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ORIGINAL ARTICLE

Oral valganciclovir as preemptive therapy is effective for cytomegalovirus infection in allogeneic hematopoietic stem cell transplant recipients

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Abstract Between March 2007 and January 2008, the safety and efficacy of oral valganciclovir (VGC) preemptive therapy for cytomegalovirus (CMV) infection was evaluated in ten consecutive patients who received allogeneic hematopoietic stem cell transplantation (HSCT). Patients were screened once or twice per week after engraftment using CMV pp65 antigenemia assay. When more than 2 CMV antigen-positive cells per 50,000 leukocytes were detected, preemptive therapy with oral VGC was initiated at a dose of 900 mg twice daily for 3 weeks. Nine patients (90%) completed the 3-week VGC treatment except for one patient who developed febrile neutropenia. There was no other significant toxicity. CMV antigen-positive cells were rapidly decreased in all nine patients and became undetectable by the end of the VGC treatment. None of the patients developed CMV disease. CMV infection relapsed in four of the ten patients (40%) after the VGC treatment. These observations suggest that preemptive therapy with VGC is effective for preventing CMV disease in allogeneic HSCT patients. Further studies with a large number of patients will be necessary to determine the optimal initial- and maintenance-dose of VGC.

Keywords Allogeneic hematopoietic stem cell transplantation · Cytomegalovirus infection · Preemptive therapy · Valganciclovir

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1 Introduction

Despite improvement in the treatment of cytomegalovirus (CMV) infection and CMV disease with ganciclovir (GCV) and/or foscarnet, CMV disease is still a major cause of morbidity and mortality after hematopoietic stem cell transplantation (HSCT) [1–4]. Major risk factors for CMV disease include CMV seropositivity before transplantation, development of graft-versus-host disease (GVHD), unrelated donor transplantation, and T cell depleted transplantation [3, 5–7]. In addition, new transplantation modalities such as nonmyeloablative conditioning regimens consisting of intensive immunosuppression increase the risk of late-onset CMV infection and CMV disease [2, 8]. Therefore, extended prevention of CMV disease may be required, especially for high-risk recipients, not only those within 100 days after HSCT but also those in the later period after HSCT [8–10]. Currently, the prevention of CMV disease involves general prophylaxis and preemptive therapy. Preemptive therapy is based on the early detection of CMV infection by virus surveillance, by monitoring with either CMV antigenemia assay or PCR techniques and followed by immediate treatment with anti-CMV drugs

[4, 11–13]. Intravenous GCV (IV-GCV) and/or foscarnet are commonly used for preemptive therapy and are effective for decreasing the incidence of early CMV disease [11, 13, 14]. However, these antiviral treatments are given intravenously and often require hospitalization, as well as high costs and IV-related complications.

Valganciclovir hydrochloride (VGC) is an oral valine-ester GCV prodrug with a tenfold higher bioavailability than oral GCV, and it is rapidly hydrolyzed to GCV after oral administration. VGC and IV-GCV have similar efficacy in the treatment of CMV retinitis in HIV-infected patients and in preemptive CMV treatment in solid organ (heart, renal, and renal-pancreas) transplant patients [15–19]. Recently, several studies have shown the efficacy of VGC for preemptive therapy in allogeneic HSCT patients [20–23]. We evaluated the safety and efficacy of oral VGC as preemptive therapy for CMV reactivation in ten allogeneic HSCT patients.

2 Patients and methods

2.1 Patients

This was a prospective multicenter study with VGC. The study patients were adults who had received an allogeneic bone marrow or peripheral blood stem cell transplant. Patients were eligible when they screened for CMV infection using CMV pp65 antigenemia assay and more than two CMV antigen-positive cells were detected. Patients unable to take oral medication, and those who impaired renal function (serum creatinine level >2.0 mg/dL) were ineligible. Patients, who developed CMV disease, had received antiviral agents other than acyclovir and who developed more than stage 2 gastrointestinal GVHD were also ineligible. Ten consecutive patients who received allogeneic HSCT at Kyushu University Hospital and Hamanomachi General Hospital between March 2007 and January 2008 were included in the study (Table 1). This study was approved by Institutional Review Board of each institute and a written informed consent was obtained from each participating patient.

Eight patients had acute myeloid leukemia, one had myelodysplastic syndrome, and one had non-Hodgkin's lymphoma. The median age of the patients at the time of transplantation was 56 years (range 33–63). They received bone marrow grafts from an HLA-matched sibling donor ($n = 1$), a matched unrelated donor ($n = 8$), or an HLA-1 locus mismatched unrelated donor ($n = 1$). All of the patients were CMV seropositive before transplantation. Nine patients received myeloablative preparative regimens including total body irradiation/cyclophosphamide (Cy) in five patients and busulfan (BU)/Cy in four patients.

Table 1 Patient characteristics

Number of patients	10
Median age, years (range)	56 (33–65)
Diagnosis	
Acute myeloid leukemia	8
Myelodysplastic syndrome	1
Non-Hodgkin's lymphoma	1
Stem cell source	
HLA-identical sibling bone marrow	1
HLA-matched unrelated bone marrow	8
HLA-mismatched unrelated bone marrow	1
CMV serologic status	
Donor + /Recipient +	9
Donor −/Recipient +	1
Preparative regimens	
TBI/Cy	5
Bu/Cy	4
Flu/Bu/TBI	1
GVHD prophylaxis	
Taclorimus + MTX	9
CSP + MTX	1
Acute GVHD prior to CMV reactivation	
Grade I	1
Grade II	7
Grade III	2
PSL treatment at the time of starting VCG	8

Bu busulfan, *CMV* cytomegalovirus, *CSP* cyclosporine, *Cy* cyclophosphamide, *Flu* fludarabine, *GVHD* graft-versus-host disease, *TBI* total body irradiation, *MTX* methotrexate, *PSL* prednisolone, *VGC* valganciclovir

The remaining patient received a fludarabine-based reduced-intensity conditioning regimen. GVHD prophylaxis consisted of taclorimus/short-term methotrexate (MTX) ($n = 9$) or cyclosporine/short-term MTX ($n = 1$). Patients who developed grade II–IV acute GVHD were given methylprednisolone (mPSL) or prednisolone (PSL) at a dose of 1 or 2 mg/kg. Acyclovir was administered orally (1,000 mg/day) or intravenously (500 mg/day) from days −7 to 35 as a prophylaxis against herpes simplex infection.

2.2 CMV antigenemia assay

CMV antigenemia assay was determined as previously described [7, 24]. In brief, peripheral blood leukocytes isolated from 3 mL of EDTA-treated blood were applied to slides by centrifugation and fixed with cold acetone. The slides were stained using a direct immunoperoxidase technique that employed the peroxidase-conjugated monoclonal antibody HRP-C7 (Teijin, Tokyo, Japan) against the CMV pp65 antigen. CMV antigen-positive cells were counted under a light microscope and the results were

expressed as the number of CMV antigen-positive cells per 50,000 leukocytes.

2.3 Definition of CMV infection and CMV disease

A positive test for CMV antigenemia was defined as the presence of one or more CMV antigen-positive cells per 50,000 leukocytes. CMV infection was considered in patients with a positive test for CMV antigenemia. CMV disease was diagnosed according to published recommendations [25]. Patients with clinical manifestations of CMV disease, such as interstitial pneumonia and gastroenteritis in the presence of CMV infection, were examined histopathologically and immunochemically from biopsy specimens.

2.4 Preemptive therapy with VGC for CMV infection

Monitoring with CMV antigenemia assay was performed at least once per week after engraftment until day 100 after HSCT and once every other week thereafter. Preemptive therapy with VGC for CMV infection was initiated at the time of the first detection of more than two CMV antigen-positive cells per 50,000 leukocytes. VGC was administered orally at a dose of 900 mg twice daily for 3 weeks. The dose was adjusted for patients with impaired renal function according to the manufacturer's recommendation. Acyclovir for the prophylaxis against herpes simplex infection was discontinued when VGC treatment was started. Supplemental immunoglobulin was administered only when a total IgG level was less than 400 mg/dL.

2.5 Endpoints and definitions

The primary endpoint was the rate of complete response of the VGC preemptive therapy to the CMV infection. The efficacy of VGC was monitored weekly using a CMV antigenemia assay. A complete response was defined as the conversion from positive to negative CMV antigenemia test results at the completion of the treatment. Patients who persistently showed positive test results for CMV antigenemia after 3 weeks of preemptive therapy or developed CMV disease during the period of preemptive therapy were considered a treatment failure.

The secondary endpoints included the safety of preemptive therapy, the incidence of CMV disease during VGC treatment, and the incidence of a recurrent CMV reactivation after the completion of VGC treatment. The patients were monitored with the CMV antigenemia assay for 5 weeks after the completion of the VGC treatment. At least once per week, a safety analysis was conducted. The analysis included the monitoring of blood counts, liver and renal function tests, and documenting other unexpected side effects. The incidence of CMV disease was evaluated for the entire period of the study. The incidence of recurrent reactivation of CMV infection after the VGC preemptive therapy was based on the conversion from negative CMV antigenemia to positive CMV antigenemia test results with more than two CMV antigen-positive cells per 50,000 leukocytes during the 5-week follow-up period.

3 Results

3.1 CMV infection and VGC preemptive therapy

Forty-seven patients received allogeneic bone marrow/peripheral blood stem cell transplants at these two institutes during the study period. Thirty-one patients showed positive CMV antigenemia test results after transplantation. Ten patients were enrolled into this study, but the remaining 21 patients were not enrolled mostly by their inability to take oral medication. Ten enrolled patients were given preemptive therapy with VGC for CMV infection (Table 1). All patients were CMV seropositive before transplantation, and nine donors were also CMV seropositive. In these patients, more than 2 CMV antigen-positive cells per 50,000 leukocytes were detected after a median of 69 days (range 22–252) following transplantation. The median number of CMV antigen-positive cells at the initiation of VGC therapy was 5 per 50,000 leukocytes (range 3–59). All of the patients developed acute GVHD prior to CMV infection after a median of 23 days (range 11–135). The severity of acute GVHD was grade I in one patient, grade II in seven, and grade III in two. Eight patients received mPSL or PSL for the treatment of acute GVHD. Preemptive therapy with VGC was started within five days after the detection of CMV antigen-positive cells. Nine patients completed 21 days of VGC treatment, whereas one patient failed to complete the therapy because of the development of grade 4 neutropenia and subsequent febrile neutropenia. Patients were followed at least 5 weeks after the completion of VGC preemptive therapy. The median follow-up was day 122 (range 41–355).

3.2 Response to VGC preemptive therapy

All patients showed negative test results for CMV antigenemia within 3 weeks after the initiation of the VGC treatment. In nine patients, CMV antigen-positive cells became negative within 2 weeks (Fig. 1). The remaining patient, who had 60/50,000 CMV antigen-positive cells at the time of initiation of VGC treatment, took 3 weeks to clear CMV antigen-positive cells. None of the patients required other anti-CMV agents. None of the patients developed CMV disease during the preemptive therapy or

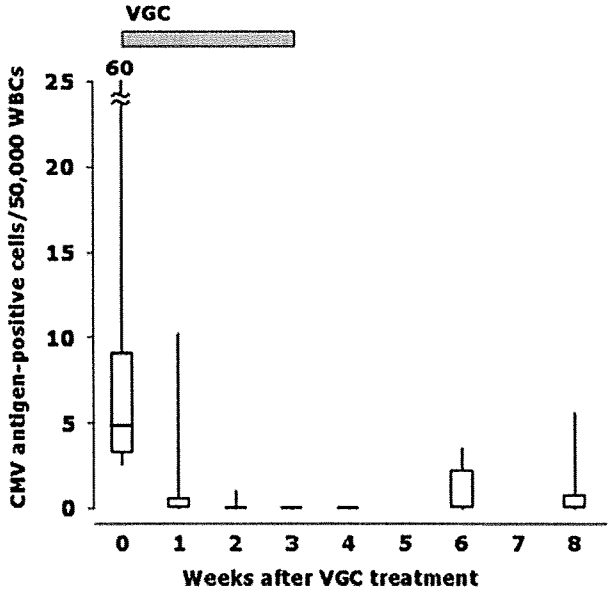

Fig. 1 Time course of the number of cytomegalovirus (CMV) antigen-positive cells after valganciclovir treatment. CMV antigenemia was reduced during treatment with valganciclovir. The *box plots* display the median, the 25th and 75th percentiles (*box*), and the smallest and largest values (*longitudinal line*). One patient discontinued valganciclovir on day 18 due to grade 4 neutropenia

in the subsequent 5 weeks after the completion of the VGC treatment.

CMV infection relapsed in four of the ten patients within 3–5 weeks after the completion of the preemptive VGC therapy. These four patients were successfully treated with IV-GCV.

3.3 Toxicity

Nine patients completed a 21-day course of VGC treatment, but one patient discontinued VGC due to grade 4 neutropenia. Due to impaired renal function (serum creatinine level, 1.68 mg/dL), this patient received a reduced VGC dose of 450 mg once per day for the first week. Renal function improved with the reduced dose, and the VGC dosage was increased to 450 mg twice per day in the second week of treatment. However, this patient developed grade 4 neutropenia (absolute neutrophil counts 0.17 × 10^9/L) after 17 days of treatment and then developed febrile neutropenia. The VGC was discontinued, and the patient immediately received granulocyte-colony stimulating factor (G-CSF) and antibiotic therapy. Neutrophil counts recovered to more than 1.0 × 10^9/L, and neutropenia resolved after five days. Recurrent CMV reactivation was not observed in this patient during the follow-up period. None of the patients developed thrombocytopenia (platelet count <30 × 10^9/L)(Fig. 2).

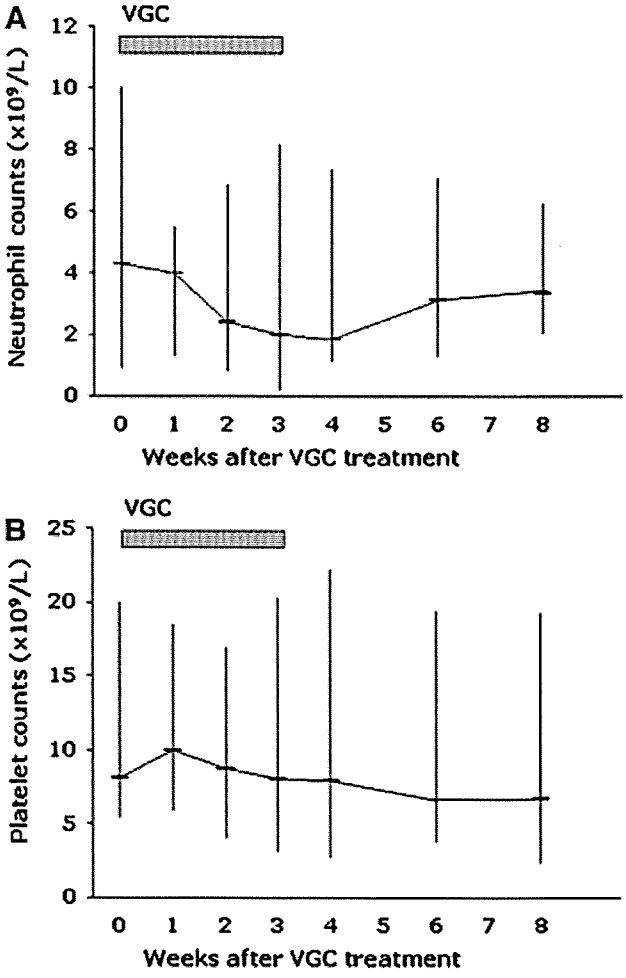

Fig. 2 Time course of neutrophils and platelets during valganciclovir treatment. Time course of neutrophil (**a**) and platelet numbers (**b**) during treatment with valganciclovir. The bar graph displays the median (*horizontal line*), and the smallest and largest values (*longitudinal line*). One patient discontinued valganciclovir on day 18 due to grade 4 neutropenia

Table 2 Adverse events other than hematological toxicities related to valganciclovir

Adverse events		No. of cases
Gastrointestinal		
Diarrhea	Grade 1	1/10
Hepatic		
AST/ALT	Grade 1	3/10

None of the patients experienced renal toxicity during the VGC treatment. Three patients developed grade 1 liver dysfunction, and one patient had grade 1 diarrhea (Table 2). However, none of these complications required discontinuation of the VGC.

4 Discussion

Effective preemptive therapy with IV-GCV reduced the incidence of early CMV disease to 5–10%; however, the risk of late CMV disease beyond day 100 after transplantation has increased over the past few years. Therefore, extended CMV monitoring beyond day 100 is currently recommended, especially in high-risk patients [2, 8]. There is a need for an effective oral anti-CMV drug that can be used for outpatient care. Oral VGC could be a useful alternative to IV-GCV in patients who require preemptive therapy for CMV infection. This study demonstrated the efficacy and safety of preemptive VGC therapy for CMV infection after allogeneic HSCT. There are four published studies that have shown the safety and the efficacy of VGC as preemptive therapy after allogeneic HSCT [20–23]. Although dosage and duration of the drug varied between studies, VGC therapy resulted in a rapid decrease of the viral load in all of the patients. In this study, we administered a dose of 900 mg twice daily for 3 weeks, and corroborated the efficacy and the tolerability of preemptive VGC therapy.

We demonstrated that VGC at a dose of 900 mg twice per day was effective and resulted in a rapid clearance of CMV antigen-positive cells in all patients. No CMV disease developed during the preemptive therapy or the subsequent 5 weeks after the completion of treatment. VGC was well tolerated as 90% of the patients completed the entire treatment course. However, four of the ten patients developed a recurrent CMV reactivation after the discontinuation of VGC treatment, and they were all successfully treated with IV-GCV. Because a guideline for preemptive VGC therapy has not been established for patients that have received allogeneic HSCT, further studies will be necessary to determine the optimal initial- and maintenance-dose of VGC.

We, and four other groups, have obtained good results with VGC starting-doses of 900 mg twice per day [20–23]. This dose was based on observations from previous pharmacokinetics studies in HIV-infected patients and liver transplant recipients. A VGC dose of 900 mg results in an area under the concentration-time curve for GCV similar to that of 5 mg/kg IV-GCV [26, 27], which is the recommended standard dose for preemptive CMV therapy [28, 29]. One of the concerns of using VGC after allogeneic HSCT is the absorption of oral VGC in patients suffering from severe gastrointestinal GVHD. Recently, Einsele et al. [30] conducted a randomized crossover clinical trial of IV-GCV and VGC in patients with or without intestinal GVHD. The results showed that patients without intestinal GVHD who took VGC were exposed to more GCV when compared to those administered IV-GCV. This was also true in patients with grade I and II intestinal GVHD. Thus, VGC may be as effective even in patients developing a mild form of intestinal GVHD as in patients without intestinal GVHD. However, a higher exposure of VGC may increase the toxicity of the drug, and the absorption of VGC was not evaluated in patients with severe intestinal GVHD. Recently, Candoni et al. [22] examined the efficacy of a lower dose of VGC. Preemptive therapy with 900 mg/day VGC was as effective for clearing CMV antigen-positive cells and preventing CMV disease as the standard dose of 1800 mg/day. These findings suggest that the initial dose of VGC could be reduced to 900 mg/day as preemptive therapy in low-risk patients.

The effective duration for preemptive VGC therapy is currently unclear. In the previous studies, patients received VGC for 2 weeks and then it was either discontinued or continued at a maintenance dose of variable duration dependant upon a negative CMV test result. Different from previous studies, we continued an initial dose of VGC for 3 weeks. The dosage and duration of VGC therapy likely affects the incidence of hematological toxicity such as neutropenia. In a study by Busca et al. [21], in which VGC was administered at a dose of 1,800 mg/day for 2 weeks, followed by 900 mg/day for an additional 2 weeks, 4 of the 15 patients failed to complete the 3-week scheduled therapy due to neutropenia and/or thrombocytopenia. In our study, only one of the ten patients failed to complete treatment. Thus, hematologic toxicity may be a significant problem after a 3 week treatment with VGC.

In our study, four of the ten patients treated with VGC developed recurrent CMV reactivation 3–5 weeks after the discontinuation of VGC. This was somewhat similar to the 10–53% recurrence rates in previous studies [20–23]. Thus, careful monitoring after the completion of VGC therapy is recommended. We continued an initial dose of VGC for 3 weeks. However, when considering hematological toxicity and frequent recurrence of CMV antigenemia, the duration of treatment and/or maintenance should be decided by monitoring CMV.

As previously reported [20–23], we found neutropenia to be the main toxic effect of VGC. One patient, who had impaired renal function before the preemptive therapy that required a dose reduction, discontinued the drug on day 17 due to grade 4 neutropenia. In high-risk patients, especially outpatient should be closely monitored, although any other toxicity profile different from IV-GCV was not observed in this study.

Our study demonstrated that the oral VGC preemptive therapy at a dose of 900 mg daily seemed to be as effective as conventional IV-GCV at a dose of 10 mg/kg daily to clear CMV antigen-positive cells. However, as shown in Fig. 1, CMV antigen-positive cells seem to decrease in numbers much faster after VGC treatment than those observed after standard dose of IV-GCV treatment.

Furthermore, hematological toxicities were considerable. Although pharmacokinetic data was not available in this study, these observations coincide with the previous pharmacokinetic study in HSCT recipients that showed the exposure of GCV after administration of 1800 mg daily VGC was significantly higher compared with 10 mg/kg IV-GCV even in patients without gastrointestinal GVHD [30]. Careful monitoring of neutrophil counts will be useful to improve the safety of VGC in HSCT recipients, especially with reduced renal function. Kanda et al. [14] showed the efficacy of response-oriented preemptive therapy using a low initial dose of IV-GCV that resulted in a successful reduction of the total dose of IV-GCV and decreased hematological toxicities. A lower dose of VGC could be also used as preemptive therapy by close CMV monitoring. Similar studies with a large number of patients will be required to define the optimal treatment schedule for preemptive VGC therapy.

Despite a limited number of patients, our results suggest that oral VGC is an effective alternative to IV-GCV for preemptive therapy to prevent CMV disease in allogeneic HSCT patients. Studies with a larger number of patients will be necessary to assess the efficacy and long-term effect of this preemptive therapy.

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Ex Vivo-Expanded Donor CD4$^+$ Lymphocyte Infusion Against Relapsing Neuroblastoma: A Transient Graft-Versus-Tumor Effect

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High-risk neuroblastoma has a poor prognosis despite multimodal treatment including high-dose chemotherapy. A 7-year-old male with neuroblastoma received ex vivo-expanded donor CD4$^+$ T lymphocyte infusion (CD4$^+$ DLI) after recurrence in the bone marrow following allogeneic hematopoietic stem cell transplantation from his HLA-identical mother. The disease transiently responded to CD4$^+$ DLI with reduction of tumor cells and a decrease of serum neuron-specific enolase. The response was associated with development of continued high fever and an increase of cytotoxic T lymphocytes in peripheral blood. This case suggests a possibility of a graft-versus-tumor effect against neuroblastoma. Pediatr Blood Cancer 2009;52:895–897.

Key words: CD4$^+$ donor lymphocyte infusion; graft-versus-tumor effect; neuroblastoma

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) can exert an immune graft-versus-tumor (GVT) effect mediated by donor lymphocytes, which plays a therapeutic role in the treatment of hematologic malignancies. The GVT effect was directly confirmed by the observation that donor lymphocyte infusion (DLI) can successfully induce remission of chronic myelogenous leukemia, which relapse after allo-HSCT [1]. Several small studies have also suggested GVT effects following allo-HSCT in patients with solid tumors [2–6]. Although allo-HSCT has been applied in a considerable number of patients with neuroblastoma (NBL) [6], there are few reports describing a GVT effect against this malignancy. Here, we describe a patient with relapsing NBL showing transient tumor regression after ex vivo-expanded donor CD4$^+$ lymphocyte infusion (CD4$^+$ DLI).

CASE REPORT

A 4-year-old male was diagnosed with stage 4 NBL (International NBL Staging System: INSS) who developed as a retroperitoneal mass with metastases to the bone marrow (BM), cervical lymph nodes and bone (orbit). Pathological studies showed poorly differentiated NBL (International NBL Pathology Classification: INPC) with Shimada's unfavorable histology without amplified N-myc expression. He was initially treated with combination chemotherapy consisting of cyclophosphamide, vincristine, pirarubicin (THP-adriamycin), cisplatin, and etoposide. He then received high-dose chemotherapy (HDC) consisting of thio-TEPA and melphalan with autologous peripheral blood stem cell transplantation (auto-PBSCT), followed by surgical removal of primary tumor [7,8].

The disease recurred in the BM, right mandible, bilateral cervical lymph nodes, and right iliac and inguinal lymph nodes at 6 years of age, 13 months after HDC with auto-PBSCT. Following combination chemotherapy consisting of topotecan, cyclophosphamide, and cisplatin, he received an allogeneic bone marrow transplantation (allo-BMT) from his HLA-identical mother. The conditioning regimen consisted of busulfan (16 mg/kg) and fludarabine (180 mg/m^2) preceded by topotecan (30 mg/m^2). Prophylaxis for graft-versus-host disease (GVHD) was short-term methotrexate and tacrolimus. Engraftment was prompt and no acute GVHD was observed. He was also treated with radiotherapy to lymph nodes of the neck and pelvis after allo-BMT, which led to successful renewed remission. However, he developed a recurrence in BM with elevation of serum neuron-specific enolase (NSE) 1 month after completion of radiotherapy, for which he received two courses of conventional DLI [1–5 × 10^6/kg CD3$^+$ T-lymphocytes] from his mother (Fig. 1). However, tumor cells in BM increased and

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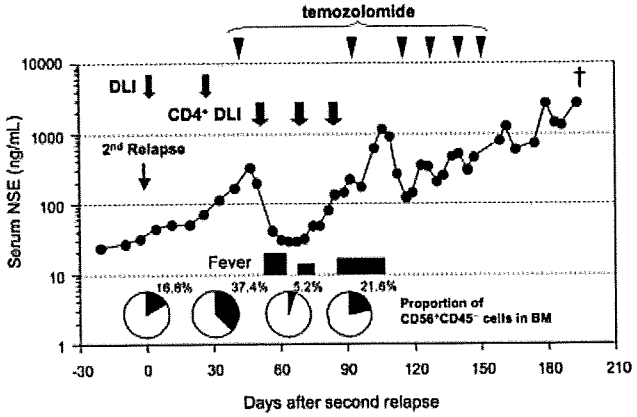

Fig. 1. Clinical course and changes in serum NSE. NSE, neuron-specific enolase; BM, bone marrow. DLI indicates donor lymphocyte infusion: 1st dose, 1×10^6/kg and 2nd dose, 5×10^6/kg $CD3^+$ T lymphocytes. $CD4^+$ DLI indicates ex vivo-expanded donor $CD4^+$ lymphocyte infusion: 1st and 2nd dose, 1×10^7/kg; and 3rd dose, 5×10^7/kg. The purity of CD4-single positive cells was 93.4%, 95.6%, and 90.9%, respectively. The majority of contaminating cells were $CD4^+CD8^+$. Temozolomide was administered at 150 mg/m^2 daily for five consecutive days for each cycle.

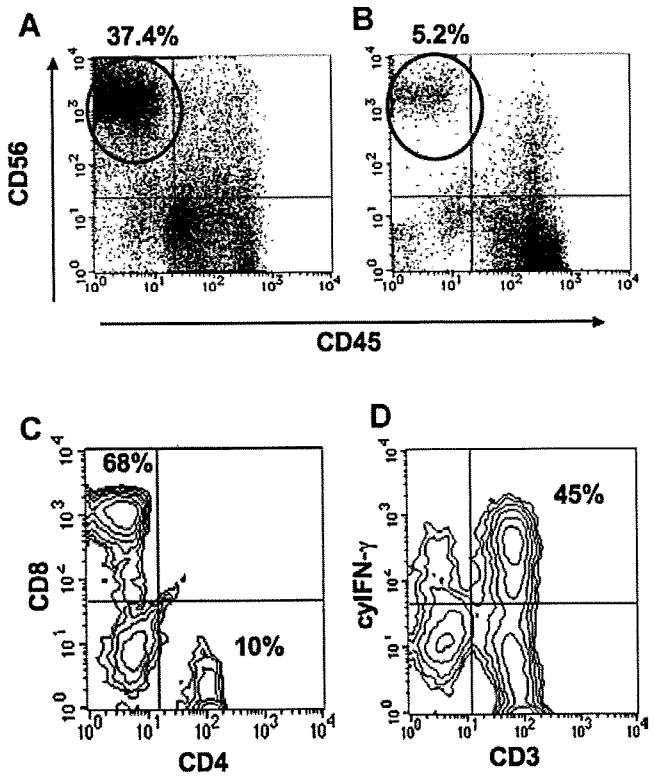

Fig. 2. Flow cytometric analysis. Tumor cells ($CD56^+CD45^-$) in iliac bone marrow before (**A**) and 12 days after (**B**) the first $CD4^+$ donor lymphocyte infusion (DLI). Proportion of $CD4^+$ or $CD8^+$ T lymphocytes (**C**) and $CD3^+$ T lymphocytes producing cytoplasmic IFN-γ (**D**) in peripheral blood mononuclear cells after $CD4^+$ DLI.

associated with increased serum NSE but without development of GVHD. We therefore infused ex vivo-expanded donor $CD4^+$ T lymphocytes ($CD4^+$ DLI) with the aim of accelerating allogeneic immunoreaction without eliciting GVHD.

Mononuclear cells were isolated from his mother. $CD4^+$ T lymphocytes were purified by CD4 monoclonal antibody (mAb)-coated magnetic beads and cultured for 1 week in the presence of recombinant IL-2 (350 IU/ml; Proleukin, Chiron BV, Amsterdam, The Netherlands) in a flask with immobilized anti-CD3 mAb, OKT3 (5 μg/ml; Jansen-Kyowa, Tokyo, Japan) [9]. This trial and culture procedure were approved by the Institutional Review Boards of Tokyo Medical and Dental University, and Osaka University Hospital. Written informed consent was obtained from the parents of the patient. The patient, then 7 years of age, was treated with $CD4^+$ DLI following administration of temozolomide (Fig. 1). Shortly after the first $CD4^+$ DLI (1×10^7/kg) with 93.4% purity of CD4-single positive cells, he developed high fever of 40°C without other GVHD signs such as skin rash, jaundice, or diarrhea. High fever continued for 2 weeks with reduction of serum NSE levels from 325.5 to 29.2 ng/ml. Iliac BM aspiration showed a decrease in the ratio of the tumor cells ($CD56^+CD45^-$ cells) from 37.4% to 5.2% (Fig. 2A,B). Twelve days after $CD4^+$ DLI, $CD8^+$ T lymphocytes with IFN-γ production predominated in peripheral blood (Fig. 2C,D). However, serum NSE increased after the second $CD4^+$ DLI. Despite the third $CD4^+$ DLI at an increased dose of 5×10^7/kg, the disease continued to progress. He then received temozolomide but without response and died 7 months after the second relapse.

DISCUSSION

The prognosis of high-risk NBL, characterized by an older age, metastases, N-myc amplification, and unfavorable histologic findings, remains poor [10,11]. More than half of these high-risk patients relapse despite strategies involving HDC followed by auto-HSCT, which indicates a need for novel strategies to eradicate residual disease. Allo-HSCT has been already used for adult patients with solid tumors [4,6], in particular renal cell carcinoma [2,5] and breast cancer [3,5]. Recent trials using allo-HSCT, mostly following non-myeloablative preconditioning, showed a response rate of up to 57% against renal cell carcinoma [2,3,5].

A dramatic reduction of tumor cells was observed in our patient following $CD4^+$ DLI. The clinical response with the development of high fever immediately after $CD4^+$ DLI combined with an increase of IFN-γ-producing $CD8^+$ T lymphocytes, that is, cytotoxic T lymphocytes (CTLs), suggests a GVT effect. Moreover, we observed no increase of NK cells in peripheral blood nor increase of expression of HLA-A24 (the patient's and the donor's HLA-A type) on residual tumor cells (data not shown). Taken together, the immunoreaction against NBL cells was presumably caused by CTLs, not by NK cells. $CD8^+$ T lymphocytes (CTLs) were increased following $CD4^+$ DLI. Expanded and activated $CD4^+$ helper T lymphocytes might have produced cytokines that stimulated CTL differentiation and enhanced the ability of antigen-presenting cells to stimulate CTL differentiation through a CD40-CD40L interaction [12].

An immunological response due to lymphocytes might be attributable in our case to scattered tumor cells in BM, which were abundant in bloodstream, as is more frequently seen in leukemia. Although the administration of temozolomide shortly before $CD4^+$ DLI might have affected the clinical response, there was no response

during the second course of temozolomide during the final course of the disease, which suggests that the first course was not associated with a reduction of tumor cells.

In 1994 Matthay et al. [13] reported no advantage of allo-HSCT over auto-HSCT in patients with NBL and few reports suggest a GVT effect against NBL. Inoue et al. [14] reported a case showing the disappearance of NBL within 3 years after allo-HSCT from an HLA haploidentical donor. Although a considerable number of patients with NBL has been treated with allo-HSCT [6], detailed analysis has not been performed regarding its efficacy. Dykes et al. [15] have recently used $CD3^+$ T-cell depleted allo-PBSCT from HLA-haploidentical donor to patients with NBL.

The response in our patient suggests a transient GVT effect against NBL cells. Immunotherapy with allogeneic lymphocytes might open new avenues for overcoming the dismal prognosis of high-risk NBL.

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ORIGINAL PAPER

Successful cord blood transplantation for a CHARGE syndrome with *CHD7* mutation showing DiGeorge sequence including hypoparathyroidism

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Abstract It is rare that coloboma, heart anomalies, choanal atresia, retarded growth and development, and genital and ear anomalies (CHARGE) syndrome patients have DiGeorge sequence showing severe immunodeficiency due to the defect of the thymus. Although the only treatment to achieve immunological recovery for these patients in countries where thymic transplantation is not ethically approved would be hematopoietic cell transplantation, long-term survival has not been obtained in most patients. On the other hand, it is still not clarified whether hypoparathyroidism is one of the manifestations of CHARGE syndrome. We observed a CHARGE syndrome patient with chromodomain helicase DNA-binding protein 7 mutation showing DiGeorge sequence including the defect of T cells accompanied with the aplasia of the thymus, severe hypoparathyroidism, and conotruncal cardiac anomaly. He received unrelated cord blood transplantation without conditioning at 4 months of age. Recovery of T cell number and of proliferative response against mitogens was achieved by peripheral expansion of mature T cells in cord blood without thymic output. Although he is still suffering from severe hypoparathyroidism, he is alive without serious infections for 10 months.

Keywords CHARGE syndrome · DiGeorge sequence · *CHD7* mutation · Hypoparathyroidism · Cord blood transplantation

Abbreviations

CHD7	Chromodomain helicase DNA-binding protein 7
CBT	Cord blood transplantation
TCR	T cell receptor
PHA	Phytohemagglutinin
Con A	Concanavalin A
ABR	Auditory brainstem response
GVHD	Graft versus host disease

Introduction

Coloboma, heart anomalies, choanal atresia, retarded growth and development, and genital and ear anomalies (CHARGE) syndrome is a distinctive clinical entity with multiple congenital anomalies [12]. Mutations in the gene chromodomain helicase DNA-binding protein 7 (*CHD7*) were identified as a cause of CHARGE syndrome [25]. *CHD7* on chromosome 8 (8q12.1) is a member of the chromodomain helicase DNA binding domain family [25]. Chromatin remodeling is a recognized mechanism of gene expression regulation, and the *CHD7* gene is likely to play a significant role in embryonic development and cell cycle regulation [29]. CHD7 is expressed throughout the neural crest containing mesenchyme of the pharyngeal arches. Mouse embryo at 10.5 days postcoitum expressed *Chd7* in

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the cardiac outflow tract, truncus arteriosus, facio-acoustic preganglion complex, hindbrain, forebrain, mandibular component of the first branchial arch, otic vesicle, optic stalk/optic vesicle, and olfactory pit [12]. Thus, CHARGE syndrome has the potential of multiple presentations.

Cellular immunodeficiency due to the lack of the thymus is not widely recognized as a manifestation of CHARGE syndrome. Recently, severe hypoparathyroidism and conotruncal cardiac anomaly were reported in patients with CHARGE syndrome caused by *CHD7* mutations having DiGeorge sequence characterized by the defect of T cells accompanied by thymus aplasia [21, 28, 30]. Although thymus hypoplasia or agenesis is rare in postnatal CHARGE syndrome cases [3], Sanlaville et al. reported that it was observed in seven of ten CHARGE syndrome fetuses [22]. Recently, Jyonouchi et al. reported that 8% (two of 25) of CHARGE syndrome patients had a phenotype of severe combined immunodeficiency with defect of T cells [10]. On the other hand, it is still not clarified whether hypoparathyroidism is one of the manifestations of CHARGE syndrome since only three CHARGE syndrome patients with *CHD7* mutation were reported to have hypoparathyroidism [21, 28, 30]. It is suggested that neural crest defect underlies the clinical overlap of both chromosome 22q11 deletion and CHARGE syndrome [22]. Accordingly, a case manifesting the CHARGE syndrome with deletion in chromosome 22q11 was reported [7].

Here, we report a patient with CHARGE syndrome with a *CHD7* mutation, who had severe T cell immune deficiencies due to thymic aplasia, severe limb anomalies, and congenital hypoparathyroidism. He was successfully treated with cord blood transplantation (CBT).

Case report

The patient was born at 39 weeks of gestational age. His birth weight was 2,488 g. Cardiac anomaly and polyhydramnion were detected by fetal ultrasound examination during his late prenatal period. Karyotype analysis of amniotic fluid showed 46,XY. His family members were healthy without having even minor anomalies.

Shortly after birth, he was admitted to the neonatal intensive care unit (NICU) in Kyushu University Hospital. He showed the characteristic facial features such as a hypertelorism and unilateral facial palsy (Fig. 1a), asymmetry of ears with protruding, helix hypoplasia, low-set and square-shaped right ear, absent anthelix, low-set left ear (Fig. 1b, c), and bilateral coloboma of the choroid. In addition, thumb polydactyly and cleft of the right hand and cleft and cutaneous syndactyly of the bilateral feet were observed (Fig. 1d–g). He had no genital abnormalities. Hematological examinations revealed white blood cell count of 4,330/μl with severe lymphopenia (neutrophils 68.5%, lymphocytes 7%, monocytes 18%). Serum calcium, phosphorus, and parathyroid hormone levels were 7.8 mg/dl, 8.4 mg/dl, and 4.5 pg/ml, respectively, showing hypoparathyroidism. Serum thyroid hormone levels were normal. Lymphocyte surface marker analysis by a flow cytometer revealed a marked decrease of T lymphocytes: $CD3^+$ 2.8% (8 cells/μl), $CD4^+$ 2.3% (7 cells/μl), and $CD8^+$ 15.3% (46 cells/μl; Table 1). T cell receptor (TCR) $\gamma\delta^+$ cells, $CD16^+/CD56^+$ cells, and $CD19^+$ cells were 0.1%, 35.9%, and 52.9%, respectively. Proliferative response of mononuclear cells against phytohemagglutinin (PHA) and concanavalin A (Con A) was 123 %S.I. (normal controls; 254–388) and 2,530 cpm (20,300–65,700), respectively. Analysis of the TCRVβ repertoire showed an abnormal pattern with overexpansion of $V\beta21.3^+$ cells (20.9%; Fig. 2a). Serum IgG, IgA, and IgM concentrations were 899, 5, and 19 mg/dl, respectively. Fluorescent in situ hybridization analysis revealed a lack of maternal cell engraftment in peripheral blood and no deletion at 22q11.2.

Computed tomography and fiberoptic laryngoscope examination revealed left choanal atresia with posterior choanal stenosis and laryngomalacia, respectively. Auditory brainstem response revealed bilateral severe sensorineuronal hearing loss. An echocardiogram and chest computed tomography scan revealed truncus arteriosus (Van Praagh type A4) and interruption of aortic arch (type B) with aberrant right subclavian artery. At 14 days of age, he underwent bilateral pulmonary artery banding operation because he was too small to receive the radical correction of truncus arteriosus and interruption of aortic arch at that time. Thymus was not detected at the time of operation.

Thus, we made the clinical diagnosis of CHARGE syndrome with manifestations of complete-type athymic DiGeorge sequence. The *CHD7* gene of the patient was analyzed according to the method described previously [2], and heterozygous c.1036A > T (R346X) mutation was observed in exon 2. He received unrelated CBT without conditioning at 4 months of age (Fig. 2b). Human leukocyte antigen full-matched female cord blood cells (28.03×10^7 cells/kg) were infused. FK506 and short-term methotrexate were used for graft versus host disease (GVHD) prophylaxis. He had only mild skin manifestation of GVHD, which resolved by prednisolone (1 mg/kg/day). On day 25 after CBT, $CD3^+$ cells increased to 60.1% of lymphocytes (1,471 cells/μl), 93.8% of which were positive for CD45RO. Analysis of the TCRVβ repertoire on day 27 showed an abnormal pattern with overexpansion of $V\beta16^+$ cells (7.3%) and $V\beta17^+$ cells (9.7%), and a different profile was observed between pre-CBT and post-CBT (Fig. 2a). Proliferative response to Con A and PHA normalized on day 50 (20,500 cpm) and on day 174 (284 %S.I.), respectively. Chimerism analysis on day 173 showed that most of the $CD3^+$

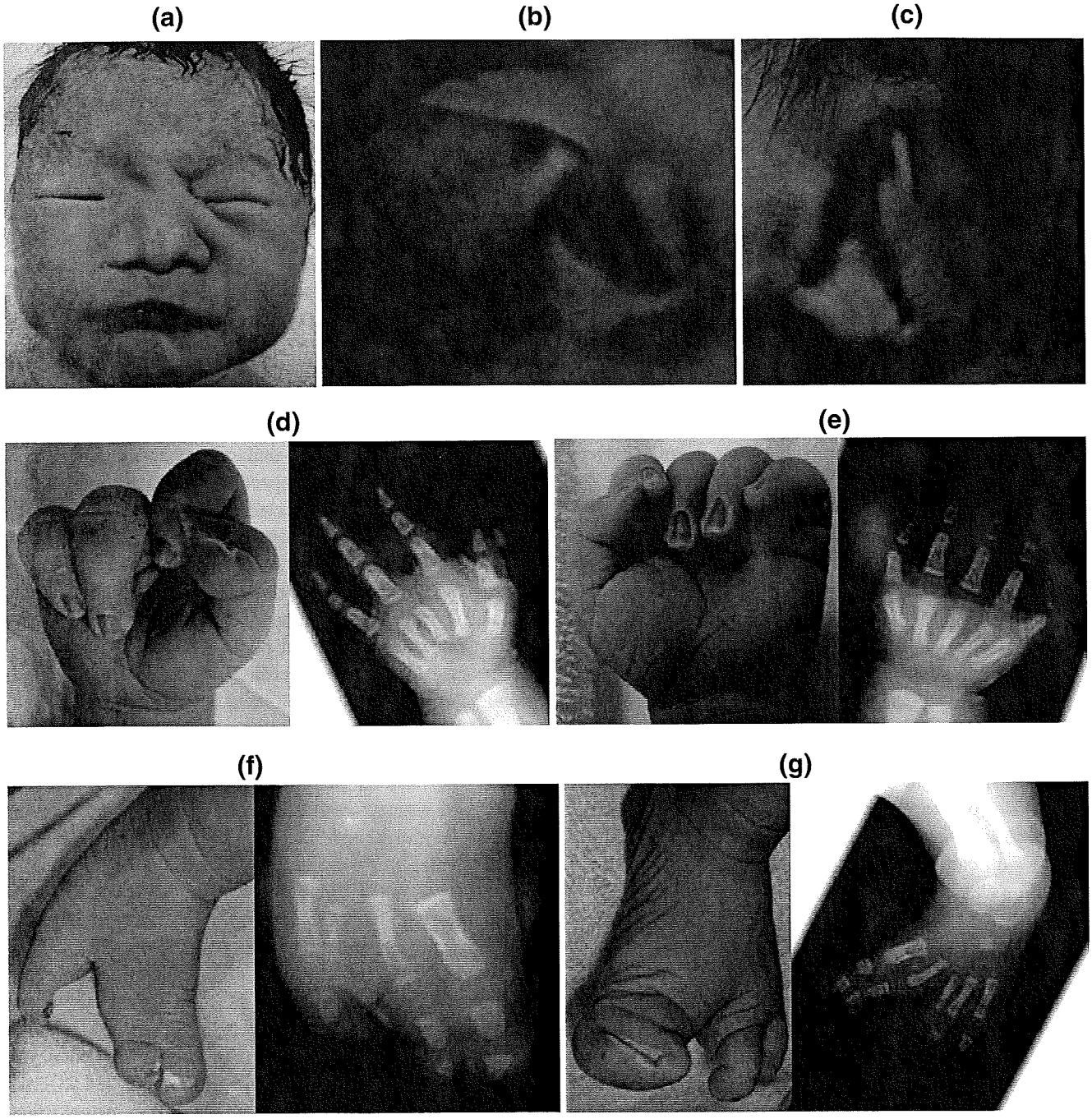

Fig. 1 Clinical manifestations of the patients. **a** Frontal view of the face showing hypertelorism and right facial palsy. **b** Lateral view of the right ear showing protruding, helix hypoplasia, and low-set ear. **c** Lateral view of the left ear showing square-shaped, absent anthelix, and low-set ear. Note asymmetry of ears. **d** Thumb polydactyly and cleft of the right hand. **e** Normal left hand. **f, g** Cleft and cutaneous syndactyly of the bilateral foots. Written consent was obtained for publication of these pictures

cells were of donor origin (94.5% of $CD3^+$ cells were XX, 5.5% were XY). At 10 months of age (day 169 after CBT), $CD3^+$ cells were 36.3% of lymphocytes (973 cells/μl), and 86.2% of T cells were positive for CD45RO. T cell receptor excision circles were below the detection limit before CBT, confirming the lack of thymic output (data not shown).

He is alive without serious infections with regular administration of immunoglobulin and prophylactic antibiotics. At 10 months of age, serum calcium, phosphorus, and parathyroid hormone levels are 7.2 mg/dl, 6.1 mg/dl, and 3.0 pg/ml, respectively. He is still receiving calcium preparation and alfacalcidol.

Table 1 Immunological studies

	Pretransplantation	Posttransplantation
$CD3^+$ cells (% lymphocytes)	2.8	36.3
$CD3^+$ cells (cells/μl)	8	973
$CD45RO^+/CD3^+$ (%)	87.7	86.2
$CD45RO^-/CD3^+$ (%)	12.4	10.9
$CD4^+$ cells (% lymphocytes)	2.3	24.2
$CD4^+$ cells (cells/μl)	7	648
$CD8^+$ cells (% lymphocytes)	15.3	12.1
$CD8^+$ cells (cells/μl)	46	324
TCR $\gamma\delta^+$ (%)	0.1	0.2
$CD19^+$ (%)	52.9	33.3
$CD16^+/CD56^+$ (%)	35.9	29.7
Proliferative response		
Against PHA (%S.I.)	123	284
Against Con A (cpm)	2,530	20,500
IgG (mg/dl)	899	425
IgM (mg/dl)	19	83
IgA (mg/dl)	5	66
Karyotype of $CD3^+$ cells	99.5% of 46,XY	5.5% of 46,XY
	0.5% of 46,XX	94.5% of 46,XX

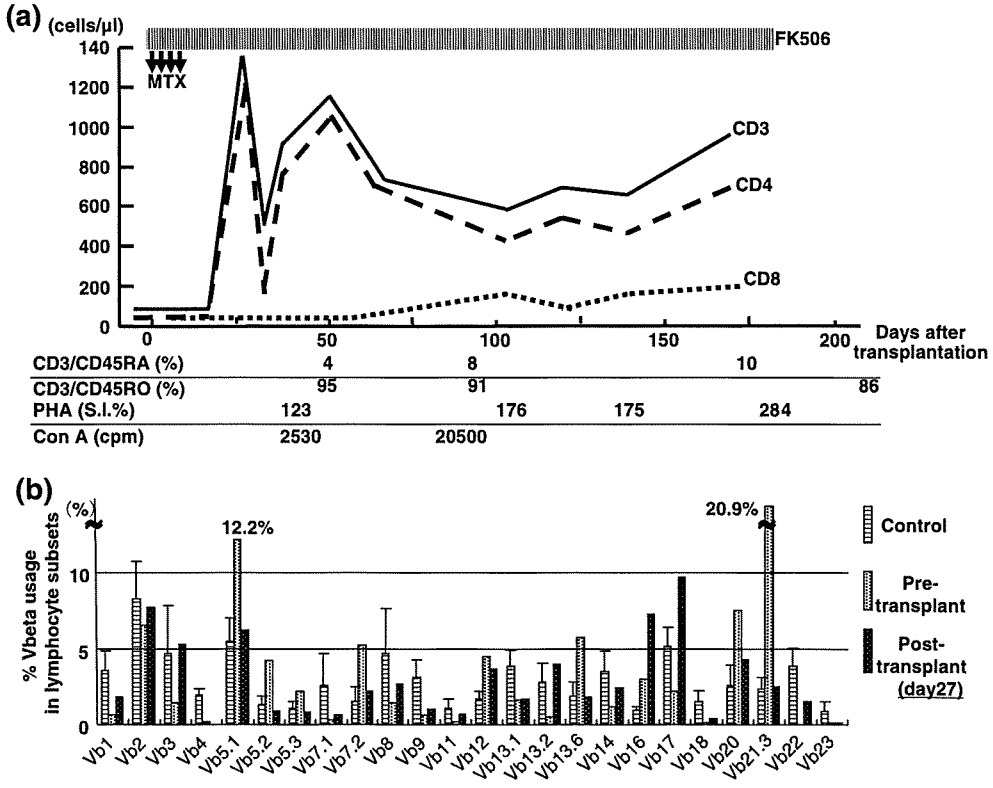

Fig. 2 Clinical course and immunological recovery after the cord blood transplantation. **a** Clinical course of the cord blood transplantation. *MTX* methotrexate, *PHA* phytohemagglutinin, *Con A* concanavalin A. **b** TCRVβ repertoire profile on the patient and control subjects. Note the skewing in the TCR repertoire before and after transplantation. *TCR* T cell receptor

Discussion

Our patient showed absence of T lymphocytes accompanied with aplasia of the thymus manifesting complete-type DiGeorge sequence, a rare complication of CHARGE syndrome [1, 30]. T cell number of the patient was recovered by CBT, although most of the T cells showed memory phenotype reflecting peripheral expansion of donor cord blood-derived mature T cells and the lack of the thymic output. He presented with additional rare manifestations, severe limb anomalies, and congenital hypoparathyroidism. DiGeorge sequence is associated with a deletion of chromosome 22q11.2 in approximately 80% of patients [23]. Interestingly, Markert et al. reported that only 52% of 54 patients with DiGeorge sequence had a deletion of 22q11, and 26% had CHARGE phenotype without the deletion [15]. A number of genes have been identified in the 22q11.2 region [31], including *TBX1* that is a major genetic determinant of del22q11.2 syndrome. As TBX1 is a transcription factor that contains a DNA binding domain, it is possible that TBX1 is a functional target for CHD7.

Thymic hypo/agenesis was observed in 70% of fetuses with CHARGE syndrome [22]. The high frequency of thymic defect in fetuses suggests that accompanying immune deficiency may be more common in this disease than previously reported. It is possible that many of athymic patients were counted on DiGeorge syndrome, rather than CHARGE syndrome. Otherwise, CHARGE syndrome patients with thymic defect may more often die during perinatal period because of the immunodeficiency or other accompanying anomalies such as severe cardiac defect. Although there have been a few reports of stem cell transplantation for the treatment of T cell deficiency in complete-type DiGeorge sequence, this is the first case of CBT for the treatment of CHARGE syndrome with *CHD7* mutation manifesting T cell defect [8, 14, 18]. The optimal treatment for patients with complete-type DiGeorge sequence has not been established. In the absence of treatment, patients usually die in the first 2 years of life [16]. Therefore, prompt reconstitution of the immune function is required to prevent fatal infectious complications. The common treatments for immunological reconstitution in complete-type DiGeorge sequence are thymic and bone marrow transplantation [13, 15]. Although thymic transplantation would be more reasonable from the physiological point of view, it is not ethically approved in Japan. We selected CBT without conditioning regimen for our patient because of the following reasons: (1) lack of sibling donors, (2) more noninvasive procurement and more rapid availability than the matched unrelated donors, (3) lower risk of GVHD or viral transmission in CBT compared with bone marrow or peripheral blood stem cells [5], and (4) higher frequency of naïve T cells in cord blood [6], which have a longer lifespan than their memory counterparts [26]. Because of the lack of thymic output after the transplantation in this disease, the high frequency of naïve T cells in the donor cells may be an important factor to avoid early immune senescence. On the other hand, it may take more time for the recovery of neutrophils in CBT leaving a higher risk of infection compared with bone marrow or peripheral stem cell transplantation [5]. In addition, naïve T cells in cord blood may require a longer time to mature into effecter memory cells and thus do not provide immediate defense against microbial agents [11]. Our patient received CBT in the NICU and has been bred in a closed infant incubator since birth. This might in part contribute to the decrease of the risk of infections and to the success of CBT.

Ryan et al. [20] reported that only a few patients (1–4%) had mild limb abnormalities in 548 patients with chromosome 22q11 deletions. Limb anomalies were not initially described in CHARGE syndrome [4]. Recently, limb anomalies have been reported as a rare manifestation in CHARGE syndrome [3, 17, 19]. On the other hand, Brock et al. [4] reported that limb anomalies occurred in about 30% of patients with definite or probable CHARGE syndrome. It is interesting that limb anomalies with DiGeorge sequence are more frequently observed in male (P value <0.034), and limb anomalies were observed in 70.0% of male DiGeorge sequence with definite CHARGE syndrome [4]. Williams proposed that CHARGE syndrome is caused by a disruption of mesenchymal–epithelial (including ectoderm and endoderm) interaction [27]. Sanlaville et al. [22] showed that the *CHD7* gene is also expressed in the limb bud mesenchyme during embryogenesis. Van de Laar et al. [24] reported that three CHARGE syndrome patients with CHD7 mutation had severe limb anomalies. Therefore, it is possible that *CHD7* mutation itself is responsible for limb defects, and limb anomalies are more strongly associated with *CHD7* mutation than 22q11 deletion.

In patients with 22q11 deletion, 203 of 340 (60%) had hypoparathyroidism and hypocalcaemia, and the hypocalcaemia resolved in 70% [20]. On the other hand, only three CHARGE syndrome patients with *CHD7* mutations had hypoparathyroidism [21, 28, 30]. It is interesting that the three patients had severe T cell deficiency [21, 28, 30]. As TBX1 might be a functional target of CHD7, it is possible that hypoparathyroidism may be more common in CHARGE syndrome than previously recognized. Günther et al. showed by using *glial cells missing2*-deficient mice that thymus had a backup mechanism of parathyroid gland and thymus itself secreted parathyroid hormone when parathyroid glands was absent [9]. It is possible that intractable hypocalcemia continues when both parathyroid gland and thymus are absent.

CHARGE syndrome is a complex of congenital malformations, and the acronym CHARGE presents the cardinal features of the disorder. Recently, there are several reports about complications with CHARGE syndrome such as immunodeficiency, limb anomaly, and parathyroid gland deficiency, which were not well recognized previously. In particular, immunodeficiency caused by thymus defect as well as the heart anomalies may be a fatal complication in this syndrome. This disease should be diagnosed as early as possible, and the immunological evaluation should be performed carefully. When the patients have severe T cells deficiency, prompt immunological reconstitution should be undertaken appropriately. Careful observation would be necessary for the gradual exhaustion of T cells and the development of autoimmune and malignant diseases in these patients after stem cell transplantation.

Conflict of interest The authors declare that there was no financial support that might pose a conflict of interest in connection with the submitted article.

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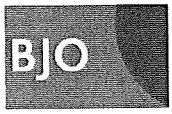

A significant association of viral loads with corneal endothelial cell damage in cytomegalovirus anterior uveitis

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A significant association of viral loads with corneal endothelial cell damage in cytomegalovirus anterior uveitis

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