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Table 2. The incidence of adverse event

Adverse event	Total (%)	Grade I (%)	Grade II (%)	Grade III (%)
Any event	33 (100)	9 (27.3)	20 (60.6)	4 (12.1)
Any immune-related event	33 (100)	27 (81.8)	6 (18.2)	0
Drug fever	8 (24.2)	4 (12.1)	4 (12.1)	0
Rash or flushing	27 (81.8)	24 (72.7)	3 (9.1)	0
Injection site reaction	33 (100)	33 (100)	0	0
Pruritus	6 (18.2)	6 (18.2)	0	0
Blood	15 (45.4)	6 (18.2)	9 (27.3)	0
Leukopenia	6 (18.2)	2 (6.1)	4 (12.1)	0
Neutropenia	8 (24.2)	5 (15.2)	3 (9.1)	0
Anemia	5 (15.2)	2 (6.1)	3 (9.1)	0
Thrombopenia	3 (9.1)	1 (3.0)	2 (6.1)	0
Increase in PT-INR	2 (6.1)	2 (6.1)	0	0
Hepatic	23 (69.7)	10 (30.3)	9 (27.3)	4 (12.1)
Hyperbilirubinemia	9 (27.3)	3 (9.1)	4 (12.1)	2 (6.1)
Increase in aspartate aminotransferase	14 (42.4)	4 (12.1)	6 (18.2)	4 (12.1)
Increase in alanine aminotransferase	12 (36.4)	10 (30.3)	1 (3.0)	1 (3.0)
Renal	9 (27.3)	6 (18.2)	3 (9.1)	0
Increase in creatinine	4 (12.1)	2 (6.1)	2 (6.1)	0
Proteinuria	6 (18.2)	4 (12.1)	2 (6.1)	0
Other laboratory				
Increase in alkaline phosphatase	9 (27.3)	4 (12.1)	4 (12.1)	1 (3.0)
Hypoalubuminemia	10 (30.3)	7 (21.2)	3 (9.1)	0
Hyponatremia	13 (39.4)	12 (36.4)	1 (3.0)	0
Hyperkalemia	4 (12.1)	4 (12.1)	0	0

Abbreviation: PT-INR, prothrombin time-international normalized ratio.

liver tumors. The effect and safety evaluation committee, including the external members, judged that these events were not related to the treatment, but rather to disease progression. All patients experienced grades I or II local skin reactions at the injection site. Transient immune-related events, including drug fever, rash, and flushing, were observed in most patients. Crotamiton, a scabicidal and antipruritic agent, was prescribed to the 5 patients who had mild itching, but no antipyretic analgesics were prescribed. These results suggest that GPC3 peptide vaccine therapy was well-tolerated.

GPC3 peptide vaccination could induce peptide-specific CTLs in most patients

To determine whether the GPC3 peptide vaccine could induce a specific immune response, PBMCs, obtained from all patients before and after vaccination, were examined by *ex vivo* IFN-γ ELISPOT assay. After the second vaccination, the number of GPC3 peptide–specific CTLs in 5×10^5 PBMCs was increased from 0 to 441 in case 32 (Fig. 1A). As shown in Table 1, we found that the GPC3 peptide vaccine induced a GPC3-specific CTL response in 30 of the 33 patients (91%). GPC3-specific CTL frequency increased in a peptide dose-dependent manner (Fig. 1B). Generally, CTLs for some tumor antigens cannot be directly detected *ex vivo*; they can only be detected after expansion by repeated *in vitro* stimulation with the antigenic peptide on appropriate antigen-presenting cells. This finding can be attributed to the sensitivity of the assay and the low frequency of tumor antigen–specific CTLs (23). Surprisingly, GPC3-specific CTLs were directly detected *ex vivo* without *in vitro* peptide stimulation in almost all patients after GPC3 peptide vaccination.

We also analyzed the GPC3-specific CTL frequency by flow cytometry using the GPC3 peptide, Dextramer. The GPC3-specific CTL frequency is indicated as the percentage of both Dextramer-positive and CD8-positice cells before and after vaccination, as shown in Fig. 1C. After the second vaccination, the frequency of GPC3-specific CTLs increased from 0% to 0.12% in case 32.

In many patients who were vaccinated only 3 times, the GPC3-specific CTL frequency decreased within 2 months after the third vaccination. We could vaccinate 4 or more times in 12 cases. In 9 of these, the GPC3-specific CTL frequency increased after the fourth vaccination (data not shown).

CTLs infiltrated the tumor after GPC3 peptide vaccination

Tumor biopsy was carried out (with informed consent) in 7 patients to evaluate the therapeutic effect after vaccination. We evaluated infiltration of CD8-positive T cells by

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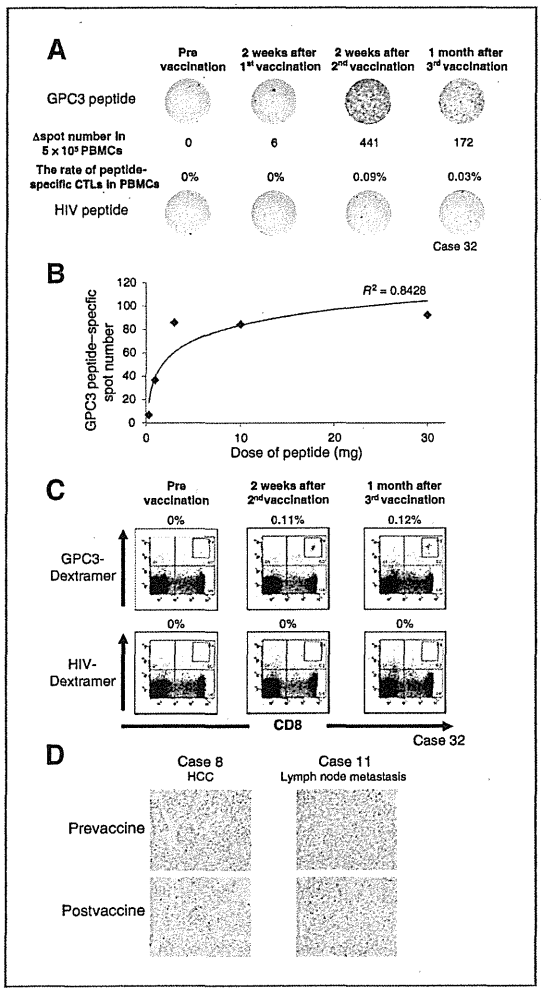

Figure 1. Immunologic monitoring of GPC3 peptide–specific T-cell responses. A, *ex vivo* IFN-γ ELISPOT assay for GPC3 in 5×10^5 PBMCs was carried out before and after vaccination in case 32. The Δspot number indicates the number of GPC3 peptide–specific CTLs. The number of IFN-γ–positive spots increased from 0 to 441 in the wells preincubated with GPC3 peptide. B, median spot number in *ex vivo* IFN-γ ELISPOT assay for GPC3 for each peptide dosage. GPC3-specific CTL frequency increased in a peptide dose-dependent manner. C, *ex vivo* GPC3 Dextramer staining before and after vaccination in case 32. GPC3 peptide–specific CTL frequency is indicated as the percentage of Dextramer-positive CTLs among PBMCs. The frequency of GPC3 peptide–specific CTLs increased from 0% to 0.12% in case 32. D, immunohistochemical staining showing CD8-positive lymphocytes infiltrating tumors before and after vaccination. In cases 8 and 11, CD8-positive T cells (brown) did not infiltrate the tumors before vaccination; in contrast, many CD8-positive T cells infiltrated the tumor after vaccination. Magnification, ×200.

immunohistochemical staining. In case 8, liver biopsy was carried out before and after vaccination. In case 11, neck lymph node metastasis was resected after vaccination. The specimen was compared with an abdominal lymph node metastasis sample obtained by a diagnostic biopsy that this patient underwent before vaccination. While CD8-positive T cells did not infiltrate the tumor before vaccination, marked infiltration of CD8-positive T cells into the tumor was observed after vaccination in both cases (Fig. 1D). In 5 of 7 cases, infiltration of CD8-positive T cells into the tumor was increased after vaccination.

Clinical responses

Patient characteristics and clinical responses in relation to GPC3-specific CTLs are shown in Table 1. Among the 33 patients, one (case 24) was judged to have a partial response (PR) and 19 patients stable disease (SD) for 2 months, according to RECIST. The assessment of tumor response according to mRECIST was the same as that according to RECIST in all 33 patients. The disease control rate (PR + SD) was 60.6% after 2 months. The median time to tumor progression (TTP) was 3.4 months [95% confidence interval (CI), 2.1–4.6]. The median OS was 9.0 months (95% CI, 8.0–10.0).

In case 24, supraclavicular lymph node metastases markedly regressed, 2 liver tumors disappeared, and the thoracic bone metastasis showed necrosis after the third vaccination (Fig. 2A and B). We carried out a biopsy of the remaining liver tumor and the thoracic bone metastasis after obtaining informed consent. Immunohistochemical staining showed expression of GPC3 and HLA class I on cells in the remaining liver tumor (Fig. 2C). Surprisingly, we detected massive infiltration of CD8-positive T cells into the remaining liver tumor by immunohistochemical staining. No viable tumor cells were found in the biopsy specimens of the thoracic bone metastasis.

Four other patients (cases 1, 15, 16, and 17) had tumor necrosis or partial tumor reduction that did not meet the PR criteria.

Serum levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are useful tumor markers of HCC (24). The levels of AFP or DCP decreased temporarily at least once in 9 of the 33 patients during the 2-month period (Supplementary Table S1). In 7 of these 9 patients, the levels of DCP fell to less than 30% of baseline values. In 15 of 32 patients, GPC3 protein was detectable in serum before vaccination. The serum levels of GPC3 temporarily decreased at least once in 12 of these 15 patients (data not shown).

These results suggest that there is not the duration of the responses in regards to CTL induction and tumor responses in this phase I trial.

OS was correlated with GPC3-specific CTL frequency

We also examined prognostic factors (Table 3). Fifty GPC3 peptide–specific CTL spots were detected in an *ex vivo* IFN-γ ELISPOT assay conducted using 5×10^5 PBMCs, which means that the GPC3 peptide–specific CTL frequency in peripheral lymphocytes was is 1×10^{-4}%. We focused on these 50 spots to elucidate prognostic factors. Univariate analysis indicated that distant metastasis (−; $P = 0.032$), invasion of the inferior vena cava (IVC) or portal vein (PV;

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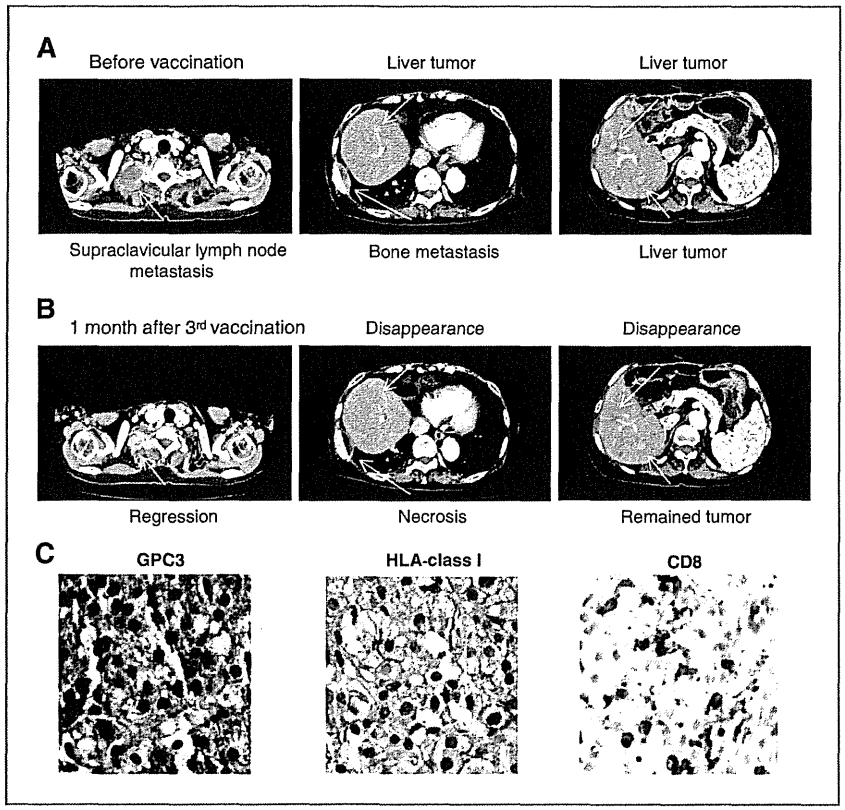

Figure 2. Response assessment in case 24. A, CT imaging, showing liver, pleura, and supraclavicular lymph node metastases before vaccination. B, CT imaging after vaccination was judged as an indicator of a PR. The supraclavicular lymph node metastasis and multiple liver tumors regressed markedly. The pleura metastasis was necrotic. C, we biopsied the remaining liver tumor after vaccination. Immunohistochemical staining showed expression of GPC3 and HLA class I on tumor cells. There was massive infiltration of CD8-positive T cells. Magnification, ×200.

$P = 0.040$), AFP ≥ 100 ng/mL ($P = 0.003$), tumor size ≥ 10 cm ($P = 0.003$), and GPC3-specific CTL frequency < 50 were prognostic factors for OS. Furthermore, AFP ≥ 100 ng/mL ($P = 0.004$; HR = 4.66; 95% CI, 1.61–13.19), tumor size ≥ 10 cm ($P = 0.003$; HR = 4.36; 95% CI, 1.58–12.05), and GPC3-specific CTL frequency < 50 ($P = 0.032$; HR = 2.71; 95% CI, 1.09–6.72) were prognostic factors for OS in a multivariate analysis. We showed that GPC3-specific CTL

Table 3. Prognostic factors of OS

	P univariate	*P* multivariate	HR (95% CI)
Sex (male/female)	0.991		
Age (≥65/<65)	0.608		
Performance status (0/1)	0.707		
Child–Pugh (A/B)	0.063		
Virus infection (+/−)	0.956		
Distant metastasis (+/−)	0.032	0.284	1.71 (0.64–4.54)
Invasion of IVC or PV (+/−)	0.040	0.706	1.21 (0.45–3.30)
AFP (≥100/<100 ng/mL)	0.003	0.004	4.66 (1.61–13.19)
Tumor size[a] (≥10/<10 cm)	0.003	0.005	4.36 (1.58–12.05)
GPC3-specific CTL[b] (≥50/<50)	0.033	0.032	2.71 (1.09–6.72)
HLA (A2/A24)	0.091		
Vaccine[c] (≥1/<1 mg)	0.053		

[a]Tumor size estimated by the RECIST.
[b]The GPC3 peptide–specific CTL frequency examined with *ex vivo* IFN-γ ELISPOT assay in 5×10^5 PBMCs.
[c]The dosage of one vaccine.

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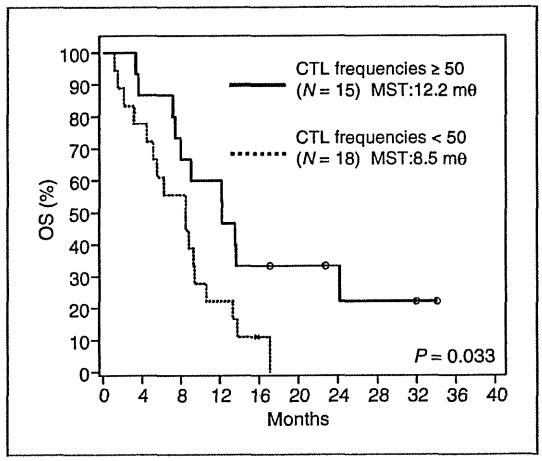

Figure 3. Kaplan–Meier curves for OS. Patients with GPC3-specfic CTL frequencies ≥50 had a longer survival than those with GPC3-specfic CTL frequencies <50 ($P = 0.033$). MST, median survival time.

frequency could be a predictive marker of the effects of GPC3 peptide vaccination. We compared patients with GPC3-specfic CTL frequencies ≥ 50 (N = 15) with those with GPC3-specific CTL frequencies < 50 (N = 18) and found that there was no significant difference in clinical background. We only found a significant difference ($P = 0.004$) for vaccine consumption (≥1.0 vs. <1.0 mg; Supplementary Table S2). Analysis of all 33 patients showed that the median OS was 12.2 months (95% CI, 6.5–18.0) in patients with GPC3-specfic CTL frequencies ≥50, compared with 8.5 months (95% CI, 3.7–13.1) in those with GPC3-specfic CTL frequencies <50 ($P = 0.033$; Fig. 3).

Discussion

We did not observe dose-limiting toxicity in this study. It was difficult to determine the maximum tolerated dose of peptide. A peptide dose of greater than 1.0 mg was required for adequate induction of GPC3-specific CTLs. However, it was complicated to inject more than 10 mg of peptide intradermally because injection mixtures contained both peptide and IFA, and doses of peptide vaccine >10 mg emulsified with IFA (consisting of 2 mL of fluid, including 1 mL of IFA), increased local skin reactions (induration, blushing) at the injection site (Supplementary Fig. S2). Therefore, a dose of peptide of 3.0 mg is recommended for future clinical trials.

We evaluated the expression of GPC3 in the primary tumors of 26 patients by immunohistochemistry. Among the 21 patients with low GPC3 expression (degree of staining − or 1+), one patient was judged to have a PR, and 3 patients have shown long-term survival. We do not suggest that only patients with high GPC3 expression (degree of staining 2+) should be enrolled in further clinical trials.

We studied immunologic responses using an *ex vivo* IFN-γ ELISPOT assay. The GPC3 peptide vaccine induced GPC3-specific CTL responses in 30 of the 33 patients. In contrast, clear immune responses were not observed in patients with HCC in another vaccination trial (9). Differences in tumor antigen may account for the differences in immune response between the 2 vaccination trials. Previous studies have shown that GPC3 is also overexpressed in other malignant tumors, including melanomas, Wilms' tumor, hepatoblastoma, ovarian clear cell carcinoma, and lung squamous cell carcinoma (12, 25–28). GPC3 might also be an effective target for immunotherapy against these tumors (29, 30).

In our study, none of the patients in the 0.1 mg dose group showed more than 50 GPC3 peptide-specific CTL spots. GPC3-specific CTL frequency increased in a peptide dose-dependent manner. Previously, Salgaller and colleagues reported no dose dependency in the capacity of the gp100 peptide to enhance immunogenicity in humans (at doses 1.0–10 mg; ref. 31). In contrast, our data indicate dose dependency in CTL induction, consistent with a previous report using a mouse model (20).

Ten of the 25 patients who received a dose higher than 1.0 mg did not exhibit GPC3-specfic CTL frequencies ≥50. There was no significant difference in the clinical background of patients with GPC3-specific CTL frequencies ≥50 and those with <50. However, GPC3-specific CTL frequency tended to correlate with the serum level of AFP or summed intrahepatic tumor size (Supplementary Table S2). In this study, several patients with advanced HCC exhibited a poor immunologic response to GPC3 peptide vaccination. There are several possible explanations for this poor immunogenicity. HCC is frequently accompanied by cirrhosis, which creates an immunosuppressive environment. There is impairment of the function and maturation of dendritic cells, which has been shown to be related to an imbalance in the extracellular amino acid profile (32). In progressive HCC, the induction of CTL may be suppressed by regulatory T cells or immunosuppressive cytokines (33). It has been reported that GPC3-specific CTLs become exhausted in HCC, and that this exhausted state cannot be reversed by blocking the CTLA-4 and PD-1 inhibitory costimulation pathways (34). Further studies will be necessary to increase the clinical efficacy of immunotherapy for advanced HCC.

The primary endpoint of this study was assessment of the safety of vaccination, but we also showed that tumor antigen–specific CTLs had a crucial role in the immunotherapy against GPC3. GPC3-specific CTL frequency was correlated with OS in this study. Peptide-specific IgG and delayed-type hypersensitivity postvaccination have been reported as potential predictive makers of prolonged survival in patients with advanced cancer vaccinated with peptides (35, 36). However, correlations between immune responses and OS have not been reported in other immunotherapy trials for HCC (7–9, 37). We found that patients with GPC3-specfic CTL frequencies ≥ 50 had a longer survival than those with GPC3-specfic CTL frequencies <50. There was no significant difference in the clinical backgrounds of patients with GPC3-specific CTL frequencies ≥50 and those with <50.

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We clearly showed the presence of GPC3 peptide–specific CTLs in peripheral blood, and showed that many CD8-positive T cells infiltrated tumors after GPC3 peptide vaccination. The evidence in this study serves as a proof-of-concept for immunotherapy using tumor antigen–specific CTLs. However, we did not confirm that the tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide–specific CTLs. We are currently initiating a pilot study of liver biopsies carried out before and after GPC3 peptide vaccination for advanced HCC to determine whether tumor-infiltrating lymphocytes are indeed GPC3 peptide–specific CTLs.

No complete responses were observed when GPC3 peptide vaccination was used as the sole therapy for advanced HCC. To-date, there has been no report of an adequate antitumor efficacy of immunotherapy in clinical trials involving patients with advanced HCC; however, immunotherapy, as an adjuvant after surgical resection, is expected (38). On the basis of this study, we have begun a phase II study of the GPC3-derived peptide vaccine as an adjuvant therapy for patients with HCC and have also planned combinatorial approaches with chemotherapy.

In conclusion, this phase I clinical trial of a GPC3-derived peptide vaccine showed the vaccination to be safe and indicated a plethora of immunologic responses. This study also showed that GPC3-specific CTL frequency was correlated with OS in patients with advanced HCC who received the GPC3 peptide vaccine.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: J. Furuse

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Sawada, T. Yoshikawa, D. Nobuoka, H. Shirakawa, Y. Motomura, H. Ishii, K. Nakachi, M. Konishi, S. Takahashi, N. Gotohda, J. Furuse, T. Kinoshita, T. Nakatsura

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Sawada, S. Mizuno, J. Furuse, T. Nakatsura

Writing, review, and/or revision of the manuscript: Y. Sawada, T. Yoshikawa, D. Nobuoka, T. Takayama, J. Furuse, T. Nakatsura

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Furuse

Study supervision: T. Nakagohri, T. Takayama, K. Yamao, J. Furuse

Monitoring and evaluation of data and safety of the study: K. Uesaka

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Carcinoma of Donor Origin After Allogeneic Peripheral Blood Stem Cell Transplantation

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Abstract: Secondary cancers developing after allogeneic hematopoietic stem cell transplantation generally originate from recipient-derived cells. In this study, we analyzed the tumor cell origin of 5 epithelial malignant tumors (esophageal squamous cell carcinoma, lung adenocarcinoma, gastric adenocarcinoma, pharyngeal squamous cell carcinoma, and thyroid papillary carcinoma) that developed after allogeneic peripheral blood stem cell transplantation using anti-AE1/3 immunofluorescence with fluorescence in situ hybridization analysis for sex chromosomes and/or short-tandem repeat microsatellite analysis of laser-microdissected tumor cells. The results revealed that 1 of these 5 cancers was derived from donor cells. In this case, transfused pluripotent cells, which include both mesenchymal stem cells and hematopoietic stem cells, might have given rise to epithelial malignant cells. Our observations suggest that transfused peripheral blood cells may be involved in the development of cancers after allogeneic peripheral blood stem cell transplantation.

Key Words: allogeneic PBSCT, secondary cancer, donor-derived cell, STR, FICTION

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Secondary malignancies developing after allogeneic hematopoietic stem cell transplantation (HSCT), including posttransplant lymphoproliferative disorders, hematological disorders, and nonhematopoietic solid tumors, are among the late complications observed in long-term survivors of allogeneic HSCT. Analysis of these tumors may help us understand the origin of tumor cells and the mechanism of carcinogenesis. Although these secondary malignancies developing after allogeneic HSCT are generally considered to be derived from recipient cells, sporadic cases of donor cell–derived leukemias have been reported since the original description of a case in 1971.[1,2]

In contrast, reports of secondary nonhematopoietic solid tumors derived from donor cells are extremely rare. In 2002, Okamoto et al[3] revealed that damaged epithelia were regenerated by bone marrow–derived cells in the human gastrointestinal tract after allogeneic HSCT. In 2004, Houghton et al[4] reported for the first time that gastric adenocarcinoma in an animal model, induced by *Helicobacter felis* infection after total-body irradiation and bone marrow reconstitution, originated from bone marrow–derived cells. These results suggest that bone marrow–derived cells play an important role in the regeneration of inflammatory tissue and in carcinogenesis. Although there have been reports on the involvement of bone marrow cells in human cancers developing after allogeneic HSCT,[5–8] the contribution of bone marrow cells to the pathogenesis of cancers remains controversial because of the heterogeneity of the patients' backgrounds and treatments (myeloablative or nonmyeloablative conditioning regimen, stem cell source, etc.). HSCT includes bone marrow transplantation (BMT), peripheral blood stem cell transplantation (PBSCT), and cord blood transplantation. There are several differences among these 3 types of HSCT in terms of their clinical courses, especially the time for engraftment and the frequency of acute or chronic graft-versus-host disease (GVHD). Reports on identification of the tumor cell origin of secondary cancers after allogeneic PBSCT are extremely rare. In this study, we demonstrated the tumor cell origin of secondary cancers developing after allogeneic PBSCT using 2 different methods, namely, anti-AE1/3 immunofluorescence with fluorescence in situ hybridization (FISH) analysis for sex chromosomes [Fluorescence Immunophenotyping and interphase Cytogenetics as a Tool for the Investigation Of Neoplasm (FICTION)][9] and/or

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short-tandem repeat (STR) analysis of laser-microdissected tumor cells.

MATERIALS AND METHODS

Patients

From January 2001 to December 2009, 8 patients who had undergone allogeneic HSCT were diagnosed as having secondary cancers. In all the cases, the tumor developed at least 1 year after the HSCT. Formalin-fixed paraffin-embedded (FFPE) samples for analysis of tumor cell origin were available in 5 of these cases. The donor was sex mismatched in 4 of these cases of allogeneic PBSCT and was sex matched in the remaining 1 case. Their hematological status was assessed as full donor chimera without relapse at the time of diagnosis of the secondary cancers. This study was conducted with the approval of the Institutional Review Board of the National Cancer Center.

Before transplantation, the STR profiles of the recipients and donors were determined using multiplexed PCR amplification systems. Peripheral blood genomic DNA extracted from both patients and donors was subjected to examination with a multiplexed PCR amplification kit targeting a total of 15 autosomal STR markers and 1 STR marker of the X and Y chromosomes (Amp FLSTR Identifiler kit, Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. After hematological recovery, peripheral blood DNA was again extracted, and STR analysis was performed; in addition, whether the recovered cells were of the donor type, recipient type, or the mixed type was determined. The information on loci that differed between the donors and recipients was utilized in the ensuing study.

FICTION Analysis

FICTION analysis of all the 5 cases was performed using anti-AE1/3 and sex chromosome probes. Paraffin sections of 4 μm thickness were cut from each FFPE block and were immunostained with an anti-AE1/3 antibody to reveal epithelial origin. The first antibody was the mouse anti-human AE1/3 (Dako, Glossary, Denmark) diluted 100-fold and incubated overnight at 4°C. As the secondary, tertiary, and quaternary antibodies, 3 fluorescence-labeled antibodies—Alexafluor 647 rabbit anti-mouse IgG, Alexafluor 647 goat anti-rabbit IgG, and Alexafluor 647 donkey anti-goat IgG, respectively (Molecular probes, Life Technologies Corporation, CA)–were used. Each antibody was diluted 1000-fold and incubated for 30 minutes at room temperature.

FISH analysis was performed using the Histology FISH Accessory kit (Dako) in accordance with the manufacturer's recommendations. We used chromosome enumeration probes for chromosomes X and Y labeled with spectrum orange and spectrum green, respectively (Vysis; Abbott Laboratories, Abbott Park, IL). For observation, we used BIOREBO (Keyence Corporation, Osaka, Japan) and observed 4-colored fluorescence at the same time.

Laser Microdissection of Tumor Cells and DNA Extraction

Laser microdissection (LMD) was performed using a Leica AS LMD system (Leica Microsystems, Wechsler, Germany). In accordance with the manufacturer's instructions, 10-μm-thick FFPE sections were mounted on Membrane Slides (Leica Microsystems) and stained with hematoxylin and eosin. The dissected areas that comprised tumor cells were identified by 2 pathologists (A.M.M. and H.T.).

Microdissected tumor cells were collected in 50 μL of 1 × TE buffer with proteinase K and incubated overnight at 60°C. After inactivation of proteinase K, these solutions were centrifuged, and the supernatant were used for further STR analysis.

DNA Extraction From Bone Marrow Smears

To analyze the PCR product size from the tumor and to compare it with that of the donor or recipient, the bone marrow smears that had been collected before transplantation and after complete engraftment were used. These smears had been fixed using May-Giemsa stain, and were free of any malignancies, both relapsed hematopoietic diseases and cancers, as confirmed by morphologic diagnosis and flow cytometry.

DNA extraction from the bone marrow smears was performed with a QIAamp DNA micro kit (Qiagen, Hamburg, Germany) according to the blood and body fluid protocol. Finally, all the samples were eluted into 100 μL of buffer AE after 1-minute incubation at room temperature and used for further PCR and STR analysis.

STR Analysis

In the tumor samples that developed after sex-matched PBSCT, STR analysis was performed using Phusion High-Fidelity DNA polymerase (Finnzymes, Vantaa, Finland). In addition, when the results of FICTION analysis suggested donor-type sex in the sex-mismatched PBSCT cases, STR analysis was performed to confirm the result of FICTION analysis.

STR loci were selected on the basis of the availability of PCR. Tumor samples were derived from FFPE tissues; therefore, the amplification efficacy was less as compared with that obtained using the multiplexed PCR amplification kit. The information on the STR loci, the PCR product sizes of which differed between the donor and the recipient, facilitated the selection of loci by which the origin of the tumors could be discerned. From these loci, we selected loci that could be successfully amplified by PCR because the FFPE samples can generate only smaller DNA fragments. Thus, only 1 or 2 STR loci were subjected to this analysis. The primers used in this study are shown in Table 1.

All the PCR products were purified using the QIA quick PCR purification kit (Qiagen). Differences in base pair lengths were visualized by capillary electrophoresis (Bioanalyzer; Agilent technologies, Santa Clara, CA) in parallel comparison with those of the amplified products

TABLE 1. The List of Primers Utilized in This Study

STR loci	Forward Primer	Reverse Primer
D7S820	5′-ATGTTGGTCAGGCTGACTATG-3′	5′-GATTCCACATTTATCCTCATTGAC-3′
D13S317	5′-ACAGAAGTCTGGGATGTGGAG-3′	5′-GCCCAAAAAGACAGACAGAAAG-3′
D16S539	5′-GATCCCAAGCTCTTCCTCTTC-3′	5′-ACGTTTGTGTGTGCATCTGTAAG-3′
VWA	5′-CCCTAGTGGATGATAAGAATAATCAGTATG-3′	5′-GGACAGATGATAAATACATAGGATGGATGG-3′
TH01	5′-GTGATTCCCATTGGCCTGTTC-3′	5′-ATTCCTGTGGGCTGAAAAGCTC-3′
Amelogenin	5′-CCCTGGGCTCTGTAAAGAATAGTG-3′	5′-ATCAGAGCTTAAACT-GGGAAGCTG-3′

of bone marrow smear samples obtained before and after the transplantation.

RESULTS

From January 2001 to December 2009, 8 patients were diagnosed with secondary cancers at least 1 year after allogeneic HSCT. FFPE samples for analysis of the tumor cell origin were available for 5 of these cases. The primary sites and types of carcinoma were as follows: esophageal squamous cell carcinoma, lung adenocarcinoma, gastric adenocarcinoma, pharyngeal squamous cell carcinoma, and thyroid papillary carcinoma. All the cases were negative for Epstein-Barr virus on the basis of in situ hybridization. The patient and transplant characteristics of these 5 cases are shown in Table 2. All the 5 transplantations were allogeneic PBSCT. The donor was a sex-mismatched donor in 4 of the cases of PBSCT and was sex matched in 1 of the cases.

In the 4 sex-mismatched cases, FICTION analysis performed using the anti-AE1/3 antibody and sex chromosome probes showed that 3 patients had cancers with the recipient-type sex chromosomes in the cells. Two patients were male with tumor cells of the male genotype, and 1 patient was female with tumor cells of the female genotype (Figs. 1–3). One patient had a tumor with the donor-type sex chromosomes in the cells (Fig. 4). This patient was a male with tumor cells of the female genotype. The specimen showed 1 or 2 signals of the X chromosome(s) but no Y signals, which suggested a tumor of donor cell origin. To confirm the results of this FICTION analysis, STR analysis was performed. Tumor cells were dissected from the FFPE samples by LMD (Fig. 5), and DNA was extracted. As we were aware that there were differences in the sizes of the PCR products between the donor cells and recipient cells in D7S820, D13S317, D16S539, VWA, TH01, and amelogenin, we tried PCR amplification of these loci; however, only D7S820 and amelogenin were successfully amplified and evaluable. The STR profiles of these 2 loci of the tumor cells were the same as those of the bone marrow after engraftment (Fig. 5). Because the other 4 loci could not be amplified, we could not evaluate them (data not shown). These results showed that the pharyngeal squamous cell carcinoma had arisen from donor-derived cells.

There was only 1 sex-matched case for which it was impossible to determine the origin by FICTION analysis (Fig. 6). Therefore, STR analysis was performed. Again, DNA was extracted from the tumor cells that were dissected by LMD. The size of the D7S820, D13S317, and D16S539 loci differed between the donor and recipient cells, and among these only D13S317 and D16S539 could be successfully amplified. The remaining single locus could not be evaluated (data not shown). The STR profiles of D13S317 and D16S539 in the tumor cells suggested that the cells were of recipient origin; they were the same as those of the bone marrow cells before allogeneic PBSCT (Fig. 7).

DISCUSSION

Allogeneic HSCT is a curative treatment option for a number of hematological disorders.[10] However, long-term survivors often suffer from life-threatening late

TABLE 2. Patient and Transplant Characteristics

UPN	Age at HSCT (y)	Recipient's Sex	Donor's Sex	Primary Disease	Type of HSCT	Site of Secondary Malignancy	Histology	Latency From HSCT (y)
1	52	M	F	CML	RPBSCT	Esophagus	Squamous cell carcinoma	7.1
2	32	M	F	PTCL-NOS	RPBSCT	Lung	Adenocarcinoma	3.1
3	54	F	M	Tf-FL	RPBSCT	Stomach	Adenocarcinoma	4.0
4	42	M	F	FL G3	RPBSCT	Pharynx	Squamous cell carcinoma	6.3
5	55	F	F	CML	RPBSCT	Thyroid	Papillary carcinoma	2.4

CML indicates chronic myeloid leukemia; FL G3, follicular lymphoma grade 3; PTCL-NOS, peripheral T-cell lymphoma, not otherwise specified; RPBSCT, related PBSCTs; Tf-FL, transformed follicular lymphoma; UPN, unique patient number.

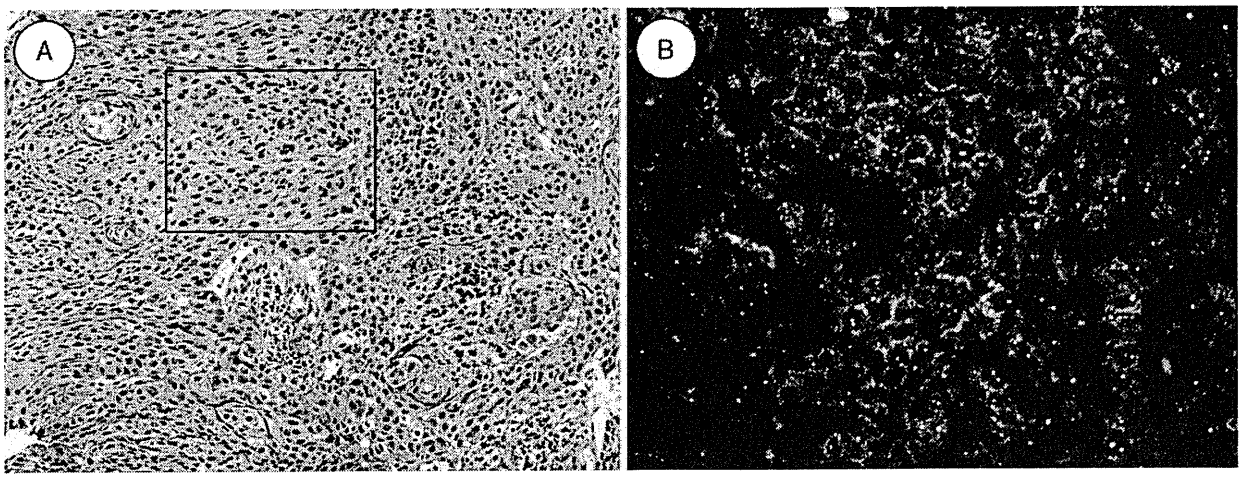

FIGURE 1. Case 1, esophageal squamous cell carcinoma after sex-mismatched transplantation (female to male). A, Hematoxylin and eosin-stained sections. Well-differentiated squamous cell carcinoma is shown in the tumor specimen obtained by endoscopic biopsy. The framed rectangle corresponds to the area shown in Fig. B. B, FICTION analysis. Approximately 90% of this specimen is occupied by the tumor cells. Tumor cells are positive for AE1/3 (yellow). The X chromosome (orange) and Y chromosome (green) are identified in tumor cells. These findings show that these tumor cells are of epithelial and recipient origin.

complications, including secondary malignancies.[11,12] It had generally been accepted that secondary cancers arise from recipient-derived cells until the first report of a donor-type cancer in an animal model was published.[4]

To the best of our knowledge, there are only a few reports of secondary cancers arising from donor-derived cells in humans undergoing allogeneic HSCT.[5–7] The tumor cell origin in these cases was investigated mainly by FISH analysis. However, various cancers and premalignant lesions have been shown to carry hyperploidies or hypoploidies.[13–16] Therefore, a false-positive donor-type result can be obtained only when sex chromosome analysis is carried out. If both chromosomal loss of a Y chromosome and duplication of an X chromosome occur in a tumor developing in a male patient, the resultant tumor would generate XX signals, suggestive of a female donor–type tumor.

Indeed, our FICTION analysis of case 4 also showed tumor cells with XX and XO in a male patient, which could be explained by the loss of a Y chromosome and duplication of an X chromosome in the tumor cells. Therefore, we performed STR analysis after the results of

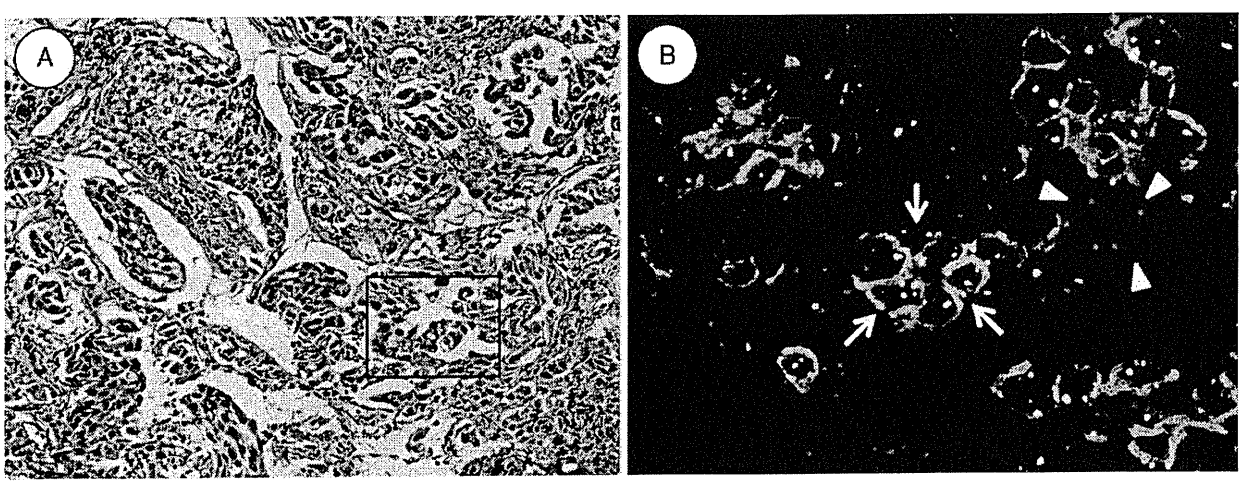

FIGURE 2. Case 2, lung adenocarcinoma after sex-mismatched transplantation (female to male). A, Hematoxylin and eosin-stained sections. Adenocarcinoma with papillary proliferation is shown in the tumor specimen obtained by transbronchial lung biopsy. The framed rectangle corresponds to the area shown in Fig. B. B, FICTION analysis. Approximately 30% of this specimen is occupied by tumor cells. The X chromosome (orange) and Y chromosome (green) are identified in AE1/3-positive tumor cells (yellow), indicating that epithelial tumor cells are of recipient origin (arrows). No Y chromosome is identified in AE1/3-negative cells, indicating donor origin of nonepithelial hematopoietic cells (shown by arrowheads).

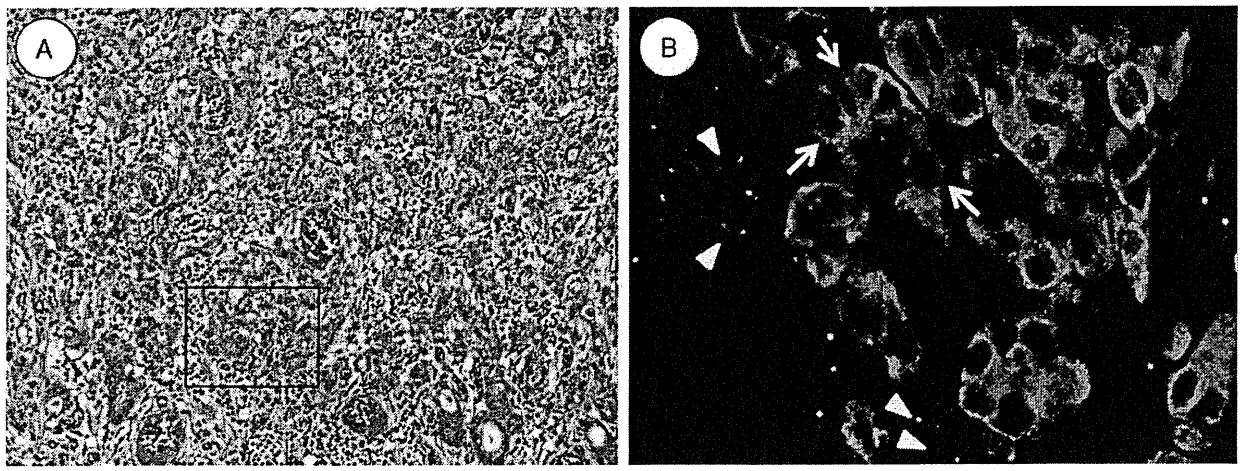

FIGURE 3. Case 3, gastric adenocarcinoma after sex-mismatched transplantation (male to female). A, Hematoxylin and eosin-stained sections. A poorly differentiated adenocarcinoma with signet ring cells is shown in the tumor specimen obtained by endoscopic resection. The framed rectangle corresponds to the area shown in Fig. B. B, FICTION analysis. Approximately 50% of this specimen is occupied by tumor cells. Two X chromosomes (orange) are identified in AE1/3-positive tumor cells (yellow), which form glandular structures, indicating epithelial tumor cells of recipient origin (shown by arrows). Epithelial tumor cells are surrounded by X and Y chromosome-positive (green), AE1/3-negative cells, indicating nonepithelial hematopoietic cells of donor origin (shown by arrowheads).

FICTION analysis, which suggested donor-type sex in the sex-mismatched PBSCT cases. The result is indicative of a true donor cell origin of the tumor.

STRs are highly polymorphic markers and allowed a clear discrimination between the donor and the recipient. Although single nucleotide polymorphism analysis may be a better and more recently developed modern technology for identifying donor or recipient type, STR analysis is routinely used in clinical settings to assess the engraftment status of donor cells in allogeneic HSCT. Thus, we selected STR analysis in this study. The information helped to identify the markers that could distinguish between donor and recipient types. We found that, among these markers, the size of the longest fragment was 240 bp, which is informative in samples obtained from formalin-fixed DNA.

For accurate interpretation of the result of STR analysis, we needed to exclude the possibility of fusion of tumor cells with bone marrow–derived cells. If the recipient-type tumor cells are fused with bone marrow–derived cells, chromosomal analysis might mistakenly conclude a donor origin. In numerous animal models and

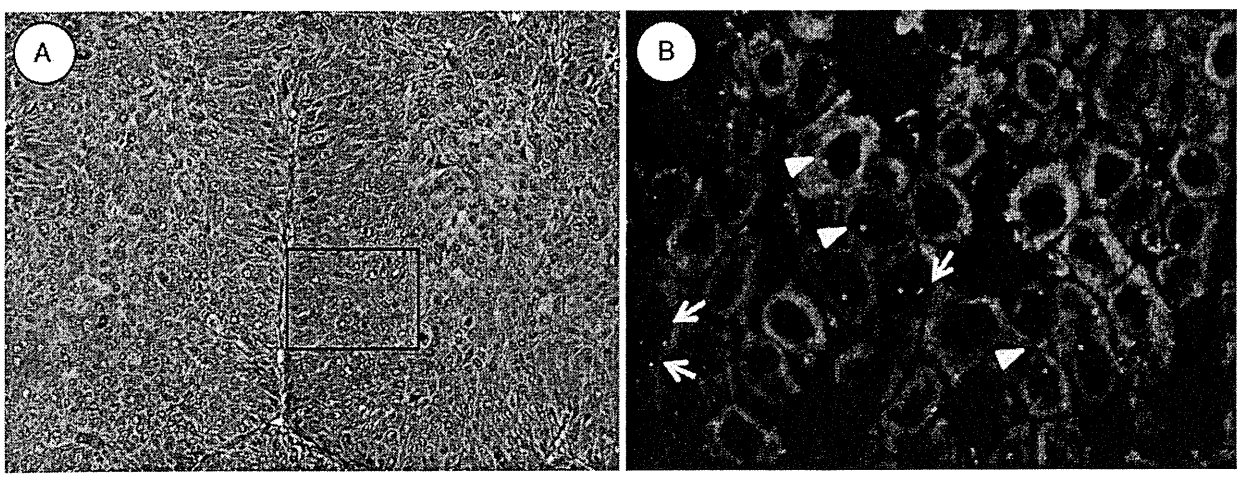

FIGURE 4. Case 4, pharyngeal squamous cell carcinoma after sex-mismatched transplantation (female to male). A, Hematoxylin and eosin-stained sections. A well-differentiated squamous cell carcinoma composed of atypical tumor cells with enlarged nuclei is shown in the tumor specimen obtained by surgical resection. The framed rectangle corresponds to the area shown in Fig. B. B, FICTION analysis. Approximately 100% of this specimen is occupied by tumor cells. All tumor cells are positive for AE1/3 (yellow). Both XX cells (shown by arrows) and XO cells (shown by arrowheads) are detected. A missing Y chromosome suggests that epithelial tumor cells originated from the donor.

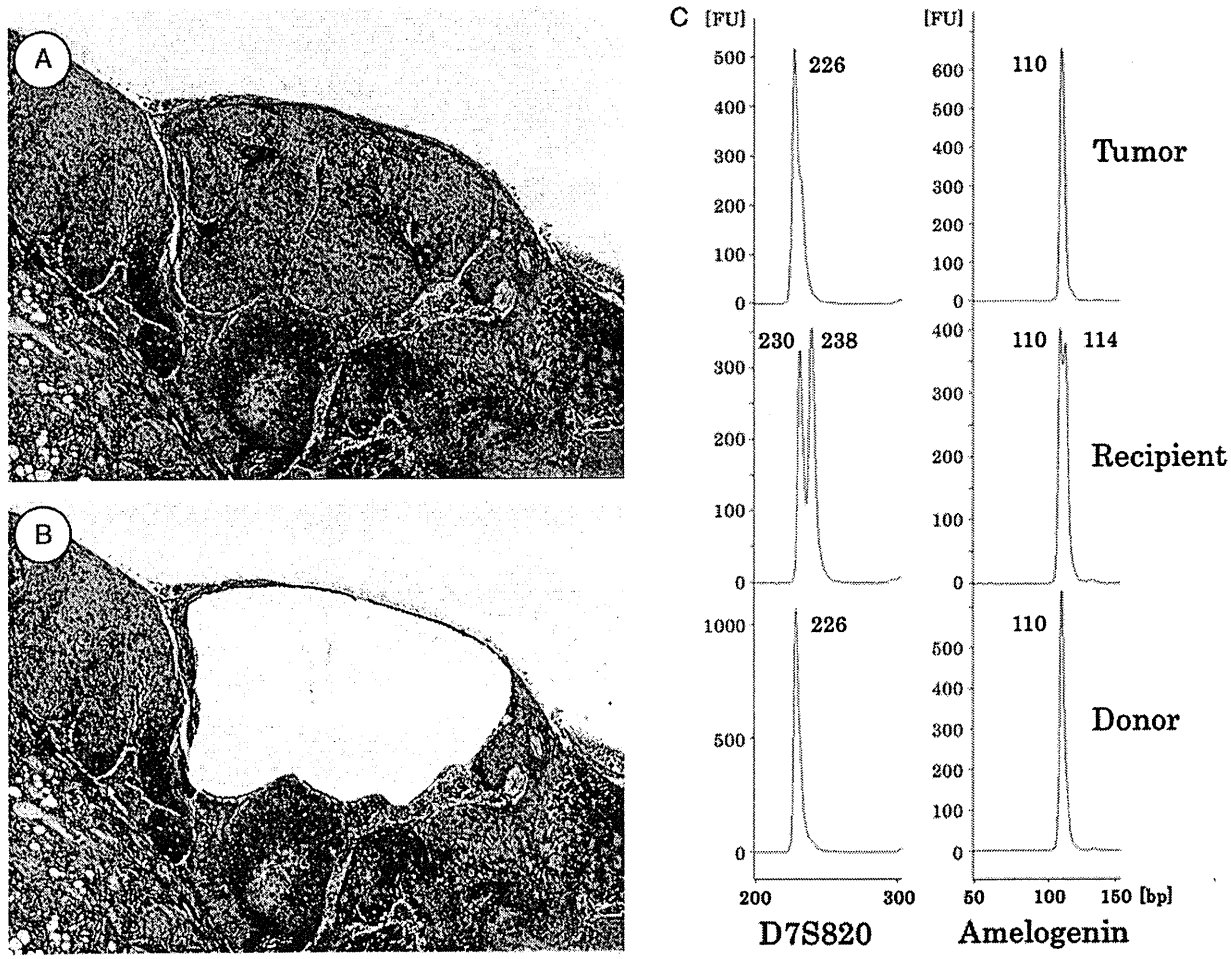

FIGURE 5. Case 4, STR analysis of laser-microdissected pharyngeal squamous cell carcinoma. A, A squamous cell carcinoma before microdissection. B, The same area after microdissection using a Leica AS LMD system (Hematoxylin and eosin staining on Membrane Slides without cover). C, Comparison of the STR profiles of the laser-microdissected tumor cells, BM before transplantation (recipient type), and BM after engraftment (donor type). The STR profile of the microdissected tumor cells reveal homozygosity at the D7S820 locus (226 base peak), heterozygosity in the recipient (230 and 238 base peaks), and homozygosity in the donor (226 base peak). STR analysis at the D7S820 locus shows that these tumor cells are of donor origin. BM indicates bone marrow.

human cancers, bone marrow–derived cell/tumor cell hybrids have been detected,[17] and in these cells the gene expression reflects a combination of 2 lineage genes. In our patient, STR analysis demonstrated loss of the recipient allele pattern, excluding the aforementioned possibility.

Because of the small number of tested samples in our study, we could not show clinical features and the incidence rate of donor-type cancers among all the secondary cancers developing after allogeneic HSCT. Five secondary cancers that developed after allogeneic HSCT were included in this study, and in only 1 case was the tumor of donor cell origin. In a previous study using STR analysis, among the 8 cases examined after allogeneic BMT, the tumors in 4 cases were identified as being recipient cell derived and in the remaining 4 cases as being donor cell derived.[8] STR analysis of stored FFPE samples would allow analysis of the tumor origin in virtually all secondary cancers developing after allogeneic HSCT, which would in turn show the true incidence of donor-type tumors and their clinical characteristics.

We have reported a case in which a tumor developing after allogeneic PBSCT and not BMT was identified as being of donor origin. The sequence of development of cancer in this unique case needs to be elucidated. We cannot exclude the possibility that the donor's peripheral blood contained occult cancer cells at the time of stem cell collection. However, the donor had not complained of any symptoms possibly associated with cancer when we evaluated donor eligibility, and we have received no feedback information on the occurrence of cancer in the donor from the donor registry data of the Japan Society for Hematopoietic Cell Transplantation until the preparation of this manuscript. In addition, as

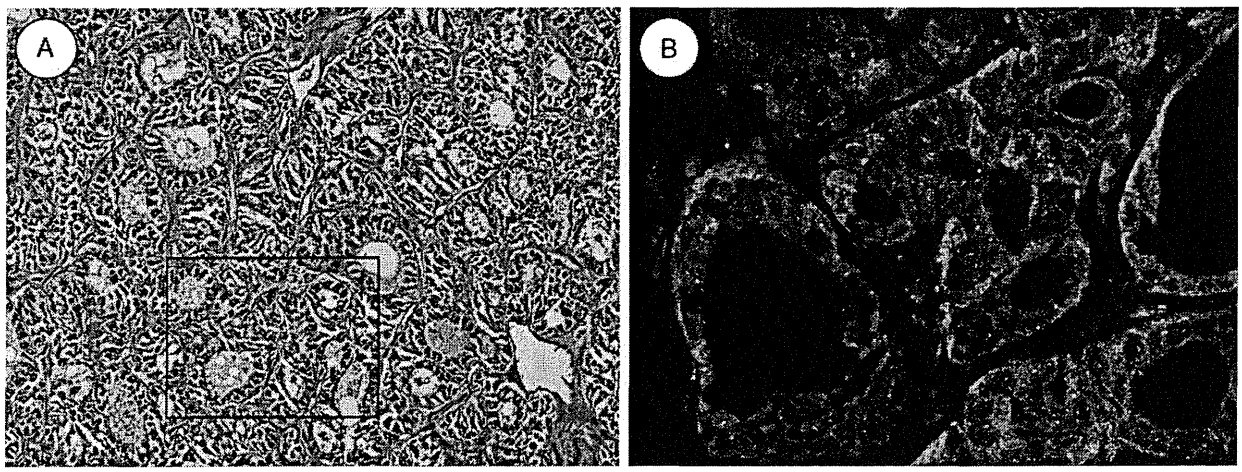

FIGURE 6. Case 5, thyroid papillary carcinoma after sex-matched transplantation (female to female). A, Hematoxylin and eosin-stained sections. Papillary carcinoma with various sizes of follicles is shown in the tumor specimen obtained by surgical resection. The framed rectangle corresponds to the area shown in Fig. B. B, FICTION analysis. Approximately 95% of this specimen is occupied by tumor cells. All the tumor cells are positive for AE1/3, and 2 X chromosomes (orange) can be detected. It is impossible to determine the origin because the donor and the recipient are sex matched.

5 years have already passed since the diagnosis of this secondary cancer, this possibility is unlikely.

Recently, it was shown that tumor cells circulating in the blood stream are associated with metastasis and a poorer prognosis.[18–20] In addition, a proportion of these circulating tumor cells show tumor stem cell characteristics.[21] Therefore, theoretically, a small number of precancerous cells may have contaminated the infused peripheral blood stem cells and contributed to the emergence of cancerous cells in the immunocompromised background of the recipient associated with radiation and immunosuppressant treatment. However, this possibility is again unlikely, because so far circulating tumor cells have been identified mostly in advanced or metastatic cancer patients.[18–24]

An alternative explanation is that the infused cells transformed into cancer cells. Bone marrow–derived cells have an important role in regeneration and repair of damaged epithelium.[3] Okamoto and colleagues reported that bone marrow–derived cells were involved in the repair of damaged epithelium in the gastrointestinal tract. Hutchinson[25] et al reported that myofibroblasts and endothelium also arose from bone marrow–derived cells in Barrett adenocarcinoma of the esophagus. These reports suggest that pluripotent cells in the bone marrow are recruited to the damaged sites and are transformed into epithelial or stromal cells. In this process, DNA damage or mutations might have occurred and promoted carcinogenesis. An emerging hypothesis is that chronic inflammation triggers carcinogenesis[26] and that the recruitment of bone marrow cells is caused mainly by chronic inflammation.[4] In patients who have undergone allogeneic HSCT, chronic GVHD (cGVHD) is associated with chronic inflammation and serves as a risk factor for the development of secondary cancers.[27,28] Oral mucosa, gastrointestinal tract, and skin are well-known target organs of cGVHD, which are also frequent sites of secondary cancers.[27] Janin and colleagues showed that 4 of 8 oral squamous cell carcinomas that developed after allogeneic BMT arose from engrafted bone marrow cells. They concluded that bone marrow–derived cells have a major role in the onset of cancer in the target organs of cGVHD.[8] In our study, the patient suffered from cGVHD in the oral cavity and skin, and the possibility that the patient had cGVHD in the pharynx is also likely.

No skin cancers were found in our archives. Skin is another lesion in which GVHD occurs. Incidence of skin cancers was recently reported by a Japanese study for HSCT.[29] The incidence of skin cancer among transplanted cases has increased compared with that of the general population. However, because of the low incidence of skin cancer in the general population in Japan, the incidence is still low, which might have contributed to the absence of skin cancer in our archives.

The impact of determining the origin of secondary cancers should be further investigated from the clinical point of view. Secondary cancers can be divided into 2 categories: one is cancer that originates from cGVHD and the other is that which originates from immunodeficiency. If the donor-type cancer arises from the cGVHD state, as shown in this study, more immunosuppression to prevent cGVHD is warranted, whereas if the secondary cancer is related to immunosuppression, as shown in posttransplant lymphoproliferative disorders, immune reconstruction should be considered as a therapy.

In conclusion, we identified a secondary cancer arising from engrafted donor peripheral blood cells. Our observations suggest that transfused peripheral blood cells and bone marrow cells are involved in the development of cancer after allogeneic PBSCT. As methods that have been developed in this study enable determination of the origin of secondary tumors that develop after

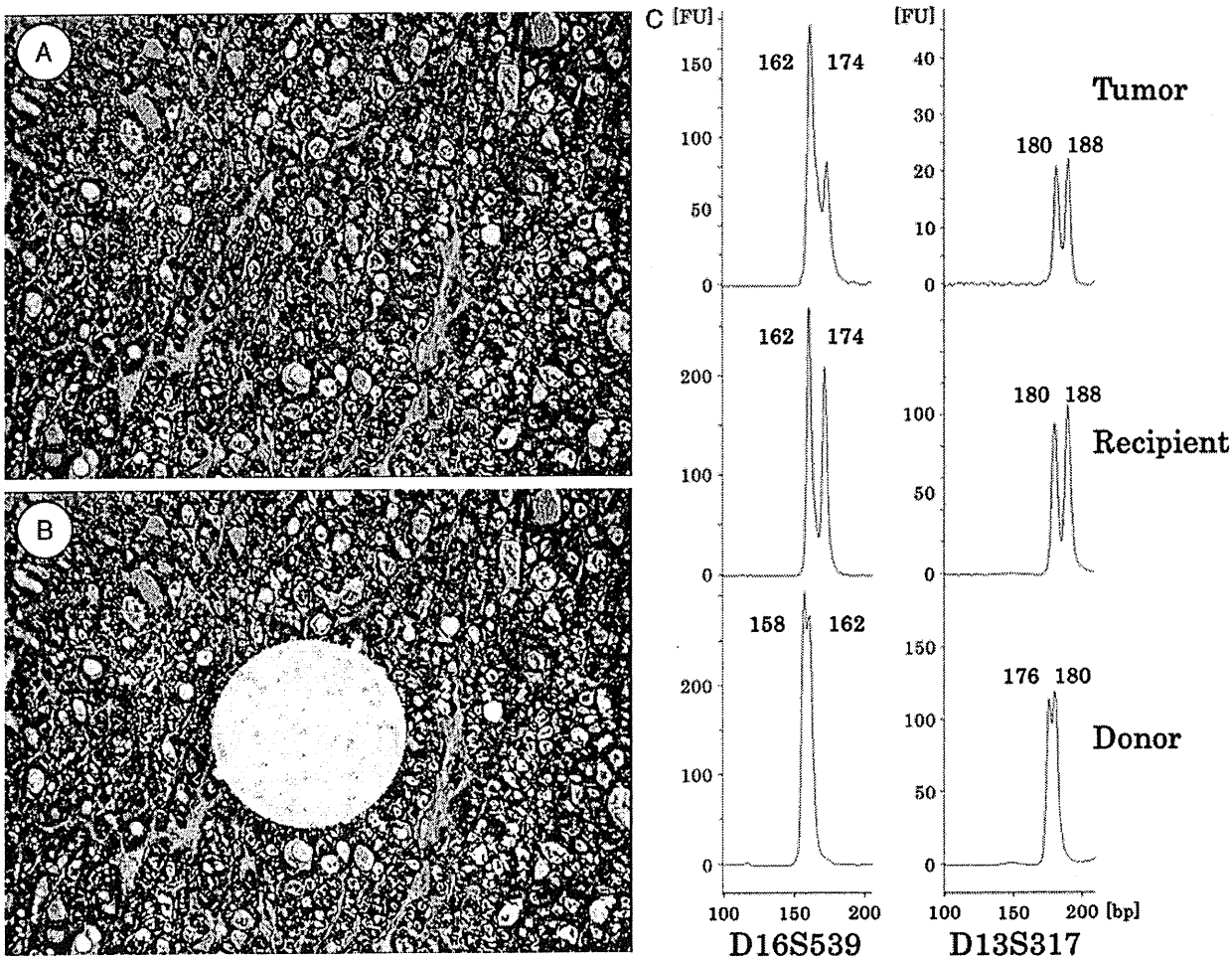

FIGURE 7. Case 5, STR analysis of a laser-microdissected thyroid papillary carcinoma. A, A thyroid papillary carcinoma before microdissection. B, The same area after microdissection using a Leica AS LMD system (hematoxylin and eosin staining on Membrane Slides without cover). C, Comparison of the STR profiles of the laser-microdissected tumor cells, BM before transplantation (recipient type), and BM after engraftment (donor type). STR analysis at the D7S820 and D13S317 loci shows that these tumor cells are of recipient origin BM indicates bone marrow.

allogeneic HSCT, further analysis is warranted in a larger number of cases with secondary cancer developing after HSCT. The analysis of tumor cell origin may contribute to formulating good prevention, screening, and treatment strategies.

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Comparison of outcomes after allogeneic hematopoietic stem cell transplantation in patients with follicular lymphoma, diffuse large B-cell lymphoma associated with follicular lymphoma, or *de novo* diffuse large B-cell lymphoma

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The outcome after allogeneic hematopoietic stem cell transplantation (allo-HCT) for diffuse large B-cell lymphoma (DLBCL) associated with follicular lymphoma (FL), which includes DLBCL with pre- or co-existing FL, remains controversial, and few previous reports have compared the outcomes after allo-HCT for FL, DLBCL associated with FL, and *de novo* DLBCL. We retrospectively analyzed 97 consecutive patients with FL (n = 46), DLBCL associated with FL (n = 22), or *de novo* DLBCL (n = 29) who received allo-HCT at our institute between 2000 and 2010. With a median follow-up of 53 months, the 5-year overall survival (OS) and progression-free survival (PFS) were, respectively, 77% and 70% for FL, 62% and 57% for DLBCL associated with FL, and 26% and 23% for *de novo* DLBCL. The 5-year cumulative incidences of non-relapse mortality and disease progression/relapse were, respectively, 16% and 15% for FL, 19% and 24% for DLBCL associated with FL, and 36% and 41% for *de novo* DLBCL. By a multivariate analysis, the OS and PFS for DLBCL associated with FL were significantly better than those for *de novo* DLBCL, whereas they were not significantly different from those for FL. These results suggest that allo-HCT may be a promising option for patients with not only advanced FL but also DLBCL associated with FL. Am. J. Hematol. 87:770–775, 2012. © 2012 Wiley Periodicals, Inc.

Introduction

Follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL) are the most common lymphoid tumors [1]. Although these two malignancies have quite different clinical presentations, FL sometimes transforms and acquires the morphological and clinical features of DLBCL. Some patients with a history of indolent lymphoma eventually progress to histological transformation over time, whereas histological transformation may be the first manifestation of lymphoma in patients without a history of indolent lymphoma. A proportion of the latter patients could be identified when FL and DLBCL components co-exist at the initial diagnosis [2,3]. According to the recently updated World Health Organization (WHO) classification, 4th edition [4], the presence of diffuse areas containing >15 centroblasts per high power field indicates a primary diagnosis of DLBCL, with the proportion of FL Grades 1, 2, 3a, or 3b reported separately.

In practice, DLBCL associated with FL, which means DLBCL with a pre- or co-existing FL component, is usually treated as aggressive B-cell non-Hodgkin lymphoma (B-NHL) in the same way as *de novo* DLBCL. However, little is known about the fate of DLBCL associated with FL when treated with allogeneic hematopoietic stem cell transplantation (allo-HCT) [5], because it is likely that most previous studies of allo-HCT for aggressive B-NHL included both DLBCL associated with FL and *de novo* DLBCL as a single group. The study by Ramadan et al. [6] was the only previous study which reported the outcomes of allo-HCT for DLBCL associated with FL. They discussed that both relapse and non-relapse mortality (NRM) remain significant problems in patients with DLBCL associated with FL after allo-HCT mostly using a myeloablative conditioning (MAC) regimen compared with FL. In this study, we compared the outcomes of allo-HCT among the three different histological subgroups: FL, DLBCL associated with FL, and *de novo* DLBCL.

Methods

Patients. We retrospectively analyzed 97 consecutive patients with FL (n = 46), DLBCL associated with FL (n = 22), or *de novo* DLBCL (n = 29) who received allo-HCT at our institute between January 2000 and December 2010. Data were collected from medical records and our original database. All available biopsy specimens (59/97, 61%) were reviewed by hematopathologists in our institute based on the WHO classification, whereas non-available biopsy specimens (38/97, 39%) were locally reviewed at the transferring institutes. Distribution of the cases without central review was not significantly different among the three groups (43% in FL, 23% in DLBCL associated with FL, and 45% in *de novo* DLBCL, P = 0.2). All of the patients gave their informed consent. The study protocol was reviewed and approved by the institutional ethics committee.

Definitions. DLBCL associated with FL consisted of two groups: DLBCL that was histologically proven in patients with pre-existing FL and DLBCL that was histologically proven in patients with co-existing FL in the same or a separate anatomical site at the initial diagnosis or relapse. The term of transformed lymphoma is used only for DLBCL

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that was histologically proven in patients with pre-existing FL. Clinical staging at allo-HCT was based on the Ann Arbor classification [7]. All patients were assigned a score using the International Prognostic Index (IPI) and the age-adjusted IPI (aaIPI) [8]. Disease response was assessed according to the international workshop to standardize response criteria for non-Hodgkin lymphoma [9]. Chemosensitive disease included all patients who had shown a response to the last chemotherapy prior to transplantation (partial response [PR], complete response [CR] unconfirmed, and CR), whereas chemoresistant disease included those with primary refractory disease or refractory relapse prior to transplantation. Acute and chronic graft-versus-host disease (GVHD) was graded according to the consensus criteria [10,11]. Overall survival (OS) was measured as the time from the day of transplantation until death from any cause, and progression-free survival (PFS) was the time from the day of transplantation until disease progression (PD)/relapse or death from any cause. Any death in the absence of documented disease progression was considered NRM.

Statistical analysis. OS and PFS were calculated using the Kaplan–Meier method. Surviving patients were censored on the day of the last follow-up. The cumulative incidences of NRM and PD/relapse were calculated by using Gray's method [12], considering each other risk as a competing risk. Risk factors for OS and PFS were evaluated using Cox proportional hazard models. Risk factors for NRM and PD/relapse were evaluated using Fine and Gray proportional hazard model, considering each other risk as a competing risk [13]. The statistical analysis was performed with the SPSS 11.0 statistical package (SPSS Inc), except for the competing analysis for NRM and PD/relapse, which was performed with R-Project (version 2.2.1; http://www.r-project.org/). *P*-values of less than 0.05 were considered statistically significant.

Results

Patient, disease, and transplantation characteristics

The patient, disease, and transplantation characteristics are listed in Table I. The median age of the 97 patients was 49 years (range, 24–67). Thirty-two patients (33%) had received high-dose chemotherapy with autologous HCT prior to allo-HCT. The disease status at allo-HCT was CR/PR in 45 (46%) patients and PD/refractory in 52 (53%) patients. Reduced-intensity conditioing (RIC) regimens for patients with an HLA-identical sibling donor consisted of fludarabine (Flu, 180 mg/m^2) or cladribine (CdA, 0.66 mg/kg) in combination with busulfan (Bu, p.o. 8 mg/kg or i.v. 6.4 mg/kg), regimens for those who had an alternative donor were the same dose of Flu and Bu in combination with total body irradiation (2 or 4 Gy) or antithymocyte globulin (ATG; ATG-Fresenius, 5–10 mg/kg or thymoglobulin, 2.5–5 mg/kg). For GVHD prophylaxis, cyclosporine was used in 54 (56%) and tacrolimus in 43 (44%) with (60%) or without (40%) methotrexate. No patients received mycophenolate mofetil.

When the characteristics among the three histological subgroups were compared, patients with FL and DLBCL associated with FL tended to be older, had a longer duration from the initial diagnosis to allo-HCT, and received more prior chemotherapy regimens than those with *de novo* DLBCL. The cohort of DLBCL associated with FL included a smaller proportion of prior autograft compared with *de novo* DLBCL ($P = 0.002$).

Clinicopathologic characteristics of DLBCL associated with FL

Clinicopathologic characteristics and outcomes of DLBCL associated with FL are shown in Table II. FL at the initial diagnosis transformed to DLBCL at relapse in nine patients (Cases 1–9) and FL had transformed to DLBCL with a FL component at relapse in one patient (Case 10). Eleven patients had DLBCL with co-existing FL at the initial diagnosis (Cases 11–21). In one patient (Case 22), pure DLBCL at the initial diagnosis led to DLBCL with co-existing FL at relapse. Five out of the eight patients with DLBCL associated with FL who did not have "last biopsies" prior to transplant had clinical signs which suggested aggressive nature of the disease, such as elevated lactate dehydrogenase levels, rapid growing of lymphoma lesion, or B symptoms [14]. There was no significant difference in OS or PFS after allo-HCT between DLBCL with pre-existing FL and DLBCL with co-existing FL.

TABLE I. Patient, Disease, and Transplantation Characteristics

	FL (n = 46)	DLBCL/FL (n = 22)	*De novo* DLBCL (n = 29)	Total (n = 97)
Age, years, median (range)	50 (31–67)	52 (30–62)	38 (24–62)	49 (24–67)
Duration from diagnosis to HCT, months, median (range)	51 (13–175)	46 (11–162)	23 (6–74)	41 (6–175)
Prior chemo regimens >4	24 (52%)	14 (64%)	9 (31 %)	47 (48%)
Prior rituximab	41 (89%)	22 (100%)	18 (62%)	81 (83%)
Prior purine analogue	20 (43%)	6 (27%)	0	26 (27%)
Prior autograft	10 (22%)	5 (23%)	17 (59%)	32 (33%)
HCT-CI <3	41 (89%)	19 (86%)	25 (86%)	85 (88%)
Disease status				
CR	11 (24%)	9 (41%)	10 (34%)	30 (31%)
PR	6 (13%)	4 (18%)	5 (17%)	15 (15%)
Resistant relapse	24 (52%)	8 (36%)	8 (28%)	40 (41%)
Primary refractory	5 (11%)	1 (5%)	6 (21%)	12 (12%)
Sensitivity to salvage therapy				
Sensitive	17 (37%)	13(59%)	15 (52%)	45 (46%)
Refractory	26 (57%)	9 (41%)	13 (45%)	48 (49%)
Untested	3 (6%)	0	1 (3%)	4 (4%)
Stage at HCT				
I	7 (15%)	3 (13%)	5 (17%)	15 (15%)
II	5 (11%)	1 (5%)	4 (14%)	10 (10%)
III	8 (17%)	1 (5%)	4 (14%)	13 (13%)
IV	15 (33%)	8 (36%)	6 (21%)	29 (30%)
No lesion (CR)	11 (24%)	9 (41%)	10 (34%)	30 (31%)
AaIPI HI/H	13 (28%)	5 (23%)	9 (31%)	27 (28%)
LN size >5 cm	8 (17%)	2 (9%)	6 (21%)	16 (16%)
Involved nodal sites				
1–4	19 (41%)	8 (36%)	14 (48%)	41 (42%)
5$^+$	14 (30%)	4 (18%)	3 (10%)	21 (22%)
Extranodal invasion	16 (35%)	8 (36%)	7 (24%)	31 (32%)
Stem cell source				
Bone marrow	21 (46%)	11 (50%)	7 (24%)	39 (40%)
Peripheral blood	23 (50%)	10 (45%)	19 (66%)	52 (53%)
Cord blood	2 (4%)	1 (5%)	3 (10%)	6 (6%)
Donor				
Related	23 (50%)	11 (50%)	21 (72%)	55 (57%)
Unrelated	23 (50%)	11 (50%)	8 (28%)	42 (43%)
Conditioning				
Myeloablative[a]	7 (15%)	5 (23%)	5 (17%)	17 (18%)
Reduced-intensity	39 (85%)	17(77%)	24 (83%)	80 (82%)
GVHD prophylaxis				
CSP-based	23 (50%)	10(45%)	21 (72%)	54 (56%)
TAC-based	23 (50%)	12(55%)	8 (28%)	43 (44%)

Abbreviations: FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; DLBCL / FL, DLBCL associated with FL; HCT, hematopoietic stem cell transplantation; CI, comorbidity index; CR, complete response; PR, partial response; aaIPI, age-adjusted international prognostic index; HI, high-intermediate risk; H, high risk; LN, lymph node; GVHD, graft-versus-host disease; CSP, cyclosporine; TAC, tacrolimus

[a] Myeloablative conditioning consisted of cyclophosphamide (120 mg/kg) with total body irradiation (12 Gy) or busulfan (p.o. 16 mg/kg or i.v. 12.8 mg/kg).

Engraftment

Neutrophil engraftment (absolute count > 500 cells/μL) was initially performed in all patients. The median day of neutrophil engraftment was Day 13 after HCT (range, 7–39). Two patients (2%) with FL experienced secondary graft failure; however both patients received second allo-HCT and achieved sustained engraftment.

GVHD

Grades II–IV acute GVHD occurred in 49 patients (51%): 22 (48%) in FL, 13 (59%) in DLBCL associated with FL, and 14 (48%) in *de novo* DLBCL. Grades III–IV acute GVHD occurred in 21 patients (22%): 6 (13%) in FL, 7

TABLE II. Clinicopathologic Characteristics and Clinical Outcomes in DLBCL Associated with FL

case	Age/ Gender	Initial diagnosis	Last diagnosis	aaIPI at HCT	OS (days)	Clincal outcome
1	45/M	FL	DLBCL	LI	31	TRM
2	53/F	FL	DLBCL	L	2570+	AWOD
3	55/F	FL,g1	DLBCL	L	1693+	AWOD
4	58/F	FL, g 3a	DLBCL	HI	407	TRM
5	47/M	FL	DLBCL	HI	1774	TRM
6	51/M	FL	DLBCL	HI	79	DOD
7	53/M	FL	DLBCL	LI	1196+	AWOD
8	52/M	FL, g 2	DLBCL	L	153	DOD
9	62/F	FL	DLBCL	LI	532+	AWOD
10	30/F	FL, g 3a	DLBCL (50%) + FL, g 1 (50%)	L	1183+	AWD
11	32/M	DLBCL (70%) + FL, g 3b (30%)	DLBCL + FL	L	3277+	AWOD
12	44/F	DLBCL + FL	DLBCL	L	140	TRM
13	47/M	DLBCL + FL		HI	64	DOD
14	46/M	DLBCL (75%) + FL, g 3b (25%)		LI	2498+	AWOD
15	55/M	DLBCL (25%) + FL, g 3a (75%)		L	2441+	AWOD
16	57/M	DLBCL (10%) + FL, g 3a (90%)		HI	2232+	AWOD
17	38/F	DLBCL + FL		L	3053+	AWOD
18	56/F	LN: DLBCL BM: DLBCL (10%) + FL, g 2 (90%)		L	1076+	AWOD
19	56/M	DLBCL (20%) + FL, g 3a (80%)		LI	545+	AWOD
20	52/M	DLBCL (20%) + FL, g 3a (80%)		LI	438+	AWOD
21	39/M	DLBCL (30%) + FL, g 3a (70%)	DLBCL	L	336+	AWOD
22	48/M	DLBCL	DLBCL (80%) + FL, g 3b (20%)	L	1444+	AWOD

Abbreviations: DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; aaIPI, age-adjusted international prognostic index; HCT, hematopoietic stem cell transplantation; OS, overall survival; M, male; F, female; (%), a proportion of area; g, grade; L, low risk; LI, low-intermediate risk; HI, high-intermediate risk; AWOD, alive without disease; AWD, alive with disease TRM, treatment-related mortality; DOD, dead of disease; LN, lymph node; BM, bone marrow.

(32%) in DLBCL associated with FL, and 8 (28%) in *de novo* DLBCL.

Chronic GVHD occurred in 57 (66%) of 86 patients who survived 100 days or longer, with extensive type in 42 (49%). Extensive chronic GVHD occurred in 17/45 (38%) in FL, 11/19 (58%) in DLBCL associated with FL, and14/22 (64%) in *de novo* DLBCL.

OS and PFS

With a median follow-up of 53 months in surviving patients, the 3-year and 5-year estimated OS were, respectively, 84% and 77% for FL, 72% and 62% for DLBCL associated with FL, and 44% and 26% for *de novo* DLBCL (Fig. 1A). The 3-year and 5-year estimated PFS were, respectively, 84% and 70% for FL, 73% and 57% for DLBCL associated with FL, and 30% and 23% for *de novo* DLBCL (Fig. 1B). The 10 clinical factors shown in Table III were assessed with regard to their relation to OS. To evaluate the effect of histology, we considered DLBCL associated with FL as a reference. A univariate analysis revealed that four factors, including histology (*de novo* DLBCL), no prior rituximab therapy, aaIPI at HCT (high or high-intermediate risk), and Grades III–IV acute GVHD were associated with a significantly worse OS. By a multivariate analysis, the OS for *de novo* DLBCL was significantly worse than that for DLBCL associated with FL [Hazard ratio (HR) 3.7 (1.1–11.8), $P = 0.03$]. OS for FL was not significantly different from that for DLBCL associated with FL [HR 0.6 (0.2–1.6), $P = 0.3$]. Another factor that influenced OS was aaIPI at HCT [high or high-intermediate risk, HR 3.6 (1.7–7.6), $P < 0.001$].

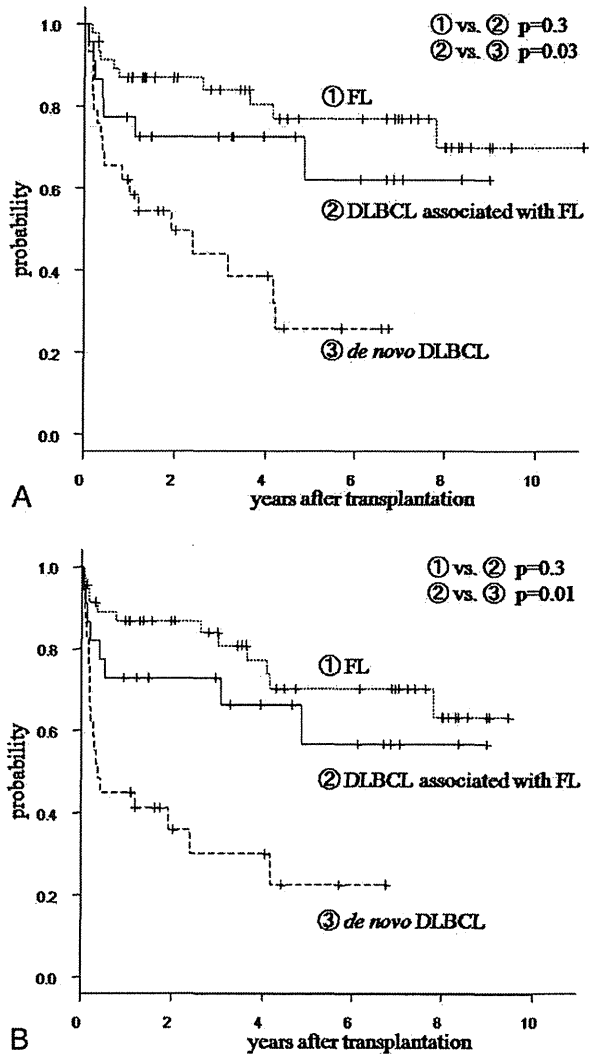

Figure 1. Kaplan–Meier curves of the (A) overall survival and (B) progression-free survival.

The same 10 clinical factors were assessed with regard to their relation to PFS. By a multivariate analysis, the PFS for *de novo* DLBCL was significantly worse than that for DLBCL associated with FL [HR 3.7 (1.3–10.7), $P = 0.02$]. PFS for FL was not significantly different from that for DLBCL associated with FL [HR 0.7 (0.3–1.8), $P = 0.4$]. AaIPI at HCT also influenced PFS [high or high-intermediate risk, HR 4.5 (2.1–9.5), $P < 0.001$].

NRM and PD/relapse

NRM occurred in 18 patients (19%): 7 in 46 FL, 3 in 22 DLBCL associated with FL, and 8 in 29 *de novo* DLBCL, and its cumulative incidence is shown in Fig. 2A. The 3- and 5-year cumulative incidences of NRM were, respectively, 10% and 16% in FL, 9% and 19% in DLBCL associated with FL, and 29% and 36% in *de novo* DLBCL. Only five patients (5%) died of NRM within day 100 after HCT, and 9 (9%) died of NRM within 1 year after HCT. The cause of NRM included GVHD in five, infection in five, multiple organ failure in two, cerebral infarction or hemorrhage in two, non-infectious lung complication in one, and unknown in three.

TABLE III. Univariate and Multivariate Analyses of Overall Survival

		Univariate analysis			Multivariate analysis		
	Variable	HR	95% CI	P	HR	95% CI	P
Histology	DLBCL/FL	1			1		
	FL	0.6	0.2–1.5	0.3	0.6	0.2–1.5	0.3
	De novo DLBCL	2.6	1.1–6.3	0.03	3.7	1.1–11.8	0.03
Age at HCT	≦ 50 y	1			1		
	> 50 y	1.2	0.6–2.3	0.6	1.6	0.8–3.5	0.2
Chemosensitivity	Sensitive	1			1		
	Refractory/ Untested	1.7	0.8–3.7	0.1	1.8	0.7–4.3	0.2
Prior rituximab	Yes	1			1		
	No	2.4	1.2–5.0	0.01	0.9	0.4–2.4	0.9
Prior autograft	Yes	1			1		
	No	0.6	0.3–1.2	0.2	1	0.5–2.3	0.9
AaIPI at HCT	L/LI	1			1		
	HI/H	3.9	2.0–7.5	<0.001	3.6	1.7–7.6	<0.001
Conditioning	RIC	1			1		
	MAC	1.9	0.9–3.9	0.1	2.1	0.9–5.1	0.09
Donor	Unrelated	1			1		
	Related	1.8	0.9–3.8	0.1	1.1	0.5–2.5	0.7
Acute GVHD	None/Grade I-II	1					
	Grade III-IV	2.5	1.2–5.0	0.01			
Chronic GVHD	None/Limited	1					
	Extensive	0.4	0.05–2.8	0.3			

Abbreviations: HR, hazard ratio; CI, confidence interval; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; DLBCL/FL, DLBCL associated with FL; HCT, hematopoietic stem cell transplantation; aaIPI, age-adjusted international prognostic index; L, low risk; LI, low-intermediate risk; HI, high-intermediate risk; H, high risk; RIC, reduced-intensity conditioning; MAC, myeloablative conditioning; GVHD, graft-versus-host disease.

The same 10 clinical factors were assessed with regard to their relation to NRM. By a multivariate analysis, MAC regimen was associated with a trend for a higher NRM [HR 2.1 (0.8–5.5), $P = 0.1$].

PD or relapse of lymphoma after allo-HCT was observed in 23 patients (24%): 6 in FL, 5 in DLBCL associated with FL, and 12 in *de novo* DLBCL. The 3- and 5-year cumulative incidences of PD/relapse were, respectively, 9% and 15% in FL, 18% and 24% in DLBCL associated with FL, and 41% and 41% in *de novo* DLBCL (Fig. 2B).

The same 10 clinical factors were assessed with regard to their relation to PD/relapse. By a multivariate analysis, aaIPI (high or high-intermediate risk) at allo-HCT was significant unfavorable factors [HR 4.6 (1.7–12.1), $P = 0.008$].

Discussion

This report describes the outcomes of patients with FL, DLBCL associated with FL, or *de novo* DLBCL who underwent allo-HCT mostly with a RIC regimen, with particular focus on the differences in outcomes among the three different histological subgroups. Our study revealed that DLBCL associated with FL showed better OS and PFS after allo-HCT than *de novo* DLBCL, and no significant difference was observed in OS or PFS between FL and DLBCL associated with FL. Since this was a retrospective study, three cohorts displayed some degree of heterogeneity in terms of clinical presentation at allo-HCT, i.e., the cohort with DLBCL associated with FL included a larger proportion of prior treatment with rituximab and a smaller proportion of prior autograft compared to the cohort with *de novo* DLBCL. Additionally, reflecting the clinical or biological differences among the three histological subgroups, patients with FL and DLBCL associated with FL tended to be older, had a longer duration from the initial diagnosis to allo-HCT, and had received more prior chemotherapy regimens. However, with regard to chemosensitivity and the disease status, which have been previously identified as important factors that influence the outcome after allo-HCT in most previously published studies for aggressive B-NHL [15–18],

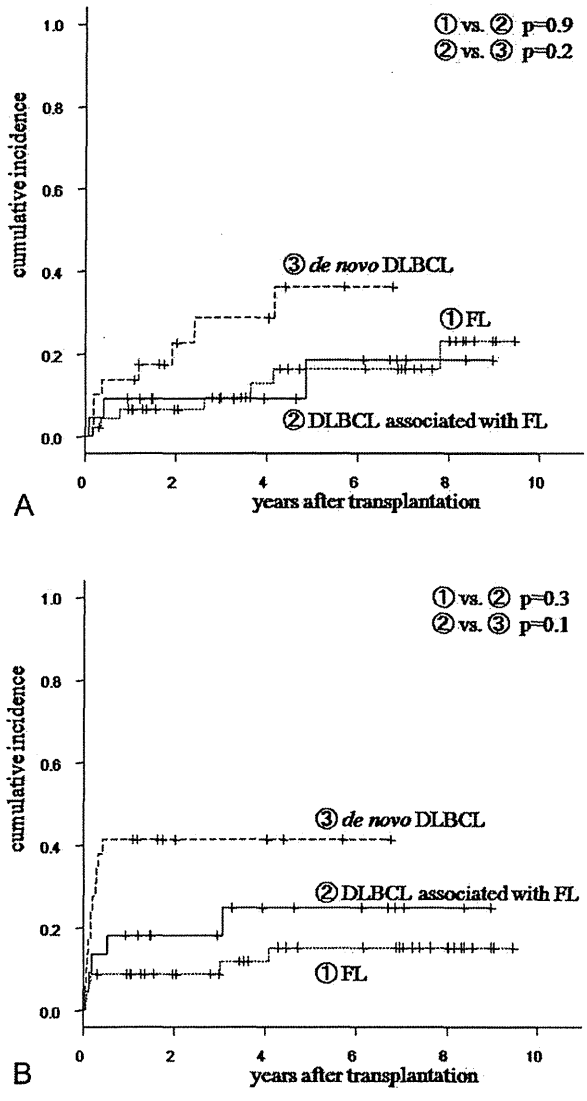

Figure 2. Cumulative incidences of the (A) non-relapse mortality and (B) disease progression/relapse.

there was no obvious difference between DLBCL associated with FL and *de novo* DLBCL. By a multivariate analysis including these pre-transplant clinical factors as covariates, DLBCL associated with FL was clearly associated with longer OS and PFS after allo-HCT compared with *de novo* DLBCL.

The outcomes of allo-HCT for DLBCL associated with FL or transformed lymphoma have been previously reported with conflicting results (Table IV). Rezvani et al. [15] and Ramadan et al. [21] reported that patients with transformed lymphoma had unfavorable outcomes compared with those with *de novo* DLBCL, whereas Thomson et al. [17] reported that there was a trend toward improved survival in transformed lymphoma compared with *de novo* DLBCL. Doocey et al. [20] and Sirvent et al. [16] reported that there were no significant differences between the outcomes of transformed lymphoma and *de novo* DLBCL. On the other hand, Rezvani et al. [19] and Ramadan et al. [6,22] reported that patients with transformed lymphoma had unfavorable outcomes compared with those with FL. Although differences in the definition of transformation, patient selection, and

TABLE IV. Outcomes After Allo-HCT for DLBCL Associated with FL or Transformed Lymphoma

Study	Regimen	Histology	*n*	Outcome
Our study	MAC, *n* = 5; RIC, *n* = 17	DLBCL/FL	22	62% OS (at 5 years)
Rezvani et al. [19]	RIC	Transformed lymphoma	16	18% OS (at 3 years)
Thomson et al. [17]	RIC	Transformed lymphoma	18	60% OS (at 4 years)
Doocey et al. [20]	MAC	Transformed lymphoma	16	38% EFS (at 5 years)
Sirvent et al. [16]	RIC	Transformed lymphoma	14	64% OS (at 2 years)
Ramadan et al. [6]	MAC, *n* = 39; RIC, *n* = 1	DLBCL/FL	40	23% OS (at 5 years)

Abbreviations: HCT, hematopoietic stem cell transplantation; DLBCL/FL, diffuse large B-cell lymphoma associated with follicular lymphoma; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; OS, overall survival; EFS, event-free survival

transplant procedures may make direct comparisons difficult, the outcomes in our study of DLBCL associated with FL seem to be favorable.

Our favorable outcomes for DLBCL associated with FL are due to both the low NRM and low PD/relapse rate. DLBCL associated with FL was heavily pretreated by conventional chemotherapy, but patients who had received prior high-dose chemotherapy with auto-graft were less common than those with *de novo* DLBCL. This may help to explain the low NRM observed in DLBCL associated with FL. One possible explanation for the lower PD/relapse rate in DLBCL associated with FL is that DLBCL associated with FL may be more strongly affected by the graft-versus-lymphoma (GVL) effect in the same way as FL than *de novo* DLBCL. In addition, the favorable outcomes for DLBCL associated with FL might be due to the heterogeneity of its aggressiveness. A proportion of DLBCL associated with FL might potentially relapse with only a low-grade lymphoma component. Since some patients were not biopsied prior to allo-HCT, it is possible that some patients but not all with undocumented pure FL relapse were categorized as DLBCL associated with FL and vice versa. Therefore, the liberal use of biopsy before allo-HCT might be useful for predicting the outcome following allo-HCT.

We confirmed long-term OS and PFS for patients with FL as previously reported [23–25], and further suggest that allo-HCT for FL can be a curative therapeutic option with a generated GVL effect. Additionally, our study showed that allo-HCT for FL, with mostly a RIC regimen, was effective even in cases that were refractory to prior chemotherapy, including with a purine analogue. This finding implies that our RIC regimens containing a purine analogue had an anti-lymphoma effect and secured a sufficient duration to express a GVL effect after allo-HCT.

We also confirmed that *de novo* DLBCL had a worse outcome after allo-HCT because of a higher NRM and PD/relapse rate. There are two possible explanations for the higher NRM in patients with *de novo* DLBCL. One is that 59% of the patients with *de novo* DLBCL had undergone prior high-dose chemotherapy with auto-graft. Another possibility is that an immunosuppressive agent might have been rapidly tapered to induce GVL effect in patients with aggressive lymphoma such as *de novo* DLBCL. A study by Gisselbercht et al. [26] revealed that patients who first relapsed with adverse prognostic factors had a worse prognosis even if they received high-dose chemotherapy with auto-graft. Hence, the timing of allo-HCT should be individually determined based on adverse prognostic factors. The PD/relapse rate in the early phase after allo-HCT was also higher in *de novo* DLBCL patients. To secure a sufficient duration to express a GVL effect, the conditioning regimen should be improved, i.e., rituximab or more potent anti-lymphoma agents instead of busulfan should be added to the conditioning regimen.

The inclusion of rituximab in pre-transplant salvage therapy has been associated with improved survival in patients undergoing autologous stem cell transplantation [27,28]. However, the impact of pre-transplant rituximab on survival in the allo-HCT setting is uncertain. While our data showed that no pre-transplant rituximab was associated with a significantly worse OS and PFS in a univariate analysis, this difference did not remain significant in a multivariate analysis. This finding suggests that histology might be more important than the history of pre-transplant rituximab.

Recent studies using gene expression analysis have demonstrated that DLBCL can be divided into two groups: germinal center B (GCB)-like DLBCL and activated B-like DLBCL [29]. Transformed FL has also been demonstrated to have a gene expression pattern most similar to GCB-like type [30], and DLBCL with co-existing FL has been shown to have a GCB-like phenotype in studies using immunohistochemical and cytogenetic analyses [2,31]. FL and DLBCL associated with FL may have a common molecular target, including a GCB-like phenotype for a GVL effect. To identify aggressive B-NHLs that may show favorable outcomes under treatment with allo-HCT, further molecular studies are warranted.

Major limitations of our study are small cohort sizes. The present results warrant confirmation through large prospective studies. In the absence of such prospective data, the indications for allo-HCT should be determined individually based on histology, disease status, comorbidities, donor availability, clinical course before allo-HCT, and patient preferences.

In conclusion, this is the first report to compare the outcomes after allo-HCT in patients with FL, DLBCL associated with FL, or *de novo* DLBCL. We identified DLBCL associated with FL as a favorable group among aggressive B-NHLs under treatment with allo-HCT. Patients with DLBCL associated with FL had more favorable outcomes after allo-HCT than those with *de novo* DLBCL, similar to FL. Allo-HCT, in the expectation of a GVL effect, may be a promising option for patients with not only relapsed or refractory FL but also DLBCL associated with FL.

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