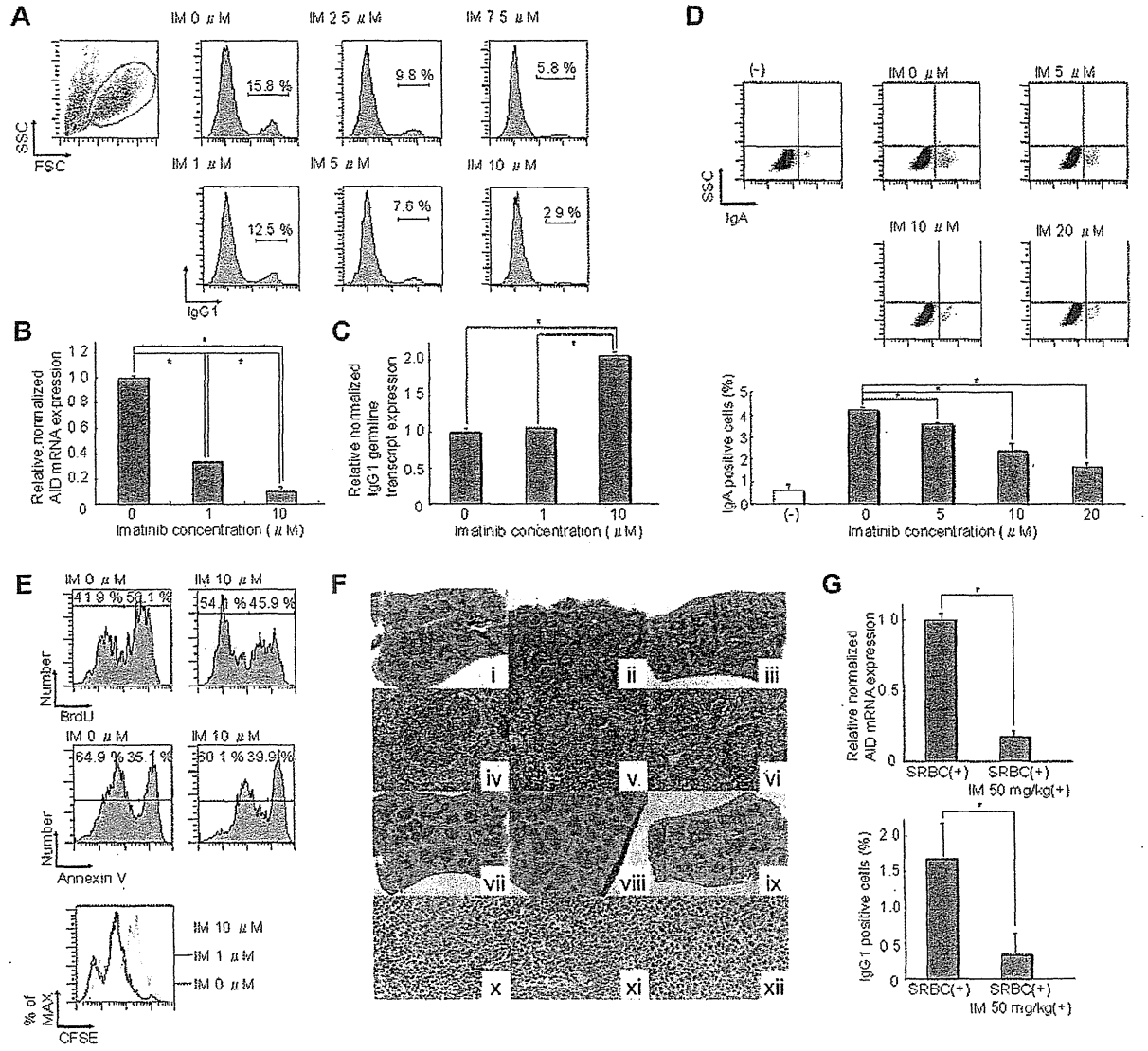

Figure 1. IM directly inhibits CSR in activated B cells through down-regulation of AID. (A) IgG1 expression levels in spleen cells cultured in conditioning medium containing 12.5 μg/mL LPS and 7.5 ng/mL IL-4 with 0, 1, 2.5, 5, 7.5, and 10μM IM for 72 hours were 15.8%, 12.5%, 9.8%, 7.6%, 5.8%, and 2.9% of untreated controls, respectively. Reduction of IgG1 expression was induced by IM dose-dependently. (B) Real-time RT-PCR in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours indicated that expression of AID was decreased by IM dose-dependently. Significant differences were found between 0 and 1 μM or 10μM IM. $^*P < .05$. The y-axis represents AID mRNA levels relative to the no-IM control. The levels of AID mRNA at each IM concentration were calculated relative to the internal control (GAPDH); n = 6. (C) The level of the germline transcript of IgG1 in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours was not decreased in contrast to AID mRNA expression levels, which were decreased by IM in a dose-dependent manner. Significant differences were found between 0, 1, and 10μM IM. $^*P < .05$. The y-axis represents expression levels of the IgG1 germline transcript relative to the no-IM control in the same manner as that in panel B; n = 4. (D) IgA expression levels in CH12F3-2A cells cultured in conditioning medium containing 7.5 μg/mL IL-4, 0.3 ng/mL TGF-β1, and 40% CD40 ligand with 0, 5, 10, and 20μM IM for 72 hours were reduced in an IM dose-dependent manner. (E) Cell proliferation, division, and apoptosis in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours were investigated using BrdU, annexin V, and CFSE assays. The BrdU incorporation rate of 10μM IM was 45.9%, whereas that of 0μM IM was 58.1%. Cell fluorescence of 10μM IM using the CFSE assay was shifted, but that of 1μM IM was not shifted to the right compared with that of 0μM IM. Annexin V analysis of 10μM IM was 39.9%, whereas that of 0μM IM was 35.1%. These results indicate that IM affects cell proliferation but not apoptosis. (F) Immunohistochemical analysis of spleens from mice that were administered SRBC with or without IM. Serial sections of spleens were prepared from nonimmunized (i,iv,vii,x), SRBC-immunized (ii,v,viii,xi), or SRBC-immunized + IM (50 mg/kg; iii,vi,ix,xii) animals. (i-vi) H&E staining. (vii-xii) Immunohistochemical analysis of AID. Low-power fields are shown in panels i to iii and vii to ix. High-power fields are shown in subpanels iv to vi and x to xii. Individual germinal centers from SRBC-immunized IM (+) mice were significantly smaller than those from SRBC-immunized IM (−) mice and were comparable with those from nonimmunized mice. AID expression, which was induced in germinal center-activated B cells, was barely detectable in spleens of IM-treated mice but was strongly positive in those of nontreated mice. Moreover, IM significantly suppressed AID expression, even in the residual germinal centers. (G) Real-time RT-PCR analysis of AID and FACS analysis of IgG1 expression of spleen cells harvested from SRBC-immunized mice with or without 50 mg/kg IM. The top panel shows relative normalized AID mRNA expression, and the bottom panel shows surface IgG1 expression of total splenocytes. A significant difference was found between SRBC (+) and SRBC (+) IM 50 mg/kg (+) for both AID and IgG1. $^*P < .05$. The y-axis represents the relative ratio of the relative expression level of AID mRNA (top panel) and the percentage of surface IgG1 expression of total splenocytes (bottom panel). Normalized values obtained for SRBC (+) IM 50 mg/kg (+) were derived from SRBC (+); n = 2.

showed that AID, but not the germline transcript, was responsible for inhibition of CSR by IM. BrdU, CFSE, and annexin V analysis revealed that IM affected proliferation but not apoptosis (Figure 1E). Importantly, 1μM of IM did not decrease proliferation but down-regulates AID (Figure 1B,E). In addition, 5-fluorouracil decreased proliferation but did not down-regulate AID (supplemental Figure 1), suggesting that proliferation is not necessarily coupled with expression of AID. Therefore, it is

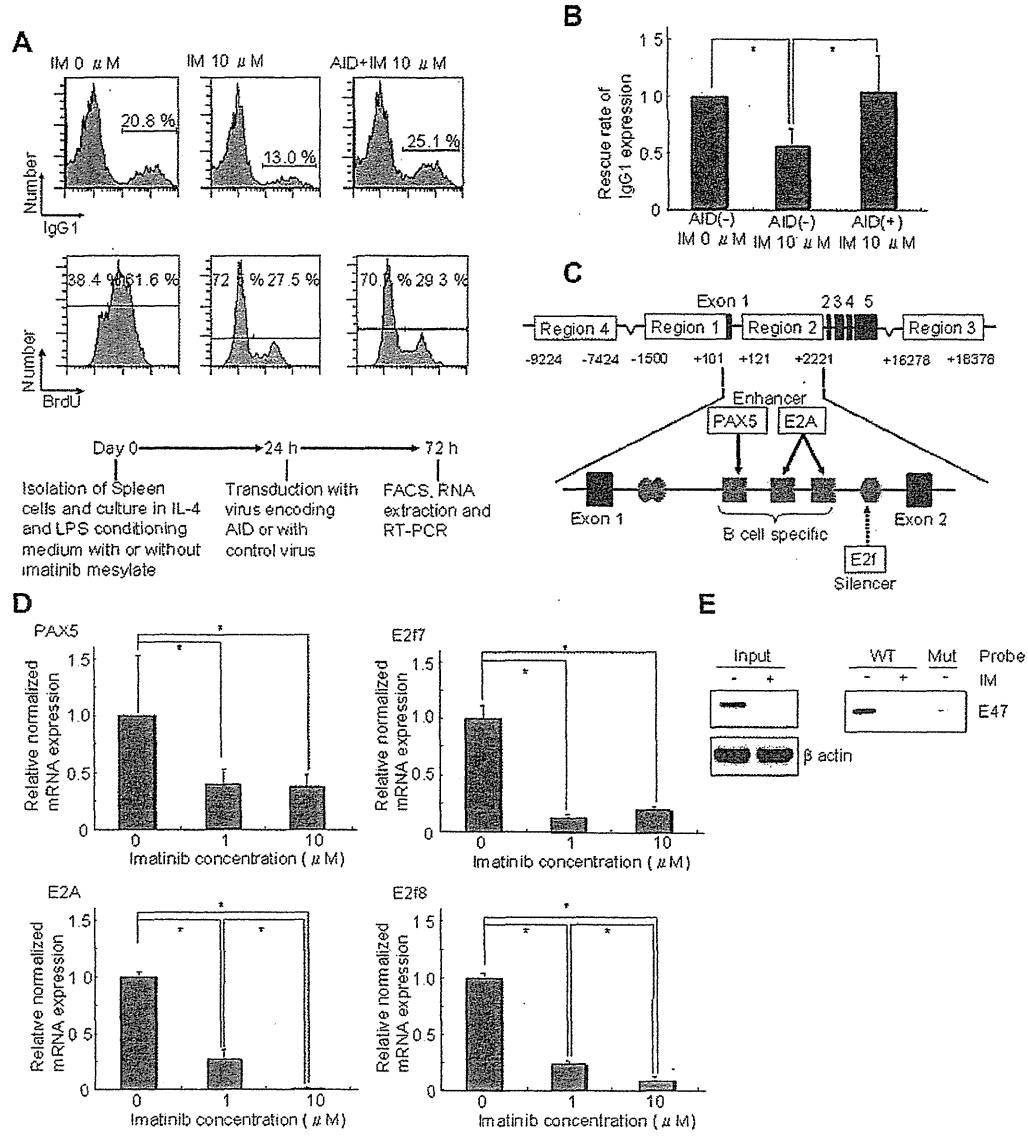

Figure 2. Down-regulation of AID mediated by E2A, Pax5, E2f7, and E2f8 is responsible for CSR impairment by IM. (A) Ectopic expression of AID completely rescued reduction of IgG1 expression caused by IM. Spleen cells were cultured in IL-4 and LPS conditioning medium with or without IM. After 24 hours of prestimulation culture, cells were transduced with retrovirus encoding AID-eGFP or retrovirus encoding eGFP only (control). After a further 48 hours, IgG1 expression was analyzed (bottom panel). A mononuclear cell fraction based on forward scatter/side scatter profiles was gated and sequentially subdivided into an eGFP-positive fraction. This eGFP-positive fraction was analyzed. Ectopic AID expression with 10μM IM increased IgG1 expression from 13.0% to 25.1% versus 20.8% without IM. The BrdU assay revealed that DNA synthesis decreased in the 10μM IM culturing condition. Although the BrdU assay was similar with or without ectopic expression of AID, IgG1 expression was completely rescued by ectopic expression of AID. (B) Average of the IgG1 expression rescue rate among 4 rescue experiments. IgG1 expression of AID(+) IM at 10μM was completely rescued by ectopic expression of AID. (C) Schema illustrating transcriptional binding sites in the *Aicda* gene promoter region, focusing particularly on region 2 in the first intron. PAX5 and E2A activate the *Aicda* promoter, whereas E2f7 and E2f8 have silencing effects. (D) The expression levels of 4 transcriptional factors (PAX5, E2A, E2f7, and E2f8) in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours were determined by real-time RT-PCR. All were reduced by IM. E2A expression was most markedly reduced.*$P < .05$. The y-axis represents mRNA levels of the PAX5, E2A, E2f7, and E2f8 relative to the no-IM control. Levels of each transcriptional factor mRNA were calculated relative to the internal control (GAPDH); n = 2. (E) Protein expression and DNA-binding activity of E2A in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours. The E2A gene encodes 2 transcription factors: E12 and E47. Western blot analysis revealed that expression of E47 in splenocytes cultured in conditioning medium containing LPS and IL-4 was down-regulated by IM to a barely detectable level. DNA affinity precipitation analysis of the same cell extracts using biotinylated E-box probe and its mutant revealed that E-box binding activity of E47 in the extracts was similarly reduced by IM.

possible to differentiate the effect of IM on proliferation and AID expression.

To further confirm that CSR is impaired by IM through down-regulation of AID in vivo, immunohistochemical analysis was performed on splenic tissues from nonimmunized and sheep red blood cells–immunized C57BL/6 mice with or without IM treatment (Figure 1F). The individual germinal centers from SRBC-immunized IM (+) mice were significantly smaller than those from SRBC-immunized IM (−) mice and comparable with those from nonimmunized mice (Figure 1F). As expected from these findings, AID expression, which is induced in germinal center-activated B cells, was barely detectable in the spleens of IM-treated mice but was strongly positive in those of nontreated mice. In addition, IM significantly suppressed AID expression,

even in the residual germinal centers. Expression of AID was confirmed by real-time RT-PCR analysis. The results of IgG1 expression did not conflict with these results (Figure 1G). Compatible with the results obtained by in vitro stimulation of spleen cells, IM down-regulated expression of IgG1 as well as AID. Although enlargement of germinal center formation has been reported in AID knockout mice,[1] it is assumed that the immunomodulatory effects of IM on B cells, T cells, and dendritic cells[6,7] resulted in impairment of germinal center formation in our system.

Furthermore, we investigated whether ectopic expression of AID could rescue inhibition of CSR by IM. IgG1 expression in spleen cells decreased with IM treatment, whereas ectopic expression of AID completely rescued impairment of CSR under the condition that cell proliferation was suppressed by IM (Figure 2A-B). The results indicated that impairment of CSR by IM was at least in part the result of down-regulation of AID.

Finally, we examined the mechanism of down-regulation of AID by IM. Recently, Tran et al reported that *Aicda* regulation involved derepression by several layers of positive regulatory elements in addition to the 5′-promoter region.[5,13] Promoter region 2 in the first intron contains the functional binding elements for the ubiquitous silencers c-Myb and E2f and for the B cell–specific activators Pax5 and E2A (Figure 2C).[5,13] Surprisingly, all of these transcription factors were down-regulated by IM. Among them, expression of E2A was most markedly reduced (to 1 of 500) by IM (Figure 2D). We further found that levels of E2A protein as well as E-box binding activity were markedly reduced by IM (Figure 2E), suggesting that down-regulation of E2A by IM causes significant suppression of AID.

For the first time, our findings elucidate a mechanism of hypogammaglobulinemia caused by IM, which has been observed frequently in IM-treated chronic myeloid leukemia patients.[8,9] Its adverse effects as well as the immunomodulatory functions of each drug and their underlying mechanisms must be examined in more extensive studies.

AID was previously reported to be induced by BCR-ABL1 in Ph1$^+$ pre B-ALL cell lines and inhibited by IM through ID2 up-regulation. Interestingly, neither PAX5 nor E2A showed changes in expression.[14] In the present study using normal mature B cells, PAX5 and E2A levels were significantly decreased by IM, whereas ID2 was not increased (data not shown). PDGFR[15] and c-kit,[16] kinases that are also inhibited by IM, were not expressed in mature B cells. Together, these results could be induced by the off-target multikinase inhibitory effects of IM. The results of microarray analysis (supplemental Table 1; supplemental Figures 2-3) are consistent with this hypothesis. All microarray data are available for viewing on the Gene Expression Omnibus under accession number GSE35559.

Inappropriate expression of AID affects many diseases, such as malignancy and autoimmune diseases.[17,18] It probably also affects allergic disorders because AID is also essential to CSR from IgM to IgE, deregulation of which is an important causative factor of allergic disorders. The results of the present study suggest that IM, which has been used safely for several decades in clinical settings, can be used for various diseases involving AID. Indeed, dramatic resolution by IM has been reported in several cases of rheumatoid arthritis or asthma complicated with chronic myeloid leukemia.[19,20]

In conclusion, suppression of AID by IM is responsible for CSR impairment, leading to the frequent adverse effects of IM. IM may also be clinically useful as an AID suppressor.

Acknowledgments

The authors thank Dr Tasuku Honjo, Dr Toru Nakano, Mrs Bidisha Chanda, Mr Haruo Onoda, and Mr Keisuke Takahashi for critical comments or technical assistance. The array analysis was performed by Mr Masayuki Tanaka and Hideki Hayashi (Education and research Support Center, Toaki University).

This work was supported by the Japan Society for the Promotion of Science, Takeda Science Foundation, Sagawa Cancer Foundation, The Foundation for Cancer Research, Kobayashi Cancer Research Foundation, Mochida Science Foundation, Sankyo Science Foundation, and Shiseido Science Grant for the female researcher (to A.K.).

Authorship

Contribution: T.K., J.L., T.S., A.K., and M.T. designed, performed, and analyzed the experiments and wrote the manuscript; T.T., H.N., Y.A., K.Y., N.O., and N.N. contributed vital reagents; K.A. collected the clinical samples; and A.T. supervised the research.

Conflict-of-interest disclosure: A.T. has received honoraria and research funding from Novartis. The remaining authors declare no competing financial interests.

Correspondence: Arinobu Tojo, Department of Hematology-Oncology, Research Hospital, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; e-mail: a-tojo@ims.u-tokyo.ac.jp; and Ai Kotani, Tokai University Institute of Innovative Science and Technology, Medical Science Division, Shimokasuya 143, Isehara City, Kanagawa Prefecture 259-1193, Japan; e-mail: ka102009@tsc.tokai-u.ac.jp.

References

1. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell.* 2000;102(5):553-563.
2. Okazaki I, Hiai H, Kakazu N, et al. Constitutive expression of AID leads to tumorigenesis. *J Exp Med.* 2003;197(9):1173-1181.
3. Kotani A, Okazaki I, Muramatsu M, et al. A target selection of somatic hypermutations is regulated similarly between T and B cells upon activation-induced cytidine deaminase expression. *Proc Natl Acad Sci U S A.* 2005;102(12):4506-4511.
4. Kotani A, Kakazu N, Tsuruyama T, et al. Activation-induced cytidine deaminase (AID) promotes B cell lymphomagenesis in Emu-cmyc transgenic mice. *Proc Natl Acad Sci U S A.* 2007;104(5):1616-1620.
5. Tran T, Nakata M, Suzuki K, et al. B cell-specific and stimulation-responsive enhancers derepress Aicda by overcoming the effects of silencers. *Nat Immunol.* 2010;11(2):148-154.
6. Gao H, Lee B, Talpaz M, et al. Imatinib mesylate suppresses cytokine synthesis by activated CD4 T cells of patients with chronic myelogenous leukemia. *Leukemia.* 2005;19(11):1905-1911.
7. Mohty M, Jourdan E, Mami N, et al. Imatinib and plasmacytoid dendritic cell function in patients with chronic myeloid leukemia. *Blood.* 2004;103(12):4666-4668.
8. Steegmann JL, Moreno G, Aláez C, et al. Chronic myeloid leukemia patients resistant to or intolerant of interferon alpha and subsequently treated with imatinib show reduced immunoglobulin levels and hypogammaglobulinemia. *Haematologica.* 2003;88(7):762-768.
9. Santachiara R, Maffei R, Martinelli S, et al. Development of hypogammaglobulinemia in patients treated with imatinib for chronic myeloid leukemia or gastrointestinal stromal tumor. *Haematologica.* 2008;93(8):1252-1255.
10. Nakayama S, Nagamura-Inoue T, Yokoyama K, et al. Cytogenetic remissions induced by interferon alpha and imatinib mesylate are immunologically distinct in chronic myeloid leukemia. *Int J Hematol.* 2007;86(3):208-211.

11. Muramatsu M, Sankaranand V, Anant S, et al. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem.* 1999;274(26):18470-18476.
12. Kinoshita K, Harigai M, Fagarasan S, Muramatsu M, Honjo T. A hallmark of active class switch recombination: transcripts directed by I promoters on looped-out circular DNAs. *Proc Natl Acad Sci U S A.* 2001; 98(22):12620-12623.
13. Nagaoka H, Tran T, Kobayashi M, Aida M, Honjo T. Preventing AID, a physiological mutator, from deleterious activation: regulation of the genomic instability that is associated with antibody diversity. *Int Immunol.* 2010;22(4):227-235.
14. Feldhahn N, Henke N, Melchior K, et al. Activation-induced cytidine deaminase acts as a mutator in BCR-ABL1-transformed acute lymphoblastic leukemia cells. *J Exp Med.* 2007;204(5):1157-1166.
15. Trink B, Wang G, Shahar M, Meydan N, Roifman CM. Functional platelet-derived growth factor-beta (PDGF-beta) receptor expressed on early B-lineage precursor cells. *Clin Exp Immunol.* 1995;102(2):417-424.
16. Busslinger M. Transcriptional control of early B cell development. *Annu Rev Immunol.* 2004;22: 55-79.
17. Pasqualucci L, Bhagat G, Jankovic M, et al. AID is required for germinal center-derived lymphomagenesis. *Nat Genet.* 2008;40(1):108-112.
18. Hsu H, Wu Y, Yang P, et al. Overexpression of activation-induced cytidine deaminase in B cells is associated with production of highly pathogenic autoantibodies. *J Immunol.* 2007;178(8):5357-5365.
19. Eklund K, Joensuu H. Treatment of rheumatoid arthritis with imatinib mesylate: clinical improvement in three refractory cases. *Ann Med.* 2003; 35(5):362-367.
20. Ramanujam D, McNicholl F, Furby D, Richardson D, Cuthbert R, McMullin M. Dramatic resolution of respiratory symptoms with imatinib mesylate in patients with chronic myeloid leukemia presenting with lower airway symptoms resembling asthma. *Leuk Lymphoma.* 2009;50(10):1721-1722.

Cancer Science
The official journal of the Japanese Cancer Association JCA

Phase I study of anti-CD22 immunoconjugate inotuzumab ozogamicin plus rituximab in relapsed/refractory B-cell non-Hodgkin lymphoma

Michinori Ogura,[1,7] Kiyohiko Hatake,[2] Kiyoshi Ando,[3] Kensei Tobinai,[4] Kota Tokushige,[5] Chiho Ono,[5] Taro Ishibashi[5] and Erik Vandendries[6]

[1]Department of Hematology and Oncology, Nagoya Daini Red Cross Hospital, Nagoya; [2]Division of Medical Oncology and Hematology, Cancer Institute Hospital, Tokyo; [3]Division of Hematology/Oncology, Department of Internal Medicine, Tokai University Hospital, Isehara; [4]Hematology and Stem Cell Transplantation Division, National Cancer Center Hospital, Tokyo; [5]Clinical Research, Pfizer Japan Inc, Tokyo, Japan; [6]Pfizer Inc, Cambridge, Massachusetts, USA

(Received November 10, 2011/Revised January 31, 2012/Accepted February 6, 2012/Accepted manuscript online February 15, 2012/Article first published online March 20, 2012)

Inotuzumab ozogamicin (CMC-544), a humanized anti-CD22 antibody conjugated to the potent cytotoxic antibiotic calicheamicin, targets the CD22 antigen expressed on the majority of B-cell non-Hodgkin lymphomas. This phase I study assessed the tolerability, safety, pharmacokinetics, and preliminary efficacy of inotuzumab ozogamicin administered intravenously in combination with rituximab in Japanese patients with relapsed or refractory B-cell non-Hodgkin lymphoma. Ten patients were administered rituximab 375 mg/m^2 followed by inotuzumab ozogamicin at the maximum tolerated dose (1.8 mg/m^2). Treatment was repeated every 28 days up to eight cycles, or until occurrence of disease progression or intolerable toxicity. The safety profile was similar to that of inotuzumab ozogamicin monotherapy, with hematologic adverse events occurring most frequently. The most common grade three or higher adverse events were thrombocytopenia (70%), neutropenia (50%), leukopenia (30%), and lymphopenia (30%). The overall response rate was 80% (8/10; 95% CI, 44–98%). Drug exposure increased with successive doses, similar to the pharmacokinetic profiles observed in previous phase I monotherapy studies. Efficacy results suggested promising antitumor activity, and the overall findings support the continued clinical development of this therapeutic regimen in patients with relapsed or refractory B-cell non-Hodgkin lymphoma. This trial was registered at www.ClinicalTrials.gov as NCT00724971. (*Cancer Sci* 2012; 103: 933–938)

CD22, a B-cell antigen expressed on >90% of B-lymphoid malignancies,[1] represents an attractive therapeutic target for treatment of B-cell non-Hodgkin lymphoma (NHL). CD22 is not routinely shed into the extracellular environment;[2] rather, CD22 is rapidly internalized upon binding with a ligand or antibody, allowing efficient delivery of targeted cytotoxic agents.[3] Inotuzumab ozogamicin (CMC-544) is a targeted chemotherapy agent composed of a humanized anti-CD22 antibody conjugated to calicheamicin, a potent cytotoxic antibiotic. *In vitro*, inotuzumab ozogamicin has demonstrated enhanced cytotoxic activity compared with untargeted uptake of calicheamicin.[2] Additionally, CD22 is expressed primarily on mature B-lymphocytes, with limited expression on lymphocyte precursor cells and no expression on memory B cells; therefore, minimal impact of inotuzumab ozogamicin on long-term immune function is expected.

Inotuzumab ozogamicin demonstrated promising cytotoxic activity both as a single agent and in combination with rituximab in xenograft models and *in vitro* studies.[2,4,5] Rituximab, an anti-CD20 monoclonal antibody, is indicated for single-agent use or in combination with chemotherapy for treatment of low-grade or follicular, CD20-positive, B-cell NHL, and in combination with chemotherapy for treatment of diffuse large B-cell, CD20-positive NHL.[6] Although rituximab has been effectively used in combination with chemotherapy for indolent and aggressive B-cell NHLs, some patients are not responsive, while those who do respond often experience relapse.[7] Mechanisms of rituximab resistance may include downregulation of CD20 and increased expression of complement inhibitory proteins.[8] Newer monoclonal antibodies that target B-cell antigens other than CD20 may be effective in rituximab-resistant B-cell NHL or work in synergistic fashion with rituximab to improve B-cell NHL treatment efficacy.[9] As both CD22 and CD20 are expressed in most patients with B-cell NHL,[1,7] inotuzumab ozogamicin and rituximab combination therapy in B-cell NHL may enhance the therapeutic advantage of each agent.[5]

Clinical activity was observed with inotuzumab ozogamicin monotherapy at the maximum tolerated dose (MTD) of 1.8 mg/m^2 i.v. every 28 days.[10,11] Results of inotuzumab ozogamicin at the MTD in combination with rituximab in non-Japanese patients with relapsed or refractory B-cell NHL has shown promising efficacy with a safety profile similar to that reported for inotuzumab ozogamicin alone.[12] The current study assessed the tolerability and initial safety profile of inotuzumab ozogamicin plus rituximab in Japanese patients with relapsed or refractory B-cell NHL. Secondary objectives included evaluating the pharmacokinetics and preliminary efficacy of this drug combination.

Materials and Methods

Patients. Eligible patients were aged 20–74 years, with a diagnosis of CD20- and CD22-positive B-cell NHL according to the World Health Organization classification.[13] The disease must have progressed after one or two prior therapies, and prior treatment must have included one or more doses of rituximab therapy (monotherapy or combined with chemotherapy). Maintenance therapy with rituximab was considered part of the preceding induction regimen, and patients could not be refractory to rituximab (i.e. progressive disease [PD] under treatment or <6 months of protocol therapy initiation). Other inclusion criteria included an Eastern Cooperative Oncology Group (ECOG) Performance Status ≤ 1; life expectancy

[7]To whom correspondence should be addressed.
E-mail: mi-ogura@naa.att.ne.jp

doi: 10.1111/j.1349-7006.2012.02241.x

$\geq$12 weeks; adequate organ function (absolute neutrophil count [ANC] $\geq 1.5 \times 10^9$/L and platelet count $\geq 100 \times 10^9$/L; serum creatinine $\leq 1.5 \times$ upper limit of normal [ULN] and urine protein to creatinine ratio of ≤ 0.2; total bilirubin $\leq 1.5 \times$ ULN, aspartate aminotransferase [AST] and alanine aminotransferase [ALT] $\leq 2.5 \times$ ULN); and ≥ 1 measurable lesion $\geq 1.5 \times 1.5$ cm by computed tomography (CT) scan. Patients who had received radioimmunotherapy or prior treatment with anti-CD22 antibodies were excluded. Prior allogeneic hematopoietic stem cell transplant was not allowed, and patients with prior autologous transplant were eligible if it occurred >6 months before the first study dose. No chemotherapy, anti-lymphoma immunosuppressive therapy, growth factors (except erythropoietin), or investigational agents <28 days before the first study dose (<6 weeks for nitrosoureas or mitomycin C) was allowed.

The study was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent, and the protocol was approved by institutional review board at each site. This trial was registered at www.ClinicalTrials.gov (Identifier: NCT00724971).

Study design. This phase I, open-label, single-arm study evaluated the tolerability, safety, pharmacokinetics, and preliminary efficacy of inotuzumab ozogamicin administered i.v. with rituximab to Japanese patients with B-cell NHL. Screening procedures were performed within 21 days of study treatment initiation and included: medical history and physical examination; ECOG Performance Status; CD20/CD22 immunophenotyping of the B-cell lymphoma; electrocardiogram (ECG) and echocardiogram; complete chemistry panel; complete blood count (CBC) with differential; chest radiograph; CT scans of the chest, abdomen, and pelvis; clinical disease and tumor site assessments; bone marrow aspiration and/or biopsy; urinalysis; and testing for serum antibodies to inotuzumab ozogamicin and rituximab.

A fixed standard dose of rituximab 375 mg/m^2 was administered i.v. on day 1 of a 28-day (±2 days) cycle followed by inotuzumab ozogamicin 1.8 mg/m^2 i.v. on day 2 of the cycle. Treatment was repeated for up to eight cycles or until the occurrence of PD, intolerable toxicity, or patient refusal. Patients were followed for a minimum period of 1 year from the first dosing to assess progression-free survival (PFS). Six patients were to be enrolled in the first cohort; if two or fewer patients experienced a dose-limiting toxicity (DLT) within 28 days after the first dose, the dose and administration schedule would be considered reasonable and an additional four patients would be enrolled in an expanded cohort (10 patients total). If more than two of six patients experienced a DLT in the first 28 days, the tolerability of lower doses of inotuzumab ozogamicin (e.g. 1.3 mg/m^2) would be explored.

A DLT was defined as any of the following that were at least possibly related to study treatment: any grade 3/4 nonhematologic toxicity (except grade 3 nausea or vomiting, unless the patient had received optimal supportive therapy); febrile neutropenia (grade 4 ANC and temperature $\geq$38.0°C); grade 4 ANC $\geq$7 days duration; grade 4 thrombocytopenia $\geq$3 days duration or grade 3/4 thrombocytopenia associated with bleeding tendency requiring platelet transfusion; and delayed recovery (to grade $\leq$1 or baseline, except alopecia) from toxicity related to the study drug that delayed initiation of the next dose by > 2 weeks.

Safety assessment. All patients who received one or more doses of inotuzumab ozogamicin or rituximab were included in the safety analysis (safety population). Safety was monitored through physical examinations, interim history, and laboratory tests. Interim physical examinations, including liver and spleen assessments, occurred every cycle before treatment administration if deemed necessary. Vital signs were measured on each day of inotuzumab ozogamicin administration. Complete chemistry panel and CBC with differential were assessed weekly during the first four cycles and biweekly during the last four cycles. Complete spot urinalysis was performed on day 1 of every other cycle beginning with cycle 2. ECG and chest radiograph were conducted at treatment completion. Adverse events (AEs) were graded using the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0. Patients were monitored for AEs for 28–42 days after the last dose of inotuzumab ozogamicin, regardless of causality, and patients with evidence of treatment-related AEs at the end of treatment visit were followed until the AEs resolved or were determined to be irreversible.

Efficacy evaluation. Efficacy analyses were based on the intention-to-treat (ITT) and evaluable populations. The ITT population included all patients who were enrolled into the intended dose scheme, and the evaluable population included all patients who received two or more cycles of the study treatment, had a baseline tumor CT scan, and had undergone one or more post-baseline tumor assessments. Tumor responses were evaluated by investigator's assessment according to the International Response Criteria for NHL.[14] Overall response rate (ORR) was defined as the proportion of patients achieving complete response (CR), unconfirmed complete response (CRu), or partial response (PR). Duration of overall response was defined as the interval between first CR, CRu, or PR, until relapse or PD. PFS was defined as the interval between the first study dose and relapse, PD, or death, censored at the last tumor evaluation date. PFS rate at 1 year, defined as the proportion of patients without an event (relapse, PD, or death), was used as an early determination of PFS. Overall survival (OS) was defined as the interval between the first study dose and death, censored at the last date known alive.

Tumor responses were determined from CT scans, clinical information (e.g. liver and spleen size), "B-symptoms" (e.g. fever, night sweats, and weight loss), laboratory assessments (e.g. bone marrow biopsies), and biochemical markers of disease activity (e.g. lactate dehydrogenase). Tumor sites and clinical disease measurements were assessed: approximately every 8 weeks during treatment (or earlier with clinical evidence of tumor response), at the end of treatment visit, and approximately every 12 weeks during follow-up visits. If clinically indicated, CT scans were performed earlier than scheduled, and a confirmatory assessment was performed within 4 weeks of a documented tumor response. Tumor assessments continued until PD or death occurred, or subsequent anti-lymphoma treatment was administered.

Pharmacokinetic analyses. Serum concentrations of inotuzumab ozogamicin, total calicheamicin, and free (unconjugated) calicheamicin were determined using a validated ELISA.[10] The quantitation ranges for inotuzumab ozogamicin, total calicheamicin, and free calicheamicin were 52.2–1400, 5–100, and 1.25–150 ng/mL, respectively. Timed blood samples for pharmacokinetic analyses were collected from cycles 1 to 3, at 0 (pre-dose), 1 (before the end of infusion), 4, 48, 144, 192, 312, 480, and 648 h relative to the start of inotuzumab ozogamicin infusion; sample collection for pharmacokinetics was not collected beyond cycle 3. Pharmacokinetic parameters of inotuzumab ozogamicin and total calicheamicin were estimated by a non-compartmental method using WinNonlin, version 5.1 (Pharsight, Mountain View, CA, USA). Validated ELISAs were used for the detection of antibodies to inotuzumab ozogamicin and rituximab. The pharmacokinetic profile of rituximab was not evaluated.

Statistics. The safety and antitumor activity of inotuzumab ozogamicin plus rituximab were evaluated on an exploratory basis and summarized using descriptive statistics. Clopper-Pearson methodology was used to estimate the confidence interval

doi: 10.1111/j.1349-7006.2012.02241.x

(CI) for the ORR, and Kaplan–Meier methodology was used to analyze the duration of overall response, PFS, and OS.

Results

Patients. Ten patients (five males, five females) were enrolled; patient characteristics are summarized in Table 1. Clinical stages at screening were IIIA in five patients, IVA in three patients, and IA and IIA in one patient each. Five patients had experienced one prior lymphoma regimen and five patients had two prior regimens (including two patients who had single-agent rituximab as the second regimen); two patients had prior radiotherapy. All 10 patients received one or more cycles of both inotuzumab ozogamicin and rituximab, with a median number of four cycles (range, 1–8 cycles), and were included in the safety and ITT populations. Eight patients were included in the evaluable population for efficacy analyses, as two patients did not complete two or more cycles of the study treatment.

Safety. In the initial cohort of six patients, two patients experienced DLTs (grade 4 thrombocytopenia persisting $\geq$3 days and grade 3 increased AST). Since two or fewer patients experienced DLTs during the first cycle, an additional four patients were enrolled at the same dose level (inotuzumab ozogamicin 1.8 mg/m^2 and rituximab 375 mg/m^2). Three of the four patients in the expanded cohort experienced DLTs (grade 4 thrombocytopenia persisting $\geq$3 days).

Of the four patients who experienced thrombocytopenia qualifying as a DLT, the patient in the initial cohort had grade 4 thrombocytopenia persisting for 5 days that required a subsequent dose reduction; the patient experienced persistent grade 1–3 thrombocytopenia thereafter but continued treatment until cycle 7. Two patients in the expansion cohort experienced grade 4 thrombocytopenia persisting for 4 days and 5 days; both experienced recovery after discontinuing treatment after cycle 1 due to neutropenia. The remaining patient experienced grade 4 thrombocytopenia persisting for 3 days that required platelet transfusion and subsequent dose reduction; this patient experienced persistent grade 1–3 thrombocytopenia thereafter but remained on therapy until cycle 5. Although an additional three patients experienced grade $\geq$3 thrombocytopenia during the study, no dose delays, dose reductions, platelet transfusions, or treatment discontinuations due to thrombocytopenia were required. In addition, no grade $\geq$3 bleeding events were reported.

All 10 patients experienced one or more treatment-emergent AE, and nine patients experienced grade three or higher treatment-emergent AEs (Table 2). The most commonly reported grade $\geq$3 treatment-emergent AEs were hematologic abnormalities; other grade $\geq$3 events included hypophosphatemia (n = 2) and increased AST (n = 1; Table 3). Neutropenia led to dose delays in two patients. AEs leading to dose reductions (to inotuzumab ozogamicin 1.3 mg/m^2) included thrombocytopenia (n = 2) and increased AST (n = 1). Five patients had AEs (neutropenia [n = 3] and hyperbilirubinemia [n = 2]) that did not recover to grade $\leq$1 within 21 days of the scheduled dosing day and were discontinued from treatment. No serious AEs or deaths occurred during the study.

Efficacy. Eight of 10 patients were followed for more than 52 weeks; one patient with mantle cell lymphoma progressed during the study, and one patient with diffuse large B-cell lymphoma discontinued due to lack of efficacy. OS at 1 year (52 weeks) was 100%, as no deaths were observed during the study. In the ITT population, the ORR was 80% (95% CI, 44–98%; Table 4). In the eight evaluable patients who received

Table 1. Patient characteristics

Characteristic	Inotuzumab ozogamicin 1.8 mg/m^2 + rituximab 375 mg/m^2 (N = 10)
Median age (range), years	60.5 (46–74)
Male sex, n (%)	5 (50)
ECOG Performance Status, n (%)	
0	10 (100)
Histologic subtype, n (%)	
Follicular lymphoma	6 (60)
Mantle cell lymphoma	2 (20)
Diffuse large B-cell lymphoma	1 (10)
Mucosa-associated lymphoid tissue lymphoma	1 (10)
Stage IIIA/IVA disease	8 (80)
No. prior anti-lymphoma regimens, n (%)	
1	5 (50)
2	5 (50)
Prior radiotherapy, n (%)	2 (20)

ECOG, Eastern Cooperative Oncology Group.

Table 2. Summary of adverse events, safety population

Event, n (%)	Inotuzumab ozogamicin 1.8 mg/m^2 + rituximab 375 mg/m^2 (N = 10)
Any TEAE	10 (100)
Grade $\geq$3 TEAE	9 (90)
AE leading to dose delays	2 (20)
AE leading to dose reduction	3 (30)
AE leading to treatment discontinuation	5 (50)
Serious AE	0
Death within 28 days from last dose	0

AE, adverse event; TEAE, treatment-emergent adverse event.

Table 3. Treatment-emergent adverse events in $\geq$30% of patients (all grades) and all grade 3/4 treatment-emergent adverse events, safety population

Event, n (%)	Inotuzumab ozogamicin 1.8 mg/m^2 + rituximab 375 mg/m^2 (N = 10)	
	All grades	Grade 3/4
Thrombocytopenia	10 (100)	7 (70)
Increased AST	9 (90)	1 (10)
Leukopenia	8 (80)	3 (30)
Nausea	8 (80)	0
Increased ALT	8 (80)	0
Neutropenia	7 (70)	5 (50)
Lymphopenia	6 (60)	3 (30)
Increased LDH	6 (60)	0
Fatigue	5 (50)	0
Increased alkaline phosphatase	5 (50)	0
Decreased appetite	5 (50)	0
Hyperbilirubinemia	4 (40)	0
Headache	4 (40)	0
Decreased hemoglobin	3 (30)	0
Increased GGT	3 (30)	0
Nasopharyngitis	3 (30)	0
Pyrexia	3 (30)	0
Stomatitis	3 (30)	0
Hypophosphatemia	2 (20)	2 (20)

Adverse events (AEs) were graded using the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0 (Bethesda, MD, USA). Patients were monitored for AEs for 28–42 days after the last dose of inotuzumab ozogamicin. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; LDH, lactate dehydrogenase.

two or more cycles of study treatment and had one or more post-baseline tumor assessment, ORR was 88% (95% CI, 47–>99%). The duration of response ranged from 346 to 540 days; median duration of response could not be estimated, as no relapse or PD was observed among responders. In the ITT population, the best overall responses (from the start of treatment until PD) were CR in six patients, CRu and PR in one patient each, and stable disease (SD) in two patients. In the evaluable population, CR was achieved in six patients and CRu and SD were achieved in one patient each. Median PFS and OS could not be estimated because the number of events observed was limited during the study. The PFS rate at 1 year was 89% (95% CI, 43–98%) in the ITT population and 88% (95% CI, 39–98%) in the evaluable population.

Pharmacokinetics. Pharmacokinetic data were collected from all 10 patients. Three patients who received AE-related dose reductions of inotuzumab ozogamicin after cycle 1 and 2 patients who discontinued treatment after cycle 1 were excluded from pharmacokinetic analyses for cycles 2 and 3. Two patients who discontinued treatment after cycle 2 were also excluded from analyses for cycle 3. Drug exposure for inotuzumab ozogamicin and total calicheamicin (peak observed concentration [C_{max}] and area under the concentration-time curve [AUC]) increased with the number of doses, coinciding with a prolonged terminal half-life ($t_{1/2}$) and a commensurate decrease in apparent clearance (Table 5). The C_{max} of inotuzumab ozogamicin was typically observed at termination or shortly after completion of infusion. The C_{max} of total calicheamicin was usually observed within 4 h after the initiation of inotuzumab ozogamicin. Mean concentrations and standard deviations of inotuzumab ozogamicin and total calicheamicin in serum over time are shown in Figure 1. Concentrations of free calicheamicin were much lower than other analytes, and pharmacokinetic parameters could not be calculated. No antibodies to inotuzumab ozogamicin or rituximab were detected during the course of the study.

Discussion

This is the first full paper to report on clinical results of inotuzumab ozogamicin therapy in combination with rituximab. The different modes of action between inotuzumab ozogamicin and rituximab may potentially provide synergistic cytotoxicity when used in combination against B-cell NHL. Upon internalization of CD22-bound inotuzumab ozogamicin, calicheamicin diffuses into the nucleus and causes cell death.[3] By contrast, CD20-bound rituximab does not undergo constitutive endocytosis, but rather induces cytotoxic mechanisms that occur at the cell surface: complement-dependent cytotoxicity and antibody-dependent cell mediated cytotoxicity.[9,15] Thus, in addition to targeting different antigens, inotuzumab ozogamicin and rituximab use non-overlapping and perhaps complementary mechanisms of action.

The safety profile of this drug combination was similar to that observed with inotuzumab ozogamicin alone;[10,11] this is consistent with the fact that safety profiles of rituximab and chemotherapy versus chemotherapy alone are similar.[16] The major treatment-related AEs in a phase I study of Japanese patients with follicular lymphoma pretreated with rituximab and administered inotuzumab ozogamicin monotherapy at the MTD of 1.8 mg/m^2 were thrombocytopenia, leukopenia, lymphopenia, neutropenia, increased AST, anorexia, and nausea.[11] A similar toxicity profile was observed during a phase I study of non-Japanese patients with B-cell NHL (predominately

Table 4. Best overall response, intention-to-treat population

Best overall response, *n* (%)	FL (*n* = 6)	DLBCL (*n* = 1)	MCL (*n* = 2)	MALT (*n* = 1)	Total (*N* = 10)
Overall response	6 (100)	0	1 (50)	1 (100)	8 (80)
Complete response (confirmed)	5 (83)	0	0	1 (100)	6 (60)
Complete response (unconfirmed)	0	0	1 (50)	0	1 (10)
Partial response	1 (17)	0	0	0	1 (10)
Stable disease	0	1 (100)	1 (50)	0	2 (20)

Tumor responses were determined by the investigator according to the International Response Criteria for Non-Hodgkin Lymphoma. Tumor assessments occurred approximately every eight weeks during treatment (or sooner), at the end of treatment visit, and every 12 weeks during follow-up visits. Overall response included complete confirmed, complete unconfirmed and partial response. DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MALT, mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma.

Table 5. Pharmacokinetic parameters† of inotuzumab ozogamicin and total calicheamicin

Treatment period (day)	C_{max} (ng/mL)	$t_{1/2}$ (h)	AUC_T (ng·h/mL)	AUC (ng·h/mL)	CL (L/h)	V_{ss} (L)
Inotuzumab ozogamicin						
Cycle 1 (day 1/2)	559 (24%) (*n* = 10)	18.8 (6%) (*n* = 2)	12 300 (51%) (*n* = 10)	22 300 (13%) (*n* = 2)	0.120 (15%) (*n* = 2)	2.33 (3%) (*n* = 2)
Cycle 2 (day 29/30)	822 (19%) (*n* = 5)	29.1 (75%) (*n* = 2)	27 000 (28%) (*n* = 5)	34 800 (35%) (*n* = 2)	0.078 (25%) (*n* = 2)	2.26 (77%) (*n* = 2)
Cycle 3 (day 57/58)	958 (7%) (*n* = 3)	51.7 (40%) (*n* = 3)	50 100 (13%) (*n* = 3)	54 800 (13%) (*n* = 3)	0.050 (2%) (*n* = 3)	3.02 (41%) (*n* = 3)
Total calicheamicin						
Cycle 1 (day 1/2)	67.7 (22%) (*n* = 10)	61.2 (57%) (*n* = 7)	2850 (49%) (*n* = 10)	4060 (27%) (*n* = 7)	0.746 (28%) (*n* = 7)	47.6 (24%) (*n* = 7)
Cycle 2 (day 29/30)	80.2 (14%) (*n* = 5)	96.4 (32%) (*n* = 5)	6490 (35%) (*n* = 5)	7360 (33%) (*n* = 5)	0.424 (31%) (*n* = 5)	45.6 (15%) (*n* = 5)
Cycle 3 (day 57/58)	96.6 (3%) (*n* = 3)	167.9 (43%) (*n* = 3)	10 700 (44%) (*n* = 3)	11 600 (35%) (*n* = 3)	0.249 (28%) (*n* = 3)	47.8 (14%) (*n* = 3)

†Data are shown as mean values at the time points indicated (CV%); the numbers of patients evaluable for each parameter or time point are also provided. AUC, area under the concentration-time curve evaluated to infinity (cycle 1) or dosing interval (672 h; cycles 2 and 3); AUC_T, area under the concentration-time curve evaluated to the last measurable observation; CL, apparent clearance; C_{max}, peak observed concentration; CV, coefficient of variation; $t_{1/2}$, terminal half-life; V_{ss}, apparent steady-state volume of distribution.

doi: 10.1111/j.1349-7006.2012.02241.x

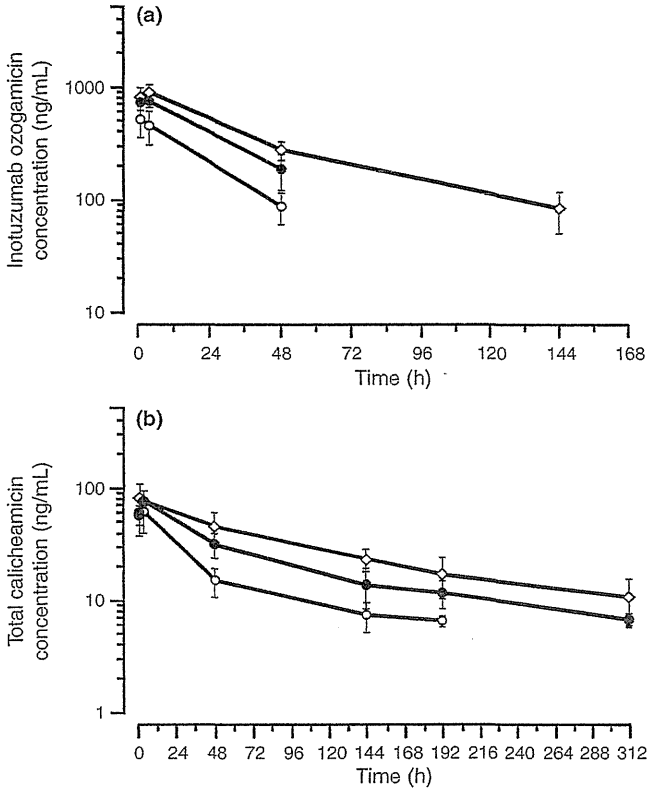

Fig. 1. Mean concentrations of inotuzumab ozogamicin (a) and total calicheamicin (b) in serum after i.v. treatment with inotuzumab ozogamicin 1.8 mg/m^2 and rituximab 375 mg/m^2, 28-day cycle. Error bars denote standard deviations. Cycle 1 (-○-); cycle 2 (-●-); cycle 3 (-◇-).

follicular lymphoma or diffuse large B-cell lymphoma) treated with inotuzumab ozogamicin 1.8 mg/m^2.[10] The most common toxicities in the current study included thrombocytopenia, leukopenia, nausea, and elevated liver function tests. Five of 10 patients had AEs that met the criteria for DLTs. However, these events were all transient laboratory abnormalities without other associated clinical sequelae. Therefore, the independent data monitoring committee considered inotuzumab ozogamicin at 1.8 mg/m^2 plus rituximab to be tolerable and safe and recommended continued clinical development with careful attention for thrombocytopenia in subsequent studies.

Patients in this study had relatively few prior treatments (one to two regimens), but included three patients with aggressive lymphoma diagnoses: two with mantle cell lymphoma and one with diffuse large B-cell lymphoma. In efficacy analyses, most patients achieved CR/CRu (7/10) or PR (1/10) and remained progression-free at 52 weeks. The clinical response in this study compares favorably to responses observed with inotuzumab ozogamicin or rituximab monotherapy. In previous phase I studies of patients with B-cell NHL administered inotuzumab ozogamicin monotherapy at the MTD, the ORRs were 39%[10] and 80%,[11] while rituximab monotherapy in patients with relapsed or refractory, low-grade or follicular, B-cell NHL was associated with an ORR of 48%.[17] The clinical activity demonstrated in this trial is consistent with the robust antitumor activity of this drug combination observed in preclinical models. *In vitro*, inotuzumab ozogamicin plus rituximab suppressed the growth of B-cell lymphoma xenografts by >90%; this effect was additive compared with either agent alone.[5]

The efficacy results were also comparable with reported results of epratuzumab, a humanized anti-CD22 monoclonal antibody, plus rituximab in patients with post-chemotherapy, relapsed or refractory, indolent B-cell NHL (ORR of 54% in 41 patients with follicular lymphoma [24% achieving a CR/CRu], and 57% in seven patients with small lymphocytic lymphoma [43% with CR/CRu]).[18] Although a definite comparison of this study with our study cannot be made due to the limited number of patients in our phase I study, the combination use of inotuzumab ozogamicin plus rituximab may have increased efficacy over combination use of two monoclonal antibodies due to the addition of a targeted chemotherapy agent.

Pharmacokinetic analyses revealed that drug exposure (C_{max}, AUC) to inotuzumab ozogamicin increased with the number of doses of combination therapy, displaying a nonlinear disposition similar to the pharmacokinetic profile observed in phase I studies of inotuzumab ozogamicin monotherapy.[10,11] No effect of rituximab on the pharmacokinetic profile of inotuzumab ozogamicin was apparent. Serum concentration increases may be partially attributable to accumulation; such nonlinearities in drug disposition are common for antibodies.[19]

Inotuzumab ozogamicin in combination with rituximab showed an acceptable safety profile in Japanese patients with relapsed or refractory B-cell NHL that is similar to the observed single-agent profile. Preliminary but encouraging evidence of clinical activity of inotuzumab ozogamicin plus rituximab was also demonstrated, and the findings support the continued clinical development of this therapeutic regimen.

Acknowledgments

This work reporting results from Study B1931005/3129K3-1104 (ClinicalTrials.gov Identifier: NCT00724971) was sponsored by Wyeth Research, which was acquired by Pfizer Inc in October 2009. Editorial/medical writing support was provided by Kimberly Brooks, PhD, at SciFluent, and was funded by Pfizer Inc. We would like to thank the patients who participated in this study and their families, as well as the research nurses, study coordinators, and operations staff. The authors also thank members of the independent data monitoring committee: Dr Ryuzo Ohno, Aichi Cancer Center; Dr Toshiyuki Takagi, Kimitsu Chuo Hospital; and Dr Noriko Usui, Jikei University Daisan Hospital. The following are the sub-investigators who enrolled patients: Toshiki Uchida, Tatsuya Suzuki, Chisako Iriyama, Yasuhito Terui, Yuko Mishima, Yukari Shirasugi, and Ken Ohmachi.

Disclosure Statement

M. Ogura, K. Hatake, K. Ando, and K. Tobinai are currently conducting research sponsored by Pfizer Inc. K. Tokushige, C. Ono, and T. Ishibashi are employees of Pfizer Japan Inc. E. Vandendries is an employee of Pfizer Inc.

References

1 Leonard JP, Coleman M, Ketas JC *et al.* Epratuzumab, a humanized anti-CD22 antibody, in aggressive non-Hodgkin's lymphoma: phase I/II clinical trial results. *Clin Cancer Res* 2004; **10**: 5327–34.
2 DiJoseph JF, Armellino DC, Boghaert ER *et al.* Antibody-targeted chemotherapy with CMC-544: a CD22-targeted immunoconjugate of calicheamicin for the treatment of B-lymphoid malignancies. *Blood* 2004; **103**: 1807–14.
3 DiJoseph JF, Khandke K, Dougher MM *et al.* CMC-544 (inotuzumab ozogamicin): a CD22-targeted immunoconjugate of calicheamicin. *Hematol Meet Rep* 2008; **5**: 74–7.
4 DiJoseph JF, Goad ME, Dougher MM *et al.* Potent and specific antitumor efficacy of CMC-544, a CD22-targeted immunoconjugate of calicheamicin,

against systemically disseminated B-cell lymphoma. *Clin Cancer Res* 2004; **10**: 8620–9.
5 DiJoseph JF, Dougher MM, Kalyandrug LB *et al.* Antitumor efficacy of a combination of CMC-544 (inotuzumab ozogamicin), a CD22-targeted cytotoxic immunoconjugate of calicheamicin, and rituximab against non-Hodgkin's B-cell lymphoma. *Clin Cancer Res* 2006; **12**: 242–9.
6 Rituxan (rituximab) Injection for Intravenous Use (package insert). South San Francisco, CA: Biogen Idec Inc. and Genentech, Inc., 2011.
7 Davis TA, Grillo-Lopez AJ, White CA *et al.* Rituximab anti-CD20 monoclonal antibody therapy in non-Hodgkin's lymphoma: safety and efficacy of re-treatment. *J Clin Oncol* 2000; **18**: 3135–43.
8 Czuczman MS, Olejniczak S, Gowda A *et al.* Acquirement of rituximab resistance in lymphoma cell lines is associated with both global *CD20* gene and protein down-regulation regulated at the pretranscriptional and posttranscriptional levels. *Clin Cancer Res* 2008; **14**: 1561–70.
9 Leonard JP, Martin P. Novel agents for follicular lymphoma. *Hematology Am Soc Hematol Educ Program* 2010; **2010**: 259–64.
10 Advani A, Coiffier B, Czuczman MS *et al.* Safety, pharmacokinetics, and preliminary clinical activity of inotuzumab ozogamicin, a novel immunoconjugate for the treatment of B-cell non-Hodgkin's lymphoma: results of a phase I study. *J Clin Oncol* 2010; **28**: 2085–93.
11 Ogura M, Tobinai K, Hatake K *et al.* Phase I study of inotuzumab ozogamicin (CMC-544) in Japanese patients with follicular lymphoma pretreated with rituximab-based therapy. *Cancer Sci* 2010; **101**: 1840–5.
12 Dang NH, Smith MR, Offner F *et al.* Anti-CD22 immunoconjugate inotuzumab ozogamicin (CMC-544) + rituximab: clinical activity including survival in patients with recurrent/refractory follicular or 'aggressive' lymphoma. *Blood* 2009; **114**: Abstract 584.
13 Harris NL, Jaffe ES, Diebold J *et al.* World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. Report of the Clinical Advisory Committee meeting, Airlie House: Virginia, November, 1997. *J Clin Oncol* 1999; **17**: 3835–49.
14 Cheson BD, Horning SJ, Coiffier B *et al.* Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. *J Clin Oncol* 1999; **17**: 1244.
15 Golay J, Manganini M, Facchinetti V *et al.* Rituximab-mediated antibody-dependent cellular cytotoxicity against neoplastic B cells is stimulated strongly by interleukin-2. *Haematologica* 2003; **88**: 1002–12.
16 Pfreundschuh M, Trumper L, Osterborg A *et al.* CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. *Lancet Oncol* 2006; **7**: 379–91.
17 McLaughlin P, Grillo-Lopez AJ, Link BK *et al.* Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 1998; **16**: 2825–33.
18 Leonard JP, Schuster SJ, Emmanouilides C *et al.* Durable complete responses from therapy with combined epratuzumab and rituximab: final results from an international multicenter, phase 2 study in recurrent, indolent, non-Hodgkin lymphoma. *Cancer* 2008; **113**: 2714–23.
19 Lobo ED, Hansen RJ, Balthasar JP. Antibody pharmacokinetics and pharmacodynamics. *J Pharm Sci* 2004; **93**: 2645–68.

doi: 10.1111/j.1349-7006.2012.02241.x

Clin Chem Lab Med 2012;50(9):1665–1670 © 2012 by Walter de Gruyter • Berlin • Boston. DOI 10.1515/cclm-2011-1854

Clinical and pathological features of B-cell non-Hodgkin lymphomas lacking the surface expression of immunoglobulin light chains

Hiromichi Matsushita[1,*], Naoya Nakamura[2], Yuzo Tanaka[1], Daisuke Ohgiya[3], Yumiko Tanaka[1], Anar Damdinsuren[1], Satomi Asai[1], Miharu Yabe[1], Hiroshi Kawada[3], Yoshiaki Ogawa[3], Kiyoshi Ando[3] and Hayato Miyachi[1]

[1] Department of Laboratory Medicine, Tokai University School of Medicine, Isehara, Kanagawa, Japan
[2] Department of Pathology, Tokai University School of Medicine, Isehara, Kanagawa, Japan
[3] Department of Hematology and Oncology, Tokai University School of Medicine, Isehara, Kanagawa, Japan

Abstract

Background: The flow cytometric analysis of surface immunoglobulin light chains (sIgL) is used as a simple method for evaluating monoclonal B-cell proliferation. However, the sIgL expression, κ or λ, is occasionally undetectable in cases with B-cell non-Hodgkin lymphomas (B-NHL). The purpose of this study was to investigate the clinical and pathological characteristics of these B-NHL cases.

Methods: We retrospectively analyzed 50 cases with previously untreated sIgL-negative B-NHL. All of these cases had been diagnosed at Tokai University Hospital between January 2001 and February 2011. Their medical charts were reviewed.

Results: These cases had several clinical features: diffuse large B-cell lymphoma (DLBCL) (72%), a high serum lactate dehydrogenase level (66%), clinical stage III and IV (68%), and complex karyotypes (58%). Seven out of eight evaluated patients (87%) did not express cytoplasmic IgL, and the DNA rearrangement pattern of IgL showed diversity in 10 analyzed patients. The 5-year event-free survival of all the sIgL-negative B-NHL cases was significantly better with rituximab-containing chemotherapies in comparison to the regimens without it (57.9% vs. 17.9%, p=0.0207), although there was no statistical significance when the DLBCL cases were analyzed (56.6% vs. 22.2%, p=0.1530).

Conclusions: These findings suggest that sIgL-negative B-NHL cases predominantly developed DLBCL in advanced disease, but were heterogeneous at the molecular level.

*Corresponding author: Hiromichi Matsushita, MD, PhD, Department of Laboratory Medicine, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
Phone: +81 463 931121, Fax: +81 463 938607,
E-mail: hmatsu@is.icc.u-tokai.ac.jp
Received November 17, 2011; accepted February 21, 2012; previously published online March 16, 2012

Keywords: diffuse large B-cell lymphoma; non-Hodgkin lymphoma; rituximab; southern blotting analysis; surface immunoglobulin light chains.

Introduction

B-cell non-Hodgkin lymphomas (B-NHL) characteristically have a unilateral expression of a surface immunoglobulin light chain (sIgL), κ or λ, reflecting the monoclonal proliferation of malignant B-cells. Because the antibodies against immunoglobulin light chains work in flow cytometric analyses, and often fail in immunohistochemical analyses, the flow cytometric analysis of sIgL is used as a rapid and simple technology to confirm a diagnosis of B-NHL (1). However, the expression of sIgL is occasionally undetectable including the cases with chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and mediastinal diffuse large B-cell lymphoma (mediastinal DLBCL) (2, 3), and it is sometimes difficult to discriminate these B-NHL cases from reactive diseases, even after the histopathological examination. A delayed diagnosis may lead to a delay in treatment, thus leading to unfavorable results.

Such cases comprise 3.4%–12.2% of all B-NHL cases, of which 30%–50% of cases have diffuse large B-cell lymphoma (DLBCL) (4–7). Because DLBCL is more common in Japan than in Western populations (8), Japanese physicians confront this problem more frequently. In addition, the predominance of sIgL-negative B-NHL in cases with DLBCL led us to hypothesize that sIgL-negative lymphoma may include a pathological or clinical entity reflecting a specific developmental stage of B-cells during lymphomagenesis.

In this study, we investigated the molecular-pathological features of sIgL-negative B-NHL in our institute including the clinical manifestations, DNA rearrangement of immunoglobulin genes, and the therapeutic prognosis, particularly the response to rituximab.

Materials and methods

The records of patients who underwent a flow cytometric analysis at Tokai University Hospital between January 2001 and March 2011 were reviewed. All B-NHL cases diagnosed histopathologically before any specific therapies were selected. The cases with CLL/SLL and mediastinal DLBCL were excluded. Thereafter, the clinical, laboratory and pathological information of these cases were retrospectively analyzed using their medical charts.

The expression of cellular surface markers on the B-NHL cells was evaluated by flow cytometry with a standard protocol using FACSCalibur (BD, Franklin Lakes, NJ, USA). The monoclonal antibodies for flow cytometry were CD5, CD10, CD19, CD20, CD22, CD25, CD30, CD38, HLA-DR, κ and λ (BD Pharmingen, San Diego, CA, USA). The sIgL expression in the lymphoid cells from the FSC/SSC gate was evaluated in two-dimensional (2D) dot plots using a FITC-conjugated anti-κ antibody and a PE-conjugated anti-λ antibody. If the lymphoma cells were limited in the morphological evaluation, the CD20-expressing cells selected by a Cy5-conjugated anti-CD20 antibody (BD Pharmingen) were applied for the analysis. The sIgL-negative B-NHL cases were extracted, according to the criteria in which a negative expression of sIgL was defined as <15% κ- and <10% λ-type sIgL, respectively (5).

An immunohistochemical analysis with formalin-fixed, paraffin-embedded sections was performed using an avidin-biotin-peroxidase complex method. The monoclonal antibodies for immunohistochemistry were CD5, CD10, p53 (Leica Microsystems, Milton Keynes, UK), CD20, BCL-2 (Dakopatts, Glostrup, Denmark), BCL-6, IRF-4 for MUM1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The presence of EBV small RNA in formalin-fixed paraffin-embedded sections was detected using in situ hybridization with a digoxigenin-labeled Epstein-Barr virus (EBV)-encoded small RNA 1(EBER1) antisense probe (9). Based on the immunohistochemical results, the cases were sub-classified into either Germinal Center B-Cell like (GCB) or non-GCB DLBCL, according to the criteria described by Hans et al. (10).

The genomic DNA rearrangement of immunoglobulin heavy and light chains (IgH and IgL, respectively) was evaluated by a Southern blot analysis using frozen tumor sections or cell pellets at a commercial laboratory (SRL, Inc., Tokyo, Japan).

The Kaplan-Meier method was used to estimate the overall survival (OS) and event-free survival (EFS). Log-rank p-values were used for descriptive comparisons of the survival outcomes among the subgroups of patients and treatments.

This retrospective single-center study was approved by the Institutional Review Board of Tokai University Hospital.

Results

Fifty sIgL-negative B-NHL cases were extracted from the 456 B-NHL cases analyzed by a flow cytometric analysis (11.0%). There were no cases with either an HIV infection or who were post-transplantation. Most of the sIgL-negative B-NHL (42 cases, 84%) were found using suspended cells from the involved lymph node, spleen and tumor, followed by bone marrow (5 cases, 10%), peripheral blood (1 case, 2%), pleural effusion (1 case, 2%) and ascites (1 case, 2%).

The majority of sIgL-negative B-NHL cases had diffuse large B-cell lymphoma (DLBCL) (72%) followed by follicular lymphoma (FL) (20%), marginal zone B-cell lymphoma (MZBCL)/mucosa-associated lymphoid tissue (MALT) lymphoma (6%) and lymphoplasmic lymphoma (LPL) (2%) (Table 1). Among the analyzed DLBCL cases, the frequencies of the germinal center B-cell-like subgroup (GCB) and non-GCB were 28% and 72%, respectively.

All of the sIgL-negative B-NHL cases were characterized with regard to age (older than 60 years; 72%), gender (female:male; 1.8:1), an elevated serum lactate dehydrogenase (LDH) level (66%), and clinical stage (CS III plus IV; 68%). DLBCL cases had similar clinical features as described above: older than 60 (72%), female-dominant (female:male; 1.8:1), an elevated serum LDH level (79%) and advanced clinical stage (CS III plus IV; 73%). The DLBCL cases were at a high IPI risk, in comparison to all B-NHL cases (46% vs. 37%). However, FL cases had a normal serum LDH level (70%) and a lower clinical stage (CS I plus II; 60%), thus 50% of them was classified as having low FLIPI risk.

A chromosomal analysis in the 24 evaluated sIgL-negative B-NHL cases showed that 14 cases (58%) had a complex karyotype, in contrast to five cases (21%) with a normal karyotype (Tables 1 and 2). In DLBCL patients, two out of 15 cases (13%) had t(3;14)(q27;q32), which has been reported to induce aberrant expression of BCL6. Four cases (27%) had hyperploidy, but five cases (33%) had a normal karyotype. In the FL patients, six out of seven cases (86%) had t(14;18)(q32;q21), which is characteristic for this type of NHL. None of the FL patients had a normal karyotype.

Flow cytometric immunophenotyping (FCI) revealed that CD19, CD20, CD22, CD38 and HLA-DR were commonly expressed in sIgL-negative B-NHL cells. CD10 was frequently expressed in FL. Sixteen percent of DLBCL cases expressed CD5, and 29% expressed CD10, which was consistent with previous studies (11, 12). Seven out of eight (87%) analyzed cases did not express cytoplasmic IgL.

An immunohistochemical analysis of the tumor confirmed the FCI findings. BCL-2 and BCL-6 were commonly detected, whereas few cases were positive for p53 or EBV. The frequency of CD5 and CD10 expression in DLBCL was 7% and 13%, respectively.

The immunoglobulin DNA rearrangement was analyzed in 10 cases (Table 3). IgH rearrangement was found in all the nine evaluated cases. Regarding IgL κ and λ, all four rearrangement patterns were evenly detected.

The 5-year overall and event-free survivals (OS and EFS) were 58.8% and 36.1% in all cases: 46.0% and 36.8% in DLBCL and 100% and 56.0% in FL, respectively (Figure 1A and B). The 5-year EFS of the cases treated with rituximab-combined regimens was higher than those treated without rituximab (57.6% vs. 17.9%, p=0.0207; Figure 1C). The FL cases also showed a significant difference, according to the treatment (80.0% for 5-year EFS in a rituximab-treated group, p=0.0495, Figure 1D). Although the addition of rituximab also tended to improve the prognosis of DLBCL, the improvement was not statistically significant (56.6% vs. 22.2% for 5-year EFS, p=0.1530, Figure 1E).

Discussion

Our study showed that 72% of sIgL-negative B-NHL cases had DLBCL. The high frequency, approximately 1.5 times higher than that in Western populations, is supposed to reflect the high frequency of DLBCL in B-NHL cases in Japan (8). These DLBCL cases had characteristic clinical features, when compared to previous DLBCL studies (11–13), with an advanced clinical stage, higher IPI, high serum LDH level and complex karyotypes. We also found female-dominancy

Table 1 *Patient characteristics.*

Characteristics	Number of cases, %		
	All cases	DLBCL	FL
Diagnosis	n=50	–	–
DLBCL	36 (72)	–	–
FL	10 (20)	–	–
MZBCL/MALT	3 (6)	–	–
LPL	1 (2)	–	–
GCB or non-GCB in DLBCL	–	n=18	–
GCB	–	5 (28)	–
Non-GCB	–	13 (72)	–
Age	n=50	n=36	n=10
Distribution, years	31–93 (median: 65)	31–93 (median: 67)	40–86 (median: 63)
≤60	14 (28)	10 (28)	4 (40)
>60	36 (72)	26 (72)	6 (60)
Sex	n=50	n=36	n=10
Male	18 (36)	13 (36)	3 (30)
Female	32 (64)	23 (64)	7 (70)
Serum level of lactate dehydrogenase	n=47	n=33	n=10
Normal	16 (34)	7 (21)	7 (70)
Elevated	31 (66)	26 (79)	3 (30)
Clinical stage	n=46	n=33	n=10
I	7 (15)	3 (9)	4 (40)
II	8 (17)	6 (18)	2 (20)
III	11 (24)	8 (24)	2 (20)
IV	20 (44)	16 (49)	2 (20)
International Prognostic Index (IPI)	n=46	n=33	–
Low	13 (28)	8 (24)	–
Low-intermediate	10 (22)	4 (12)	–
High-intermediate	6 (13)	6 (18)	–
High	17 (37)	15 (46)	–
Follicular Lymphoma International Prognostic Index (FLIPI)	–	–	n=10
Low	–	–	5 (50)
Intermediate	–	–	2 (20)
High	–	–	3 (30)
Chromosomal analysis	n=24	n=15	n=7
t(14;18)(q32;q21)	8 (33)	2 (13)	6 (86)
t(3;14)(q27;q32)	2 (8)	2 (13)	0 (0)
t(8;14)(q24;q32)	1 (4)	1 (7)	0 (0)
Complex	14 (58)	9 (67)	5 (71)
Hyperploidy	4 (17)	4 (27)	0 (0)
Normal karyotype	5 (21)	5 (33)	0 (0)
Flow cytometry	Positive cases/analyzed cases, %		
CD19	46/49 (94)	32/35 (91)	10/10 (100)
CD10	17/49 (35)	10/35 (29)	7/10 (70)
CD20	46/49 (94)	29/34 (85)	10/10 (100)
CD22	41/44 (93)	28/31 (90)	10/10 (100)
CD23	13/45 (29)	4/32 (13)	8/10 (80)
HLA-DR	42/43 (98)	29/30 (97)	10/10 (100)
CD38	42/42 (100)	29/29 (100)	10/10 (100)
CD5	6/45 (13)	5/32 (16)	1/10 (10)
CD25	8/43 (19)	7/30 (23)	0/10 (0)
CD30	6/43 (14)	5/30 (17)	1/10 (10)
Surface κ	0/50 (0)	0/36 (0)	0/10 (0)
Surface λ	0/50 (0)	0/36 (0)	0/10 (0)
Cytoplasmic κ	1/8 (13)	0/5 (0)	1/3 (33)
Cytoplasmic λ	0/8 (0)	0/5 (0)	0/3 (0)
Immunohistochemistry	Positive cases/analyzed cases, %		
CD10	9/35 (26)	3/24 (13)	6/8 (75)
CD20	41/44 (93)	26/28 (93)	7/7 (100)
CD5	2/38 (5)	2/27 (7)	0/7 (0)
BCL-2	32/37 (86)	22/27 (81)	8/8 (100)
BCL-6	18/26 (69)	11/17 (65)	7/7 (100)
MUM-1	14/26 (54)	12/17 (71)	2/7 (29)
p53	3/22 (14)	3/20 (15)	0/2 (0)
EBV	1/19 (5)	1/15 (7)	0/3 (0)

DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma grade 1–3a; FLIPI, Follicular Lymphoma International Prognostic Index; LPL, lymphoplasmic lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; MZBCL, marginal zone B-cell lymphoma; –, not determined.

Table 2 Karyotypic analysis in each sIgL-negative B NHL.

Diagnosis	Age	Sex	Specimen[a]	Karyotype
DLBCL	49	F	LN	46,XX [3]
DLBCL	57	F	BM	46,XX [20]
DLBCL	59	F	LN	47,XX,t(3;14)(q27;q32),del(15)(q25),del(18)(q21),–20,+mar2 [5]/48,–X,X,+7,add(14)(q32), del(18)(q21),+mar2 [1]
DLBCL	59	F	BM	46,XX [20]
DLBCL	60	M	Tumor	80–84<4n>XY,+X,–Y,add(1)(q21),add(1)(q23),–5,del(6)(q15),–7,–7,add(9)(p24),+10, add(11)(q13),–13,–14,add(14)(q32),+15, add(15)(p12),–16,–17,add(18)(p11.3),–21,–21, +mar4–5,dms [20]
DLBCL	62	M	LN	46,XY,del(10)(q24),del(13)(q12q22),t(14;18)(q32;q21) [4]/46,XY [16]
DLBCL	63	F	BM	47,XX,del(1)(q23),+del(1)(p11),t(8;14)(q24;q32),–13,t(14;18)(q32;q21),del(15)(q11q14),–22, +mar2 [17]/46,XX [3]
DLBCL	63	F	BM	78<3n>,X,–X×3,–1×2,–4,–5,del(6)(q21)×2,–7×2,–8,–9×2,–11×2,–12,–13,–14,add(14)(q32), ?del(15)(q21),–22,+5mar [6]/46,XX [14]
DLBCL	64	F	LN	45,X,–X,del(6)(q12),t(18;21)(q21;q22) [5]/46,XX [3]
DLBCL	67	F	LN	46,XX [20]
DLBCL	68	M	PB	46,XY [11]
DLBCL	68	F	LN	46,X,–X,t(3;14)(q27;q32),del(8)(q11q13),del(10)(p12),–13,add(21)(q22),+mar2 [20]
DLBCL	72	M	LN	46,X,–Y,del(1)(p11),del(1)(q23),add(2)(p25),add(2)(q21),der(3)add(3)(p21)add(3)(q21), +4,del(4)(q31.1)×2,–5,–8,del(8)(p21),–13,add(14)(p11.2),add(15)(p11.2),–19,–20, add(21)(q22),+5mar [14]/92<4n>,idem×2 [3]/46,XY [3]
DLBCL	81	M	LN	89<4n>,XY,+X,+Y,+del(1)(p22),+del(1)(p22),del(2)(q31),–2,add(3)(p26),+add(3)(q21),–4, –4,–5,–5,–5,+del(6)(q21),+del(6)(q21), del(7)(q32),del(7)(q32), –8,–8,+9,+9,+9, add(10)(p15), +add(10)(p15),+11,+12,+13,+14,–15,–15,–16,–16,–16,–17,–17,–19 [9]/46,XY [11]
DLBCL	93	M	Tumor	46,XY,der(2)t(2;12)(p21;q13) [20]
FL	40	F	LN	46,XX,del(6)(q21),del(10)(q22),t(14;18)(q32;q21) [2]
FL	54	M	LN	46,XY,add(2)(p25),+7,–13,t(14,18)(q32;q21) [8]/46,idem,–8,der(17)t(1;17)(q21;q25), +mar,1dim [7]/46,XY [5]
FL	58	F	SP	46,X,–X,del(10)(q24),+16 [14]/46,XX [6]
FL	63	F	LN	46,XX,add(3)(q21),–4,der(8)t(4;8)(q12;p23),t(14;18)(q32;q21),del(15)(q24), +der(18)t(14;18) [8]/46,idem,+7,–13 [9]/46,XX [3]
FL	64	M	LN	46,XY,t(14;18)(q32;q21) [10]/46,XY [10]
FL	75	F	LN	46,XX,del(10)(q24),t(14;18)(q32;q21) [17]
FL	86	M	LN	52–53,XY,+X,+9,+12,?inv(13)(q12q34),t(14;18)(q32;q21),+add(14)(q32),add(17)(q25), +mar2–3 [7]/53,XY,+X,ins(7;?)(q22;?), +add(8)(q21.2),+9,+12,?inv(13)(q12q34), t(14;18)(q32;q21),+add(14)(q32),add(17)(q25),+mar [4]/46,XY [3]
MZBCL	74	F	Tumor	46,XX,del(6)(q21q23),t(12;18)(q22;q21) [20]
LPL	61	F	LN	46,XX,add(6)(p22),t(14;22)(q32;q11) [20]

[a]The involvement of lymphoma cells in the specimen used for the karyotype analysis was confirmed using tissue specimens or smears. BM, bone marrow; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LN, lymph node; LPL, lymphoplasmic lymphoma; MZBCL, marginal zone B-cell lymphoma; PB, peripheral blood; PE, pleural effusion; SP, spleen.

Table 3 Rearrangement of immunoglobulin DNA.

Diagnosis	Sex	Age	Southern blot analysis		
			IgH	IgLκ	IgLλ
DLBCL	F	62	R	R	NR
DLBCL	F	69	R	R	NR
DLBCL	F	72	R	R	NR
DLBCL	F	63	R	NR	R
FL	F	75	R	NR	R
DLBCL	M	71	R	R	R
DLBCL	M	93	R	R	R
DLBCL	M	70	NT	NR	NR
FL	F	49	R	NR	NR
MZBCL	F	74	R	NR	NR

DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MZBCL, marginal zone B-cell lymphoma; NR, not rearranged; NT, not tested; R, rearranged.

in sIgL-negative B-NHL and DLBCL; however, the previous studies reported male-dominancy in DLBCL in cases both with or without sIgL expression (11–13).

Most of the analyzed cases were negative for cytoplasmic IgL expression, suggesting that the rearranged IgL proteins are not produced in sIgL-negative B-NHL. DNA analysis of tumor samples from this study showed the diversity of IgL rearrangement in sIgL-negative B-NHL. During B-cell development, IgH DNA is rearranged prior to the rearrangement of IgL κ DNA, followed by that of IgL λ DNA after segmental deletion through κ deleting elements (κde). Therefore, sIgL-negative B-NHL with the unilateral DNA rearrangement of IgL κ or IgL λ was typical B-NHL, but with unstable or untranslated IgL. However, the cases without IgL rearrangement or with both types of IgL rearrangement were difficult to explain. All of the DNA samples were extracted from the exact tumors that were utilized for

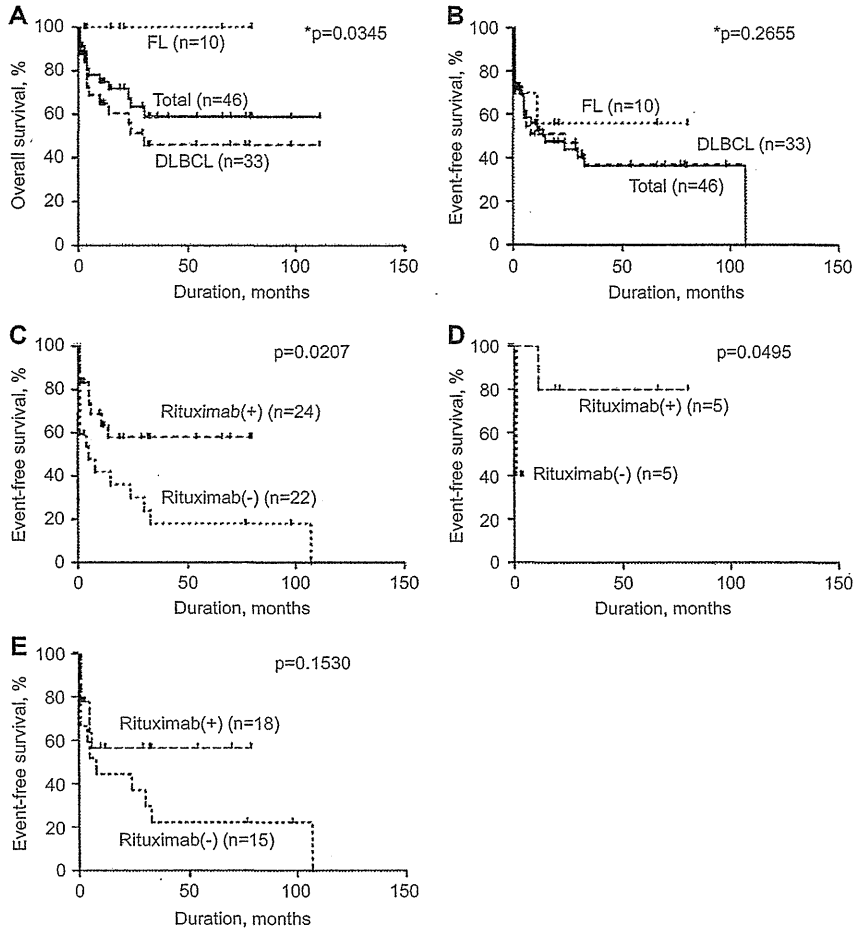

Figure 1 Prognosis of patients with surface immunoglobulin light chain (sIgL)-negative B-cell non-Hodgkin lymphoma (B-NHL) shown by Kaplan-Meier curves.
(A) Overall survival (OS) and (B) event free survival (EFS) of cases with sIgL-negative B-NHL. DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; *, these p-values are calculated based on comparisons of OS or EFS between DLBCL and FL. (C) EFS of sIgL-negative B-NHL with or without rituximab-containing regimens. (D) EFS of sIgL-negative FL with or without rituximab-containing regimens. (E) EFS of sIgL-negative DLBCL with or without rituximab-containing regimens.

the histological and immunohistochemical evaluation by hematopathologists. In addition, a karyotype analysis using the exact tumors revealed that monoclonal chromosomal abnormalities were detected in the two evaluable cases of the unexplained cases (one case without IgL rearrangement and the other with it). These facts convinced us that the DNA samples were actually derived from the lesion involved with the lymphoma. The possible explanations were as follows; the former might be due to deletion of rearranged IgL DNA by unknown causes, and the latter might be due to the presence of two different clones or to rearrangement of both subtypes of IgL by altered functions of κde in one clone. Our overall findings suggested that sIgL-negative B-NHL cells were heterogeneous at the molecular level, and that this could occur as a result of different causes similar to the generation of sIgL-positive B-NHL. The frequencies of CD5, CD10 and GCB in sIgL-negative DLBCL in this study, which were comparable to those in the literature, supported this notion (11, 12).

The proliferation of sIgL-negative B-cells was previously recognized in HIV-related benign follicular hyperplasia and lymphoma (7, 14), thus suggesting that T-cell immunodeficiency may be a cause of sIgL-negative B-NHL in Western populations. In Eastern countries, the EB virus (EBV) is recognized as a causal virus for B- and NK/T-lymphomas (15, 16). However, only a case of the sIgL-negative B-NHL were EBV positive in this study, thus suggesting that EBV is not involved in tumorigenesis of sIgL-negative NHL.

The prognosis was comparable to the previously reported data about sIgL-negative DLBCL cases (13). The present study also showed that rituximab-containing regimens improved the 5-year EFS in all sIgL-negative B-NHL and FL patients, but not in DLBCL patients. However, these findings were not based on the comparison between sIgL-negative and positive cases in the same patient cohort. Further studies are

therefore required to evaluate the difference in the efficacy of rituximab between these cases.

In conclusion, our findings suggest that sIgL-negative B-NHL cases predominantly develop DLBCL with diversity in their IgL DNA rearrangement pattern. This is the first report which has systematically evaluated the DNA rearrangement of immunoglobulins in sIgL-negative B-NHL. Most of these cases usually had advanced stage disease, but the administration of chemotherapeutic regimens with rituximab may improve their prognosis.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

References

1. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. Blood 2008;111:3941–67.
2. Müller-Hermelink HK, Montserrat E, Catovsky D, Campo E, Harris NL, Stein H. Chronic lymphocytic leukemia/small lymphocytic lymphoma. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. WHO classification of tumors of hematopoietic and lymphoid tissues. Lyon: International Agency for Research on Cancer, 2008:180–2.
3. Gaulard P, Harris NL, Pileri SA, Kutok JL, Stein H, Kovrigina AM, et al. Primary mediastinal (thymic) large B-cell lymphoma. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. WHO classification of tumors of hematopoietic and lymphoid tissues. Lyon: International Agency for Research on Cancer, 2008:250–1.
4. Liendo C, Kanieu L, Al-Katib A, Koziner B. Phenotypic analysis by flow cytometry of surface immunoglobulin light chains and B and T cell antigens in lymph nodes involved with non-Hodgkin's lymphoma. Am J Med 1985;79:445–55.
5. de Martini RM, Turner RR, Boone DC, Lukes RJ, Parker JW. Lymphocyte immunophenotyping of B-cell lymphomas: a flow cytometric analysis of neoplastic and nonneoplastic cells in 271 cases. Clin Immunol Immunopathol 1988;49:365–79.
6. Kaleem Z, Zehnbauer BA, White G, Zutter MM. Lack of expression of surface immunoglobulin light chains in B-cell non-Hodgkin's lymphomas. Am J Clin Pathol 2000;113: 399–405.
7. Li S, Eshleman JR, Borowitz MJ. Lack of surface immunoglobulin light chain expression by flow cytometric immunophenotyping can help diagnose peripheral B-cell lymphoma. Am J Clin Pathol 2002;118:229–34.
8. Lymphoma Study Group of Japanese Pathologists. The world health organization classification of malignant lymphomas in Japan: incidence of recently recognized entities. Pathol Int 2000;50: 696–702.
9. Kuze T, Nakamura N, Hashimoto Y, Abe M, Wakasa H. Clinicopathological, immunological and genetic studies of CD30$^+$ anaplastic large cell lymphoma of B-cell type; association with Epstein-Barr virus in a Japanese population. J Pathol 1996;180:236–42.
10. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 2004;103:275–82.
11. Seki R, Ohshima K, Fujisaki T, Uike N, Kawano F, Gondo H, et al. Prognostic impact of immunohistochemical biomarkers in diffuse large B-cell lymphoma in the rituximab era. Cancer Sci 2009;100:1842–7.
12. Niitsu N, Okamoto M, Tamaru JI, Yoshino T, Nakamura N, Nakamura S, et al. Clinicopathologic characteristics and treatment outcome of the addition of rituximab to chemotherapy for CD5-positive in comparison with CD5-negative diffuse large B-cell lymphoma. Ann Oncol 2010;21:2069–74.
13. Tomita N, Takeuchi K, Hyo R, Hashimoto C, Takemura S, Taguchi J, et al. Diffuse large B cell lymphoma without immunoglobulin light chain restriction by flow cytometry. Acta Haematol 2009;121:196–201.
14. Zhao XF, Cherian S, Sargent R, Seethala R, Bonner H, Greenberg B, et al. Expanded populations of surface membrane immunoglobulin light chain-negative B cells in lymph nodes are not always indicative of B-cell lymphoma. Am J Clin Pathol 2005;124: 143–50.
15. Aozasa K, Takakuwa T, Hongyo T, Yang WI. Nasal NK/T-cell lymphoma: epidemiology and pathogenesis. Int J Hematol 2008;87:110–7.
16. Wada N, Ikeda J, Hori Y, Fujita S, Ogawa H, Soma T, et al. Epstein-barr virus in diffuse large B-cell lymphoma in immunocompetent patients in Japan is as low as in Western countries. J Med Virol 2011;83:317–21.

Int J Hematol (2012) 95:652–659
DOI 10.1007/s12185-012-1065-2

ORIGINAL ARTICLE

An open-label extension study evaluating the safety and efficacy of romiplostim for up to 3.5 years in thrombocytopenic Japanese patients with immune thrombocytopenic purpura (ITP)

Yukari Shirasugi · Kiyoshi Ando · Koji Miyazaki · Yoshiaki Tomiyama · Koji Iwato · Shinichiro Okamoto · Mineo Kurokawa · Keita Kirito · Satoshi Hashino · Haruhiko Ninomiya · Shinichiro Mori · Yuji Yonemura · Kensuke Usuki · Helen Wei · Richard Lizambri

Received: 7 August 2011 / Revised: 15 March 2012 / Accepted: 22 March 2012 / Published online: 25 April 2012

Abstract Long-term use of the thrombopoietin mimetic romiplostim was examined in Japanese patients with chronic immune thrombocytopenic purpura (ITP) in this open-label extension. The starting dose of romiplostim was the previous trial dose or 3 μg/kg/week, which was titrated up to 10 μg/kg/week to maintain platelet counts between 50 and 200 × 10^9/L. As of April 2010, 44 patients had enrolled; 71 % women, median age 55.5 years, with five patients discontinuing romiplostim due to patient request (2), administrative decision (2), or not achieving study-defined platelet response (1). Median treatment duration was 100 weeks; median average weekly dose was 3.8 μg/kg. Twenty-eight patients (64 %) self-injected romiplostim. The most frequent adverse events were nasopharyngitis and headache. Nine patients (20 %) had a total of 14 serious adverse events (0.31/100 patient-weeks); of these, only oral hemorrhage was considered treatment related. Fifty hemorrhagic adverse events were reported in 20 patients (46 %) (1.12/100 patient-weeks). Ninety-six percent of patients had a platelet response (doubling of baseline platelet count and platelet count ≥50 × 10^9/L). Of the 25 patients receiving concurrent ITP therapy at baseline, all reduced or discontinued the therapy. Eight patients (18 %) received rescue medications. Administration of up to

Y. Shirasugi (✉) · K. Ando
Department of Hematology and Oncology,
Tokai University Hospital, Kanagawa, Japan
e-mail: whiteced@is.icc.u-tokai.ac.jp

K. Miyazaki
Department of Hematology, Kitasato University Hospital,
Kanagawa, Japan

Y. Tomiyama
Department of Blood Transfusion, Osaka University Hospital,
Osaka, Japan

K. Iwato
Department of Blood Transfusion, Hiroshima Red Cross
Hospital and Atomic-bomb Survivors Hospital,
Hiroshima, Japan

S. Okamoto
Division of Hematology, Keio University Hospital,
Tokyo, Japan

M. Kurokawa
Department of Hematology and Oncology,
University of Tokyo Hospital,
Tokyo, Japan

K. Kirito
Department of Hematology and Oncology,
University of Yamanashi Hospital, Yamanashi, Japan

S. Hashino
Department of Gastroenterology and Hematology,
Hokkaido University Hospital, Hokkaido, Japan

H. Ninomiya
Division of Hematology, Tsukuba University Hospital,
Ibaraki, Japan

S. Mori
Department of Hematology and Oncology,
Kansai Medical University Hirakata Hospital, Osaka, Japan

Y. Yonemura
Department of Transfusion Medicine and Cell Therapy,
Kumamoto University Hospital, Kumamoto, Japan

K. Usuki
Division of Hematology, NTT Kanto Medical Center,
Tokyo, Japan

H. Wei · R. Lizambri
Amgen Inc., Thousand Oaks, CA, USA

3.5 years of romiplostim increased platelet counts and was well tolerated in Japanese patients with chronic ITP.

Keywords Immune thrombocytopenic purpura (ITP) · Romiplostim · Thrombopoietin receptor agonists · Thrombopoietin mimetic

Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by isolated thrombocytopenia (i.e., no other hematologic abnormality) with platelet counts below 100×10^9/L, due to both increased platelet destruction and a relatively low level of platelet production [1–5]. Incidence of ITP in Japan is 2.16/100,000/year, with approximately 70 % of cases occurring in patients older than 50 years [6, 7]. Treatment is typically not recommended for patients with platelet counts $>50 \times 10^9$/L [2, 8, 9]. When treatment is necessary, options include corticosteroids and other immunosuppressive agents, splenectomy, and immunoglobulins [8, 9]. However, a significant proportion of ITP patients either will not respond to or will not have a sustained platelet response with these agents, many of which are accompanied by significant side effects [8, 15, 16]. For those ITP patients who have active *Helicobacter pylori* infection, *H. pylori* eradication therapy appears to improve thrombocytopenia in some [6, 10–14].

While, traditionally, options such as those listed above aim to limit platelet destruction, a new class of agents addresses the now understood relative deficiency in platelet production. Romiplostim, a thrombopoietin (TPO) mimetic with no structural overlap with TPO, increases platelet production by a mechanism similar to that of endogenous TPO [5, 17, 18]. Romiplostim, which has been shown to be effective for the treatment of chronic ITP with good tolerability, has been approved in many countries for the treatment of chronic ITP in adult patients with an insufficient response to previous treatments [19]. Specifically, romiplostim (Nplate®) is indicated in the United States for the treatment of thrombocytopenia in patients with chronic ITP who have had an insufficient response to corticosteroids, immunoglobulins, or splenectomy [20]. Romiplostim should be used only in patients with ITP whose degree of thrombocytopenia and clinical condition increases the risk for bleeding, but not to normalize platelet counts [20]. In Europe, romiplostim is indicated for the treatment of splenectomized adult chronic ITP patients who are refractory to other treatments and may be considered as second-line treatment for adult non-splenectomized ITP patients for whom surgery is contraindicated [21]. As of January 21, 2011, the Japanese regulatory agency, the Ministry of Health, Labour, and Welfare, approved romiplostim (brand name Romiplate®) for the treatment of thrombocytopenia in adult chronic ITP in patients who have had an inadequate response to or are intolerant of other therapies for ITP [22]. Romiplostim should be used only in those patients with ITP whose degree of thrombocytopenia and clinical condition increase the risk for bleeding [22].

Disease presentation, pharmacokinetics, pharmacodynamics, and safety may be affected by ethnic background [23, 24]. Therefore, the use of romiplostim in Japanese patients with ITP was assessed in clinical studies in Japanese patients with ITP. Similar to early phase studies in other populations, romiplostim was found to be well tolerated and effective at increasing platelet counts in a dose-dependent manner with good tolerability in Japanese patients with ITP [25–27]. Likewise, romiplostim significantly increased and maintained platelet counts and was well tolerated in a phase 3 study of 34 Japanese patients with ITP, with similar dosing to that seen in non-Asian patients [27, 28]. However, there are few reports of the long-term safety and efficacy of romiplostim in clinical trials. We describe here the results of patients from the phase 2 and phase 3 studies who then continued into an open-label extension study for up to 3.5 years of romiplostim treatment.

Materials and methods

Study design

This was an open-label extension study designed to assess the safety and efficacy of long-term romiplostim dosing in thrombocytopenic Japanese patients with ITP (Fig. 1). If patients entered the extension study within 12 weeks of receiving the last romiplostim dose in the previous study and had shown an increase in platelet counts $\geq 20 \times 10^9$/L from baseline at least once during the 13-week treatment period of the original trial (excluding 4 weeks after rescue medication), they were treated with romiplostim at the same weekly dose last received in the previous study. Otherwise, patients were treated with romiplostim at a starting dose of 3 μg/kg. Romiplostim was administered by subcutaneous (SC) injection once per week. Dose adjustment based on platelet counts was permitted throughout the treatment period to allow patients to maintain platelet counts in the target range of $50–200 \times 10^9$/L, up to a maximum permitted dose of 10 μg/kg. Patients who achieved a stable dose of romiplostim for at least 3 consecutive weeks were allowed to self-inject romiplostim away from the clinic. The study began in October 2006 and is ongoing.

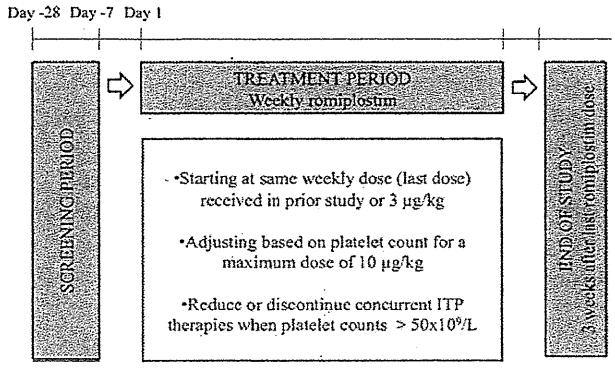

Fig. 1 Study design. This was an open-label extension study of long-term romiplostim dosing in thrombocytopenic Japanese patients with ITP

Eligibility

Patients who had completed any previous romiplostim ITP study in Japan (a phase 2 open-label study and a phase 3 randomized study) were eligible to screen for this study. Additionally, patients were required to provide written informed consent before any study-specific procedures were performed and must have had a platelet count at screening of $<50 \times 10^9$/L. Patients were excluded from the study if they had any significant change in medical history since completion of the previous romiplostim ITP study, including bone marrow stem cell disorders or new active malignancies; tested positive for neutralizing antibodies to romiplostim in the previous romiplostim ITP study; were receiving any treatment for ITP except oral corticosteroids, azathioprine, and/or danazol administered at a constant dose and schedule for at least 4 weeks prior to the screening visit; were pregnant, breastfeeding, or of reproductive potential and not using adequate contraception; had a known severe drug hypersensitivity; or were unlikely to comply with the protocol.

Study endpoints

The primary endpoint of this study was to determine the safety of romiplostim as a long-term treatment in thrombocytopenic Japanese patients with ITP, as measured by the incidence of adverse events, including clinically significant changes in laboratory values. Additional endpoints included incidence of anti-romiplostim antibody formation, incidence of platelet response (doubling of the baseline platelet count at study entry of the previous study and platelet counts $\geq 50 \times 10^9$/L), and proportion of patients able to reduce or discontinue their concurrent ITP therapies (for patients who were receiving oral corticosteroids at a constant dose and schedule at the screening visit). Anti-romiplostim antibodies were assayed at week 1, every 12 weeks during the study and at end of study. Specifically, two validated assays were used: a Biacore 3000 (Biacore International, AB, Uppsala, Sweden) immunoassay and a cell-based bioassay to detect neutralizing or inhibitory effects in vitro [29–31]. If a sample was positive in both assays, a subject was defined as positive for neutralizing antibodies. Throughout the study, investigators could perform a bone marrow biopsy when deemed medically appropriate.

Statistics

The statistical analyses in this open-label extension study were descriptive in nature. Categorical endpoints were summarized by the number and percentage of patients in each category. Continuous endpoints were summarized by number in an eligible subset (n), mean, standard deviation, median, Q1 (25th percentile), Q3 (75th percentile), and minimum and maximum values.

Results

Patient characteristics, disposition, and exposure

As of April 2010, 44 patients who had previously completed either a phase 2 or phase 3 study in Japan [25, 27] enrolled in this open-label extension study. These patients had baseline characteristics of 71 % women, median age 55.5 years (ranging from 25 to 81 years), and median (Q1, Q3) platelet count of 16.5 (6.0, 23.0) $\times 10^9$/L (Table 1). Past treatments included corticosteroids (98 %), IVIg (57 %), *H. pylori* eradication (48 %), splenectomy (39 %), azathioprine (25 %), danazol (23 %), cyclophosphamide (11 %), vincristine/vinblastine (7 %), and rituximab (7 %). Two patients had a past medical history of hepatitis B virus (HBV), three of hepatitis C virus (HCV), and one of HBV and HCV. As of this data cutoff, 5 patients (11 %) discontinued romiplostim due to patient request (2, after 85 and 183 days of treatment, respectively), administrative decision secondary to platelet counts $>200 \times 10^9$/L (2, after 281 and 583 days of treatment, respectively) and platelet counts $\leq 20 \times 10^9$/L after 4 weeks of dosing with 10 μg/kg (1, after 247 days of treatment). The patients who discontinued romiplostim completed an end of study visit 3 weeks after the last administration of romiplostim. All patients received at least one dose of romiplostim, with the mean (SD) treatment duration being 102 (47) weeks (ranging from 12 to 184 weeks) and the mean (SD) average weekly dose being 4.3 (2.7) μg/kg (ranging from 0 to 10 μg/kg). The overall mean weekly dose increase around week 150 corresponds to when the study population consisted of patients from the phase 2 study only (i.e., none

Table 1 Baseline characteristics

	Phase 2 ($N = 11$)	Phase 3 ($N = 33$)	Total ($N = 44$)
Age (years)			
Mean ± SD	55.5 ± 9.8	54.7 ± 13.9	54.9 ± 12.9
Median (min, max)	62.0 (32, 63)	54.0 (25, 81)	55.5 (25, 81)
Sex, *n* (%)			
Female	7 (63.6)	24 (72.7)	31 (70.5)
Male	4 (36.4)	9 (27.3)	13 (29.5)
Baseline platelet count ($\times 10^9$/L)[a]			
Mean ± SD	11.5 ± 10.1	17.7 ± 8.5	16.1 ± 9.2
Median (min, max)	5.5 (3, 31)	19.5 (3, 32)	16.5 (3, 32)
Platelet count prior to the treatment of this study ($\times 10^9$/L)[b]			
Mean ± SD	10.7 ± 8.6	16.3 ± 11.9	14.9 ± 11.3
Median (min, max)	6.0 (3, 25)	11.0 (3, 53)	11.0 (3, 53)

[a] Baseline platelet count in this study was baseline platelet count obtained in the previous study

[b] Platelet count of week 1 or pre-treatment platelet count closest to the first dose of romiplostim in this study if platelet count of week 1 was missing

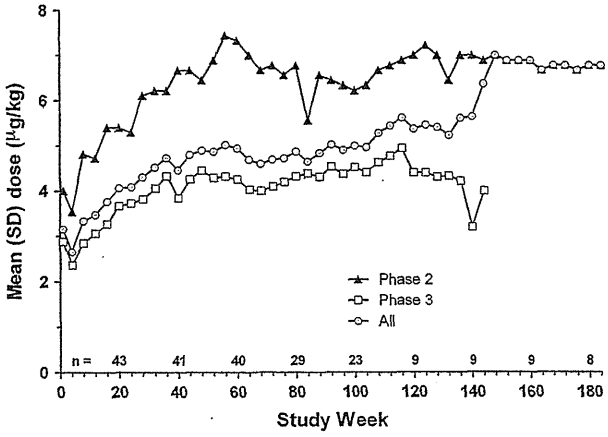

Fig. 2 Mean dose over time. Mean doses for all of the patients, as well as for those who were originally from the phase 2 trial or the phase 3 trial prior to entering the extension, are shown

from the phase 3 study) (Fig. 2). The patients in the phase 2 study had received higher doses throughout this extension. Twenty-eight patients (64 %) received romiplostim by self-injection, beginning after a median (Q1, Q3) of 21 (8.5, 29.0) weeks on study and continuing self-injection for a median (Q1, Q3) duration of 60.0 (28.5, 87.5) weeks. The median (Q1, Q3) percent of weeks these patients were self-injecting was 65 % (42, 81 %). Twelve of these 28 patients (43 %) discontinued self-injection.

Safety

All patients reported at least 1 adverse event after beginning treatment with romiplostim, with 27 patients (61 %) reporting adverse events that were considered by the investigator to be related to the treatment with romiplostim (Table 2). The most frequent adverse events were nasopharyngitis (2.1/100 patient-weeks), headache (0.7/100 patient-weeks), back pain (0.3/100 patient-weeks), contusion (0.3/100 patient-weeks), and malaise (0.3/100 patient-weeks). All nasopharyngitis cases were considered by investigators to not be related to romiplostim, and they were generally mild common upper respiratory tract infections; 6 cases (of 101) were rated as moderate in severity. The most frequently reported treatment-related adverse events were headache (0.52/100 patient-weeks), back pain (0.13/100 patient-weeks), malaise (0.13/100 patient-weeks), and vertigo (0.09/100 patient-weeks).

Nine patients (20 %) reported serious adverse events (duration-adjusted rate of 0.31/100 patient-weeks), with one serious adverse event, mouth hemorrhage, considered by the investigator to be related to the treatment with romiplostim. Other reported serious adverse events included one event each of hemorrhagic anemia, thrombocytopenia, appendicitis, grand mal convulsion, transient ischemic attack, epistaxis, intracranial aneurysm, lumbar spinal stenosis, allergic transfusion reaction, melena, mouth hemorrhage, subcutaneous hematoma, wound, and spinal compression fracture (Table 3). The event of mouth hemorrhage occurred 17 months after initiation of romiplostim in this study. Platelet counts in this patient during romiplostim treatment fluctuated greatly, and thus the dose was frequently adjusted. During one of the times of low platelet counts, the mouth hemorrhage occurred, thus the investigator indicated that there was a reasonable possibility that the hemorrhage was due to romiplostim. As the investigator judged the romiplostim as being effective, treatment with romiplostim was continued. The event of transient ischemic attack occurred 59 days after initiation of romiplostim in this study. The patient had a history of paroxysmal atrial fibrillation, hyperlipidemia, and hyperbilirubinemia. Three days prior to the event, the platelet count was 206 $\times$ 10^9/L. The patient went to the emergency room, where the platelet count was measured at 135 $\times$ 10^9/L. He was not hospitalized, returned home, and the event resolved the next day. Platelet count 4 days after the event was 70 $\times$ 10^9/L. As the investigator judged that the event was caused by transient cerebral hypoperfusion and cerebrovascular spasm, it was considered to not be due to romiplostim, and romiplostim treatment continued. Each of these serious adverse events occurred at a rate of 0.02/100 patient-weeks. There were