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LETTER TO THE EDITOR

Allogeneic stem cell transplantation for hepatosplenic gammadelta T-cell lymphoma

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Hepatosplenic gammadelta T-cell lymphoma (HSTCL) was first described by Farcet et al. in 1990 [1]. Most cases of HSCTL occur in young men. Patients typically present with hepatosplenomegaly and bone marrow infiltration with resultant cytopenias but comparatively little lymphadenopathy. The prognosis of HSCTL is poor with reported median survivals of 8–16 months and few instances of long-term disease free survival with conventional chemotherapy [2,3]. Recently, a limited number of cases treated with allogeneic SCT for HSTCL have been reported [2–15]. We previously reported a patient of HSTCL successfully treated with allogeneic bone marrow transplantation (BMT) from an HLA-identical sibling [6]. In this report, we provide an update of this patient with 7-years follow-up and review the literature for allogeneic SCT for HSTCL.

A 23-year-old Japanese male was admitted to our hospital in June 1999 with abdominal distension, malaise, and night sweats. As previously described, the patient was diagnosed with hepatosplenic gamma/delta T-cell lymphoma and treated with intensive B-NHL86 protocol chemotherapy. After the two courses of chemotherapy, he achieved complete remission (CR). Thereafter, he received allogeneic BMT in August 1999 from an HLA-identically matched younger sister. The conditioning regimen consisted of four fractionated 12-Gy total body irradiation (TBI) on Day −9 and Day −8 and high-dose etoposide (60 mg/kg), which was administered as 24-h continuous intravenous infusion on Day −4. Graft-versus-host disease (GVHD) prophylaxis consisted of intravenous cyclosporine and short-term methotrexate. He had evidence of grade II acute GVHD of the skin and gut, which required no steroid treatment. Chimerism evaluation assessed by fluorescent in situ hybridization (FISH) using a mixture of X and Y chromosome-specific probes revealed complete donor chimerism of bone marrow cells on Day +28. Because of high risk of disease relapse after transplantation, cyclosporine was tapered rapidly and finished on Day +70. Thereafter, mild chronic GVHD of the liver and skin developed at 4 months after BMT, requiring treatment with oral cyclosporine for 8 months. At 5 years post-BMT, bone marrow examination revealed complete donor chimerism by sex mismatched FISH. Seven years after BMT, the patient is alive and free of disease.

Treatment modalities for HSTCL have considerable heterogeneity [3], including splenectomy, corticosteroids, purine analogue, anthracycline containing regimens such as CHOP (cyclophosphamide, hydroxydaunomycin, vincristine and prednisone) or CHOP-like regimen, second or third generation aggressive lymphoma regimen such as IEV (ifosphamide, epirubicin and etoposide) or modified MACOP-B (methotrexate, etoposide instead of adriamycin, cyclophosphamide, vincristine, predonisone and bleomycin), alemtuzumab and autologous and allogeneic stem cell transplantation. However, such treatments have limited efficacy and the vast majority of patients will die from progressive disease. Although allogeneic SCT, which is the only potentially curative therapy, has been attempted to treat

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Table I. Summary of published cases of allogeneic transplantation for hepatosplenic gammadelta T-cell lymphoma.

Author/published year	Age/sex	Prior treatment	Disease status at SCT	Conditioning regimen	Source of stem cells/donor type	Outcome
Cooke 1996 [2]	19/M	2-CdA, CHOP	PD	NA	BM/sibling	Alive in remission at 12 months after SCT
Jonveaux 1996 [5]	25/M	NA	NA	NA	BM/donor NA	Relapse at 1 month after SCT and death of disease
	21/M	NA	NA	NA	BM/donor NA	Alive in remission at 3 months after SCT
Weidmann 2000 [7]	41/F	Chemotherapy for previous AML, HD-Ara-C + VP-16, FLAG-Ida	PR	TBI12.5Gy + TT	PBSC/sibling	Relapse at 13 months after SCT and death of disease
Aldinucci 2000 [8]	34/F	CHOP like, α-IFN, HD-CY, Splenectomy, Pentostatin	PR	NA	BM/donor NA	TRD at 2 months after SCT
Przybylski 2000 [9]	46/M	CHOP	PD	NA	BM/donor NA	TRD at 3 weeks after SCT
Rossbach 2002 [10]	9/F	MSKNYII protocol	NA	NA	BM/donor NA	Alive in remission at published time
Belhadj 2003 [4]	19/M	CHOP like, Splenectomy	CR	NA	BM/donor NA	Relapse at 15 months after SCT and death of disease at 25 months after diagnosis
	21/M	CHOP like, Auto PBSCT	PD	NA	Allo SC/donor NA	Relapse at 4 months after SCT and death of disease at 19 months after diagnosis
	28/M	CHOP like, Splenectomy	CR	NA	BM/donor NA	TRD at 6 months after diagnosis
	44/M	CHOP like	PR	NA	BM/donor NA	TRD at 9 months after diagnosis
Gassas 2004 [11]	10/M	ALL protocol, ICE, mini BEAM	NA	TBI + CY	Allo SC/MUD	Alive in remission at 12 months after SCT. (He is alive in remission on November 15, 2006.)
Domm 2005 [12]	8/F	CHOP like, PSL	PD	TBI13.2Gy + CY	BM/sibling	Alive in remission at 30 months after SCT
Takaku 2005 [13]	35/M	PSL, CSP, Splenectomy, LSG15 regimen	PR	NA	Allo SC/MUD	Relapse at 11 months after SCT and alive at published time
Sakai 2006 [14]	25/F	CHOP, HD-CY, HD-MTX, AutoPBSCT	PR	TBI12Gy + TT + CY	UCB	Alive in remission at 58 months after diagnosis
Mittal 2006 [15]	18/M	AZT for previous crohn's disease, IVE, ESHAP, Splenectomy, Alemtuzumab, Flu, Pentostatin	PR	NA	Allo SC/MUD	TRD at 6 weeks after SCT
Our case 2006	23/M	B-NHL 86 protocol	CR	TBI12Gy + VP-16	BM/sibling	Alive in remission at 86 months after SCT

SCT, stem cell transplantation; 2CdA, cladribine; CHOP, cyclophosphamide, hydroxydaunomycin, vincristine and predonisone; NA, information not available; AML, acute myeloid leukemia; HD, high dose; Ara-C, cytosine arabinoside; VP-16, etoposide; FLAG-Ida, fludarabine, cytosine arabinoside, granulocyte colony-stimulating factor and idarubicin; IFN, interferon; CY, cyclophosphamide; Auto PBSCT, autologous peripheral blood stem cell transplantation; ALL, acute lymphoblastic leukemia; ICE, Ifosfamide, carboplatin and etoposide; BEAM, carmustine, etoposide, cytosine arabinoside and melphalan; MTX, methotrexate; PSL, predonisone; Flu, fludarabine; ESHAP, etoposide, methylpredonisolone, cisplatin and cytosine arabinoside; CSP, cyclosporine; AZT, azathioprine; IVE, Ifosfamide, epirubicine, etoposide; PD, progressive disease; PR, partial remission; CR, complete remission; TBI, total body irradiation; TT, thiotepa; BM, bone marrow; PBSC, peripheral blood stem cell; Allo SC, unspecified allogeneic stem cell; MUD, matched unrelated donor; DLI, donor lymphocyte infusion; UCB, unrelated cord blood; TRD, treatment related death.

HSTCL, the exact role of allogeneic SCT in the treatment of HSTCL remains unclear. The published cases of allogeneic SCT for HSTCL are presented in Table I. Most of these reports are single case reports or single cases within series of cases. Literature review reveals 17 cases treated with allogeneic SCT (Table I). Of the 17 cases reported, 12 of the patients were male and 5 female. The median age was 23 years (range, 8–46 years). Ten of 13 patients evaluable for disease status at transplantation had refractory disease, and two had experienced disease relapse after a previous autologous transplantation. Conditioning regimen was based on TBI in all of five evaluable cases. Thirteen patients were evaluable for source of transplanted stem cell and different kinds of stem cell sources were used, including bone marrow ($n=11$), peripheral blood stem cell ($n=1$), and cord blood ($n=1$). On the basis of the reported follow-up, 7 of 17 patients were alive in remission. These cases indicate prolonged remission duration in some patients with HSCTL. Compared with the prognosis of HSTCL with conventional chemotherapy, the outcome with allogeneic SCT might be significantly better, with approximately 40% chance of being curable. For patients who lack an HLA-identical sibling donor, the role of SCT from unrelated-donor or cord blood transplantation should be explored. In conclusion, these results suggest that allogeneic SCT is potentially curative in patients with HSCTL. Therefore, allogeneic SCT for HSTCL patients needs to be considered early in the disease course.

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References

1. Farcet JP, Gaulard P, Marolleau JP, Le Couedic JP, Henni T, Gourdin MF, et al. Hepatosplenic T-cell lymphoma: sinusal/sinusoidal localization of malignant cells expressing the T-cell receptor gamma delta. Blood 1990;75:2213–2219.
2. Cooke CB, Krenacs L, Stetler-Stevenson M, Greiner TC, Raffeld M, Kingma DW, et al. Hepatosplenic T-cell lymphoma: A distinct clinicopathologic entity of cytotoxic gammadelta T-cell origin. Blood 1996;88:4265–4274.
3. Weidmann E. Hepatosplenic T cell lymphoma. A review on 45 cases since the first report describing the disease as a distinct lymphoma entity in 1990. Leukemia 2000;14:991–997.
4. Belhadj K, Reyes F, Farcet JP, Tilly H, Bastard C, Angonin R, et al. Hepatosplenic gammadelta T-cell lymphoma is a rare clinicopathologic entity with poor outcome: report on a series of 21 patients. Blood 2003;102:4261–4269.
5. Jonveaux P, Daniel MT, Martel V, Maarek O, Berger R. Isochromosome 7q and trisomy 8 are consistent primary, non-random chromosomal abnormalities associated with hepatosplenic T gamma/delta lymphoma. Leukemia 1996; 10:1453–1455.
6. Ooi J, Iseki T, Adachi D, Yamashita T, Tomonari A, Tojo A, et al. Successful allogeneic bone marrow transplantation for hepatosplenic gammadelta T cell lymphoma. Haematologica 2001;86:E25.
7. Weidmann E, Hinz T, Klein S, Schui DK, Harder S, Kriener S, et al. Cytotoxic hepatosplenic gammadelta T-cell lymphoma following acute myeloid leukemia bearing two distinct gamma chains of the T-cell receptor. Biologic and clinical features. Haematologica 2000;85:1024–1031.
8. Aldinucci D, Poletto D, Zagonel V, Rupolo M, Degan M, Nanni P, et al. In vitro and in vivo effects of 2′-deoxycoformycin (Pentostatin) on tumour cells from human gammadelta+ T-cell malignancies. Br J Haematol 2000;110:188–196.
9. Przybylski GK, Wu H, Macon WR, Finan J, Leonard DG, Felgar RE, et al. Hepatosplenic and subcutaneous panniculitis-like gamma/delta T cell lymphomas are derived from different V delta subsets of gamma/delta T lymphocytes. J Mol Diag 2000;2:11–19.
10. Rossbach HC, Chamizo W, Dumont DP, Barbosa JL, Sutcliffe MJ. Hepatosplenic gamma/delta T-cell lymphoma with isochromosome 7q, translocation t(7;21), and tetrasomy 8 in a 9-year-old girl. J Pediatr Hematol Oncol 2002;24:154–157.
11. Gassas A, Kirby M, Weitzman S, Ngan B, Abla O, Doyle JJ. Hepatosplenic gammadelta T-cell lymphoma in a 10-year-old boy successfully treated with hematopoietic stem cell transplantation. Am J Hematol 2004;75:113–114.
12. Domm JA, Thompson M, Kuttesch JF, Acra S, Frangoul H. Allogeneic bone marrow transplantation for chemotherapy-refractory hepatosplenic gammadelta T-cell lymphoma. J Pediatr Hematol Oncol 2005;27:607–610.
13. Takaku T, Miyazawa K, Sashida G, Shoji N, Shimamoto T, Yamaguchi N, et al. Hepatosplenic gammadelta T-cell lymphoma with myelodysplastic syndrome. Int J Hematol 2005;82:143–147.
14. Sakai R, Fujisawa S, Fujimaki K, Kanamori H, Ishigatsubo Y, et al. Long-term remission in a patient with hepatosplenic gammadelta T cell lymphoma after cord blood stem cell transplantation following autologous peripheral blood stem cell transplantation. Bone Marrow transplant 2006;37:537–538.
15. Mittal S, Milner BJ, Johnston PW, Culligan DJ, et al. A case of hepatosplenic gamma-delta T-cell lymphoma with a transient response to fludarabine and alemtuzumab. Eur J Haematol 2006;76:531–534.

Comparative single-institute analysis of cord blood transplantation from unrelated donors with bone marrow or peripheral blood stem-cell transplants from related donors in adult patients with hematologic malignancies after myeloablative conditioning regimen

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We studied the clinical outcomes of 171 adults with hematologic malignancies who received unrelated cord blood transplantation (CBT) as a primary unrelated stem-cell source (n = 100), or bone marrow transplant (BMT) or peripheral blood stem-cell transplant (PBSCT) from related donors (n = 71, 55 BMT and 16 PBSCT). All patients received myeloablative regimens including 12 Gy total body irradiation. We analyzed the hematologic recovery, and risks of graft-versus-host disease (GVHD), transplantation-related mortality (TRM) and relapse, and disease-free survival (DFS) using Cox proportional hazards models. Significant delays in engraftment occurred after cord blood transplantation; however, overall engraftment rates were almost the same for both grafts. The cumulative incidences of grades III to IV acute and extensive-type chronic GVHDs among CBT recipients were significantly lower than those among BMT/PBSCT recipients. Multivariate analysis demonstrated no apparent differences in TRM (9% in CBT and 13% in BMT/PBSCT recipients), relapse (17% in CBT and 26% in BMT/PBSCT recipients), and DFS (70% in CBT and 60% in BMT/PBSCT recipients) between both groups. These data suggest that unrelated cord blood could be as safe and effective a stem-cell source as related bone marrow or mobilized peripheral blood for adult patients when it is used as a primary unrelated stem-cell source. (Blood. 2007; 109:1322-1330)

Introduction

Recently, cord blood has been increasingly used in adults as a stem-cell source for allogeneic transplantation to treat hematologic malignancies.[1-5] We previously reported on a comparative analysis of cord blood transplant (CBT) versus bone marrow transplant (BMT) from unrelated donors in our institute.[6] The overall results for CBT recipients were better than for BMT recipients in terms of graft-versus-host disease (GVHD), transplant-related mortality (TRM), and disease-free survival (DFS). In our previous assessments, the availability of grafts containing sufficient cell numbers, the shorter time from donor search to transplantation, the low requirements of steroid therapy for GVHD, the conditioning regimen, the GVHD prophylaxis used in our institution, and Japanese genetic issues regarding low alloreactivity[7-9] might have contributed to our favorable results of cord blood transplantation in adults.

Two other registration-based studies comparing both CBT and BMT from unrelated donors in adult patients with acute leukemia have recently been published; both studies showed almost the same results between cord blood transplantation and bone marrow transplantation.[10,11] However, some results in those reports were conflicting, especially for TRM. The US study demonstrated a poor outcome for TRM in CBT recipients compared with HLA (human leukocyte antigen)–matched BMT recipients.[10] The European study[11] showed similar TRM in both groups.

We speculated that the key difficulty in interpreting retrospective comparative studies, including ours, may be related to patient selection. Most recipients of CBT did not have an HLA-matched unrelated donor, and their disease tended to progress to advanced or high-risk stage while searching, unsuccessfully, for marrow donors.[12] However, when a patient was eligible for allogeneic transplantation but did not have a related donor, we performed cord blood transplantation at the same timing as for patients who had a related donor.

In the present study, we compared our results of CBT from unrelated donors with those of BMT or peripheral blood stem-cell transplant (PBSCT) from related donors in our hospital; all patients received essentially the same supportive care. The main purpose of this analysis was to assess the safety and efficacy of unrelated CBT compared with BMT or PBSCT from related donors in adult patients in the setting of a comparable situation regarding patient selection.

Patients, materials, and methods

Patients and controls

The study included data from 171 consecutively treated patients, 16 years of age or older, who received BMT or PBSCT from related donors (n = 71, 55 BMT and 21 PBSCT recipients) or unrelated CBT (n = 100) for acute

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myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML), myelodysplastic syndrome (MDS), or malignant lymphoma (ML) between January 1997 and August 2005 at the Institute of Medical Science, University of Tokyo. T-cell depletion was not performed in either group. Patients qualified as being standard risk if they were in first or second complete remission (CR), had chronic-phase CML or refractory anemia MDS, or had no high-risk cytogenetics (eg, ALL with t(4;11) or t(9;22), or AML with complex karyotype, −5, del(5q), −7, or abnormalities of 3q). Patients in third CR, in relapse, with CML beyond chronic phase, or with high-risk cytogenetics were classified as being high risk. Patients receiving BMT, PBSCT, or CBT as a second transplant following relapse after a first allogeneic transplantation were excluded. Median follow-up was 32 months (range, 1-110 months; 39 survivors and 32 censored) for BMT and 22 months (range, 0-91 months; 72 survivors and 28 censored) for CBT recipients ($P = .77$). The clinical protocol was approved by the institutional review board of the Institute of Medical Science, University of Tokyo, and written informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

HLA typing and donor selection

HLA-A and HLA-B antigens were identified by serologic typing. HLA-DRB1 alleles were determined by high-resolution molecular typing using polymerase chain reaction sequence-specific primers (PCR-SSPs). Patients without a suitable closely HLA-matched related donor, namely, with 5 of 6 or 6 of 6 matching HLA loci, were eligible for cord blood transplantation as a first treatment option, because most patients eligible for allogeneic stem-cell transplantation were thought to have insufficient time for an unrelated bone marrow donor search and early timing of transplantation was preferable. On the other hand, if those patients had any type of anti-HLA antibody, we generally attempted to locate bone marrow grafts from unrelated donors because most cord blood grafts were HLA mismatched and the predictable risk of poor engraftment results after cord blood transplantation. All cord blood grafts were evaluated by HLA-A and HLA-B typing serologically, by HLA-DRB1 typing at high resolution, and by nucleated cell counts. Preferred cord blood units matched 4 of 6 to 6 of 6 HLA loci and contained a minimal cell count of 1.5×10^7 nucleated cells/kg body weight before freezing. T-lymphocyte depletion was not performed on cord blood or bone marrow grafts.

Conditioning regimen, GVHD prophylaxis, and supportive care

All patients received a total body irradiation (TBI)–containing myeloablative pretransplantation conditioning regimen of 12 Gy, fractionated in 4 or 6 doses. The TBI + cytosine arabinoside (Ara-C: total dose 24 g/m^2) combined with G-CSF (lenograstim) regimen[13-15] was chosen for patients with myeloid leukemias who had an HLA-matched related donor (n = 31). Additionally, cyclophosphamide (CY) was administered to patients who received transplants from HLA-mismatched cord blood donors or HLA-mismatched related donors while reducing the Ara-C dose to a total dose of 12 g/m^2 (n = 82) as reported previously.[6] CY was avoided in favor of 120 mg/m^2 fludarabine (n = 6) in the case of recipients who had risk of organ dysfunction, especially in the heart. Thirty-one patients received TBI + CY (n = 23) or TBI + CY + one cytotoxic drug (12 g/m^2 Ara-C in 6 patients, 60 mg/kg etoposide in 1 patient, or 300 mg/m^2 thiotepa in 1 patient). Fifteen patients received TBI + 60 mg/kg etoposide. Six additional patients received TBI + 90 mg/m^2 fludarabine + one drug (12 g/m^2 Ara-C in 3 patients or 140 mg/m^2 melphalan in 3 patients) (Table 1).

One hundred and sixty-three (95%) of all 171 patients received a standard cyclosporine (CsA) and methotrexate (MTX) combination as GVHD prophylaxis. CsA was administered daily from day −1 at 3 mg/kg per day intravenously and MTX at 15 mg/m^2 intravenously on day 1, followed by 10 mg/m^2 on days 3 and 6. MTX on day 11 was given only to patients receiving HLA-mismatched bone marrow or peripheral blood from related donors. Six patients received only CsA, 1 received tacrolimus (FK-506) combined with a short course of MTX, and 1 received CsA plus mycophenolate mofetil (MMF). Once oral intake could be tolerated, patients were administered oral CsA at a dose ratio of 1:2.5, in 2 divided doses/d based on the last intravenous dose. In the absence of GVHD, CsA was tapered beginning between weeks 6 and 9 until it could be discontinued, depending on the degree of GVDH severity. Corticosteroid-based treatment was considered when grade II or higher severe acute GVHD occurred (1 to 2 mg/kg).

The supportive-care regimen, including prophylaxis, for infection was the same as previously reported.[6] All patients after cord blood transplantation and 60 of 71 after bone marrow transplantation/peripheral blood stem-cell transplantation received G-CSF (lenograstim, 5 μg/kg per day, intravenous infusion) starting on day 1 until durable granulocyte recovery was achieved. The same supportive care, except for the G-CSF administration, was given to both groups.

End points, definitions, and assessments of hematopoietic recovery, GVHD, TRM, disease relapse, and DFS

We focused on hematologic recovery, acute and chronic GVHD, TRM, disease relapse, and DFS after unrelated cord blood transplantation compared with related bone marrow transplantation/peripheral blood stem-cell transplantation. The primary measure of hematopoietic recovery was the time required for myeloid and platelet recovery. The myeloid-cell recovery time was defined as the first of 3 consecutive days during which the absolute neutrophil count in the blood was at least 0.5×10^9/L.[3] Platelet recovery time was achieved on the first of 3 days when the platelet count was higher than 2×10^9/L (or 5×10^9/L) without transfusion support. Primary engraftment failure was defined as the absence of donor-derived myeloid cells on the day of death, the day of relapse, or day 60 in patients surviving beyond day 28 after transplantation. Patients were also defined as having had primary engraftment failure when either a second allogeneic transplantation before donor-derived myeloid recovery or reconstitution with autologous cells was required. Chimerism was evaluated by fluorescence in situ hybridization for the Y chromosome or quantitative PCR analysis for microsatellite DNA markers. Acute GVHD was graded 0 to IV according to the criteria of Glucksberg et al,[16] and chronic GVHD was defined as none, limited, or extensive.[17] The incidence of and time to acute GVHD development were evaluated in patients surviving 21 days or longer with evidence of engraftment. Time to occurrence of any chronic GVHD disease was evaluated in patients surviving 100 days or longer after transplantation with allogeneic engraftment. TRM was defined as death from any cause except relapse. Relapse was defined by morphologic evidence of disease in peripheral blood, marrow, or extramedullary sites, or the recurrence and sustained presence of pretransplantation chromosomal abnormalities on cytogenetic analysis of bone marrow cells. Patients showing minimal residual disease (eg, the presence of bcr/abl RNA transcripts by PCR) were not classified as having relapsed. DFS was defined as survival in continuous CR.

Statistical analysis

The probability of DFS was estimated from the time of transplantation according to the Kaplan-Meier product limit method. Cumulative incidences were estimated for hematopoietic recovery, GVHD, TRM, and relapse in order to take competing risks into account. Associations between graft type and outcome were evaluated using Cox proportional hazard regression models. In addition to the hematopoietic stem-cell source, the following variables were considered as covariates: recipient age at transplantation; weight; status regarding cytomegalovirus (CMV, determined by serologic testing); recipient and donor sex; degree of ABO matching; degree of HLA matching; type (ALL, AML, CML, MDS, or malignant lymphoma) and pretransplantation duration of the underlying disease; disease status at transplantation (standard or high risk); conditioning regimen; GVHD prophylaxis used; use or nonuse of G-CSF during the first 7 days after transplantation; and time of transplantation (between 1997 and 2000, or 2001 and 2005). We used backward and stepwise procedures at a significance level of 5% to construct prognostic models, in which we tried to maintain the graft source (cord blood from an unrelated donor or bone marrow/peripheral blood from a related donor) as a variable until the final step of the procedures. The proportional hazard assumption of the Cox model was assessed essentially by a graphic approach. When groups were compared according to continuous covariates, we calculated the mean or

Table 1. Characteristics of patients and grafts

Characteristic	BMT or PBSCT recipient	CBT recipient	P
Recipients, n	71	100	—
Age, y			.83
Median	40	38	
Range	16-58	16-55	
Weight, kg			.01
Median	59	55	
Range	35-85	36-76	
Sex of donor and recipient, no. (%)			.69
Male/male	26 (37)	29 (29)	
Female/female	15 (21)	20 (20)	
Female/male	16 (23)	27 (27)	
Male/female	14 (20)	24 (24)	
CMV serologic status, no. (%)			.37
Negative	6 (9)	13 (13)	
Positive	64 (91)	87 (87)	
Diagnosis, no. (%)			.10
AML			
CR1, CR2	8 (11)	26 (26)	
Advanced	23 (32)	31 (31)	
ALL			
CR1, CR2	7 (10)	9 (9)	
Advanced	10 (14)	11 (11)	
CML			
CP	7 (10)	1 (1)†	
Advanced	3 (6)	5 (5)	
MDS			
RA	4 (6)	3 (3)	
Advanced	1 (1)	8 (8)	
ML			
CR1, CR2	1 (1)	5 (5)†	
Advanced	6 (9)	2 (2)	
Duration from diagnosis to transplantation, mo			.30
Median	15	17.5	
Range	2-177	2-223	
Conditioning, no. (%)			<.01
TBI + Ara-C/G-CSF	31 (44)	0	
TBI + Ara-C/G-CSF + CY	14 (20)	68 (68)	
TBI + CY	10 (14)	13 (13)	
TBI + other combination‡	16 (23)	19 (19)	
GVHD prophylaxis, no. (%)			.39
CsA	2 (3)	4 (4)	
CsA + sMTX	67 (94)	96 (96)	
FK506 + sMTX	1 (1)	0	
CsA + MMF	1 (1)	0	
G-CSF administration during first 7 d, no. (%)			<.01
Yes	60 (85)	100 (100)	
No	11 (15)	0	
Year of transplantation, no. (%)			<.01
1997 to 2000	50 (70)	21 (21)	
2001 to 2005	21 (30)	79 (79)	
No. of leukocytes for transplantation, $\times 10^7$/kg			<.01
Median	33.0	2.43	
Range	6.6-50	1.1-5.29	
No. of HLA-A, B, and DRB1 mismatches, no. (%)			<.01
0	54 (76)	0 (0)	
1	11 (15)	16 (16)§	
2	6 (8)	54 (54)§	
3	0	28 (28)§	
4	0	2 (2)§	
Extent of ABO match, no. (%)			<.01
Match	39 (55)	29 (29)	
Minor mismatch	15 (21)	31 (31)	

Table 1. Characteristics of patients and grafts (continued)

Characteristic	BMT or PBSCT recipient	CBT recipient	P*
Major mismatch	17 (24)	40 (40)	

Italicized *P* values are ___.

CMV indicates cytomegalovirus; AML, acute myelogenous leukemia; CR1 and CR2, 1st and 2nd complete remission, respectively: advanced, patients in third complete remission, in relapse, in CML beyond chronic phase, or who had high-risk cytogenetics were classified as high risk; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; CP, chronic phase; MDS, myelodysplastic syndrome; RA, refractory anemia; ML, malignant lymphoma; TBI, total body irradiation: Ara-C, cytosine arabinoside; G-CSF, granulocyte colony-stimulating factor; CY, cyclophosphamide; CsA, cyclosporine; sMTX, short-term methotrexate; FK506, tacrolimus; —, not applicable; and MMF, mycophenolate mofetil.

*The Chi-square test was used for categoric variables; the Mann-Whitney test was used for continuous variables. †One patient with ALL in chronic phase and non-Hodgkin lymphoma in first complete remission received cord blood for the treatment of both diseases and was categorized as standard risk.

‡All conditioning regimens included 12 Gy TBI. In BMT/PBSCT recipient group, 15 patients received TBI + etoposide (60 mg/kg). One patient received TBI + G-CSF–combined Ara-C + fludarabine (90 mg/m^2). In CBT recipient group, 5 patients received TBI + G-CSF–combined Ara-C + fludarabine (90 mg/m^2), 3 patients received TBI + Ara-C + fludarabine (90 mg/m^2), 6 patients received TBI + Ara-C + CY, 2 patients received TBI + one drug (etoposide or thiotepa) + CY, and 3 patients received TBI + fludarabine (90 mg/m^2) + melphalan (140 mg/m^2).

§In 16 patients receiving 1-HLA-antigen-mismatched cord blood grafts, 7 had a mismatch antigen in class I and 9 had a mismatch antigen in class II. In 54 patients receiving 2-antigen-mismatched grafts, 14 had mismatched antigens in class I, 37 had mismatched antigens both in classes I and II, and 3 had mismatched antigens in class II. In 28 patients receiving 3-antigen-mismatched grafts, 18 had 2 class I mismatched antigens and a class II mismatched antigen, and 10 had a class I mismatched antigen and 2 class II mismatched antigens.

median of each group, and the Student *t* test or Mann-Whitney *U* test was used. A Chi-square test was used to compare categoric covariates: SAS version 8.2 (SAS Institute, Cary, NC) and S Plus 2000 (Mathsoft, Seattle, WA) were used for all analyses. End points were calculated at the last contact, the date of the latest follow-up being March 1, 2006.

Results

Characteristics of patients and donors

The patients' age, sex, CMV serology, diagnosis, the ratio of standard-risk versus high-risk, duration from diagnosis to transplantation, and GVHD prophylaxis regimen were almost the same between the BMT/PBSCT and CBT recipients. Overall rates of high-risk patients were 62% for BMT/PBSCT recipients and 57% for CBT recipients ($P = .57$). On the other hand, there were significant differences in the following variables (Table 1). Patients receiving CBT had lower body weight and received transplants in a later calendar year. Sixty-seven percent of BMT/PBSCT recipients were administered a conditioning regimen without CY, and 91% of CBT recipients were administered a conditioning regimen with CY. The 6 possible matches between the recipient and the donor were scored serologically for HLA-A and HLA-B and genetically for DRB1 alleles, and the results showed 54 (76%) matched grafts in BMT/PBSCT recipients and no complete matches in CBT recipients. Details of HLA disparities between CBT recipients and grafts are described in the footnote to Table 1. Although the number of leukocytes for CBT recipients was 1 log lower than in BMT/PBSCT recipients, 93 of 100 cord blood grafts contained more than 2.0×10^7 cells/kg. The median number of CD34$^+$ progenitor cells was 0.93×10^5/kg (range, 0.15×10^5 to 8.97×10^5/kg) before freezing of cord blood grafts.

Engraftment, hematopoietic recovery, GVHD, and length of hospitalization

Four patients (4%) died within 28 days of cord blood transplantation, and primary graft failure occurred in 5 of the surviving 96 in the CBT recipient group. There was one early death (2%) on day 7 due to multiple organ failure in the BMT/PBSCT recipient group, but no patients had primary graft failure.

Patients receiving CBT had significantly slow neutrophil and platelet recovery in multivariate analysis (Table 2), in contrast with almost comparable recoveries in hematopoietic engraftment with longer-term follow-up. The overall myeloid engraftment rates on day 60 were 91% (95% confidence interval [CI], 85% to 97%) for CBT recipients and 96% (95% CI, 91% to 100%) for BMT/PBSCT recipients. Platelet counts of more than 2×10^9/L on day 100 were 85% (95% CI, 78% to 92%) and 94% (95% CI, 89% to 100%), and platelet counts of more than 5×10^9/L on day 180 were 83% (95% CI, 75% to 91%) and 92% (95% CI, 85% to 98%) for CBT and BMT/PBSCT recipients, respectively. One hundred percent of donor chimerisms were confirmed in all recipients after hematopoietic recovery by the techniques described in "Patients, materials, and methods."

More than 90% of patients in both groups received CsA plus a short-term MTX regimen as GVHD prophylaxis. The tapering rate of immunosuppressant drugs differed among individual patients due to variations in GVHD severity, renal function, and primary disease risk, although the protocol was assigned as described previously.[6] Consequently, the rate of decreasing immunosuppressants and discontinuation for CBT recipients was faster than those for BMT/PBSCT recipients (Table 3). The cumulative incidence of grades II to IV acute GVHD in both groups was almost equivalent (Figure 1A). On the other hand, despite the rapid tapering of prophylactic drugs for GVHD and the high degree of HLA disparity among CBT recipients, the cumulative incidence of grades III and IV acute GVHD was significantly lower in multivariate analysis (hazard ratio: 0.38; 95% CI: 0.15 to 0.95; $P = .04$; Table 2 and Figure 1B). The cumulative incidence of requiring steroids for treating acute GVHD among CBT recipients was significantly lower than among BMT/PBSCT recipients (hazard ratio: 0.25; 95% CI: 0.13 to 0.50; $P < .01$; Table 2 and Figure 1C).

Chronic GVHD affected 73 of 82 CBT and 49 of 55 BMT/PBSCT recipients surviving more than 100 days. Twenty-three CBT and 30 BMT/PBSCT recipients developed extensive GVHD. The incidence of overall chronic GVHD in CBT recipients tended to be higher than that in BMT/PBSCT recipients (Table 2; Figure 1D); however, the cumulative incidence of extensive-type GVHD among CBT recipients was significantly lower than that among recipients using grafts from related donors (hazard ratio: 0.49; 95% CI: 0.29 to 0.85; $P = .01$; Table 2 and Figure 1E).

Eighty-three CBT recipients and 59 BMT or PBST recipients were discharged from the hospital. The median number of days of hospitalization for CBT recipients was 121 and tended to be longer than that for BMT/PBSCT recipients, which was 89 days after transplantation (hazard ratio: 0.73; 95% CI: 0.450 to 1.06; $P = .10$; Table 2).

Table 2. The results of multivariate analysis of time to engraftment, GVHD, and length of hospitalization

	BMT or PBSCT recipient	CBT recipient	Hazard ratio (95% CI)*	P
Recipients, n	71	100	—	—
Absolute neutrophil count higher than 0.5 × 10^9/L			0.14 (0.10-0.22)	< .01
No. of patients to achieve (%)	69 (97)	91 (91)	—	—
Median	17	22	—	—
Range	10-35	16-46	—	—
Platelet count higher than 20 × 10^9/L			0.19 (0.13-0.28)	< .01
No. of patients to achieve (%)	68 (96)	85 (85)	—	—
Median	22.5	40	—	—
Range	12-122	13-99	—	—
Platelet count higher than 50 × 10^9/L			0.25 (0.17-0.36)	< .01
No. of patients to achieve (%)	67 (94)	84 (84)	—	—
Median	27	46	—	—
Range	13-453	25-263	—	—
Acute GVHD			—	—
No. of evaluable patients†	66	85	—	—
Grade, no. (%)			—	—
0	6 (9)	4 (5)	—	—
I	24 (36)	30 (35)	—	—
II	23 (35)	45 (53)	—	—
III	8 (12)	4 (5)	—	—
IV	5 (8)	2 (2)	—	—
II-IV	36	51	1.09 (0.71-1.68)	.69
III-IV	13	6	0.38 (0.15-0.95)	.04
Patients treated with steroids	28	18	0.25 (0.13-0.50)	< .01
Chronic GVHD			—	—
No. of evaluable patients‡	55	82	—	—
Limited + extensive	49	73	1.43 (0.95-2.14)	.09
Extensive	30	23	0.49 (0.29-0.85)	.01
Length of hospitalization			0.73 (0.50-1.06)	.10
No. of evaluable patients	59	83	—	—
Median	89	121	—	—
Range	39-355	58-608	—	—

CI indicates confidence interval; GVHD, graft-versus-host disease; and —, not applicable.

*The hazard ratio is for cord blood transplantation compared with bone marrow or peripheral blood stem-cell transplantation.

†Acute GVHD was evaluated in patients surviving 21 days or longer after transplantation with evidence of engraftment.

‡Chronic GVHD disease was evaluated in patients surviving 100 days or longer after transplantation with engraftment.

In CBT recipients, HLA disparities did not have any significant effects on engraftment, hematopoietic recovery, GVHD, or length of hospitalization.

TRM, relapse, and DFS

The respective 100-day and 1-year cumulative incidences of TRM were 8% (95% CI, 3% to 14%) and 9% (95% CI, 3% to 15%) among CBT recipients, and 4% (95% CI, 0 to 9%) and 13% (95% CI, 4% to 21%) among BMT/PBSCT recipients (Figure 2A). Higher age and higher risk of disease had significant impacts, as shown in Table 4, but the source of graft did not. Higher risk of disease and the diagnosis of ALL were significantly poor factors for relapse. Higher risk of disease was also a significant risk factor on DFS results in the multivariate analysis, as shown in Table 4.

There was no apparent difference between the risk of relapse and DFS in both groups in multivariate analysis (Table 4). The 3-year cumulative incidence of relapse was 17% (95% CI, 9% to 25%) in CBT recipients and 26% (95% CI, 15% to 37%) in BMT/PBSCT recipients (Figure 2B). The 3-year probabilities of Kaplan-Meier–estimated DFS were 70% (95% CI, 61% to 80%) after cord blood transplantation and 60% (95% CI, 49% to 72%) after bone marrow transplantation/peripheral blood stem-cell transplantation (Figure 2C). Because the ratio of high-risk disease in the BMT/PBSCT recipient group (62%) was slightly higher than in the CBT recipient group (57%, $P = .57$), we compared DFS rates of both groups for each disease risk. DFS of both groups was also equivalent in standard-risk patients and high-risk patients. The 3-year probabilities of DFS were 93% (95% CI, 85% to 100%) after cord blood transplantation and 85% (95% CI, 71% to 99%) after bone marrow transplantation/peripheral blood stem-cell transplantation in recipients in the standard-risk disease category (Figure 2D), and those in recipients in the high-risk disease category were 56% (95% CI, 42% to 70%) after cord blood transplantation and 45% (95% CI, 30%

Table 3. The results of multivariate analysis of tapering rate of immunosuppressants after transplantation

	Median d (no. of patients to achieve, range)			
	BMT or PBSCT recipient	CBT recipient	Hazard ratio (95% CI)	P
Decreased to 50% dose	47 (57, 7-1910)	44 (83, 7-149)	1.49 (1.05-2.12)	.03
Termination	383 (37, 51-1910)	188.5 (58, 50-1197)	4.10 (2.17-7.72)	< .01

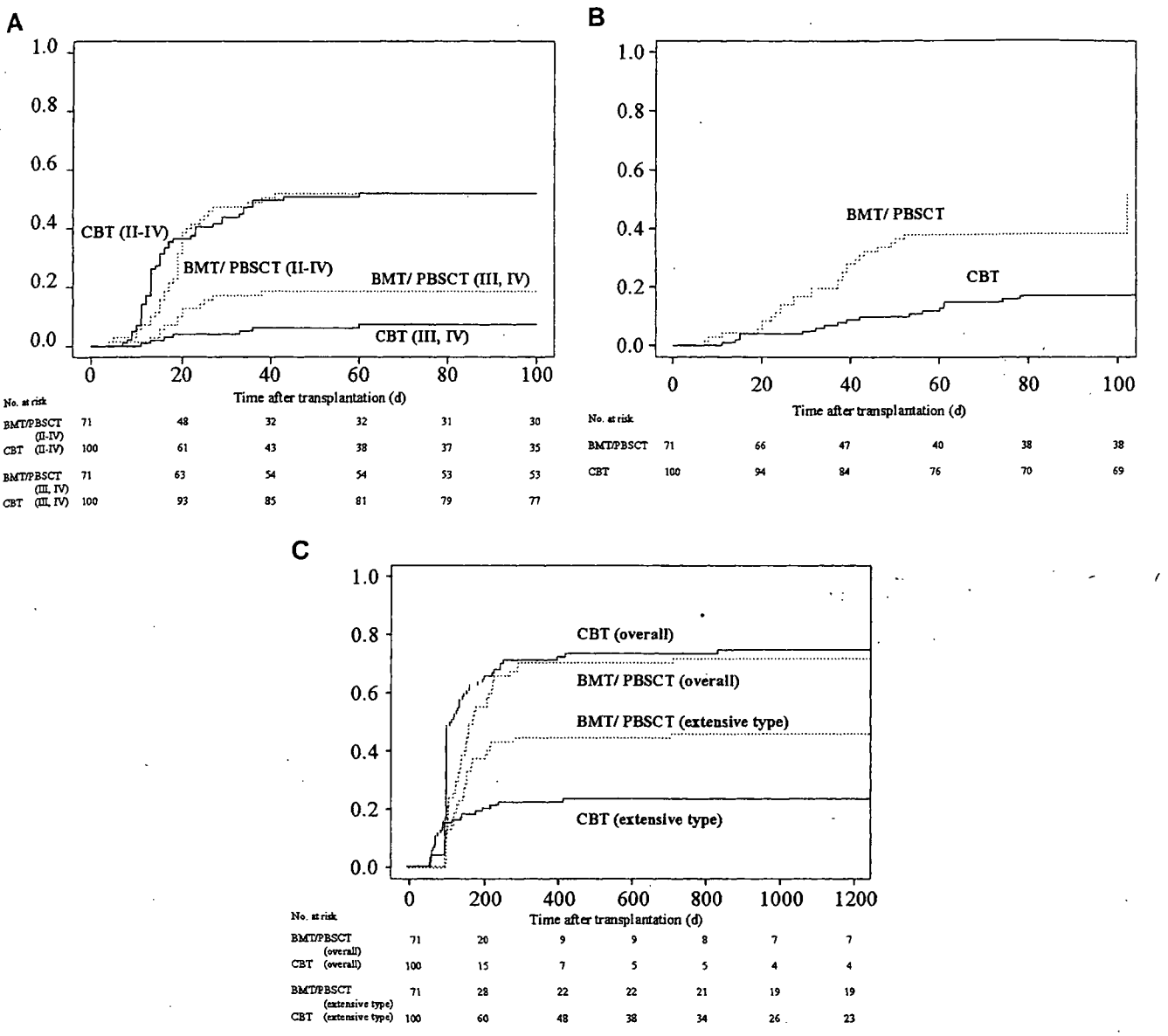

Figure 1. Cumulative incidences of acute and chronic GVHD after transplantation and kinetics of immunosuppressant use after transplantation. (A) Cumulative incidence of acute GVHD. The values of grades II to IV acute GVDH on day 100 were 52% (95% CI, 42% to 62%) for CBT and 52% (95% CI, 40% to 64%) for BMT/PBSCT recipients ($P = .69$). The values of grades III and IV acute GVDH on day 100 were 7% (95% CI, 2% to 13%) for CBT and 19% (95% CI, 19% to 28%) for BMT/PBSCT recipients ($P = .04$). (B) The cumulative incidence of requiring steroid therapy in patients after cord blood transplantation and bone marrow transplantation/peripheral blood stem-cell transplantation. The values on day 100 were 17% (95% CI, 10% to 24%) for CBT and 38% (95% CI, 27% to 49%) for BMT/PBSCT recipients ($P < .01$). (C) Cumulative incidence of chronic GVHD in patients surviving more than 100 days. The values for overall chronic GVHD were 71% (95% CI, 62% to 80%) at 1 year and 74% (95% CI, 40% to 64%) at 3 years after cord blood transplantation, in contrast to 68% (95% CI, 56% to 79%) at 1 year and 69% (95% CI, 58% to 80%) at 3 years after bone marrow transplantation/peripheral blood stem-cell transplantation ($P = .09$). The values of the cumulative incidence of extensive-type GVDH were 22% (95% CI, 14% to 30%) at 1 year and 25% (95% CI, 15% to 32%) at 3 years after cord blood transplantation, in contrast to 44% (95% CI, 32% to 55%) at 1 year and 45% (95% CI, 33% to 57%) at 3 years after bone marrow transplantation/peripheral blood stem-cell transplantation ($P = .01$).

to 60%) after bone marrow transplantation/peripheral blood stem-cell transplantation.

The proportion of causes of death was equivalent between CBT and BMT recipients (Table 5): almost 10% of deaths in both groups were GVHD related. The major cause of death in both recipient groups was relapse.

Discussion

Recently, 2 registration-based retrospective studies comparing the results of unrelated cord blood transplantation and unrelated bone marrow transplantation in adults have been published.[10,11] The investigators concluded that unrelated cord blood is an acceptable alternative source of hematopoietic stem cells for adults with acute leukemia who do not have an HLA-matched marrow donor. We have also reported on a comparative analysis of CBT versus BMT from unrelated donors for adult patients at our institute.[6] The overall results for CBT recipients were better than for BMT recipients in terms of GVHD, TRM, and DFS; moreover, our results for CBT recipients were better than those reported in European or US studies.

In this analysis, we compared updated results of unrelated cord blood transplantation with those of related transplantations in our hospital and demonstrated an equivalent safety and efficacy

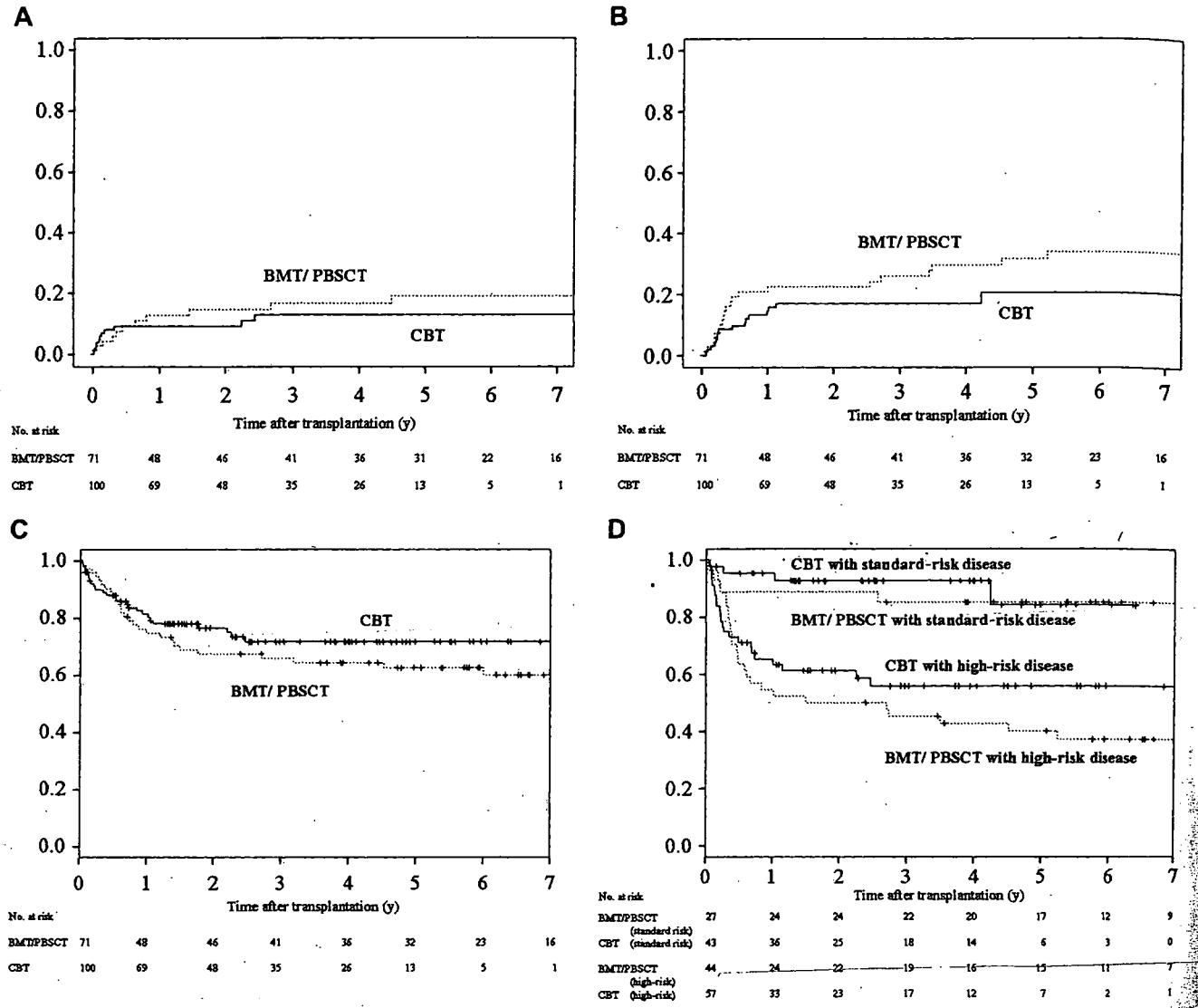

Figure 2. Outcomes among CBT and BMT/PBSCT recipients. (A) The 1-year and 3-year cumulative incidences of TRM were 8% (95% CI, 3% to 14%) and 9% (95% CI, 3% to 15%) among CBT recipients, respectively, in contrast to 4% (95% CI, 0 to 9%) and 13% (95% CI, 4% to 21%) among BMT/PBSCT recipients, respectively. The differences between the 2 groups were not significant ($P = .13$). (B) The 3-year cumulative incidences of relapse among recipients were 17% (95% CI, 9% to 25%) after cord blood transplantation and 26% (95% CI, 15% to 37%) after bone marrow transplantation/peripheral blood stem-cell transplantation. The differences between the 2 groups were not significant ($P = .34$). (C) The 3-year Kaplan-Meier estimate of DFS was 70% (95% CI, 61% to 80%) after cord blood transplantation and 60% (95% CI, 49% to 72%) after bone marrow transplantation/peripheral blood stem-cell transplantation. The differences between the 2 groups were not significant ($P = .26$). (D) The 3-year Kaplan-Meier estimate of DFS in patients with standard-risk disease was 93% (95% CI, 85% to 100%) after cord blood transplantation and 85% (95% CI, 71% to 99%) after bone marrow transplantation/peripheral blood stem-cell transplantation. The differences between the 2 groups were not significant by nonadjusted comparison ($P = .72$). The 3-year Kaplan-Meier estimate of DFS in patients with high-risk disease was 56% (95% CI, 42% to 70%) after cord blood transplantation and 45% (95% CI, 30% to 60%) after bone marrow transplantation/peripheral blood stem-cell transplantation. The differences between the 2 groups were not significant by nonadjusted comparison ($P = .26$).

between both. Hematologic recovery was slower after cord blood transplantation, although the overall engraftment rates were not significantly different, with more than 80% of patients in both groups achieving myeloid and platelet engraftment. Incidences of severe acute GVHD and extensive-type chronic GVHD were significantly lower in CBT recipients.

Patients receiving related grafts were treated at earlier dates, whereas CBT recipients were treated more recently. We used almost the same supportive care during the period for both recipients of BMT/PBSCT and CBT. On the other hand, with the anticipated improved survival rate overall in allogeneic transplantation patients over time, we thought this factor of treatment dates may have in part contributed to the equivalent survival observed in patients receiving related versus cord blood allogeneic grafts. However, this factor did not affect any clinical results in our multivariate analysis.

In the multivariate analysis, older age and high risk of disease had significant impacts in terms of TRM. High risk of disease was also a significant risk factor for relapse and DFS. The diagnosis of ALL was another significantly poor indicator of relapse. However, there was no apparent difference in the risks of TRM, relapse, and rate of DFS between the CBT and BMT/PBSCT recipient groups. DFS in both groups was also comparable among standard-risk and high-risk groups.

We speculated on several reasons for our favorable results in CBT recipients. One of the reasons might be the availability of grafts containing sufficient numbers of cells and because Japanese body size is relatively small. In fact, there were only 7 patients who received cord blood grafts containing less than 2.0×10^7 nucleated cells/kg body weight among our 100 CBT recipients. Secondly, Japanese patients might have some advantages in the setting of HLA-mismatched transplantation due to HLA or non-HLA immune

genetics.[7-9,18] In particular, there is mounting evidence indicating that polymorphisms in non-HLA immune mediators and host defense genes, such as tumor necrosis factor, interleukin-10, or their receptor genes, could affect the severity of GVHD.[19-21] The immunogenetics in Japanese[7-9,22] may also have contributed to the favorable results in related stem-cell transplantation[23,24] and unrelated bone marrow transplantation compared with reports from Western countries. This racial advantage might be significantly observed in the setting of allogeneic transplantation using HLA-mismatched grafts such as cord blood transplantation. Thirdly, the preparative conditioning and GVHD prophylaxis regimens used in our study might also have been favorable factors.

In addition to the just-mentioned reasons, the timing of transplantation is a very important factor relating to clinical results. The quick availability of cord blood as a stem-cell source is thought to be one of the most important advantages compared with unrelated bone marrow grafts.[6,25] If the patient was eligible for allogeneic transplantation but had no related donor, we generally selected a cord blood graft first, rather than waiting for the results of an unrelated marrow donor search. In fact, duration from diagnosis to transplantation was almost the same between the BMT/PBSCT and CBT recipients in the study. This might be one of the reasons for our favorable results in adult CBT recipients, especially in terms of TRM, compared with most previously published studies. The earlier timing contributes to better clinical outcomes in both patients with stable or advanced-stage disease at transplantation because disease progression can be prevented and accumulation of chemotherapy-induced tissue toxicity can be decreased. The rapid preparation of grafts may also increase the likelihood of achieving successful transplantation in patients.

Table 4. Multivariate analysis of factors associated with TRM, relapse, and DFS

Outcome/variable	Hazard ratio (95% CI)	P
TRM		
Age		.03
45 y or older	2.66 (1.12-6.30)	
Younger than 45 y	1.0	
Disease status		.01
High-risk disease	4.91 (1.43-16.8)	
Standard risk	1.0	
Graft source		.13
CBT	0.49 (0.19-1.24)	
BMT/PBSCT	1.0	
Relapse		
Disease status		< .01
High-risk disease	5.00 (2.07-12.1)	
Standard risk	1.0	
Diagnosis of primary disease		.03
ALL	2.37 (1.20-4.70)	
Other than ALL	1.0	
Graft source		.34
CBT	0.72 (0.37-1.41)	
BMT/PBSCT	1.0	
DFS		
Disease status		< .01
High-risk disease	5.37 (2.54-11.4)	
Standard risk	1.0	
Graft source		.26
CBT	0.74 (0.44-1.25)	
BMT/PBSCT	1.0	

TRM indicates transplant-related mortality; ALL, acute lymphoblastic leukemia; and DFS, disease-free survival.

Table 5. Cause of death

	BMT or PBSCT recipient, no. (%)	CBT recipient, no. (%)
Total	26	25
Relapse/refractory disease	13 (50)	14 (56)
Infection	7 (27)	4 (16)
GVHD with or without infection	3 (12)	3 (12)
Organ failure with or without infection	3 (12)	4 (16)

GVHD indicates graft-versus-host disease.

Once a patient was considered eligible for allogeneic transplantation and did not have a related donor, we performed cord blood transplantation at the same timing as bone marrow transplantation/peripheral blood stem-cell transplantation from a related donor. On the other hand, in most institutes, including those in Japan, a cord blood graft was not selected as a primary graft for patients who did not have a family donor, which might be a reason for the different clinical results for cord blood transplantation among centers in Japan.[26]

In this analysis, we compared the clinical outcomes of CBT from unrelated donors with BMT/PBSCT from related donors including HLA-matched siblings and also HLA-mismatched relatives, the latter 2 types of donors being quickly available. We took this approach because HLA closely-matched relatives were previously considered as acceptable donors in some clinical settings in patients without HLA-matched sibling donors.[24,27-29]

Our clinical results suggest that cord blood from unrelated donors could be as safe and effective a stem-cell source as bone marrow or mobilized peripheral blood from related donors for adult patients when used as a primary unrelated stem-cell source.

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Authorship

Contribution: S.T. and S.A. designed the study; J.O., A.T., T.K., N.T., K.F., M.U., K.T., T.I., and A.T. performed patients' care; M.O.M. performed data management; T.Y. analyzed data statistically; and S.T. wrote the paper.

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References

1. Laughlin MJ, Barker J, Bambach B, et al. Hematopoietic engraftment and survival in adult recipients of umbilical-cord blood from unrelated donors. N Engl J Med. 2001;344:1815-1822.
2. Sanz MA, Sanz GF. Unrelated donor umbilical cord blood transplantation in adults. Leukemia. 2002;16:1984-1991.
3. Long GD, Laughlin M, Madan B, et al. Unrelated umbilical cord blood transplantation in adult patients. Biol Blood Marrow Transplant. 2003;9:772-780.
4. Ooi J, Iseki T, Takahashi S, Tomonari A, Tojo A, Asano S. Unrelated cord blood transplantation for adult patients with acute lymphoblastic leukemia. Leukemia. 2004;18:1905-1907.
5. Ooi J, Iseki T, Takahashi S, et al. Unrelated cord blood transplantation for adult patients with advanced myelodysplastic syndrome. Blood. 2003; 101:4711-4713.
6. Takahashi S, Iseki T, Ooi J, et al. Single-institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematologic malignancies. Blood. 2004;104:3813-3820.
7. Lin M-T, Storer B, Martin PJ, et al. Genetic variation in the IL-10 pathway modulates severity of acute graft-versus-host disease following hematopoietic cell transplantation: synergism between IL-10 genotype of patient and IL-10 receptor {beta} genotype of donor. Blood. 2005;106:3995-4001.
8. Lin M-T, Storer B, Martin PJ, et al. Relation of an interleukin-10 promoter polymorphism to graft-versus-host disease and survival after hematopoietic-cell transplantation. N Engl J Med. 2003; 349:2201-2210.
9. Tegoshi H, Hasegawa G, Obayashi H, et al. Polymorphisms of interferon-γ gene CA-repeat and interleukin-10 promoter region (-592A/C) in Japanese type I diabetes. Human Immunol. 2002;63: 121-128.
10. Laughlin MJ, Eapen M, Rubinstein P, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. N Engl J Med. 2004;351:2265-2275.
11. Rocha V, Labopin M, Sanz G, et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. N Engl J Med. 2004;351:2276-2285.
12. Brunstein CG, Wagner JE. Umbilical cord blood transplantation and banking. Ann Rev Med. 2006; 57:403-417.
13. Takahashi S, Okamoto SI, Shirafuji N, et al. Recombinant human glycosylated granulocyte colony-stimulating factor (rhG-CSF)-combined regimen for allogeneic bone marrow transplantation in refractory acute myeloid leukemia. Bone Marrow Transplant. 1994;13:239-245.
14. Takahashi S, Oshima Y, Okamoto S, et al. Recombinant human granulocyte colony-stimulating factor (G-CSF) combined conditioning regimen for allogeneic bone marrow transplantation (BMT) in standard-risk myeloid leukemia. Am J Hematol. 1998;57:303-308.
15. Okamoto S, Takahashi S, Wakui M, et al. Treatment of advanced myelodysplastic syndrome with a regimen including recombinant human granulocyte colony-stimulating factor preceding allogeneic bone marrow transplantation. Brit J Haematol. 1999;104:569-573.
16. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation. 1974;18:295-304.
17. Shulman HM, Sullivan KM, Weiden PL, et al. Chronic graft-versus-host syndrome in man: a long-term clinicopathologic study of 20 Seattle patients. Am J Med. 1980;69:204-217.
18. Oh H, Loberiza FR Jr, Zhang M-j, et al. Comparison of graft-versus-host-disease and survival after HLA-identical sibling bone marrow transplantation in ethnic populations. Blood. 2005;105: 1408-1416.
19. Mullighan CG, Petersdorf EW. Genomic polymorphism and allogeneic hematopoietic transplantation outcome. Biol Blood Marrow Transplant. 2006;12:19-27.
20. Dickinson AM, Middleton PG. Beyond the HLA typing age: genetic polymorphisms predicting transplant outcome. Blood Rev. 2005;19:333-340.
21. Dickinson AM, Middleton PG, Rocha V, Gluckman E, Holler E, Eurobank members: genetic polymorphisms predicting the outcome of bone marrow transplants. Brit J Haematol. 2004;127:479-490.
22. Hattori H, Matsuzaki A, Suminoe A, et al. Polymorphisms of transforming growth factor-1 and transforming growth factor-1 type II receptor genes are associated with acute graft-versus-host disease in children with HLA-matched sibling bone marrow transplantation. Bone Marrow Transplant. 2002;30:665-671.
23. Ichinohe T, Uchiyama T, Shimazaki C, et al. Feasibility of HLA-haploidentical hematopoietic stem cell transplantation between noninherited maternal antigen (NIMA)-mismatched family members linked with long-term fetomaternal microchimerism. Blood. 2004;104:3821-3828.
24. Kanda Y, Chiba S, Hirai H, et al. Allogeneic hematopoietic stem cell transplantation from family members other than HLA-identical siblings over the last decade (1991-2000). Blood. 2003;102: 1541-1547.
25. Barker JN, Krepski TP, DeFor TE, Davies SM, Wagner JE, Weisdorf DJ. Searching for unrelated donor hematopoietic stem cells: availability and speed of umbilical cord blood versus bone marrow. Biol Blood Marrow Transplant. 2002;8:257-260.
26. Nishihira H, Kato K, Isoyama K, et al. The Japanese cord blood bank network experience with cord blood transplantation from unrelated donors for haematological malignancies: an evaluation of graft-versus-host disease prophylaxis. Brit J Haematol. 2003;120:516-522.
27. Ottinger HD, Ferencik S, Beelen DW, et al. Hematopoietic stem cell transplantation: contrasting the outcome of transplantations from HLA-identical siblings, partially HLA-mismatched related donors, and HLA-matched unrelated donors. Blood. 2003;102:1131-1137.
28. Beelen DW, Ottinger HD, Elmaagacli A, et al. Transplantation of filgrastim-mobilized peripheral blood stem cells from HLA-identical sibling or alternative family donors in patients with hematologic malignancies: a prospective comparison on clinical outcome, immune reconstitution, and hematopoietic chimerism. Blood. 1997;90:4725-4735.
29. Anasetti C, Amos D, Beatty PG, et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. N Engl J Med. 1989;320:197-204.

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ORIGINAL ARTICLE

New highly potent and specific E6 and E7 siRNAs for treatment of HPV16 positive cervical cancer

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Persistent infection by high-risk types of human papillomaviruses (HPV) is a necessary cause of cervical cancer, with HPV16 the most prevalent, accounting for more than 50% of reported cases. The virus encodes the *E6* and *E7* oncoproteins, whose expression is essential for maintenance of the malignant phenotype. To select efficacious siRNAs applicable to RNAi therapy for patients with HPV16+ cervical cancer, E6 and E7 siRNAs were designed using siDirect computer software, after which 10 compatible with all HPV16 variants were selected, and then extensively examined for RNAi activity and specificity using HPV16+ and HPV16− cells. Three siRNAs with the highest RNAi activities toward E6 and E7 expression, as well as specific and potent growth suppression of HPV16+ cancer cells as low as 1 nM were chosen. Growth suppression was accompanied by accumulation of p53 and $p21^{WAF1/CIP1}$, as well as morphological and cytochemical changes characteristic of cellular senescence. Antitumor activity of one of the selected siRNAs was confirmed by retarded tumor growth of HPV16+ cells in NOD/SCID mice when locally injected in a complex with atelocollagen. Our results demonstrate that these E6 and E7 siRNAs are promising therapeutic agents for treatment of virus-related cancer.

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Introduction

Cervical cancer is the second most common type of cancer encountered in females, with an incidence rate of up to 18.7 per 100 000.[1] Epidemiological and experimental studies have shown that persistent infections with high-risk types of human papillomaviruses (hrHPVs; HPV16, 18, 31, 33, 45) are a necessary cause of cervical cancer, with HPV16 accounting for approximately 50% of reported cases of HPV-related cervical cancer. These viruses encode the *E6* and *E7* oncogenes, whose expression is essential for virus replication.[2–4] Furthermore, deregulated E6 and E7 expression leads to malignant transformation, and is pivotal for maintenance of the malignant phenotype of cervical cancer.

Cellular targets for these hrHPV oncogenes have been investigated, with the p53 tumor suppressor protein the first identified E6 target and still the most important. The E6 protein forms a tertiary complex with E6AP functioning as a ubiquitin ligase and its substrate, p53, which leads to degradation of p53. Other E6 targets include the PDZ family of proteins, such as PSD-95, hDlg and ZO-1, as well as the co-activator p300/CBP. E6 also induces the expression of human telomerase reverse transcriptase (hTERT), a catalytic subunit of human telomerase, whose activity may be involved in immortalization.

Targets of the E7 protein include the pocket protein family of retinoblastoma protein (Rb) and the cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$.[2–4] Since these molecules are negative regulators of the cell cycle, their inhibition by E7 results in upregulation of genes required for G_1/S transition and DNA synthesis, which is essential for viral genome replication and amplification.[5] However, E7 also induces genome instability

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and genetic alterations, which subsequently accumulate and cause transformation of infected cells into malignant cells. Recently, E7 was shown to form a complex with promyelocytic leukemia protein (PML), which has been implicated in controlling cellular senescence as a pro-senescence factor.[6]

Because of the strong relationships between the expression of HPV oncogenes and cervical cancer carcinogenesis, several technologies have been applied to target virus oncogenes to develop novel therapies for cervical cancer. Antisense oligo-deoxynucleotides and ribozymes targeting virus oncogenes were found to suppress growth of virus-related cancer cells both *in vitro* and *in vivo*.[7–9] Introduction of a short double-stranded RNA with a duplex region of 19 base pairs and two nucleotide 3′ overhangs, known as a short-interfering RNA (siRNA), induces sequence-specific destruction of endogenous mRNA in mammalian cells, a phenomenon known as RNA interference (RNAi). Since the discovery of siRNA-induced RNAi in 2001,[10,11] siRNAs have been utilized in reverse genetics and gene-targeting therapy. siRNA technology is completely different from others, in that siRNAs utilizes endogenous RNAi machineries that exert normal cellular functions. Studies have shown that introduction of those targeting E6 and E7 of hrHPVs resulted in growth suppression of virus-positive cancer cells.[12–19] These results indicate the possibility of applying RNAi technology to therapeutic options for HPV + cancer.

Although siRNA-induced target gene suppression was initially assumed to be highly sequence specific, it has become clear that siRNAs suppress unintended genes through RNA-induced silencing complex (RISC)-mediated cleavage of target mRNA or translation suppression, which is analogous to miRNA-mediated RNAi,[20–22] and stimulation of innate immune by type I interferon (IFN-I) and inflammatory cytokine responses.[23,24] Suppression of such unintended genes by siRNAs is known as an off-target effect. RISC-mediated off-target effects are caused by a partial sequence complementarity between the guide strand and unintended genes, especially when excessive RISC is present.[25] Furthermore, some siRNAs have been reported to induce innate immune responses in a sequence-specific manner.[23,24] For the purpose of applying siRNA technology to therapy for hrHPV-related cancers, it is mandatory to select siRNA sequences targeting E6 and E7 that have high levels of RNAi activity and minimal off-target effects. However, to date, no known HPV16 E6 or E7 siRNA sequence has been analyzed in detail in regard to those factors.

For the present study, we selected siRNA sequences targeting mRNA coding HPV16 E6 and E7 using siDirect software, based on guidelines established by Ui-Tei *et al*.[26,27] From those findings, we developed highly effective siRNA sequences with maximum target specificity, and examined the siRNAs for RNAi activity and specificity using HPV16 + and HPV16− cancer cells. Our results identified three E6 and E7 siRNAs compatible with most HPV16 variants, which were potent and specific in suppression of E6 and E7 expression, as well as growth of HPV16 + cancer cells.

Materials and methods

Cell lines and plasmid transfection

SiHa (HPV16 + cervical cancer), CaSki (HPV16 + cervical cancer), HeLa (HPV18 + cervical cancer) and SK-OV-3 (HPV− ovarian cancer) cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). SiHa, CaSki and HeLa cells were maintained in DMEM containing 10% fetal calf serum (FCS), while SK-OV-3 cells were cultured in RPMI1640 containing 10% FCS. The incubations were performed at 37 °C in an atmosphere of 5% CO_2. Plasmid transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions.

siRNAs and transfection

The siRNAs used in this study were as follows: control siRNA passenger strand, 5′-GUACCGCACGUCAUUC GUAUC-3′; control siRNA guide strand, 5′-UACGAAU GACGUGCGGUACGU-3′; FLuc siRNA passenger strand, 5′-CCGUGGUGUUCGUGUCUAAGA-3′; FLuc siRNA guide strand, 5′-UUAGACACGAACACCACG GUA-3′; lamin A/C siRNA passenger strand, 5′-CU GAAAGCGCGCAAUACCAAG-3′; lamin A/C siRNA guide strand, 5′-UGGUAUUGCGCGCUUUCAGC U-3′; 222 passenger strand,[12] 5′-GAGGUAUAUGACU UUGCUUUU-3′; 222 guide strand, 5′-AAGCAAAGU CAUAUACCUCAC-3′; 660 passenger strand,[12] 5′-AGG AGGAUGAAAUAGAUGGUC-3′; 660 guide strand, 5′-CCAUCUAUUUCAUCCUCCUCC-3′; 375 passenger strand,[14] 5′-UACAACAAACCGUUGUGUGAU-3′; 375 guide strand, 5′-CACACAACGGUUUGUUGUAU U-3′; 186 passenger strand,[13] 5′-GAAUGUGUGUACU GCAAGCAA-3′; 186 guide strand 5′-GCUUGCAGUA CACACAUUCUA-3′; 203 passenger strand,[16] 5′-GCAA CAGUUACUGCGACGUGA-3′; 203 guide strand, 5′-A CGUCGCAGUAACUGUUGCUU-3′; 535 passenger strand,[16] 5′-CACGUAGAGAAACCCAGCUGU-3′; and 535 guide strand, 5′-AGCUGGGUUUCUCUACGU GUU-3′.

The sequences of siRNAs 233, 243, 244, 493, 497, 501, 573, 698, 707 and 752 are shown in Table 1. Twenty-one-base ribonucleotides were synthesized, annealed in buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) and column-purified (Proligo Co. Ltd, Boulder, CO).

One day before transfection, the cells were plated at a density of 5×10^4 to 1×10^5 cells per well in a six-well plate containing 2 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% FCS. Various amounts of the siRNA were added with Opti-MEM I reduced serum medium (Invitrogen Corporation) up to 50 μl. For SiHa cells, SK-OV-3 cells, and their derivatives, 1.6 μl of Lipofectamine 2000 was diluted with Opti-MEM I reduced serum medium to 50 μl. For HeLa cells and their derivatives, 0.4 μl of Lipofectamine 2000 was diluted with Opti-MEM I reduced serum medium to 50 μl. Diluted siRNA and Lipofectamine 2000 were combined, incubated at room temperature for 20 min and then added to a 2-ml

Table 1 siDirect-selected siRNAs targeting HPV16 E6 and E7

siRNA	*Nucleotide position*	*Mismatch tolerance*	*Sequence (upper, passenger strand; lower, guide strands)*	*Incompatible HPV16 class and subclass*	*Mismatched nucleotide*
117	117–139	3	5′-ACUUUCUGGGUCGCUCCUGUG-3′	EG(131)	131
			5′-CAGGAGCGACCCAGAAAGUUA-3′	Af1	132, 136
				Af2	132
128	128–150	3	5′-CAGAAAGUUACCACAGUUAUG-3′	EG(131)	131
			5′-UAACUGUGGUAACUUUCUGGG-3′	Af1	132, 136, 143, 145
				Af2	132, 143, 145
233	233–255	3	5′-CUUUGCUUUUCGGGAUUUAUG-3′		
			5′-UAAAUCCCGAAAAGCAAAGUC-3′		
243	243–265	3	5′-CGGGAUUUAUGCAUAGUAUAU-3′		
			5′-AUACUAUGCAUAAAUCCCGAA-3′		
244	244–266	2	5′-GGGAUUUAUGCAUAGUAUAUA-3′		
			5′-UAUACUAUGCAUAAAUCCCGA-3′		
324	324–346	3	5′-GAGUAUAGACAUUAUUGUUAU-3′	Af1, Af2, NA1, AA	335
			5′-AACAAUAAUGUCUAUACUCAC-3′		
326	326–348	3	5′-GUAUAGACAUUAUUGUUAUAG-3′	Af1, Af2, NA1, AA	335
			5′-AUAACAAUAAUGUCUAUACUC-3′		
493	493–515	3	5′-GGUGGACCGGUCGAUGUAUGU-3′		
			5′-AUACAUCGACCGGUCCACCGA-3′		
497	497–519	3	5′-GACCGGUCGAUGUAUGUCUUG-3′		
			5′-AGACAUACAUCGACCGGUCCA-3′		
501	501–523	2	5′-GGUCGAUGUAUGUCUUGUUGC-3′		
			5′-AACAAGACAUACAUCGACCGG-3′		
573	573–595	3	5′-CACCUACAUUGCAUGAAUAUA-3′		
			5′-UAUUCAUGCAAUGUAGGUGUA-3′		
583	583–605	3	5′-GCAUGAAUAUAUGUUAGAUUU-3′		
			5′-AUCUAACAUAUAUUCAUGCAA-3′		
615	615–637	3	5′-CAACUGAUCUCUACUGUUAUG-3′	As, Af2	618
			5′-UAACAGUAGAGAUCAGUUGUC-3′		
625	625–647	3	5′-CUACUGUUAUGAGCAAUUAAA-3′		
			5′-UAAUUGCUCAUAACAGUAGAG-3′		
698	698–720	3	5′-CCGGACAGAGCCCAUUACAAU-3′		
			5′-UGUAAUGGGCUCUGUCCGGUU-3′		
707	707–729	3	5′-GCCCAUUACAAUAUUGUAACC-3′		
			5′-UUACAAUAUUGUAAUGGGCUC-3′		
752	752–774	4	5′-CUUCGGUUGUGCGUACAAAGC-3′		
			5′-UUUGUACGCACAACCGAAGCG-3′		

siRNAs were designated by the nucleotide position number of the 3′ end of the guide strand. HPV16 classes and subclasses carrying a mismatch to the guide strand sequence are shown in the column of incompatible HPV16 class and subclass, with mismatched nucleotide positions also shown. The HPV16 classes and subclasses were as follows: Prototype (accession number K02718); European German 131 (EG131; accession number AF536179); East Asian (As; accession number AF534061); African 1 (Af1; accession number AF472508, AF536180); African 2 (Af2; accession number AF472509); Asian-American (AA; accession number AF402678); and North American I (NA1; accession number AF486325).
Underlines indicate an immunostimulatory motif.[24]

culture. The amount of Lipofectamine 2000 was changed in proportion to the culture medium volume. Transfection of siRNAs using Oligofectamine (Invitrogen Corporation) was performed according to the manufacturer's instructions.

Plasmid construction

The HPV16 E6E7 region from nt 231 to 858, ΔNE6E7 (231–858), was obtained by PCR amplification using pSV2-E6E7 (a gift from Dr T Kanda, National Institute for Infectious Disease, Tokyo, Japan)[28] as a template, and E6 sense and E7 antisense primers containing *Not*I sites at the 5′ end were subcloned at the *Not*I site of the psiCheck-2 plasmid, with the resultant plasmid designated as 16NΔE6E7/psiCheck-2. ΔNE6 region (231–559) and E7 region (562–858) were similarly cloned, and inserted between the *Xho*I and *Not*I sites of psiCheck-2, with the resultant plasmids designated as 16ΔNE6/psiCheck-2 and 16E7/psiCheck-2, respectively. An hLuc cDNA fragment isolated from psiCheck-2 was cloned into pcDNA3 at the *Hind*III and *Xba*I sites, and the resultant plasmid was designated as hLuc/pcDNA3. E7 of 16E7/psiCheck-2 was changed to an E7 variant carrying cytosine instead thymine at nt760 using GeneTailor Site-Directed Mutagenesis System (Invitrogen Corporation), and the resultant plasmid was designated as 16E7(760C)/psiCheck-2.

The primers used for plasmid construction were as follows: Not-HPV16_231s, 5′-GCGGCCGCATGACTT

TGCTTTTCGGGAT-3′; Xho-HPV16_231s, 5′-CTCGAG ATGACTTTGCTTTTCGGGAT-3′; Not-HPV16_E6as, 5′-GCGGCCGCTTACAGCTGGGTTTCTCTAC-3′; Xho-HPV16_E7s, 5′-CTCGAGATGCATGGAGATACACC TAC-3′; and Not-HPV16_E7as, 5′-GCGGCCGCTTAT GGTTTCTGAGAACAGA-3′. Underlines indicate the *Not*I and *Xho*I sites.

SiHa, HeLa and SK-OV-3 clones with constitutive expression of firefly luciferase

SiHa, HeLa and SK-OV-3 cells were transfected with a firefly luciferase (Fluc) expression plasmid (hLuc/pcDNA3) using Lipofectamine 2000 according to the manufacturer's instructions, and selected by cultivating in the presence of G418. The resultant stable clones expressing FLuc were designated as FL-SiHa-2, FL-SKOV-5 and FL-HeLa-1, respectively.

Reverse transcription–PCR

Isolation of DNA-free total RNA, synthesis of single-stranded cDNAs and PCR amplification were performed as previously described.[13] cDNA from mock-transfected cells was serially diluted and used as the standard. PCR assays were performed under the following conditions: denaturation at 94 °C for 5 min, followed by 22–26 PCR cycles (denaturation at 94 °C for 30 s, primer-annealing at 58 °C for 30 s, primer-extension at 72 °C for 30 s) and incubation at 72 °C for 5 min. Amplified products were separated on 2.5% agarose gels, stained with SYBR GOLD (Molecular probes, Eugene, OR) and analyzed using an FLA-2000 fluoro-image analyzer (Fuji Film, Tokyo, Japan). Band intensities were quantified using Image Gauge software (Fuji Film), and normalized to those of the β-actin band or those of 18S ribosomal RNA. PCR primers used for detection of E6 and E7 cDNAs are shown in Table 2. Those for detection of β-actin cDNA were as follows: β-actin forward primer, 5′-CTCAC CATGGATGATGATAT-3′ and β-actin reverse primer, 5′-TGGGTCATCTTCTCGCGGTT-3′. A pair of PCR primers for 18S ribosomal RNA was purchased from Applied Biosystems (Foster City, CA).

Immunoblotting

Cells at 2–5 × 10^5 were washed with Dulbecco's phosphate-buffered saline (PBS) once, directly dissolved in 100 μl of sodium dodecyl sulfate (SDS)-gel loading buffer and incubated for 5 min at 95 °C. Ten microliters of each sample was then separated on a polyacrylamide gel containing 0.1% SDS. Immunoblot analysis was performed as described previously.[13] Bands were detected using an FLA-2000 fluoro-image analyzer (Fuji Film). Band intensities were quantified using Image Gauge software (Fuji Film) and normalized to those of the lamin A/C band. Anti-$p21^{WAF1/CIP1}$ (clone 70) and anti-lamin A/C monoclonal antibodies (clone 14) were purchased from BD Biosciences (San Jose, CA), while anti-p53 monoclonal antibody (DO-1) came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Table 2 PCR primers for HPV16 E6 and E7

PCR primer	*Direction*	*Nucleotide position*	*Sequence*
E6s104	Forward	104–123	5′-ATGTTTCAGGACCCACAGGA-3′
E6as375	Reverse	375–356	5′-TGCTGTTCTAATGTTGTTCC-3′
E6as558	Reverse	558–539	5′-TACAGCTGGGTTTCTCTACG-3′
E7s562	Forward	562–581	5′-ATGCATGGAGATACACCTAC-3′
E7as855	Reverse	855–836	5′-TTATGGTTTCTGAGAACAGA-3′

Analysis of in vitro *cell growth and senescence-associated β-galactosidase staining*

Cell viability was examined with a colorimetric assay using WST-8 (Cell Counting Kit 8, Dojin Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Senescence-associated β-galactosidase staining was performed using a Senescent Cell Staining Kit (Sigma-Aldrich Corporation, St Louis, MO).

In vivo *tumor growth*

To study the effects of the siRNAs on tumor growth *in vivo*, SiHa cells (2 × 10^7 cells) were subcutaneously inoculated into 6-week-old NOD/SCID mice, after which tumors slowly formed at the injected site. Six weeks after the inoculation, siRNAs complexed with AteloGene (Koken Co. Ltd, Tokyo, Japan) according to the manufacturer's instructions were directly injected into the tumors at a dose of 500 pmole per tumor every 7 days ($n=6$ for each group). Thirty-five days after the first siRNA injection, the mice were killed according to the UKCCCR guidelines, and each tumor was excised and weighed. Tumor weight differences were analyzed for significance using a Student's t test, with $P<0.05$ considered to indicate statistical significance.

Results

Selection of HPV16 E6 and E7 siRNA sequences

A total of 17 siRNA sequences targeting the E6 and E7 coding regions of HPV16 (Table 1, Figure 1a) were selected using siDirect software,[27] which is based on guidelines for designing siRNA sequences for mammalian RNAi.[26] All of the sequences had structural characteristics of functional siRNA[26,30,31] and contained at least three mismatches in the guide strand with the nonredundant sequence set of human genes (mismatch tolerance 3).[27]

Five siRNAs (117, 128, 324, 326, 615) containing nucleotides that differed among HPV16 classes and subclasses were excluded (Table 1, Supplementary Table 1), and a total of 10 siRNAs targeting different regions of E6 and E7 were chosen from the remaining 12 for this study, as follows: 233, 243 and 244, targeting E6-E7 mRNA, but not E6*I-E7 or E6*II-E7 mRNA; 493, 497 and 501, targeting E6-E7 and E6*I-E7 mRNA, but not E6*II-E7 mRNA; 573, 698, 707 and 752, targeting all three splicing variants. After revision of the nonredundant sequence database, two siRNAs (244, 501) were reclassified as mismatch tolerance 2.

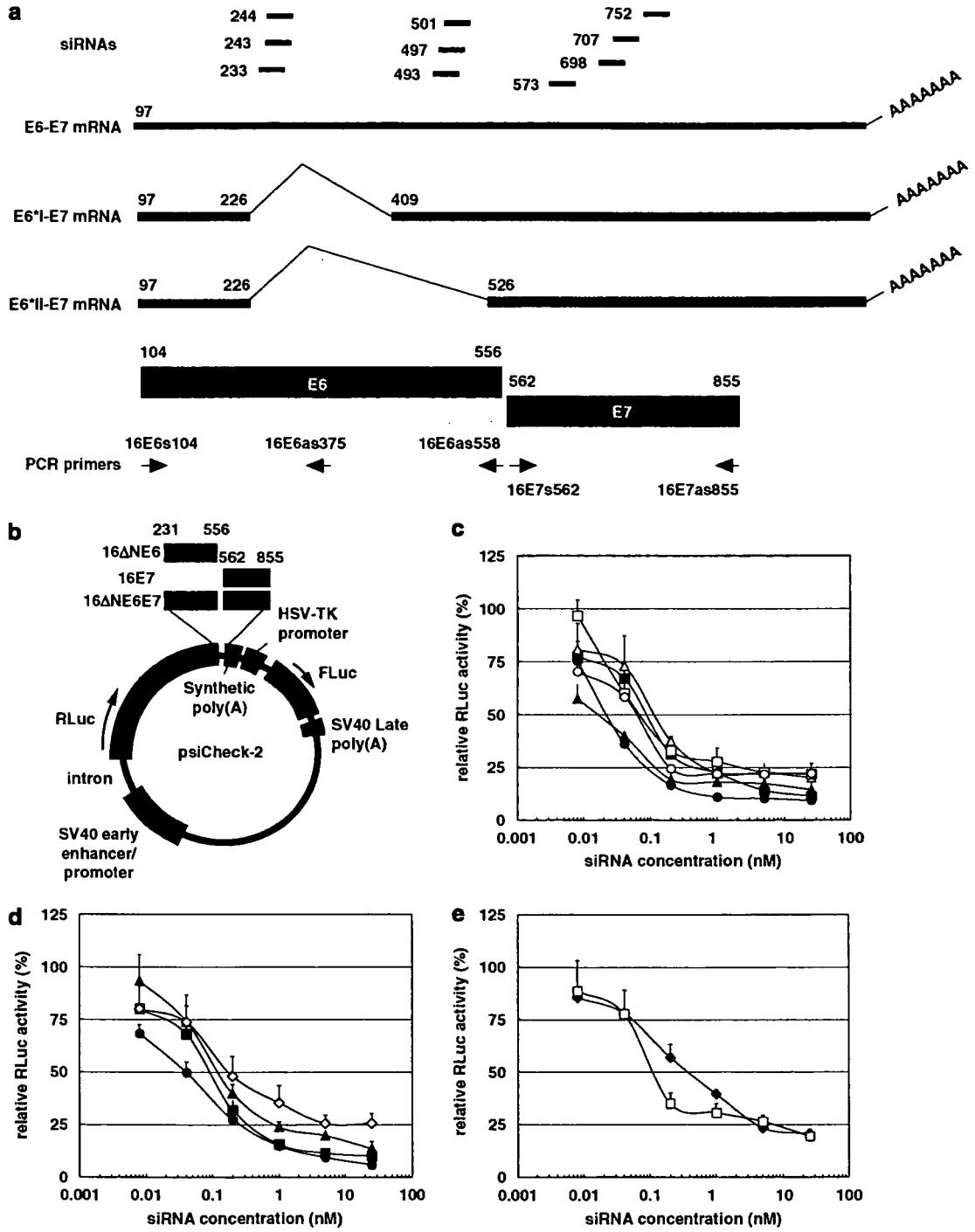

Figure 1 siDirect-selected siRNAs targeting HPV16 E6 and E7, and their effects on luciferase-E6 and -E7 fusion gene expression. (**a**) E6 and E7 siRNAs, mRNA splicing variants, genomic organization and PCR primers. siRNAs targeting E6 and E7 are depicted as short lines (top). Thick bars represent naturally occurring E6E7 mRNA species with introns within E6 (middle). The closed boxes show the locations of the E6 and E7 coding regions of the HPV16 prototype (accession number K02718) (bottom). Positions of the PCR primers used in this study are indicated as arrows. The numbers refer to the locations of the transcription initiating site and splicing junctions.[29] (**b**) Illustration of *Renilla* luciferase (Rluc)-E6E7 fusion gene expression plasmids. SiHa cells were sequentially transfected with an RLuc-ΔNE6E7 (**c**), RLuc-ΔNE6 (**d**) or RLuc-ΔNE6E7 expression plasmid (**e**) and various concentrations of E6 and E7 siRNAs. Forty-eight hours after transfection, the cells were assayed for firefly luciferase (Fluc) and RLuc activities. RLuc activity was normalized to FLuc activity, with RLuc activity of mock-transfected cells defined as 100%. Closed triangle represents siRNA 233; closed square, siRNA 243; closed circle, siRNA 244; open diagonal, siRNA 493; closed diagonal, siRNA 573; open triangle, siRNA 698; open square, siRNA 707 and open circle, siRNA 752. Experiments were performed in triplicate. Error bars represent the s.d. from the mean.

RNAi activities of E6 and E7 siRNAs toward Renilla *luciferase-E6E7 fusion mRNA*

The RNAi activities of the E6 and E7 siRNAs selected using siDirect software were examined with a reporter gene assay. SiHa HPV16+ cancer cells were transfected with a *Renilla* luciferase (RLuc)-ΔNE6E7 fusion gene expression plasmid (Figure 1b), along with various concentrations of the different siRNAs (233, 243, 244, 493, 573, 698, 707, 752). After 48 h of incubation, RLuc and FLuc activities were determined (Figure 1c). The RNAi activities of siRNA 493 and 573 toward RLuc-ΔNE6E7 were low (data not shown), and thus reevaluated using RLuc-ΔNE6 and RLuc-E7, respectively (Figures 1d and e). All the siRNAs examined showed high RNAi activities with IC_{50} values between 0.012 and 0.380 nM, which suggested that the selected siRNAs efficiently formed an RISC and degraded the artificial RLuc-oncogene fusion mRNAs.

Effects of HPV16 E6 and E7 siRNAs on endogenous mRNAs coding E6 and E7

To examine the effects of the siDirect-selected siRNAs on endogenous E6 and E7 expression, SiHa cells were transfected with the siRNAs (50 nM) for 48 h, then E6 and E7 expression was analyzed by RT–PCR. As shown in Figure 2a, PCR amplification of cDNA from control siRNA-transfected cells with a pair of E6-specific primers (E6_104s and E6_558as in Figure 1a, Table 2) gave rise to three bands representing E6, E6*I and E6*II (top panel). Transfection with siRNA 233, 243 and 244 decreased the levels of the upper band representing mRNA coding E6, but not those of the PCR bands of E6*I and E6*II. Furthermore, transfection with 493, 497 and 501 decreased the bands of E6 and E6*I, while that with 573, 698, 707 and 752 decreased all three bands. RT–PCR analysis using a pair of E7-specific primers (E7s562 and E7as855 in Figure 1a, Table 2) showed a decrease in E7 mRNA level in cells transfected with 493, 497, 501, 573, 698, 707 and 752 (middle panel). The levels of the E7 band were proportional to the sum of the E6, E6*I and E6*II levels. Each sample was also examined for β-actin mRNA level and the results were used as an internal control (bottom panel). Transfection with siRNA 497, 573, 698, 707 and 752 decreased the expression of either E6 or E7 to a level below 25%, thus they were chosen for further analyses.

Effects of HPV16 E6 and E7 siRNAs on the growth of HPV16+ and HPV16− cancer cells

HPV16+ (SiHa) and HPV16− (HeLa, SK-OV-3) cancer cells were stably transfected with a FLuc expression plasmid (hLuc/pcDNA3), and designated as FL-SiHa-2, FL-HeLa-1 and FL-SKOV-5 cells, respectively. Using an siRNA targeting FLuc mRNA (FLuc siRNA) and Lipofectamine 2000, siRNA transfection conditions for these cells were optimized to achieve comparable transfection efficiencies. Transfection with 5 nM of FLuc siRNA suppressed the FLuc activities of FL-SiH-2, FL-SKOV-5 and FL-HeLa-1 cells by 96.3 ± 0.5, 93.8 ± 0.3 and 93.3 ± 0.5%, respectively (data not shown), which demonstrated that the cells were transfected by the siRNA at similar levels of efficiency. These results enabled us to analyze the specificity of E6 and E7 siRNA-induced growth suppression.

Under the optimized transfection condition, FL-SiH-2, FL-SKOV-5 and FL-HeLa-1 cells were transfected with five different siDirect-selected siRNAs (497, 573, 698, 707, 752) as well as four others previously reported (186, 203, 222, 535)[12,14,16] and analyzed for cell viability. As shown in Figure 2b, all tested siRNAs showed moderate-to-strong growth suppression toward FL-SiHa-2 cells, while they also inhibited the growth of HPV− cells (FL-HeLa-1, FL-SKOV-5) to various degrees. Among them, 698, 186 and 535 strongly suppressed the growth of HPV16− cells, especially FL-HeLa-1 cells by more than 70%, suggesting that these siRNAs had a strong nonspecific growth inhibition characteristic.

To determine if decreased siRNA concentrations alleviated nonspecific growth suppression without compromising the specific growth inhibition, the dose effects of siRNA 497, 573, 707, 752, 203 and 222 were examined. At concentrations from 1 to 5 nM, all siRNAs suppressed the growth of FL-SiHa-2 cells in a dose-dependent manner (Figure 3c), except for 573, which exhibited similar levels of growth suppression at these concentrations. At a concentration of 1 nM, 497, 573 and 752 inhibited FL-SiHa-2 cell growth by more than 90%, while the growth suppression of HPV16− cells decreased to less than 35% (Figures 3a and b). These results suggest that 497, 573 and 752 were able to induce a potent and specific growth inhibition toward HPV16+ cells at 1 nM. In contrast, 707, 203 and 222 showed less potent growth inhibition of FL-SiHa-2 cells at 1 nM.

Dose-dependent effects of E6 and E7 siRNAs on endogenous E6–E7 mRNA

To confirm that the observed siRNA-induced growth inhibition was associated with E6 and E7 downregulation, FL-SiHa-2 cells were transfected with mock, control, E6 and E7 siRNAs (752, 203, 535) for 72 h, after which E6 and E7 expression was examined by RT–PCR using primer pairs for each (E6; 16E6s104 and 16E6as375, shown in Figure 1a, Table 2 and E7; 16E7s562 and 16E7as855). As shown in Figure 3d, siRNA 752 suppressed the level of E7 mRNA to 11, 19 and 43% at 25, 5, and 1 nM, respectively, as compared to the control siRNA-transfected cells. Furthermore, siRNA 752 decreased the E6 mRNA level to 29, 48 and 52%, respectively. In addition, two previously reported siRNAs (203, 535) showed comparable RNAi activities toward E6 and E7 mRNA. Also, 186 decreased the expression levels of both E6 and E7 mRNA, whereas 222 decreased only that of E6 (Figure 3e), which has been reported previously.[12,13] siRNA 375[14] and 660[12] were less potent and suppressed E6 and E7 expression by only 50% (data not shown).

Induction of p53 and $p21^{WAF1/CIP1}$ by E6 and E7 siRNAs

Next, we examined the downregulation of *E6* protein in the siRNA-transfected cells by analyzing the expression of

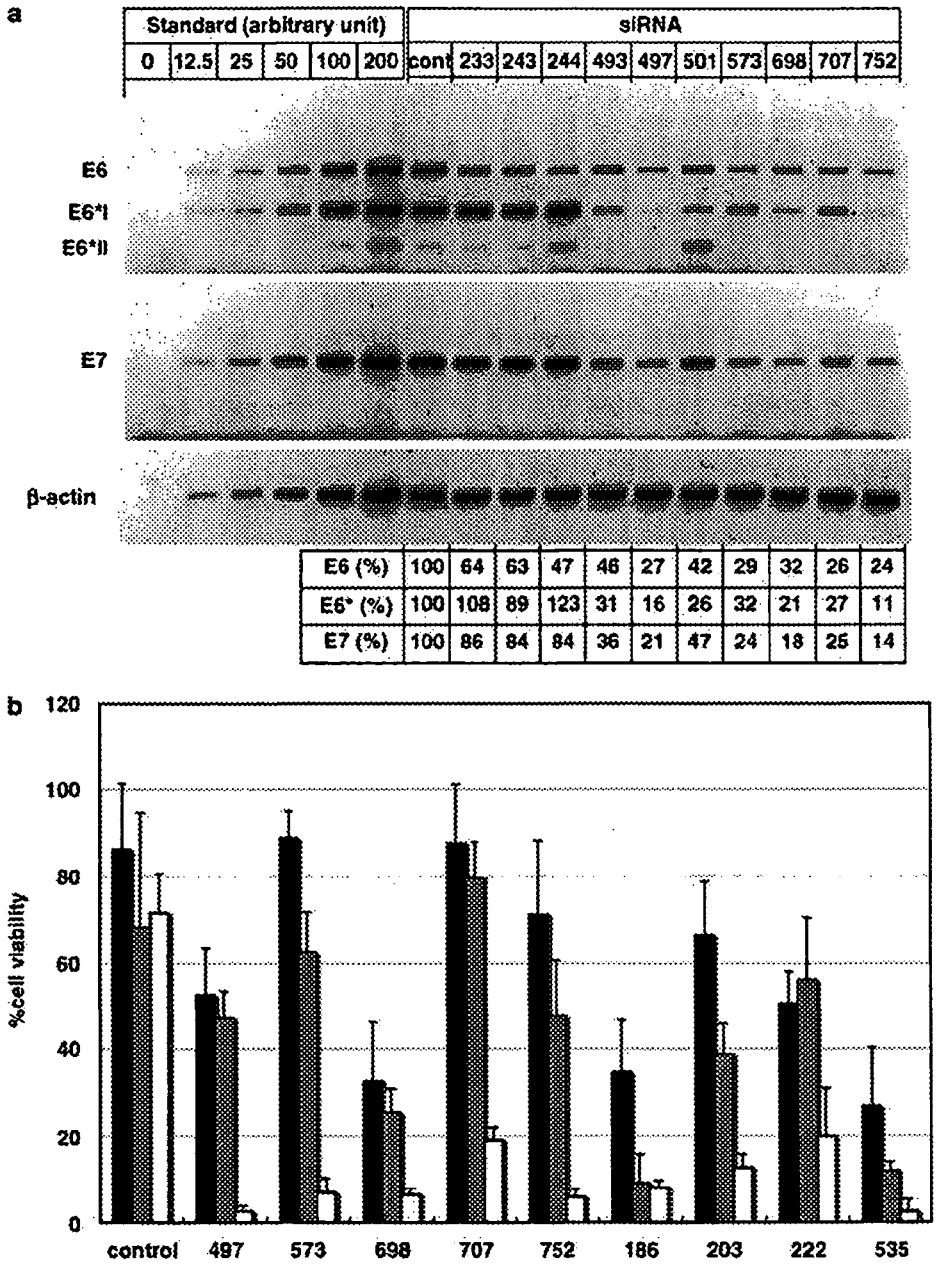

Figure 2 Effects of E6 and E7 siRNAs on E6 and E7 expression and cell growth. (**a**) Suppression of endogenous E6 and E7 expression in SiHa cells by E6 and E7 siRNAs. SiHa cells were transfected with mock, control, E6 or E7 siRNA (50 nM) for 48 h. cDNA was synthesized from total RNA and subjected to PCR. The intensity of the bands for E6 and E7 from the siRNA-treated cells were normalized to the β-actin band, and the relative E6 and E7 mRNA levels were calculated by comparing with those of serially diluted samples from mock-transfected cells. The mRNA level in control siRNA-treated cells was defined as 100%. (**b**) Effects of transfection with HPV16 E6 and E7 siRNA on growth of HPV16+ and HPV16− cells. Effects of siDirect-designed (497, 573, 698, 707, 752) and reported E6 and E7 (186, 203, 222, 535) siRNAs on the growth of HPV16− (FL-SKOV-5, FL-HeLa-1) and HPV16+ (FL-SiHa-2) cells were examined. The cells were transfected with mock, control, E6 or E7 siRNA at 5 nM, with the media changed and siRNA transfection repeated every 3 or 4 days. In 8–12 days after the first transfection, cell viability was examined using a WST-8 assay. Cell viabilities relative to mock-transfected cells are shown. Closed bar represents FL-SKOV-5; gray bar, FL-HeLa-1; open bar, FL-SiHa-2. All experiments were performed in triplicate. Error bars represent the s.d. from the mean.

p53, since there is no high affinity anti-E6 antibody capable of detecting low E6 expression presently available. p53 is an E6 target protein that degrades p53 through the ubiquitin-proteasome pathway,[2,3] while E6 knockdown restores its expression. The dose-dependent effects of siRNA 497, 573 and 752 on p53 levels were examined in FL-SiHa-2 cells using immunoblot analysis (Figures 4a–c). siRNA 497 and 573 caused a substantial accumulation of

p53 and its downstream target gene product $p21^{WAF1/CIP1}$ at concentrations from 1 to 25 nM. Transfection of siRNA 752 also caused accumulations of p53 and $p21^{WAF1/CIP1}$ at concentrations from 1 to 25 nM, with a slightly lower induction of p53 at 1 nM.

The effects of the E6 and E7 siRNAs (573, 752, 186, 222, 660, 203, 535) on the expressions of p53 and $p21^{WAF1/CIP1}$ were also examined in SiHa and CaSki cells, both of which are HPV16+ cervical cancer cell lines, using immunoblotting (Figures 4d–f). As shown in Figures 4d and e, accumulations of p53 and $p21^{WAF1/CIP1}$ were seen in SiHa cells transfected with 752, 573, 186, 222, 203 and 535, but not in those transfected with 375 and 660, which was consistent with the low RNAi activities of

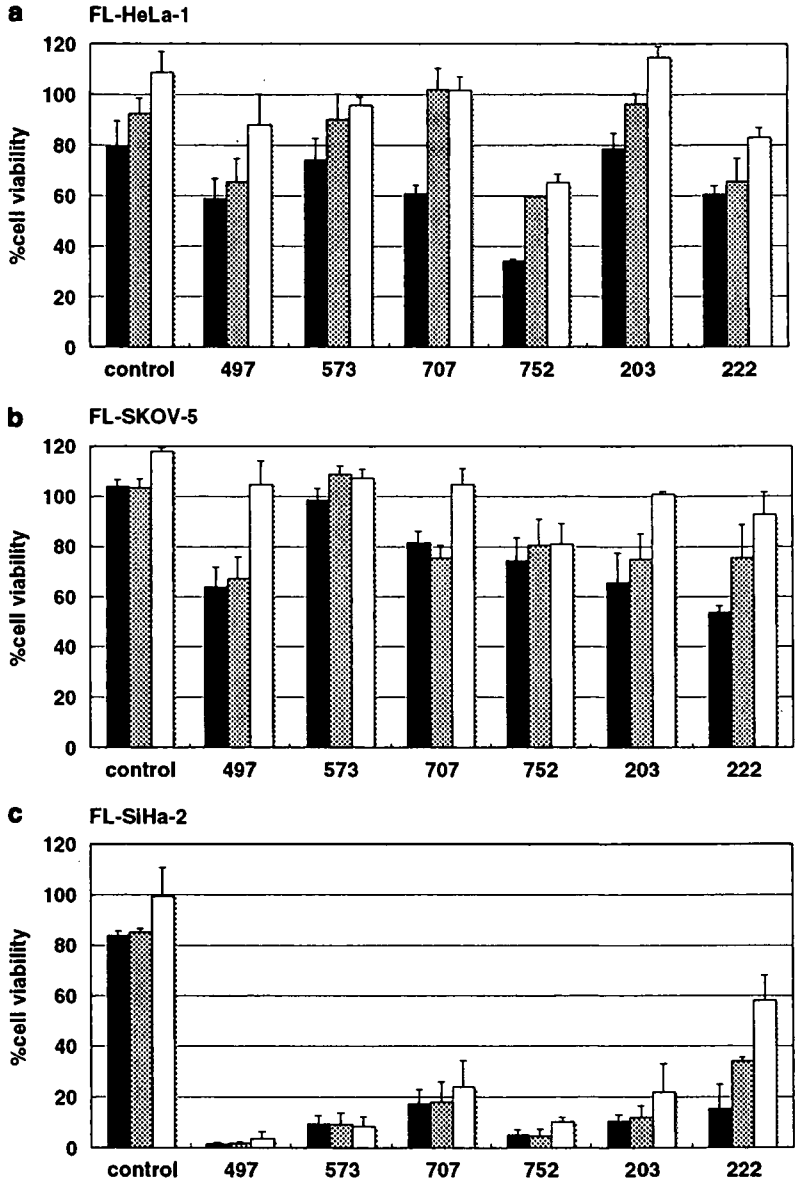

Figure 3 Dose-dependent effects of HPV16 E6 and E7 siRNAs on growth of HPV16– and HPV16+ cancer cells, and endogenous E6 and E7 expression. Dose-dependent effects of E6 and E7 siRNAs on growth of FL-HeLa-1 (**a**), FL-SKOV-6 HPV16– (**b**) and FL-SiHa-2 HPV16+ cells (**c**). Cells were plated in 96-well plates, then transfected with mock, control, E6 or E7 siRNAs (497, 573, 707, 752, 203, 222) at 5 nM (closed bar), 2 nM (gray bar) and 1 nM (open bar). Transfection was repeated every 3–4 days and a WST-8 assay was performed 7–11 days after the first transfection. Cell viabilities relative to mock-transfected cells are shown. All experiments were performed in triplicate. Error bars represent the s.d. from the mean. Dose-dependent effects of E6 and E7 siRNAs on E6 and E7 mRNA expression were analyzed in FL-SiHa-2 cells. FL-SiHa-2 cells were transfected with mock, control, E6, E7 or firefly luciferase (Fluc) siRNA at the indicated concentrations for 72 h, then analyzed for E6 and E7 expression (RT–PCR) and FLuc activity. The E6 and E7 siRNAs used in this experiment were 752, 203 and 535 (**d**), and 186 and 222 (**e**). 18S ribosomal RNA was used for normalizing the mRNA quantity. mRNA levels in siRNA-treated cells are shown relative to the level in control siRNA-treated cells. FLuc siRNA decreased FLuc activity by 96, 97 and 95% at 25, 5 and 1 nM, respectively.