Table 1 Clinical characteristics of the 89 acute myelogenous leukaemia patients according to FLT3-ITD/NPM1 mutational status

	FLT3-Wt/ NPM1-Wt	FLT3-Wt/ NPM1-Mt	FLT3-ITD/ NPM1-Wt	FLT3-ITD/ NPM1-Mt	Multivariate analysis (*P*-value)	
Number	59	11	11	8*	FLT3-ITD	NPM1-Mt
Female (%)	27.1	63.6	45.5	62.5	0.3492	0.0216
Age	46 (19-74)	49 (19-79)	52 (15-86)	51.5 (37-70)	0.6558	0.3736
FAB classification (%)						
M0	4 (6.8)	1 (9.1)	1 (9.1)	0		
M1	16 (27.1)	2 (18.2)	3 (27.3)	1 (12.5)		
M2	23 (39.0)	2 (18.2)	3 (27.3)	2 (25.0)		
M4	9 (15.3)	3 (27.3)	0	5 (62.5)		
M5	6 (10.2)	3 (27.3)	3 (27.3)	0		
M6	1 (1.7)	0	0	0		
M7	0	0	1 (9.1)	0		
M4 + M5	15 (25.4)	6 (54.5)	3 (27.3)	5 (62.5)		
others	44 (74.6)	5 (45.5)	8 (72.7)	3 (37.5)	0.7526	0.0159
Cytogenetics (%)						
Normal	25 (42.4)	9 (81.8)	8 (72.7)	7 (87.5)		
Favourable	9 (15.3)	0	0	0		
Intermediate	19 (32.2)	1 (9.1)	3 (27.3)	1 (12.5)		
Adverse	6 (10.2)	1 (9.1)	0	0		
Normal	25 (42.4)	9 (81.8)	8 (72.7)	7 (87.5)		
Others	34 (57.6)	2 (18.2)	3 (27.3)	1 (12.5)	0.0683	0.0109
WBC (10^9/L)	6.0 (1.2-362)	12.7 (1.4-92.1)	18 (4.1-200.4)	39.7 (1.9-177.3)	0.0006	0.4223
PB blast count (10^9/L)	1.64 (0-358.4)	5.8 (0-87.3)	15.6 (2.77-198.4)	33.5 (0.19-164.9)	0.0007	0.4369

*$P < 0.05$ by univariate analysis.

large products, which were cut out from the gel, purified with a QIAquick Gel Extraction kit (QIAGEN), and cloned into the PCR II-TOPO vector (Invitrogen, San Diego, CA, USA) according to the manufacturer's recommendations. Sequencing was performed using an ABI PRISM BigDye terminator V 1.1 cycle sequencing kit and 21M13 and T7 with an ABI 310 Prism sequencer (Applied Biosystems, Foster City, CA, USA).

For the detection of NPM1 mutations, we amplified genomic DNA corresponding to exon 12 of NPM1 by PCR using the NPM1 primers: forward, 5′-CTA GAG TTA ACT CTC TGG TGG-3′ and reverse, 5′-CCT GGA CAA CAT TTA TCA AAC-3′. Amplified products were confirmed by electrophoresis and directly sequenced on an ABI 310 Prism sequencer using an ABI PRISM BigDye terminator cycle sequencing kit.

Immunophenotypic analysis

In all cases, immunophenotypic analyses were performed using bone marrow samples obtained at the time of diagnosis. Mononuclear cells (MNC) were separated by density-gradient centrifugation. The number of MNC collected was adjusted to 10^6 per tube. Selected monoclonal antibodies conjugated to fluorescein isothiocyanate, phycoerythrin and peridinin-chlorophyll protein were used at concentrations titrated to attain optimal staining. Immunophenotype measurements were performed with a multi-colour flow cytometer (FACScalibur or FACScanto, Becton Dickinson, San Jose, CA, USA). Leukaemic blasts were gated according to dim CD45 vs. low-side scatter and analysed further using various combinations of conjugated monoclonal antibodies: CD19/CD13/CD45, CD7/CD33/CD45, CD22/CD33/CD45, CD34/CD56/CD45, CD10/HLA-DR/CD45, CD15/CD11b/CD45, CD3/CD5/CD45, CD4/CD8/CD45, CD2/CD38/CD45, CD41/CD14/CD45, CD16/CD56/CD45, CD45/GPA, CD20/CD10/CD45 and a negative control for autofluorescence. All bone marrow samples contained over 20% blasts, and at least 20 000 events per tube were assessed. Each antigen was considered positive if expressed at a frequency exceeding 20% of cells gated as blasts. Data were analysed with FLOWJO software (TreeStar, Ashland, OR, USA).

Statistical analysis

For univariate comparisons, including cases with FLT3-Wt vs. those with FLT3-ITD; cases with NPM1-Wt vs. those with NPM1-Mt; and cases with FLT3-Wt/NPM1-Wt vs. those with FLT3-Wt/NPM1-Mt vs. those with FLT3-ITD/NPM1-Wt vs. those with FLT3-ITD/NPM1-Mt, we examined categorical variables such as gender, FAB classification, cytogenetic findings, response to induction therapy, type of postremission therapy and immunophenotype with the chi-squared test or Fisher's

exact test. Numerical variables such as age, WBC at diagnosis and PB blast cell count at diagnosis were compared with Student's *t*-tests or analysis of variance.

To evaluate effects of FLT3 and NPM1 with adjustment for one another, logistic regression analysis and multiple linear regression analysis were carried out using each categorical or numerical variable as a dependent variable, by designating both FLT3 and NPM1 as independent variables in the model.

Overall survival (OS) was measured from the date of diagnosis until the date of death. OS was calculated according to the Kaplan–Meier method, and comparisons were made with the log-rank test in the univariate analysis. Furthermore, an analysis of survival using Cox's proportional hazard model was conducted with FLT3-ITD and NPM1-Mt as independent variables. *P*-values < 0.05 were considered to indicate statistical significance. All calculations were performed using BMDP statistical software.

Results

Incidence of FLT3-ITD and/or NPM1 mutations

FLT3-ITD was detected in 19 of 89 patients (21.3%). Although sequencing of the PCR product indicated that FLT3-ITD varied in both position and length from 21 to 102 bp, it was always in-frame and limited to the juxtamembrane domain. A representative result is shown in Fig. 1A. NPM1 mutations were detected in 19 of 89 patients analysed (21.3%). All mutations occurred at position 960 of the NPM coding sequence. Among the 19 patients with NPM1 mutations, mutation A, the most common type (6) with insertion of four bases, TCTG was detected in 16 (84.2%). The remaining three patients (15.8%) had mutation B, an insertion of CATG (Fig. 1B).

FLT3-ITD was present in eight of 19 patients with NPM1-Mt (42.1%), when compared with only 11 of 70 patients with NPM1-Wt (15.7%), ($P = 0.029$). Similarly, NPM1-Mt was detected in eight of 19 patients with FLT3-ITD (42.1%), but only 11 of 70 patients with FLT3-Wt (15.7%). These results indicate that NPM1 mutations and FLT3-ITD represent partially overlapping subgroups in AML. We therefore performed further analyses in terms of four groups: NPM1 and FLT3-ITD single mutants (FLT3-ITD/NPM1-Wt, $n = 11$ and FLT3-Wt/NPM1-Mt, $n = 11$); double mutants (FLT3-ITD/NPM1-Mt, $n = 8$); and subjects with wild-type alleles of both loci (FLT3-Wt/NPM1-Wt, $n = 59$).

Characteristics and outcome

Characteristics of patients in the four groups above are summarised in Table 1. Age distribution did not differ between groups. In agreement with some previous reports (18,21), we detected NPM1-Mt more frequently in female patients (12 of 33, 36.4%) than in men (seven of 56, 12.5%), ($P = 0.022$). By multivariate analysis, median WBC ($P = 0.0006$) and PB blast cell counts ($P = 0.0007$) at diagnosis were significantly higher in FLT3-ITD patients, while NPM1-Mt was not associated

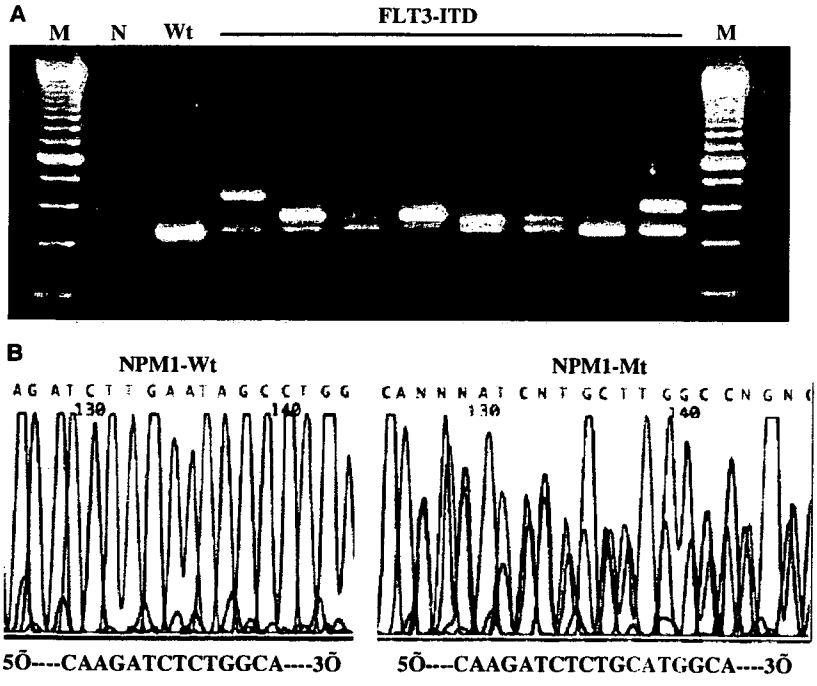

Figure 1 (A) PCR-based detection of FLT3-ITD in acute myelogenous leukaemia (AML). Single bands of 328 bp mark the wild-type FLT3 (Wt), but additional high molecular-weight bands of different sizes are found according to the length of duplicated fragments in AML with FLT3-ITD. M; 100 bp size marker, N; negative control (water). (B) Representative sequence results with and without NPM1 mutation. A 'Type-B' 4-bp insertion was detected at position 960 of the NPM1 gene in this case of mutated NPM1 (NPM1-Mt).

with high WBC or PB blast cell counts (P = 0.422 and 0.437 respectively). A significant difference also was seen in subtype of AML: NPM1-Mt was found predominantly in M4 and M5 (P = 0.016). In contrast, these subtypes did not differ in frequency of FLT3-ITD (P = 0.753).

We characterised cytogenetically all patients. As shown in Table 1, NPM1-Mt was associated with a normal karyotype (P = 0.011), and FLT3-ITD patients tended to have a normal karyotype (P = 0.068). No patient had NPM1-Mt or FLT-ITD in subgroups with t(8;21) or inv16.

All patients were treated with conventional remission induction-chemotherapy as described above. Complete remission rates were similar among between the four groups defined by mutation: 78.0% in FLT3-Wt/NPM1-Wt; 81.8% in FLT3-Wt/NPM1-Mt; 72.7% in FLT3-ITD/NPM1-Wt and 87.5% in FLT3-ITD/NPM1-Mt. Responders were treated further with various postremission therapies including consolidation chemotherapy, auto-PBSCT, and allo-SCT. OS for 5 yr was obtained in 41.65% of the FLT3-Wt/NPM1-Wt group; 64.65% of FLT3-Wt/NPM1-Mt; 44.55% of FLT3-ITD/NPM1-Wt; and 41.67% of FLT3-ITD/NPM1-Mt. Although no significant difference in OS was noted between groups, patients with FLT3-Wt/NPM1-Mt tended to have better outcomes as reported previously (16,18).

Immunophenotype

Expression of surface markers on leukaemic cells from patients in the four groups is shown in Table 2. Myeloid antigen (CD13 and CD33) and HLA-DR were detected in most cases, while T-lymphoid antigens (CD2 and CD3) and B-lymphoid antigens (CD10, CD19, and CD20) were detected in only a few cases. CD7, a pan-T-cell antigen, often is reported to be co-expressed on AML blasts; some investigators have suggested that CD7 expression on AML cells represents an unfavourable risk factor reflecting leukaemic transformation at an early stage of haematopoietic differentiation (22). Frequency of CD7 expression in our study did not differ significantly between the four groups. By multivariate analysis, myelomonocytic markers such as CD11b and CD14 were expressed more frequently on AML blasts in NPM1-Mt cases (P = 0.046 and 0.042 respectively). In addition, CD4, a marker frequently expressed at a low level in myelomonocytic leukaemias (23), was detected in 10 of 19 NPM1-Mt cases (P = 0.1079). These immunophenotypic results are compatible with a significantly high proportion of FAB subgroups M4 and M5 among NPM1-Mt cases (Table 1). Both FLT3-ITD (P = 0.005) and NPM1-Mt (P < 0.0001) were significantly associated with CD34 expression as an independent variable, with respective odds ratio of 5.7 (FLT3-ITD vs. FLT3-Wt) and 13.3 (NPM1-Mt vs. NPM1-Wt); the higher ratio indicated that NPM1 mutations had a closer relationship with low CD34 than did FLT3-ITD.

Discussion

Recent studies frequently have detected FLT3-ITD and NPM1-Mt in patients with normal-karyotype AML, with the two mutations often coinciding (6,18); accordingly,

Table 2 Surface marker profile of leukaemic blast cells in the 89 acute myelogenous leukaemia patients according to FLT3-ITD/NPM1 mutational status

	FLT3-Wt/ NPM1-Wt	FLT3-Wt/ NPM1-Mt	FLT3-ITD/ NPM1-Wt	FLT3-ITD/ NPM1-Mt	Multivariate analysis (*P*-value)	
Number	59	11	11	8	FLT3-ITD	NPM1-Mt
Antigen						
CD2	3 (5.1)	0	2 (18.2)	0	0.1709	0.0651
CD3	0	0	1 (9.1)	0	0.0523	0.2883
CD4	22 (37.3)	7 (63.6)	3 (27.3)	3 (37.5)	0.2292	0.1079
CD7	11 (18.6)	1 (9.1)	3 (27.3)	2 (25.0)	0.3041	0.4946
CD10	0	0	0	0		
CD11b	14 (23.7)	5 (45.5)	1 (9.1)	3 (37.5)	0.2779	0.046
CD13	57 (96.6)	9 (81.8)	8 (72.7)	8 (100)	0.5957	0.5957
CD14	6 (10.2)	3 (27.3)	1 (9.1)	3 (37.5)	0.7846	0.042
CD15	18 (30.5)	5 (45.5)	2 (18.2)	4 (50.0)	0.5893	0.1077
CD19	4 (6.8)	0	0	0	0.2355	0.2355
CD20	0	0	0	0		
CD33	56 (94.9)	11 (100)	9 (81.8)	8 (100)	0.1709	0.0651
CD34	47 (79.7)	3 (27.3)	5 (45.5)	0	0.0054	<0.0001
CD56	6 (10.2)	3 (27.3)	1 (9.1)	0	0.2208	0.3269
HLA-DR	56 (94.9)	8 (72.7)	8 (72.7)	6 (75.0)	0.1431	0.1431
GPA	1 (1.7)	0	0	0	0.5600	0.5600

they may be directly and co-operatively involved in the pathogenesis of AML. To more precisely identify factors associated with FLT3-ITD and/or NPM1-Mt, we performed multivariate as well as univariate analysis that included clinical and pathologic features. We detected NPM1 mutations in 21.3% and FLT3-ITD mutations in 21.3% of all AML patients analysed, a frequency essentially consistent with some previous reports (10,17,18). Double mutations were detected about three times more frequently than single mutations, with the frequency at which FLT3-ITD and NPM1-Mt occurred together with other mutated genes being about the same. In agreement with some previous univariate analyses (6,10), we observed a close association between normal karyotype and FLT3-ITD and/or NPM1-Mt by univariate analysis (FLT3-ITD vs. FLT3-Wt, $P = 0.0357$; NPM1-Mt vs. NPM1-Wt, $P = 0.0088$; comparing all subgroups, $P = 0.0081$). However, multivariate analysis disclosed that only NPM1-Mt was significantly associated with normal karyotype ($P = 0.0109$), while FLT3-ITD only tended to be associated ($P = 0.0683$); NPM1-Mt therefore showed a closer relationship. Similarly, some previous studies have shown that NPM1-Mt and FLT3-ITD each are associated with higher leucocyte and blast cell counts (15–18); while we also found significantly increased numbers of leucocytes and blast cells in patients with FLT3-ITD ($P = 0.0002$, $P = 0.0002$) and with double mutants ($P = 0.0019$, $P = 0.0018$); NPM1-Mt alone showed only a marginal relationship to higher numbers of leucocytes ($P = 0.0929$) and blast cells ($P = 0.0999$) by univariate analysis. In our multivariate analysis, a significant difference in leucocyte and blast counts was found only patients with FLT3-ITD ($P = 0.0006$, $P = 0.0007$), suggesting that FLT3-ITD mainly induces leukaemic cell proliferation. Recent researches have revealed molecular mutations play an increasing role for classification, prognostication and therapeutic strategies in AML (24,25). Despite our limitation of studying a small number of patients retrospectively, our NPM1-Mt patients showed a tend toward favourable clinical outcome, especially in patients without FLT3-ITD, in agreement with some recent investigations (16,18). More prospective studies will be required to establish an optimal method of treating AML patients with FLT3-ITD and/or NPM1-Mt.

Surface markers are a useful tool for assigning leukaemic blast cells to the myeloid or lymphoid lineage and for predicting which cells would be a target for leukaemic transformation during haematopoietic differentiation. Munoz *et al.* (26) demonstrated that AML cells with FLT3-ITD very often express myelomonocytic markers including CD11b, CD15, CD33 and CD36, and less commonly, immature markers such as CD34 and CD117. Similarly, evidence has been accumulating that antigen patterns in cases of NPM1-Mt correspond to a mature myeloid population with monocytic differentiation, showing lower expression of CD34 (6,15,18,21). In addition, M4 and M5 disease has been more frequent in FLT3-ITD (4,26) and in NPM1-Mt (16,17,21) cases. However, as these mutations often coincide with each other, which mutational event most affects the pathogenesis of AML with monocytic differentiation remains unclear. Although our univariate analysis did not reveal any significant difference in surface marker expression among the four subgroups except for CD34; multivariate analysis indicated that CD11b ($P = 0.046$) and CD14 ($P = 0.042$) were significantly associated with NPM1-Mt, independently of FLT3 status ($P = 0.2779$ and 0.7846 respectively). High expression of monocytic markers also was compatible with a more frequent finding of M4 and M5 disease in patients with NPM1-Mt ($P = 0.0159$), than in FLT3-ITD ($P = 0.7526$). Less frequent CD34 expression also was confirmed in both FLT3-ITD ($P = 0.0054$) and NPM1-Mt cases ($P < 0.0001$) independently of each other in the multivariate analyses. Less frequent CD34 expression was more closely associated with NPM1-Mt than FLT3-ITD (odds ratio, 13.3 vs. 5.7), which paralleled the more frequent diagnosis of mature myelomonocytic leukaemia, M4 and M5. Thus, our findings clearly indicated a close association between NPM1-Mt and monocytic features of AML.

Why FLT3 and NPM1 mutations often occur together remains unclear, as do which mutation precedes the other in the process of leukaemogenesis and how the two mutations function co-operatively. Multivariate analyses demonstrated distinctive pathologic and clinical features for each mutation. Significantly increased WBC and blast cell counts in FLT3-ITD may be associated with cell proliferation, while higher expression of monocytic markers and down-regulation of CD34 expression in NPM1-Mt may reflect differentiation toward the monocytic lineage. Recent findings suggest that FLT3 mutations occur in the primitive $CD34^+$ $CD38^-$ fractions, where a rare 'leukaemic stem cells' population might exist despite less frequent CD34 expression in AML with FLT3-ITD (27). Consequently, we would explain the pathogenesis of AML with FLT3-ITD and NPM1-Mt as follows: FLT3-ITD arises in the most primitive $CD34^+$ $CD38^-$ fractions and together with NPM1 mutations impairs differentiation of the accumulated blast cells, causing these cells to develop along the myelomonocytic lineage. Analyses of larger numbers of patients and detailed molecular investigations will be required to address how NPM1 and FLT3 mutations interact in leukaemogenesis to induce leukaemias specific to the myelomonocytic lineage.

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ORIGINAL ARTICLE

Toxoplasmosis encephalitis following severe graft-vs.-host disease after allogeneic hematopoietic stem cell transplantation: 17 yr experience in Fukuoka BMT group

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Abstract

Toxoplasmosis is a rare but rapidly fatal complication that can occur following hematopoietic stem cell transplantation (HSCT). Over a 17-yr period at our institutions, a definite diagnosis of toxoplasmosis was made in only two of 925 allogeneic HSCT recipients (0.22%) and none of 641 autologous HSCT recipients. These two patients received a conventional conditioning regimen followed by transplantation from an HLA-matched donor; however, they developed severe graft-vs.-host disease, which required intensive immunosuppressive therapy. Despite prophylactic treatment with trimethoprim/sulfamethoxazole, their immunosuppressive state, as indicated by a low $CD4^+$ cell count, might have resulted in toxoplasmosis encephalitis. Rapid and non-invasive methods such as a polymerase chain reaction (PCR) test of their cerebrospinal fluid for *Toxoplasma gondii* and magnetic resonance imaging of the brain were useful for providing a definitive diagnosis and prompt therapy in these patients: one patient stabilized and survived after responding to treatment with pyrimethamine/sulfodiazine whereas the other died of bacterial infection. In addition, retrospective PCR analyses of the frozen stored peripheral blood samples disclosed that detection of *T. gondii* preceded the onset of disease, indicating routine PCR testing of peripheral blood specimens may be an early diagnostic tool. It should be noted that when patients receiving HSCT have an unexplained fever and/or neurological complications, PCR tests should be considered to avoid cerebral lesions and improve the outcome of the patients.

Key words toxoplasmosis; encephalitis; graft-vs.-host disease; hematopoietic stem cell transplantation; polymerase chain reaction

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Toxoplasmosis is a rare opportunistic protozoal infection caused by the parasite *Toxoplasma gondii*. Infection is acquired mainly by ingestion of food or water contaminated with oocysts shed by cats or by eating undercooked or raw meat containing tissue cysts (1). A primary infection most often leads to an asymptomatic infection in healthy individuals, resulting in the latency of the *T. gondii* in muscles and other organs. Fatal toxoplasmosis appears to occur mainly through reactivation of these latent cysts in seropositive individuals who are immunocompromised, especially those with advanced acquired immunodeficiency syndrome (2). In this context of hematopoietic stem cell transplantation (HSCT), severe toxoplasmosis can occur in patients who have been profoundly immunosuppressed. The seroprevalence for *T. gondii* varies geographically, ranging from < 15% in

North America to 50–80% in Central Europe (3, 4). Thus, the incidence of diagnosed cases of toxoplasmosis after HSCT has also been reported to vary between 0.4% and 7%, depending on the endemicity (3, 5–7). In Japan, the seropositivity for *T. gondii* in the general population has been reported to be approximately 10–15% (8, 9); however, only a few cases of toxoplasmosis after HSCT has been reported (10).

We report on two patients who developed severe toxoplasmosis encephalitis following allogeneic HSCT, confirmed by a polymerase chain reaction (PCR) examination of their cerebrospinal fluid (CSF) for *T. gondii*. One patient survived after responding to treatment with pyrimethamine and sulfodiazine, but the other died of bacterial infection. Physicians should be alert to this rare but often fatal complication in patients receiving HSCT.

Case presentation

Case 1

A 52-yr-old Japanese man underwent allogeneic bone marrow transplantation (BMT) from an HLA-matched unrelated donor for treatment of acute myelogenous leukemia (AML) in August 2006. A preparative conditioning regimen consisted of total body irradiation (TBI) and cyclophosphamide. Short-term methotrexate (MTX) and tacrolimus were administered as prophylaxis against graft-vs.-host disease (GVHD). Engraftment was achieved on day 23 after BMT, followed by the daily administration of trimethoprim/sulfamethoxazole (TMP 80 mg/SMT 400 mg). On day 38, the patient developed skin eruptions, diagnosed as cutaneous acute GVHD (grade II, stage 3) confirmed by skin biopsy. Prednisolone (PSL) was started at a daily dose of 2 mg/kg; however, on day 45 he had massive watery diarrhea (20 times per day). A diagnosis of gastrointestinal steroid-resistant acute GVHD (grade III, stage 3) was made based on endoscopic and histologic findings. In addition to PSL, mycophenolate mofetil (MMF) was administered at a daily dose of 2 g. This treatment resulted in a gradual clinical improvement of the acute GVHD and the immunosuppressants were then tapered. MMF was discontinued 1 month later, leaving a daily maintenance dose of PSL (0.2 mg/kg) and tacrolimus. On day 78, the patient became somnolent, disoriented and suffered from short-term memory loss. A magnetic resonance imaging (MRI) of the brain revealed multiple hyperintense lesions with mild perifocal edema in the left basal nuclei and in the subcortical white matter of the frontal lobes, thalamus and cerebellum, suggestive of possible toxoplasmosis (Fig. 1A). Examination of CSF demonstrated an increase in the concentration of CSF protein (87 mg/dL)

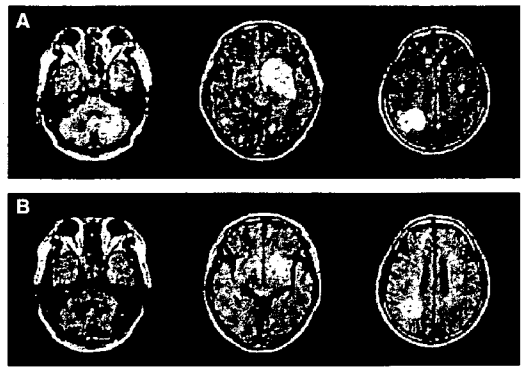

Figure 1 Serial MR maging studies on day 79 (A) and on day 164 (B) after allogeneic bone marrow transplantation in case 1. Images were obtained by the fluid-attenuated inversion recovery sequences and the postgadolinium T2-weighted sequences (T2-FLAIR). Images revealed multiple hyperintense lesions with mild perifocal edema in the left basal nuclei, subcortical white matter of the frontal lobes, thalamus and cerebellum (A). After 85 d of treatment, a marked decrease in the lesions and of the contrast enhancement was evident (B).

and white blood cell count (262 cells/μL), predominantly neutrophils. Both cultures and PCR of CSF were positive for *T. gondii* but negative for other fungus, bacteria, or viruses such as herpes simplex virus, human herpesvirus-6, cytomegalovirus and Epstein–Barr virus (Fig. 2). There was no evidence of toxoplasmosis in other organs. Based on these findings, the patient was diagnosed as having toxoplasmosis encephalitis and treatment with cotrimoxazole and clindamycin was initiated. Flow cytometric analysis of the peripheral blood demonstrated that $CD4^+$ and $CD8^+$ cells comprised 2.3% (46/μL) and 17.3% (346/μL) of mononuclear cells respectively. Despite anti-parasitic therapy with cotrimoxazole/clindamycin, his level of consciousness rapidly deteriorated and he slipped into a coma. Follow-up MR scans 12 d after the initial MRI demonstrated significant progression of the disease. On day 95, cotrimoxazole/clindamycin was

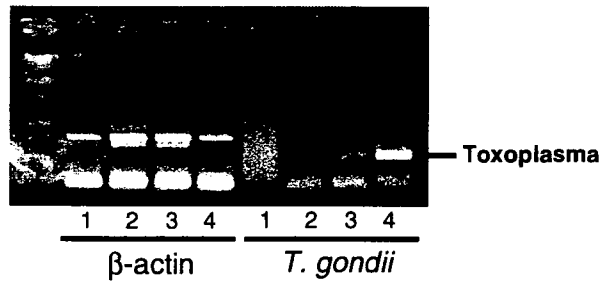

Figure 2 Polymerase chain reaction analysis for detection of *Toxoplsma gondii*. (1) negative control, (2) cerebrospinal fluid at the onset of toxoplasmosis from case 1, (3) peripheral blood samples obtained 7 d before the onset of toxoplasmosis from case 1, (4) positive control.

replaced by a combination of pyrimethamine and sulfodiazine with folinic acid, recommended as the most successful regimen (1). These orphan drugs are not yet commercially available in Japan, but were obtained from the Research Group on Chemotherapy of Tropical Diseases (Division of Infectious Diseases, Advanced Clinical Research Center, the Institute of Medical Science, University of Tokyo). Thereafter, the patient's level of consciousness improved gradually. On day 111 (33 d after starting therapy for toxoplasmosis), the result of a PCR test for *T. gondii* in the CSF became negative. On day 164, an MRI revealed a marked decrease in the lesions and of contrast enhancement (Fig. 1B). A slow but steady improvement was observed, and the patient stabilized. He had continued to receive a maintenance dose of pyrimethamine/sulfodiazine until his CD4 cell count recovered to >200 μ/L according to published recommendations (1). He was sent to a rehabilitation unit and survived 5 months after the first diagnosis of toxoplasmosis without a relapse of either the AML or toxoplasmosis encephalitis.

Case 2

A 54-yr-old Japanese woman underwent an allogeneic peripheral blood stem cell transplantation (PBSCT) from an HLA-identical sibling donor for the treatment of myeloid blastic crisis of Philadelphia-positive chronic myelogenous leukemia (CML-BC) in December 2005. The conditioning regimen consisted of TBI and cyclophosphamide. Short-term MTX and cyclosporine (CSP) were administered as prophylaxis against GVHD. Rapid engraftment was achieved without development of acute GVHD, followed by the daily administration of TMP 80 mg and SMT 400 mg. On day 42 after the allogeneic PBSCT, PCR analysis of the BM for a chimeric bcr-abl gene was negative. On day 74, however, myeloblasts were documented in the peripheral blood and a relapse of CML-BC was confirmed by bone marrow examination. CSP was discontinued to induce a graft-vs.-leukemia effect, followed by the administration of imatinib. These treatments were effective in decreasing the number of myeloblasts. On day 120, a PCR study was again negative for bcr-abl, resulting in a discontinuation of the imatinib. On day 151, her serum bilirubin (8.9 mg/dL), AST and ALT (182 IU/L and 233 IU/L, respectively) concentration were elevated. Treatment with CSP (150 mg daily) and PSL (2 mg/kg daily) against hepatic GVHD resulted in a slow clinical improvement, leaving daily maintenance doses of PSL (0.2 mg/kg) and CSP (75 mg). On day 234, she manifested a headache and nuchal rigidity. An MRI scan of the brain disclosed numerous enhancing lesions involving both cerebral and cerebellar hemispheres, suggesting toxoplasmosis or another infectious disease (Fig. 3). A lumbar puncture revealed that her CSF protein concentration was increased to 87 mg/dL and a white cell count of 262 cells/μL, predominantly neutrophils. PCR amplification for *T. gondii* performed on the CSF fluid was positive. There was no evidence of bacterial, viral, or fungal infections in the CSF. A diagnosis of toxoplasmosis encephalitis was made based on these findings. Flow cytometric analysis of her peripheral blood showed that the concentrations of $CD4^+$ and $CD8^+$ cells were 86/μL and 198/μL respectively. Treatment with cotrimoxazole/clindamycin was started; however, subsequently she developed bacterial pneumonia and sepsis (*Pseudomonas aeruginosa*) and she died of pneumonia on day 248. An autopsy was not performed.

Figure 3 Magnetic resonance imaging of case 2 by T2-FLAIR sequences on day 235 following allogeneic peripheral blood cell stem cell transplantation. Images disclosed multiple lesions involving both cerebral and cerebellar hemsispheres.

PCR analyses for *T. gondii*

Toxoplasmosis encephalitis was diagnosed on positive results from PCR analysis for *T. gondii* on CSF samples (11). Recently, several reports have suggested that prospective detection of *T. gondii* in peripheral blood by PCR precedes the onset of toxoplasmosis and might help in the early diagnosis (12–16). Therefore, PCR amplification for *T. gondii* was also retrospectively performed on the frozen stored peripheral blood samples obtained prior to allo-HSCT and 7 d before the onset of toxoplasmosis encephalitis to determine if PCR test could have contributed to the early diagnosis of toxoplasmosis. DNA was extracted from CSF and peripheral blood samples and PCR amplification was performed using primers (forward primer, GGAACTGCATCCGTTCAT GAG, and reverse primer, TCTTTAAAGCTTCGTGG TC) specific for a sequence on the B1 gene of *T. gondii* (17). β-actin DNA was amplified as a control. PCR products were electrophoresed on an ethidium bromide-stained 2% agarose gel (Fig. 2).

Toxoplasma gondii was undetectable in peripheral blood samples in both patients by PCR before allo-HSCT. In contrast, a positive signal for *T. gondii* was detected in peripheral blood obtained 7 d before the

onset of toxoplasmosis in both cases (Fig. 2). Two and four weeks after starting therapy for toxoplasmosis, patient 1 had negative results of PCR for *T. gondii* in both CSF and peripheral blood.

Discussion

Toxoplamosis is a rare but almost always fatal opportunistic infection following HSCT. In most of the published reports, the diagnosis was made at autopsy without having received specific, anti-toxoplasmosis therapy before death (4). The prognosis of toxoplasmosis is very poor with a nearly 90% case fatality rate in HSCT patients (12, 18). In addition, for direct proof of toxoplasmosis infection, an invasive procedure, such as a brain biopsy, is often performed but in HSCT recipients this usually carries a high risk of complications. However, if appropriately treated, approximately 60% of patients usually show clinical improvement (1, 12, 19). Thus, a rapid and non-invasive diagnosis as well as early treatment are extremely important because toxoplasmosis is so difficult to diagnose histologically in HSCT patients. Recently, several studies have reported that PCR test for *T. gondii* is useful for making an earlier diagnosis of toxoplasmosis encephalitis that can prove directly the presence of this parasite (4, 16, 20), as serology is of little use for diagnosing toxoplasmosis after HSCT. In our patients, typical features on their MRI scans helped us to suspect toxoplasmosis encephalitis, which led to an early diagnosis confirmed by PCR examination of the CSF. One patient survived after responding to anti-toxoplasmosis therapy while the other died of another infectious complication. Recent several reports suggested the efficacy of prospective PCR monitoring in peripheral blood samples to detect reactivation of *T. gondii* for guiding pre-emptive therapy in patients at very high risk (12–16). Retrospective PCR analysis revealed the presence of *T. gondii* in peripheral blood obtained 7 d before the onset of toxoplasmosis in our patients, indicating that PCR monitoring of peripheral blood may contribute to a rapid diagnosis and efficacy of treatments. Thus, toxoplasmosis encephalitis should be considered in the differential diagnosis of HSCT recipients who present with neurologic complications, and the use of sensitive and non-invasive PCR analysis is important for making an early diagnosis in these patients. This early detection allows therapy to be started more quickly.

As far as we know, only a few HSCT patients have been reported to have developed toxoplasmosis in Japan (10), which might depend on the relatively low seropositivity rate for *T. gondii*; seropositivity for *T. gondii* in Japan has been reported to be approximately 10% (8, 9). On the other hand, it is possible that physicians might not include this infectious disease in their differential diagnosis of HSCT patients who present with neurologic complications (21). From 1989 to 2006 at the four HSCT centers in Fukuoka Blood and Marrow Transplantation Group, 925 allografts and 641 autografts were performed. In our experience, there have been no cases to date of toxoplasmosis among autologous HSCT recipients, whereas two patients who received an allogeneic HSCT were diagnosed conclusively as having toxoplasmosis encephalitis, which corresponds to a frequency of 0.22% (two of 925) of all allogeneic and 0.13% (two of 1566) of all HSCT recipients. Our two patients were diagnosed as toxoplasmosis after 2005, which might have been influenced from advances in MRI scanning technology and PCR techniques.

It has been reported that good compliance with TMP/SMT prophylaxis might have a possible protective effect on the subsequent development of toxoplasmosis in immunocompromised hosts, even in those with a low $CD4^+$ cell count ($< 200/\mu L$) (1). Our institutions routinely give prophylactic TMP/SMT to all transplant recipients: they are given daily TMP 320 mg and SMT 1600 mg prior to HSCT, which is reduced to TMP 80 mg and SMT 400 mg daily after engraftment. Most patients have not been tested serologically for *T. gondii* prior to HSCT. Theoretically, this prophylactic treatment with TMP/SMT will prevent the occurrence of *Pneumocystis carinii* pneumonia as well as toxoplasmosis, which might be at least partially responsible for the low incidence of toxoplasmosis at our hospitals. However, there are several reports of toxoplasmosis that occurred in spite of prophylaxis with TMP/SMT (6, 14). Our two patients who developed toxoplasmosis had suffered from severe GVHD, which required intensive immunosuppressive therapy. These two patients showed delayed recovery of immune reconstitution with a low number in $CD4^+$ cells ($46/\mu L$ and $86/\mu L$ in the two patients) because of the occurrence of GVHD and/or immunosuppressive therapy against it (22). Moreover, in the first patient who had suffered from gastrointestinal GVHD, incomplete absorption in the gut might have allowed the reactivation of toxoplasmosis despite treatment with TMP/SMT. Therefore, routine PCR testing of peripheral blood specimens may help in the early diagnosis of toxoplasmosis in the patients who suffer from and treated for the severe GVHD, similar to the pre-emptive antigenemia or PCR studies detecting cytomegalovirus infection (23).

In conclusion, we described two patients who developed toxoplasmosis encephalitis following allogeneic HSCT. The onset of GVHD and the subsequent administration of immunosuppressive agents might have compromised their immune surveillance, ultimately resulting in this complication. Thus, physicians should be aware that fatal toxoplasmosis can occur in HSCT patients and

PCR test should be considered to make an early diagnosis and improve the outcome of the patients when they have an unexplained fever and/or neurological complications.

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宛先ラベルに変更･訂正がある場合ご記入下さい

（〒　　－　　）

造血幹細胞移植患者の長期フォローに関する実態調査

ご回答に際してのお願い

■ ご回答の記入は、
① 該当する項目番号に○印をつける。
② 記入欄に数字を記入する。
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以上の方法でお願いいたします。

■ ご記入頂きましたら、同封の返信用封筒にて、切手を貼らずにそのままご返送下さい。

■ このアンケートについて、ご不明な点などがございましたら、ご遠慮なく下記の担当者まで、ご連絡下さい。

■ 尚アンケート用紙は、＿＿月　日(　)までに＿＿ご投函頂きますようお願い致します。

＊本アンケートへのご回答は、移植業務に従事されている医師および看護師の方にご記入をお願いします。

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【造血幹細胞移植および移植後長期フォローの実施状況について】

【全員の先生へ】

Q1. 貴施設の（診療科ではなく施設全体の）病床数をお教え下さい。（○は１つ）

1.	300床未満
2.	300～500床
3.	500～1000床
4.	1000床以上

Q2. 貴科の移植用病床数をお教え下さい。

移植用病床数	床

Q3. 貴科の移植に携わっている医師数をそれぞれお教え下さい。

(1) 常勤医師	名
(2) レジデント	名
(3) 非常勤医師	名

Q4. 昨年（2007年度）１年間の「造血幹細胞移植」の実施総数をそれぞれお教え下さい。

(1) 自家移植	例／2007年
(2) 血縁同種移植	例／2007年
(3) 非血縁骨髄移植	例／2007年
(4) 臍帯血移植	例／2007年

Q5. 貴施設で定期的にフォローしている移植後長期生存患者（以下、長期フォロー患者と略す）の人数をお教え下さい。（○は１つ）

1.	50名未満
2.	50～99名
3.	100～299名
4.	300～499名
5.	500名以上

Q6. 長期フォローの必要な患者１人あたりの診療時間は平均何分でしょうか。

長期フォローに必要な診療時間	分／1患者

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Q7. 関係各科との連携についてお伺いします。
長期フォロー患者のコンサルトを依頼する頻度が高い順に、順位をお教え下さい。その際、付ける順位はいくつでも結構です。

消化器内科		位	脳神経外科		位
呼吸器内科		位	心臓血管外科		位
内分泌・代謝科		位	外科／乳腺外科		位
腎臓内科		位	呼吸器外科		位
循環器科		位	産婦人科		位
膠原病科		位	泌尿器科		位
精神科		位	整形外科		位
心療内科		位	耳鼻咽喉科		位
緩和ケア科		位	歯科口腔外科		位
神経内科		位	眼科		位

Q8. 医師以外のスタッフの関与についてお伺いします。
長期フォローにおいて、医師以外のスタッフが関与することはございますか？（○は１つ）

1. 関与することがある（⇒Q9へ）
2. 関与することはない（⇒Q11へ）

Q9. 【Q9＝1「関与することがある」先生へ】
長期フォローに関与する医師以外のスタッフの職種をお教え下さい。（○はいくつでも）

1. 看護師
2. 薬剤師
3. 栄養士
4. 理学療法士
5. 歯科衛生士
6. ソーシャルワーカー
7. 臨床心理士
8. その他（具体的に: ）

Q10. 【Q9＝1「関与することがある」先生へ】
長期フォローに関与する医師以外のスタッフに対して、長期フォロー患者に関する多職種カンファレンスを開いていますか？（○は１つ）

1. 開いている
2. 開いていない

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【全員の先生へ】

Q11. 移植患者のために、特化した外来枠を設けていますか？（○は１つ）

1. 設けている
2. 設けていない

Q12. 移植後、長期フォローのための外来枠を設けていますか？（○は１つ）

1. 設けている
2. 設けていない

Q13. 長期フォローは、連携病院でも行っていますか？（○は１つ）

1. 行っている（⇒Q14へ）
2. 行っていない（⇒Q15へ）

Q14. 【Q13＝1「行っている」先生へ】

関連病院と長期フォローをどのように連携していますか？（自由回答）

具体的に:

【移植後の長期フォロー患者のクリニカルプロセスについて】

【全員の先生へ】

Q15. 貴施設での、長期フォロー患者の診療指針をお教え下さい。（○は１つ）

1. 診療マニュアルを定めている
2. マニュアルはないがカンファレンス等でコンセンサスを作っている
3. 各医師の判断にまかせている
4. その他（具体的に: ）

Q16. 移植後の慢性GVHD（移植片対宿主反応病）の定義には議論があるところと思いますが、貴施設での診断基準をお教え下さい。（○は１つ）

1. NIH consensus development projectによるcriteria
2. 発症時期を問わない慢性GVHD所見
3. Day100以降のGVHD所見
4. その他（具体的に: ）

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Q17. 慢性GVHDの重症度分類は、どのようにされていますか。実際にカルテに記載している分類法をお教え下さい。（○は１つ）

1. Limited or Extensive
2. Scoring system: NIH consensus development projectによるスコア
3. その他（具体的に: ）

Q18. 貴施設での、慢性GVHDなどの晩期合併症のスクリーニング頻度を、検査や評価項目毎にそれぞれお教え下さい。（○はそれぞれ１つずつ）

		3ヵ月毎	6ヵ月毎	12ヵ月毎	症状があれば施行	施行しない
歯科口腔検査	⇒	1.	2.	3.	4.	5.
眼科検査	⇒	1.	2.	3.	4.	5.
呼吸機能検査	⇒	1.	2.	3.	4.	5.
胸部CT	⇒	1.	2.	3.	4.	5.
甲状腺機能	⇒	1.	2.	3.	4.	5.
生殖機能検査	⇒	1.	2.	3.	4.	5.
骨代謝・骨密度	⇒	1.	2.	3.	4.	5.
心機能	⇒	1.	2.	3.	4.	5.
神経学的検査	⇒	1.	2.	3.	4.	5.
皮膚生検	⇒	1.	2.	3.	4.	5.
皮膚硬化度評価	⇒	1.	2.	3.	4.	5.
婦人科がん検診	⇒	1.	2.	3.	4.	5.
乳がん検診	⇒	1.	2.	3.	4.	5.
消化器がん検診	⇒	1.	2.	3.	4.	5.
SF36/FACT等ツールによるQOL評価	⇒	1.	2.	3.	4.	5.

Q19. 慢性GVHDの病状/症状あるいは治療効果の評価の方法をお教え下さい。（○は１つ）

1. NIH consensus development projectによるcriteriaに準じた評価
2. 医師の経験に基づいた判断
3. その他（具体的に: ）

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Q20. 貴施設では、全身型慢性GVHDの治療薬として、通常カルシニューリンインヒビターを使用されますか。（○は１つ）

1. 通常使用する（⇒Q21へ）
2. 通常使用しない（⇒Q22へ）

Q21. 【Q20＝1「通常カルシニューリンインヒビターを使用する」先生へ】
では、予防薬と治療薬で使用するカルシニューリンインヒビター（CSPあるいはTacro）は、同じ薬剤を使用しますか、それとも変更しますか。（○は１つ）

1.	GVHD予防薬と治療薬は同じ （予防薬がCSPで治療薬もCSP、または、予防薬がTacroで治療薬もTacro）
2.	GVHD予防薬と治療薬は異なる （予防薬がCSPで治療薬はTacro、または、予防薬がTacroで治療薬はCSP）

【全員の先生へ】

Q22. 全身型慢性GVHDの治療において、ステロイドの代表的な使用方法を、各項目毎にお教え下さい。

種類（○は1つ）	⇒	**1.** PSL	**2.** mPSL	
初期投与量（○は1つ）	⇒	**1.** ≧2mg, /kg/day	**2.** 1mg, /kg/day	**3.** 0.5mg, /kg/day
		4. その他（具体的に: ）		
服用方法（○は1つ）	⇒	**1.** 朝1回	**2.** 朝夕2分割	**3.** 朝昼2分割
減量時の服用方法（○は1つ）	⇒	**1.** 日投与	**2.** 隔日投与	
減量開始の時期（記入）	⇒			
減量速度（記入）	⇒			
中止の目安（記入）	⇒	服用開始後		ヵ月で中止

Q23. 全身型慢性GVHDの治療において、カルシニューリンインヒビターやステロイドの他に使用経験がある薬剤をお教え下さい。（○はいくつでも）

1. サリドマイド
2. MMF
3. PUVA
4. その他（具体的に: ）

Q24. 全身型慢性GVHDの治療において、貴科で使用している代表的なレジメンをお教え下さい。

具体的に:

（資料提供）国立国際医療センター
萩原 將太郎

Q25. 慢性GVHDの以下の障害や症状について、先生が実施される「局所療法」を、それぞれ具体的にお教え下さい。

ドライアイ	⇒
口腔粘膜障害	⇒
呼吸器症状	⇒
皮膚症状	⇒

【同種移植後の長期フォロー患者の感染予防について】

【全員の先生へ】

Q26. 同種移植後のニューモシスティス肺炎の予防的抗生剤として、「バクタ」を投与していますか。

1. バクタを投与している（⇒Q28へ）
2. バクタを投与していない（⇒Q30へ）

Q27. 【Q27＝1「バクタを投与している」先生へ】
バクタの予防的投与期間をお教え下さい。（○は１つ）

1. 3ヵ月
2. 6ヵ月
3. 12ヵ月
4. ステロイド投与中
5. CSP/Tacro投与中

Q28. 【Q27＝1「バクタを投与している」先生へ】
予防投与の際のバクタの１日投与量をお教え下さい。（○は１つ）

1. 2錠　　**2.** 4錠
3. その他（具体的に:　　）

Q29. 【Q27＝1「バクタを投与している」先生へ】
その予防投与の際のバクタの投与間隔をお教え下さい。（○は１つ）

1. 毎日　　**2.** 週2日　　**3.** 週3日
4. その他（具体的に:　　）

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【全員の先生へ】

Q30. 同種移植後の肺炎球菌等の予防についておたずねします。肺炎球菌等に対して、予防的抗生剤を投与していますか（バクタ連日投与も含む）。（○は１つ）

1. 投与している(⇒Q31へ)
2. 投与していない(⇒Q33へ)

Q31. 【Q30＝1「投与している」先生へ】
予防的抗生剤をお教え下さい。

薬剤名(具体的に)：

Q32. 【Q30＝1「投与している」先生へ】
予防的抗生剤の投与期間をお教え下さい。（○は１つ）

1. 3ヵ月
2. 6ヵ月
3. 12ヵ月
4. ステロイド投与中
5. CSP/Tacro投与中

【全員の先生へ】

Q33. 同種移植後の感染予防として、抗真菌剤を投与していますか。（○は１つ）

1. 抗真菌剤を投与している(⇒Q34へ)
2. 抗真菌剤を投与していない(⇒Q36へ)

Q34. 【Q33＝1「抗真菌剤を投与している」先生へ】
その抗真菌剤をお教え下さい。

薬剤名(具体的に)：

Q35. 【Q33＝1「抗真菌剤を投与している」先生へ】
抗真菌剤の予防的投与期間をお教え下さい。（○は１つ）

1. 1ヵ月
2. 3ヵ月
3. 6ヵ月
4. 12ヵ月
5. ステロイド投与中
6. CSP/Tacro投与中

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【全員の先生へ】

Q36. 同種移植後のVZVの予防期間をお教え下さい。（○は１つ）

1. 1ヵ月	**2.** 3ヵ月
3. 6ヵ月	**4.** 12ヵ月
5. ステロイド投与中	**6.** CSP/Tacro投与中
7. VZVの予防は行っていない	

Q37. 同種移植後のＣＭＶモニタリング期間を、100日以内にCMVantigenemia(+)の場合と、100日までCMVantigenemia(-)の場合について、それぞれお教え下さい。（○はそれぞれ１つ）

100日以内にCMVantigenemia(+)	100日までCMVantigenemia(−)
↓（○は１つ）	↓（○は１つ）
1. 3ヵ月	**1.** 3ヵ月
2. 6ヵ月	**2.** 6ヵ月
3. 12ヵ月	**3.** 12ヵ月
4. ステロイド投与中	**4.** ステロイド投与中
5. CSP/Tacro投与中	**5.** CSP/Tacro投与中

Q38. 同種移植後に実施する予防接種の種類をお教え下さい。（○はいくつでも）

1. インフルエンザウイルス	**5.** 麻疹	**9.** HBV
2. インフルエンザ桿菌	**6.** ムンプス	**10.** Hlb
3. 肺炎球菌	**7.** 風疹	**11.** その他（具体的に：↓）
4. 水痘	**8.** ポリオ	

Q39. 【Q38＝1「インフルエンザウイルス」予防接種実施の先生へ】

インフルエンザ予防接種の開始時期をお教え下さい。（○は１つ）

1. 移植後半年以内	**3.** 移植後1-2年
2. 移植後1年以内	**4.** 2年以上経過後

Q40. 【Q38＝4「水痘」予防接種実施の先生へ】

水痘予防接種の開始時期をお教え下さい。（○は１つ）

1. 移植後半年以内	**3.** 移植後1-2年
2. 移植後1年以内	**4.** 2年以上経過後

Q41. 【Q38＝5「麻疹」予防接種実施の先生へ】

麻疹接種の開始時期をお教え下さい。（○は１つ）

1. 移植後半年以内	**3.** 移植後1-2年
2. 移植後1年以内	**4.** 2年以上経過後