days 3 and 6. Both acute and chronic GVHD were graded according to previously published criteria [8-10]. All patients received G-CSF (5 μg/kg per day) by intravenous infusion, starting on day 1 and continuing until durable granulocyte recovery was achieved. The cord blood unit was selected according to the number of nucleated cells per the recipient's body weight and HLA compatibility (HLA-A and HLA-B determined by serology and HLA-DRB1 determined by high-resolution DNA typing). Chimerism status after CBT was determined either by fluorescence in situ hybridization with a Y-chromosome probe for sex-mismatched CBT or by polymerase chain reaction DNA typing of HLA antigens for HLA-mismatched CBT. One patient (case 1) was included in our previous study [11], and all 3 patients were included in another study [12]. All patients were transfusion dependent before transplantation. No patient had a related or unrelated bone marrow donor available at the time of transplantation. Written informed consent for treatment was obtained from all patients. The characteristics of the 3 patients and the cord blood units are shown in Table 1. All patients had myeloid reconstitution, and the times to reach an absolute neutrophil count greater than 0.5×10^9/L were 23, 20, and 26 days. All patients showed full donor chimerism at the time of the first bone marrow examination (on day +42, +43, and +62) after CBT. Acute and chronic GVHD occurred in all patients. All patients are alive and free of disease at between 17 and 39 months after transplantation (Table 2).

3. Discussion

Although allogeneic stem cell transplantation from an HLA-identical related donor offers a potential cure for MDS-RA patients, a suitably matched related donor is unavailable for approximately two thirds of patients. In addition, the optimal timing of transplantation for low-risk MDS is unknown, and transplantation with the use of unrelated donors has remained controversial. A recent analysis by the International Bone Marrow Transplant Registry and the Fred Hutchinson Cancer Research Center group showed that delayed transplantation for the International Prognostic Scoring System (IPSS) low and intermediate-1 risk groups is associated with maximized overall survival [13]. They hypothesized that the optimal timing of transplantation for this cohort is at the time of a clinically important cytopenia or the progression from one IPSS group to a higher-risk group. Because all patients described in this report showed a clinically important cytopenia and transfusion dependence, we made the decision that all patients should receive a transplant. None of our patients had any related or unrelated bone marrow donors. Therefore, unrelated cord blood, which has the advantage of rapid availability, was used as an alternative stem cell source. Several studies that have included MDS patients have suggested promising results for unrelated CBT after myeloablative conditioning for adult patients [11,12,14-18]. In the study by Laughlin et al [14], 68 adult patients received a CBT. Of these 68 patients, 2 had MDS. In a report by the Eurocord group [16], 12 of the 108 adult patients who received a CBT had MDS. However, these 2 reports did not detail the preparative regimens, the prophylaxis therapy against

Table 1.
Characteristics of Patients and Cord Blood Units*

Case No.	Age, y/Sex	Body Weight, kg	HLA-A, HLA-B, DRB1 Mismatches, n	Cord Blood Cell Dose (Cryopreserved), $\times 10^7$/kg	Recipient CMV Status	Time from Diagnosis to Transplantation, mo	Treatment before Transplantation	Blood Counts at Transplantation† Neutrophils, $\times 10^9$/L	Hemoglobin, g/dL	Platelets, $\times 10^9$/L	Cytogenetics‡	IPSS Score at CBT
1	48/M	61.6	1 (DRB1)	2.45	Positive	23	ATG, CyA, Pred, mPred	0.04	8.0	18	Poor (−7)	Int-2
2	31/M	59.0	3 (HLA-A, HLA-B, DRB1)	2.31	Negative	146	Pred, mPred, anabolic steroid	1.94	6.3	30	Poor (complex§)	Int-2
3	28/M	49.8	2 (HLA-B, DRB1)	2.43	Positive	27	ATG, CyA, Pred, mPred, anabolic steroid	0.46	7.9	19	Good (normal)	Int-1

*CMV indicates cytomegalovirus; IPSS, International Prognostic Scoring System; CBT, cord blood transplantation; ATG, antithymocyte globulin; CyA, cyclosporin A; Pred, prednisolone; mPred, methylprednisolone; Int-2, intermediate-2.
†Blood counts were performed immediately before the conditioning regimen.
‡Cytogenetic analyses were performed before transplantation and according to IPSS classification [21].
§48,XY,+9,+15,der(18)t(1;18)(q21;p11) (1/20); 48,XY,+9,+15,der(18)t(1;18)(q21;p11),inv(12)(p13q24) (1/20); 46,XY,del(11)(q?) (9/20); 46,XY,ins(1;?)(q21;?),del(11)(q?),add(18)(p11) (3/20); 46,XY (6/20)

Table 2.
Transplantation Outcomes*

Case No.	Neutrophils >0.5 × 10^9/L, d	Reticulocytes >1%, d	Hemoglobin >8.5 g/dL, d	Platelets >50 × 10^9/L, d	Acute GVHD Grade (Organ Involvement and Stage)	Chronic GVHD	Immunosuppressive Treatment at Last Follow-up Day	Blood Counts and Performance Status at Last Follow-up Day: Neutrophils, ×10^9/L	Hemoglobin, g/dL	Platelets, ×10^9/L	PS	Survival, mo
1	23	32	55	48	I (skin 1, liver 0, gut 0)	Limited	None (Jan 13, 2005)	3292	12.9	213	0	39
2	20	32	125	131	II (skin 3, liver 0, gut 0)	Extensive	Prednisolone, 7.5 mg/d (Jan 20, 2005)	9143	13.7	209	1	30
3	26	46	126	109	I (skin 1, liver 0, gut 0)	Limited	None (Jan 13, 2005)	5001	13.9	141	0	17

*GVHD indicates graft-versus-host disease; PS, Eastern Cooperative Oncology Group performance status.

GVHD, or the results of transplantation for the MDS patients. Sanz et al [15] reported the results with 22 adult patients who received a CBT following a standardized preparative and GVHD regimen. Of the 22 patients, only 1 had MDS. Long et al [17] reported the results with 57 adult patients who underwent CBT at Duke University. Although 3 of the 57 patients had MDS, the details of the outcomes for the MDS patients were not described. Recently, Laughlin et al [18] reported the results with 150 adult patients who received a CBT. Although 10 of the 150 patients had MDS, the details of the outcomes for the MDS patients also were not described. At present, therefore, the role of unrelated cord blood as an alternative stem cell source is not well defined for adult MDS-RA patients eligible for conventional conditioning regimens. Because the safety of the preparative regimen including G-CSF has been confirmed for the treatment of myeloid malignancies [19,20], we have used the same regimen for CBT patients with myeloid malignancies, including those with MDS-RA. Because unrelated CBT can be performed without severe regimen-related toxicities and because all 3 patients are alive and free of disease after transplantation, we suggest that unrelated CBT may be beneficial for adult MDS-RA patients without a suitable related or unrelated bone marrow donor.

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Unrelated cord blood transplantation for a human immunodeficiency virus-1-seropositive patient with acute lymphoblastic leukemia

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The concurrent use of highly active antiretroviral therapy (HAART) improves results of high-dose chemotherapy with autologous stem cell transplantation (SCT) for human immunodeficiency virus-1 (HIV)-associated lymphomas.[1] Recently, successful allogeneic SCT from HLA-matched sibling donors was reported in HIV-infected patients.[2–4] Here, we describe the first case of an HIV-infected patient with acute lymphoblastic leukemia (ALL) who underwent umbilical cord blood transplantation (CBT).

In July 1996, a 23-year-old Japanese woman presented with fever and genital herpes. She was confirmed as seropositive for HIV, probably transmitted from her boyfriend. In March 2001, a real-time quantitative polymerase chain reaction (PCR) analysis showed that the HIV-RNA level was elevated to 25 000 copies/ml (lower limit of detection, 50). The CD4 count decreased to 28/μl. Therefore, HAART consisting of 60 mg stavudine, 300 mg lamivudine, and 600 mg efavirenz was initiated. In July 2001, the HIV-RNA level decreased to 220 copies/ml, and the CD4 count increased to 129/μl. In May 2003, her complete blood count tests showed a white blood cell count (WBC) of 3990/μl with 29% lymphoblasts. Bone marrow (BM) examination showed hypercellularity with 96% lymphoblasts, which were positive for CD4, CD10, CD13, CD19, CD33, CD34, and HLA-DR. Cytogenetic analysis disclosed the presence of t(9;22)(q34;q11) in 12 of 20 metaphases. The p190$^{BCR\text{-}ABL}$ transcript was shown by a reverse transcriptase (RT)-PCR analysis. She was diagnosed as Philadelphia chromosome-positive ALL. She achieved hematological complete remission after two courses of chemotherapy. She has been taking HAART during and after the chemotherapy and her HIV-RNA level continued to be below detectable levels. She was negative for hepatitis B virus surface antigen and anti-hepatitis C virus antibody, and positive for anti-cytomegalovirus antibody. As she had no HLA-matched related or unrelated BM donors, the patient underwent CBT from an unrelated donor with mismatches at two loci (HLA-B and DR) in September 2003 (Figure 1). The numbers of total nucleated cells and CD34-positive cells in the cord

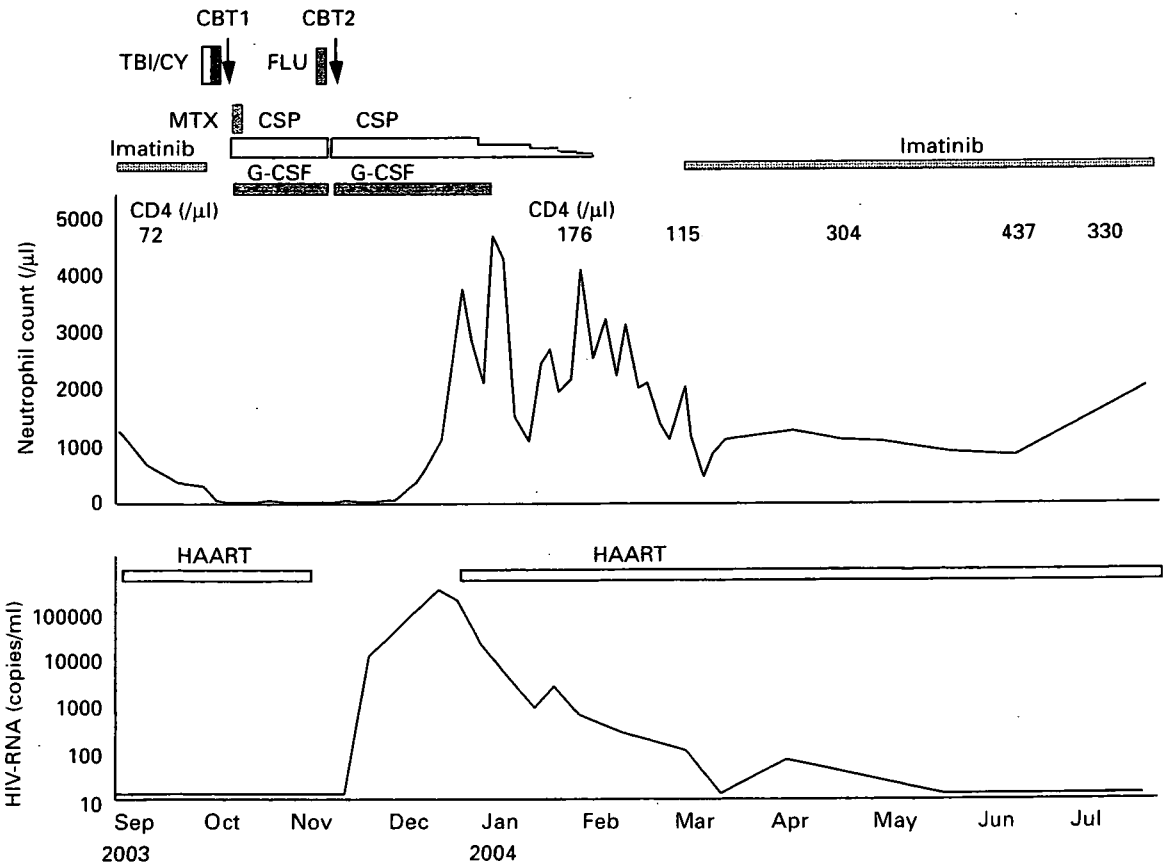

Figure 1 Clinical course of the patient.

blood (CB) unit were 2.9×10^7/kg and 0.76×10^5/kg, respectively. The conditioning regimen included 12 Gy total body irradiation and 120 mg/kg cyclophosphamide. Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine and methotrexate. The patient tolerated the procedure well with minimal regimen-related toxicity. Owing to possible myelosuppression, HAART was discontinued on day +28. On day +33, her WBC remained below 100/μl and all of the BM cells were shown to be derived from the recipient. At 40 days after the first CBT, second CBT was performed from an unrelated donor with a one-locus mismatch at HLA-DR. The numbers of total nucleated cells and CD34-positive cells in the CB unit were 2.1×10^7/kg and 0.46×10^5/kg, respectively. The conditioning regimen included 40 mg/m^2 fludarabine for 3 days. Cyclosporine was administered for GVHD prophylaxis. A neutrophil count consistently greater than 500/μl was achieved on day +27. Full donor chimerism of BM cells was shown on day +28. The HIV-RNA level increased to 3×10^6 copies/ml on day +31. After the administration of HAART from day +38, the HIV-RNA levels returned to below detectable levels from day +195, and the CD4 count increased to above 300/μl from day +170. No bacterial or fungal infections were documented during the first and second CBT processes and cytomegalovirus reactivation was successfully treated with ganciclovir and foscarnet. Grade I acute GVHD occurred, but resolved without any additional immunosuppressants. No chronic GVHD was observed. An RT-PCR analysis showed continuous negative test results for the p190$^{BCR\text{-}ABL}$ transcript until the last follow-up evaluation at 15 months post-CBT.

CBT for adults has been associated with a high rate of early transplantation-related mortality (TRM).[5,6] However, our single-institution experience showed a 1-year TRM of 9% and 2-year disease-free survival of 74% in 68 adults after CBT.[7] Both CB donors and the patient in the present study were Japanese. The lesser genetic diversity in a single ethnic population in our studies might be associated with the favorable outcomes of CBT, such as the lower rates of severe acute GVHD. Although our results suggest that CBT is feasible for HIV-infected patients on HAART, the safety and efficacy should be further examined by prospective studies.

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Human Herpesvirus 6 Variant B Infection in Adult Patients after Unrelated Cord Blood Transplantation

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Abstract

Human herpesvirus 6 variant B (HHV-6B) infection was studied in 23 adult patients who underwent cord blood transplantation (CBT). HHV-6B DNA was detected by quantitative polymerase chain reaction analysis after CBT in the sera from 15 patients (65%) at day 14 or 15 (week 2), from 16 patients (70%) at day 21 or 22 (week 3), and from 3 patients (13%) at day 28 or 29 (week 4). HHV-6B DNAemia was found in none of the 20 patients examined at day 7 or 8 (week 1). The overall incidence of HHV-6B DNAemia reached 87% (20 of 23 patients). This incidence was much higher than after unrelated bone marrow transplantation (19%, $P < .0001$). In CBT patients, positive HHV-6B DNAemia at week 3 was significantly associated with early skin rash (88% versus 14%, $P < .005$) and grade II-IV acute graft-versus-host disease (aGVHD) (69% versus 14%, $P < .05$). In contrast, positive HHV-6B DNAemia at week 2 was associated with neither skin rash nor aGVHD. Prospective large-scale studies are needed to determine the role of HHV-6 infection in CBT patients.
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Key words: Human herpesvirus 6; Cord blood transplantation; Graft-versus-host disease

1. Introduction

Human herpesvirus 6 (HHV-6) is classified into variants A and B. HHV-6B causes significant complications after hematopoietic stem cell transplantation (SCT), including encephalitis, pneumonia, and bone marrow suppression [1,2]. An association between HHV-6 infection and early skin rash or acute graft-versus-host disease (aGVHD) in SCT patients has also been reported [3-5]. The probability of HHV-6 infection after bone marrow transplantation (BMT) or peripheral blood SCT (PBSCT) ranges from 30% to 80% and may vary according to the method of detection and the type of SCT [1,2]. In previous studies, the detection of HHV-6 DNA in peripheral blood mononuclear cells (PBMCs) by polymerase chain reaction (PCR) analysis was mainly used to examine HHV-6 infection in SCT patients. However, this method may detect latent infection by viral genomes in PBMCs. In contrast, several studies have shown that the detection of HHV-6 DNA in serum or plasma is a marker of active infection [6-8]. Using a real-time quantitative PCR method on serum samples, we compared the HHV-6 DNA levels in adults after cord blood transplantation (CBT) and after BMT.

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2. Patients and Methods

2.1. Patients

Twenty-three adults who underwent CBT from HLA-mismatched unrelated donors between October 2000 and May 2003 were studied (Table 1). Transplantation procedures and supportive care were described previously [9,10]. The severity of aGVHD was graded according to the standard criteria [11]. All patients received 1000 mg/day acyclovir orally from day −3 to day 35 to prevent herpes simplex virus reactivation. After neutrophil engraftment, cytomegalovirus infection was monitored with an antigenemia assay. Preemptive ganciclovir therapy was initiated as described previously [10]. Patient no. 20 developed positive cytomegalovirus antigenemia and received 5 mg/kg ganciclovir from day 22. The remaining patients did not receive ganciclovir therapy within

Table 1.
Patient Characteristics*

	CBT (n = 23)	BMT (n = 21)
Median age (range), y	36 (18-53)	28 (17-50)
Sex (male/female), n	16/7	14/7
Disease, n		
AML	16	7
ALL	5	4
CML	1	8
MDS	1	2
Disease status, n		
Low risk	15	8
High risk	8	13
HLA matching, n†		
6/6	0	21
5/6 or 4/6	14	0
3/6 or 2/6	9	0
Preparative regimen, n		
TBI + CY + Ara-C	17	17
TBI + CY	4	4
TBI + FLU + Ara-C	2	0
GVHD prophylaxis, n		
CSP + MTX	23	9
FK + MTX	0	12
Acute GVHD, n		
0-I	11	8
II-IV	12	13

*CBT indicates cord blood transplantation; BMT, bone marrow transplantation; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; TBI, total body irradiation (12 Gy); CY, cyclophosphamide; Ara-C, cytarabine; FLU, fludarabine; CSP, cyclosporine; MTX, methotrexate; FK, tacrolimus; GVHD, graft-versus-host disease.

†The matching of HLA-A and HLA-B was confirmed by serologic typing methods, and the matching of DRB1 was confirmed by genomic typing methods.

28 days after CBT. One patient (no. 21) developed neurologic symptoms that might be attributable to HHV-6B infection. The patient died of fungal infection on day 48. Three patients (nos. 12, 14, and 18) developed leukemia relapse on days 96, 89, and 368, respectively. The remaining 19 CBT patients were alive at a median follow-up time of 30 months (range, 17-48 months). The control patients were 21 adults who underwent BMT from HLA-matched unrelated donors between March 1997 and February 2003 (Table 1). No patients developed positive cytomegalovirus antigenemia or received ganciclovir therapy within 28 days after BMT. Ten BMT patients developed leukemia relapse at a median of 236 days after BMT (range, 35-1539 days). Four patients died of transplantation-related complications on days 75, 79, 110, and 1523. The remaining 7 BMT patients were alive at a median follow-up time of 50 months (range, 21-90 months).

2.2. Real-Time Quantitative PCR

Serum samples were obtained on day 7 or 8 (week 1), day 14 or 15 (week 2), day 21 or 22 (week 3), and day 28 or 29 (week 4). Real-time quantitative PCR for HHV-6B DNA was performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described previously [12]. In brief, the reaction mixture consisted of the TaqMan Universal PCR Master Mix (Applied Biosystems) with 300 nM of each primer, 200 nM TaqMan probe, and sample DNA that had been purified with a QIAamp DNA Minikit (Qiagen, Hilden, Germany) from 200 μL serum. After 50 cycles of PCR amplification, the data were analyzed with Sequence Detection System software, version 1.6.3 (Applied Biosystems). The numbers of HHV-6B DNA copies in the samples were determined by comparing the data with the standard curves obtained with serially diluted plasmids (10^1-10^7 copies) containing the sequence of the HHV-6B immediate-early-1 gene. The forward and reverse primers were 5′-GGTCATACAAGGAAGCGTT TCG-3′ and 5′-GTACAGCCTCAGTGACAGATCTG-3′, respectively. The probe was 5′-CAGCCCCGATAAAAG GTCACAGACAAAAGA-3′ and was labeled with a fluorescent dye, 6-carboxyfluorescein (FAM) (Applied Biosystems). The assay could distinguish HHV-6B from other herpesviruses, including HHV-6A, cytomegalovirus, and Epstein-Barr virus. The minimum detectable level of this assay was 2×10^2 copies/mL.

2.3. Statistical Methods

The frequencies of categorical variables were compared with the Fisher exact test. The levels of HHV-6B DNA were compared by means of the Mann-Whitney *U* test.

3. Results

3.1. Detection of HHV-6B DNA in Sera after CBT

The presence of HHV-6B DNA in sera from 23 CBT patients at weeks 2, 3, and 4 was examined by the quantitative PCR method (Table 2). In 20 (87%) of 23 CBT patients, HHV-6B DNA was found in at least 1 serum sample. HHV-6B DNAemia was found in 15 patients (65%) at week 2, 16 patients (70%) at week 3, and 3 patients (13%) at week 4. However, HHV-6B DNAemia was found in none of the 20 patients examined at week 1. The median HHV-6B DNA level was 6×10^2 copies/mL (range, $<2 \times 10^2$ to 6×10^4 copies/mL) at week 2 and 1×10^3 copies/mL (range, $<2 \times 10^2$ to 2×10^4 copies/mL) at week 3. The difference between the HHV-6B DNA levels at weeks 2 and 3 was not statistically significant ($P = .73$).

As a control, HHV-6B DNAemia was examined in 21 BMT patients at weeks 2, 3, and 4. In 4 (19%) of 21 BMT patients, HHV-6B DNAemia was found in the serum sample. One patient developed HHV-6B DNAemia with 2×10^2 copies/mL at week 2 and 1×10^3 copies/mL at week 3, 2 patients developed HHV-6B DNAemia with 7×10^3 copies/mL and 1×10^4 copies/mL only at week 2, and the fourth patient developed DNAemia with 4×10^3 copies/mL only at week 3. HHV-6B DNAemia was not found at week 4. The probabilities for HHV-6B DNAemia at week 2, week 3, and week 2 or 3 after CBT were significantly higher than after BMT ($P < .001$, $P < .0001$, and $P < .0001$, respectively). The HHV-6B DNA levels at weeks 2 and 3 after CBT were also higher than after BMT ($P < .01$, and $P < .001$, respectively).

Table 2.
Human Herpesvirus 6 Variant B (HHV-6B) DNA Levels, Skin Rash, and Grade II-IV Acute Graft-versus-Host Disease (aGVHD) in Cord Blood Transplantation Patients*

Patient No.	HHV-6B DNA, copies/mL				Rash, d	aGVHD, grade
	Week 1†	Week 2	Week 3	Week 4		
1	—	ND	1×10^4	ND	13	II
2	ND	2×10^4	ND	ND	No	I
3	ND	ND	1×10^4	ND	13	II
4	ND	ND	ND	ND	13	I
5	ND	4×10^4	1×10^4	ND	11	II
6	ND	2×10^4	2×10^4	3×10^2	13	IV
7	ND	2×10^2	2×10^2	ND	No	I
8	ND	ND	5×10^3	ND	No	0
9	—	1×10^4	9×10^3	ND	11	I
10	ND	ND	ND	ND	No	II
11	ND	ND	ND	ND	No	0
12	ND	2×10^3	1×10^3	ND	12	I
13	ND	1×10^4	3×10^2	ND	12	II
14	ND	ND	4×10^3	ND	15	I
15	ND	1×10^4	1×10^3	ND	11	II
16	ND	2×10^4	2×10^3	ND	11	II
17	ND	ND	2×10^3	ND	16	III
18	ND	4×10^2	5×10^3	ND	8	II
19	ND	2×10^4	ND	ND	No	I
20	ND	2×10^3	2×10^4	2×10^2	10	II
21	ND	2×10^2	5×10^3	6×10^5	10	III
22	—	6×10^2	ND	ND	No	0
23	ND	6×10^4	ND	ND	No	I

*ND indicates not detected.
†The HHV-6B DNA level at week 1 was not examined in 3 patients (nos. 1, 9, and 22).

3.2. Associations of HHV-6B Infection after CBT

3.2.1. Early Skin Rash

Within 21 days after CBT, 15 (65%) of 23 patients developed an erythematous maculopapular skin rash (Table 2). The onset was a median of 12 days (range, 8-16 days) after CBT. In all of the 15 patients, the skin rash was accompanied by a high-grade fever higher than 39.0°C. A skin biopsy was performed on the onset day for 2 patients (nos. 13 and 16) and 3 days after the onset of the skin rash for 1 patient (no. 12). There was a sparse lymphocytic infiltration within the epidermis in all patients, and apoptotic figures were observed in 1 patient (no. 12). The skin rash was clinically and histologically indistinguishable from that of aGVHD. A skin rash was observed in 14 (70%) of 20 patients with HHV-6B DNAemia at any week and in 1 (33%) of the 3 patients without it. The association of skin rash with HHV-6B DNAemia at any week was not statistically significant ($P = .27$). Next, the association of skin rash with HHV-6B DNAemia at week 2 or at week 3 was examined separately. A skin rash was observed in 10 (67%) of 15 patients with HHV-6B DNAemia at week 2 and in 5 (63%) of 8 patients without it ($P = .60$). In contrast, a skin rash was observed in 14 (88%) of 16 patients with HHV-6B DNAemia at week 3 and in 1 (14%) of 7 patients without it ($P < .005$). The median HHV-6B DNA level at week 3 in patients with skin rash was 4×10^3 copies/mL (range, $<2 \times 10^2$ to 2×10^4 copies/mL), which was higher than the median level ($<2 \times 10^2$ copies/mL; range, $<2 \times 10^2$ to 5×10^3 copies/mL) found in those without a skin rash ($P < .005$).

3.2.2. Grade II-IV aGVHD

Because skin rash occurring within 21 days after CBT was not distinguishable from that of aGVHD, even by histologic examination, a skin rash was considered to be the skin manifestation of aGVHD in this study. Twelve (52%) of 23 patients developed grade II-IV aGVHD at a median of 32 days after CBT (range, 10-42 days). Grade II-IV aGVHD was observed in 11 (55%) of 20 patients with HHV-6B DNAemia at any week and in 1 (33%) of 3 patients without it. The association of grade II-IV aGVHD with HHV-6B DNAemia at any week was not statistically significant ($P = .47$). Next, the association of HHV-6B DNAemia at week 2 or at week 3 was examined separately. Grade II-IV aGVHD was observed in 8 (53%) of 15 patients with HHV-6B DNAemia at week 2 and in 4 (50%) of 8 patients without it ($P = .61$). In contrast, grade II-IV aGVHD was observed in 11 (69%) of 16 patients with HHV-6B DNAemia at week 3 and in 1 (14%) of 7 patients without it ($P < .05$). The median HHV-6B DNA level at week 3 in patients with grade II-IV aGVHD was 5×10^3 copies/mL (range, $<2 \times 10^2$ to 2×10^4 copies/mL), which was higher than the median level ($<2 \times 10^2$ copies/mL; range, $<2 \times 10^2$ to 5×10^3 copies/mL) of those with grade 0 to I aGVHD ($P < .05$).

4. Discussion

HHV-6B infection in adults after CBT was studied by means of quantitative PCR on serum samples. In 20 (87%) of 23 CBT patients, positive HHV-6 DNAemia was found in at

least 1 sample. This incidence was much higher than after BMT (19%). This report is the first to compare the incidences of HHV-6B DNAemia and the viral loads after CBT and BMT in adults. In children, Sashihara et al [12] detected HHV-6 DNA in PBMCs more frequently in CBT patients than in BMT or PBSCT patients (100% [7 of 7] versus 56% [9 of 16]). A lack of primed HHV-6–specific T-cells in the infused CB units and the immunologic immaturity of CB lymphocytes may be associated with the higher rate of HHV-6 infection after CBT.

We used a real-time quantitative PCR method on serum samples to study HHV-6 infection. In previous studies, detection of HHV-6 DNA in PBMCs by PCR had been used to examine HHV-6 infection in SCT patients. However, this method may detect both latent and active infections. In contrast, several studies have shown that the detection of viral DNA in serum or plasma is a marker of active infection [6,7]. In addition, reverse transcriptase–PCR for detecting both immediate-early and late gene transcripts can distinguish active and latent infection and may be a useful method for monitoring active viral infection [13]. For most CBT patients, however, obtaining sufficient RNA samples from PBMCs is difficult because most patients do not attain neutrophil engraftment within 3 weeks after CBT. Therefore, the measurement of viral DNA in serum or plasma by real-time PCR methods may give a clinical benefit to CBT patients.

The association between HHV-6 infection and skin rash or aGVHD has been reported [3-5]. There are 2 possible explanations for this association. First, HHV-6 infection in the skin or other organs may trigger or enhance aGVHD. Yoshikawa et al [14] suggested that the alterations in surface molecule expression caused by HHV-6 infection might enhance the infiltration of inflammatory cells into epidermal tissues and result in the skin rash. The second possible explanation is that the presence of severe aGVHD and the effects of immunosuppressive therapy may trigger or enhance HHV-6 infection. We found that HHV-6B DNAemia at week 3 was associated with skin rash and grade II-IV aGVHD after CBT. All 4 CBT patients with positive HHV-6B DNAemia only at week 2 (nos. 2, 19, 22, and 23) did not develop skin rash or grade II-IV aGVHD. These results suggest that transient HHV-6B infection prior to sufficient lymphocyte recovery may not induce strong lymphocyte reactions leading to skin rash or severe aGVHD. However, the reason for the association of skin rash and grade II-IV aGVHD with HHV-6B DNAemia only at week 3 was unclear. Because our study included only a small number of patients, prospective large-scale studies are needed to determine the role of HHV-6 infection in CBT patients.

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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.

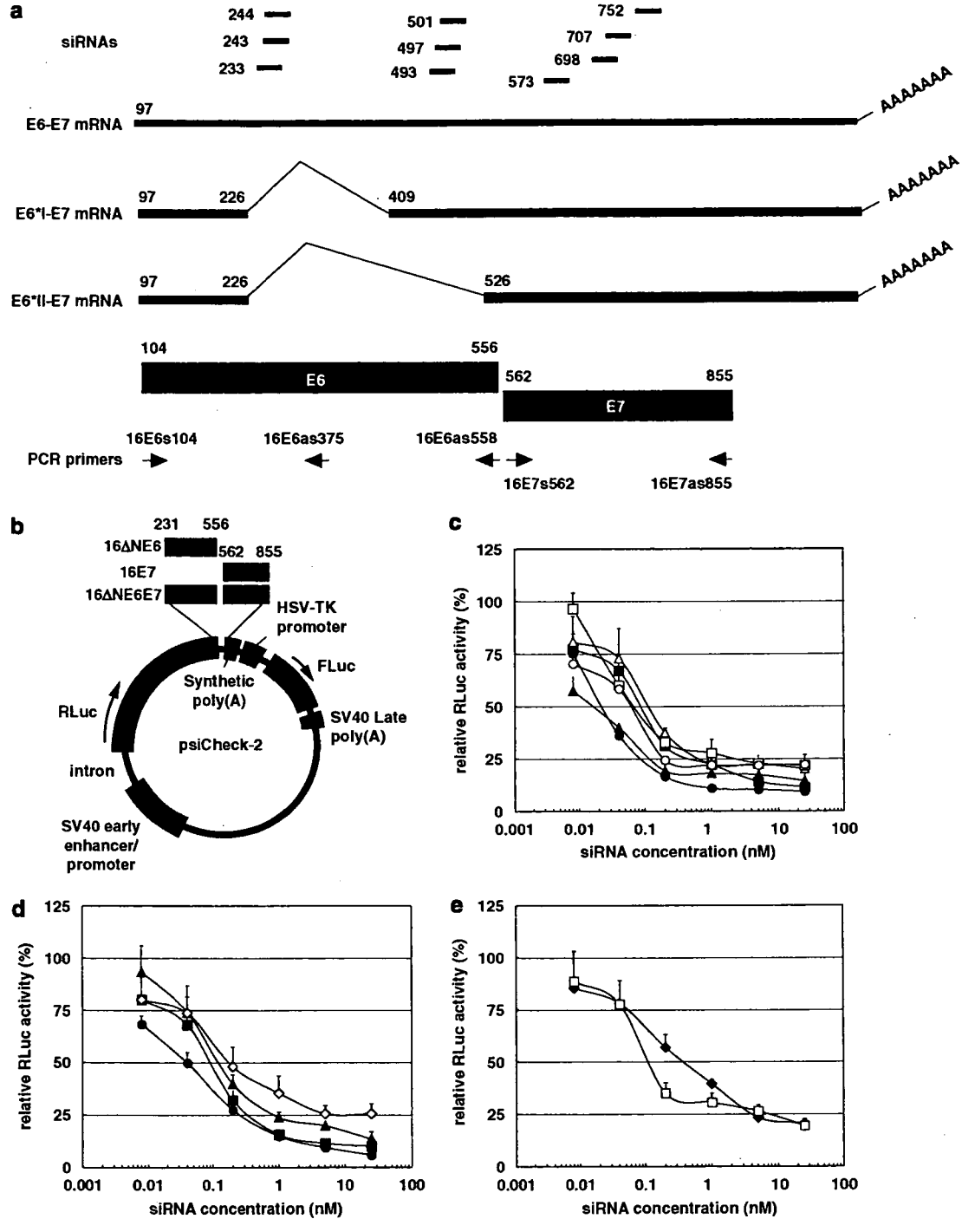


Figure 1 siDirect-selected siRNAs targeting HPV16 E6 and E7, and their effects on luciferase-E6 and -E7 fusion gene expression. (**a**) E6 and E7 siRNAs, mRNA splicing variants, genomic organization and PCR primers. siRNAs targeting E6 and E7 are depicted as short lines (top). Thick bars represent naturally 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

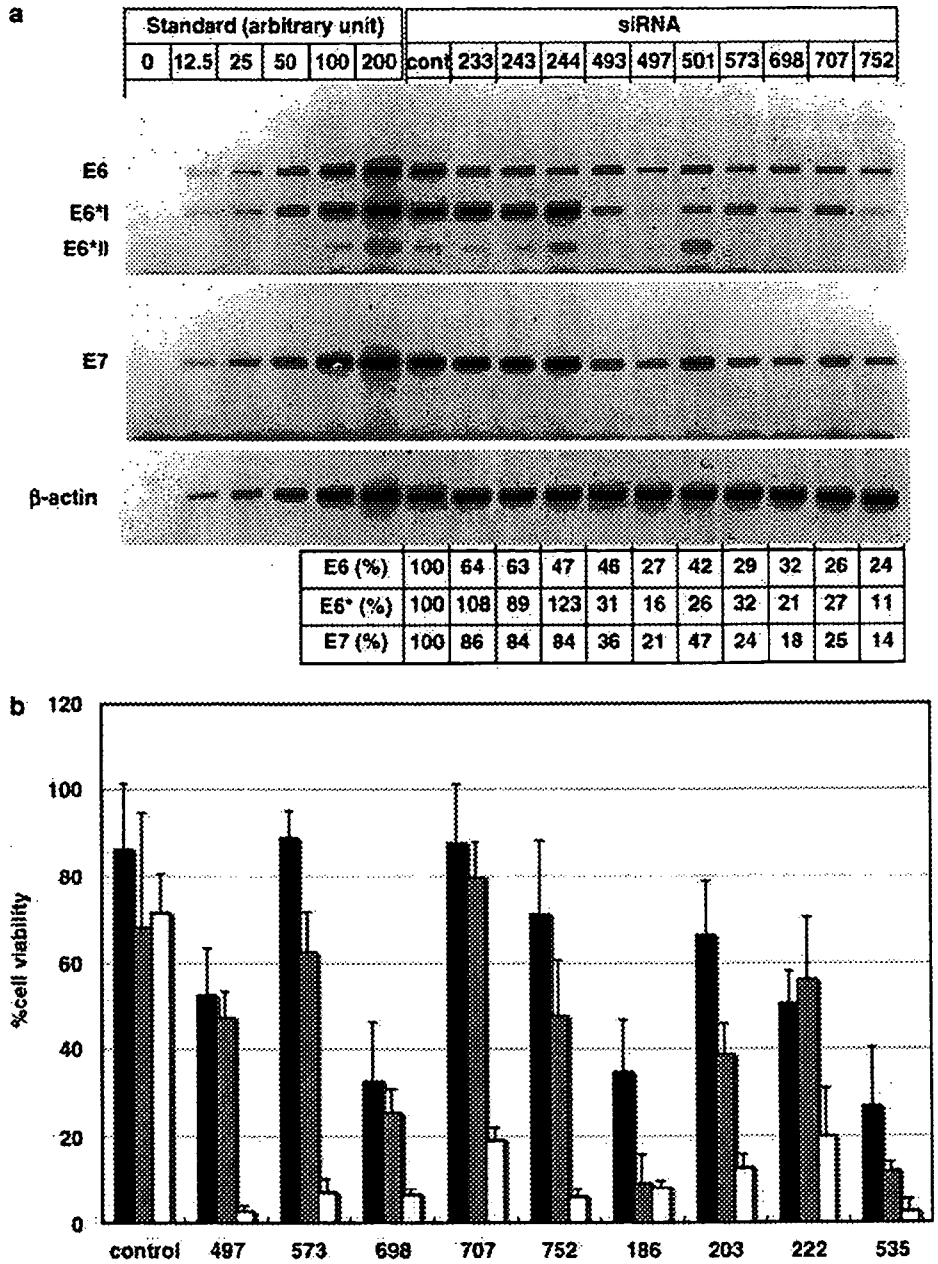


Figure 2 Effects of E6 and E7 siRNAs on E6 and E7 expression and cell growth. (a) Suppression of endogenous E6 and E7 expression in SiHa cells by E6 and E7 siRNAs. SiHa cells were transfected with mock, control, E6 or E7 siRNA (50 nM) for 48 h. cDNA was synthesized from total RNA and subjected to PCR. 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

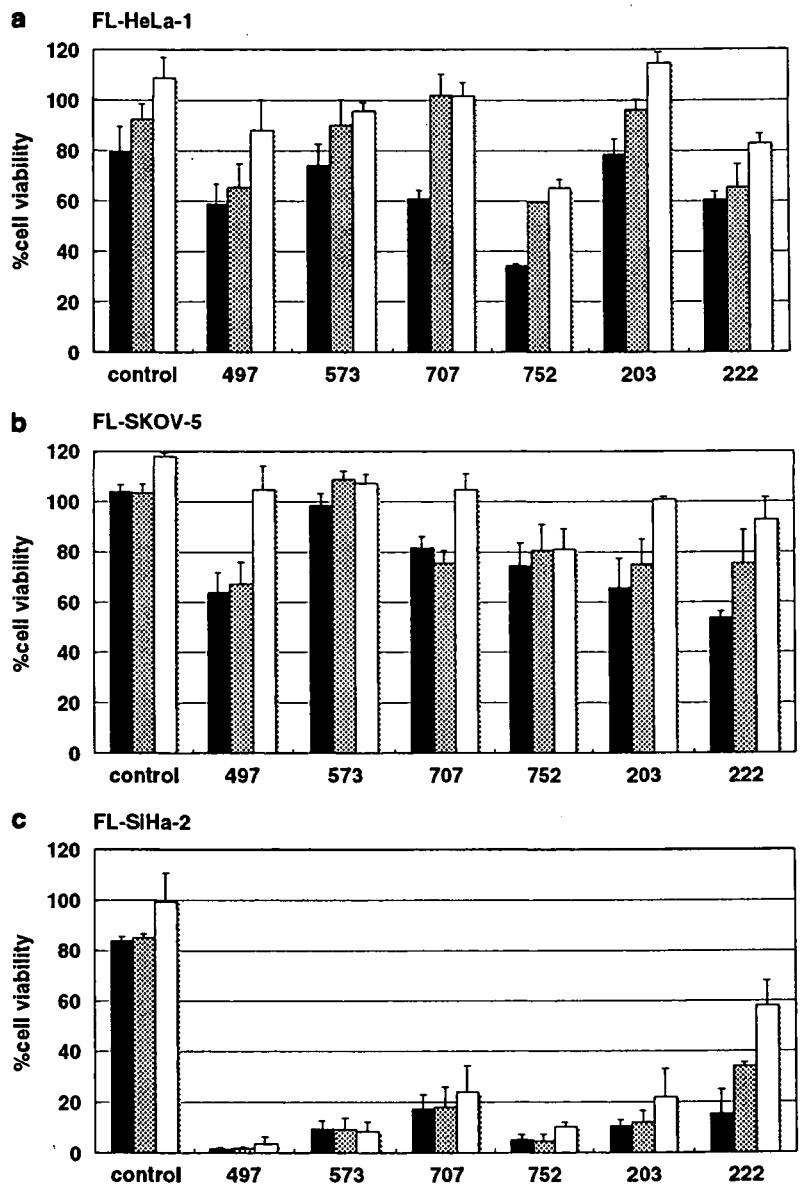
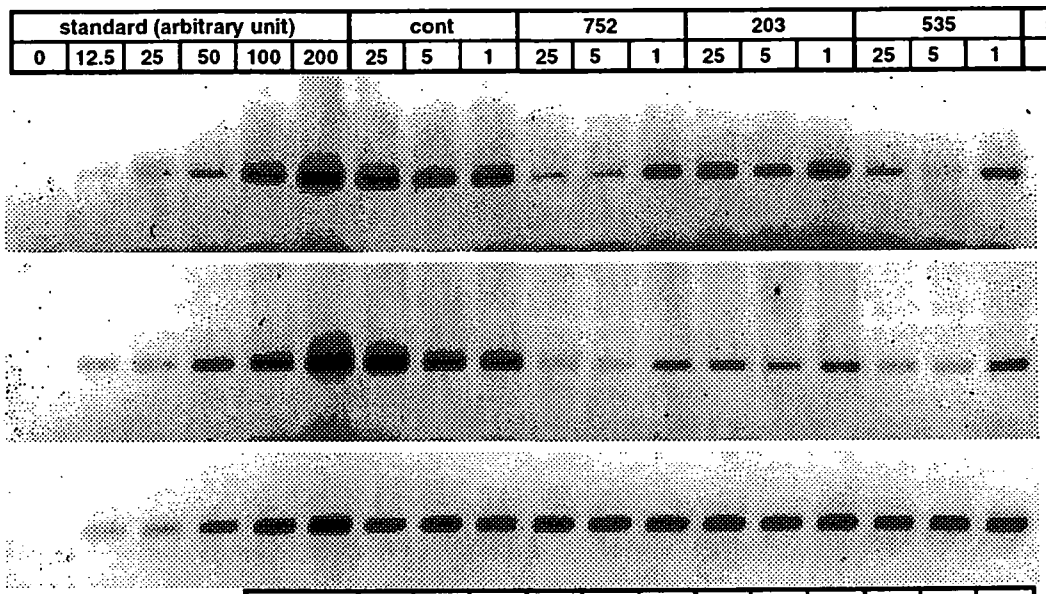


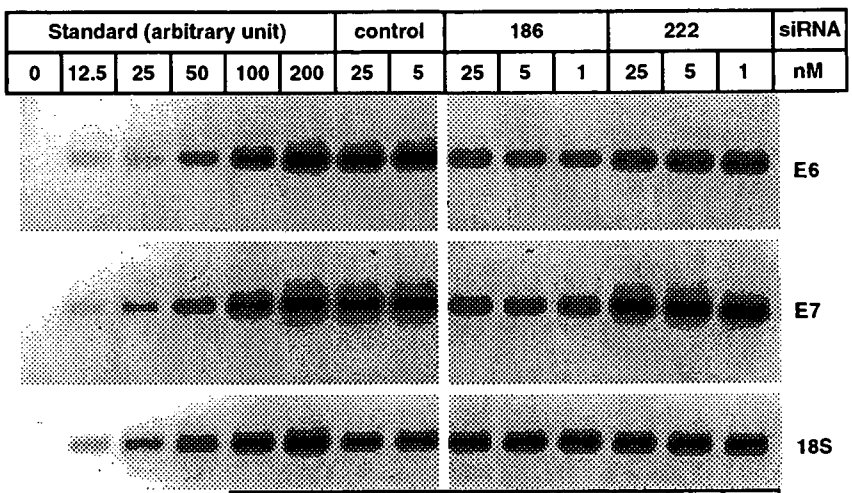
Figure 3 Dose-dependent effects of HPV16 E6 and E7 siRNAs on growth of HPV16– and HPV16+ cancer cells, and endogenous E6 and E7 expression. Dose-dependent effects of E6 and E7 siRNAs on growth of FL-HeLa-1 (**a**), FL-SKOV-6 HPV16– (**b**) and FL-SiHa-2 HPV16+ cells (**c**). Cells were plated in 96-well 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.

d



E6	120	92	99	37	40	70	77	67	94	47	29	54
E7	153	101	87	18	17	41	42	37	44	26	21	44
18S	80	91	83	86	84	90	84	72	82	80	85	85
E6(%)	100	100	100	29	48	52	61	93	96	39	34	54
E7(%)	100	100	100	11	19	43	26	46	51	17	22	50

e



E6	174	188	70	64	63	95	106	108
E7	162	178	69	65	75	176	209	206
18S	83	88	103	124	127	118	121	102
E6(%)	100	100	32	25	24	38	42	50
E7(%)	100	100	34	27	30	76	88	103

Figure 3 Continued

those siRNAs toward E6 expression. The level of p53 level induced by 535 was disproportionate to its effect on E6 mRNA, which might have been caused by an off-target effect. CaSki cells were transfected with Oligofectamine, since Lipofectamine 2000 is cytotoxic to CaSki cells. Transfection of lamin A/C siRNA (50 nM) decreased the level of lamin A/C to 35%, suggesting that at least two-thirds of the cells were transfected with Oligofectamine (Figure 4f). The accumulation of p53 by siRNA 573 and 752 was obscure in CaSki cells as compared to SiHa cells, which was likely due to dilution of accumulated p53 by nontransfected cells containing low levels of p53.

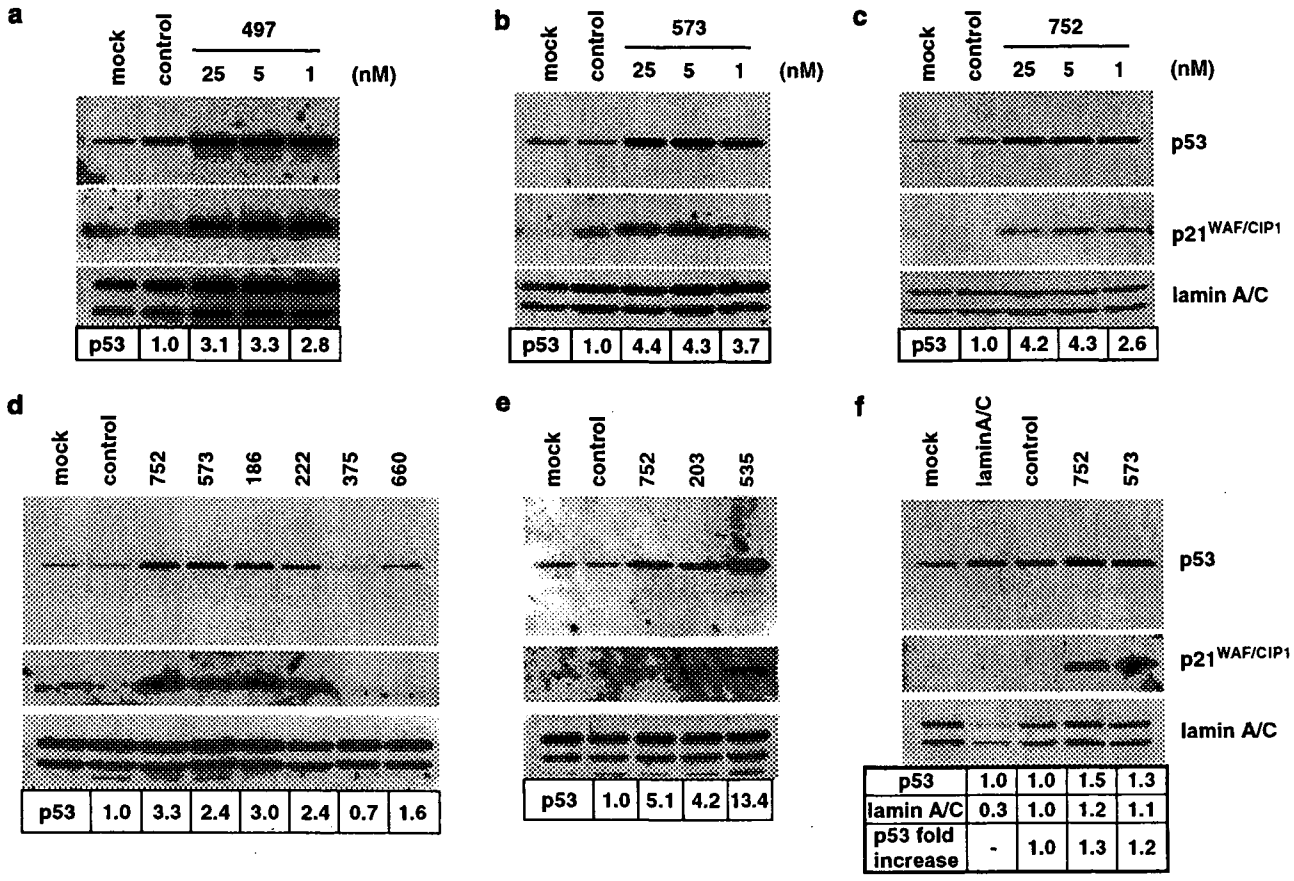


Figure 4 Effects of E6 and E7 siRNAs on p53 and p21$^{WAF1/CIP1}$ expression in HPV16+ cancer cells. FL-SiHa-2 cells were transected with mock, control, 497 (**a**), 573 (**b**) or 752 siRNA (**c**) at the indicated concentrations. SiHa cells were transfected with mock, control, 752, 573, 186, 222, 375 or 660 siRNA (**d**), or 203 or 535 siRNA (**e**) at 25 nM. CaSki cells were transfected with mock, control, 573 or 752 siRNA at 50 nM (**f**). The cells were incubated for 72 h, then analyzed for the expression of p53, p21$^{WAF1/CIP1}$ and lamin A/C by immunoblotting. p53 levels were normalized to those of lamin A/C. Lamin A/C siRNA was used to assess siRNA transfection efficiency in CaSki cells.

However, both siRNAs induced p21$^{WAF1/CIP1}$ expression in those cells, suggesting that their suppression of E6 expression caused p53 accumulation in cells effectively transfected with the siRNAs.

Induction of senescence in cervical cancer cells treated with siRNA 573 and 752

To clarify the mechanism by which E6 and E7 siRNAs induced growth suppression in cervical cancer cells, FL-SiHa-2 cells were transfected separately with siRNA 573 and 752 at 5 nM every 3 days, with cell morphological changes closely observed under an inverted microscope. Seven days after the first transfection, both 573- and 752-siRNA-transfected cells become larger and flatter, whereas mock and control siRNA-transfected cells did not change (data not shown). In 12–14 days after the first transfection, most E7 siRNA-treated cells were found to have long cytoplasmic projections and became positive for senescence associated β-galactosidase (SA β-gal) staining (Figure 5a). Furthermore, SA β-gal activity was detected in the perinuclear areas of the E7 siRNA-treated cells. These results suggested that the E7 siRNAs caused growth inhibition in HPV16+ cells by inducing cellular senescence.

Inhibition of tumor growth initiated by SiHa cells in NOD/SCID mice by siRNA 752

SiHa cells at 2×10^7 were subcutaneously inoculated into NOD/SCID mice. Six weeks after inoculation, palpable tumors had formed in all of the mice, and control or 752 siRNA complexed with atelocollagen was directly injected into each tumor every 7 days. Thirty days after the first siRNA injection, the tumors were excised and weighed. As shown in Figure 5b, tumors treated with siRNA 752 had significantly lower weights than those treated with control siRNA ($P<0.001$, mean weight: 2.73 ± 0.66 g in the control siRNA-treated group; $n=6$ versus 1.03 ± 0.29 g in the 752-treated group; $n=6$), which showed that treatment with siRNA 752 decreased *in vivo* tumor growth caused by SiHa cells.

Effects of E7 siRNAs on E7 variant expression

Three siRNAs (497, 573, 752) were chosen from 10 siDirect-selected siRNAs based on their high levels of RNAi activity and specificity. We then performed a

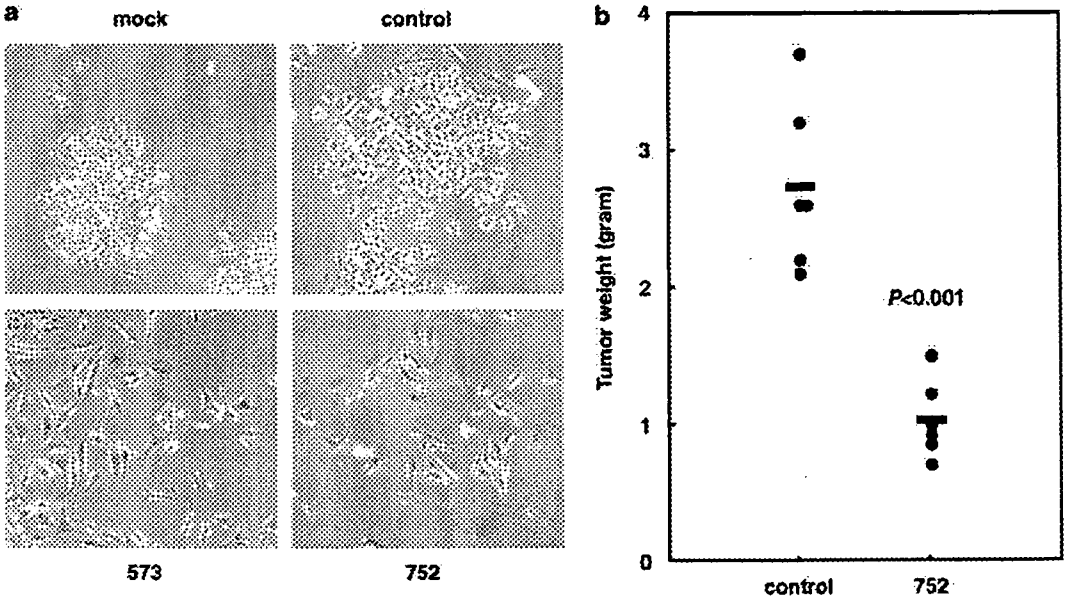


Figure 5 Induction of senescence and *in vivo* tumor growth of HPV16+ cancer cells by HPV16 E7 siRNA. (**a**) FL-SiHa-2 cells were transfected with mock, control, 573 or 752 siRNA at 5 nM using Lipofectamine 2000 every 3 days for 14 days, and then stained for senescence-associated β-galactosidase activity. Representative microscopy photographs are shown. (**b**) Suppression of *in vivo* tumor growth of SiHa cells by E7 siRNA. SiHa cells were subcutaneously inoculated into NOD/SCID mice ($n=6$). Six weeks after transplantation palpable tumors had developed, and then the control or 752 siRNA (500 pmole) complexed with atelocollagen was directly injected into the tumors every 7 days. Thirty-five days after the first siRNA injection, all tumors were excised and weighed. Bars represent the mean results ($n=6$).

literature search for E6 and E7 variant sequences that had a mismatch with these siRNAs.[32–37] A minor E7 variant, E7(760c), which contained cytosine instead of thymine at nucleotide 760,[35] was found to have a mismatch with siRNA 752. We tested the RNAi activity of 752 toward the expression of E7(760c) using a reporter gene assay. SiHa cells were transfected with the RLuc-E7(760c) fusion mRNA expression plasmid along with siRNA 752 or 752c, which was perfectly matched to E7(760C) (Supplementary Figure 1). Although both 752 and 752c suppressed the expression of RLuc-E7(760c), the 1-base mismatch decreased the IC_{50} value by fivefold (0.021 nM of 752c siRNA versus 0.107 nM of 752 siRNA). This result suggested that perfect sequence complementarity between the guide strand and target mRNA, especially in the middle portion of the guide strand, is important for RISC enzyme efficiency.

Discussion

With the aim of identifying new drug candidates for HPV16 infection and virus-related cancer, we designed siRNAs targeting HPV16 E6 and E7 oncogenes using siDirect computer software,[27] and analyzed their RNAi activities and specificity. The siDirect software is based on guidelines for the selection of effective siRNA sequences developed by Ui-Tei *et al.*[26] and enables selection of siRNAs with structural advantages for efficient loading of guide strands into RISC,[26,30,31] as well as a minimal number of off-target candidates from a database of nonredundant sequence sets of human genes. In the present study, reporter gene assays using luciferase-target fusion gene expression plasmids and our designed siRNAs revealed that all siDirect-designed siRNAs possessed high RNAi activity, as expected. However, the levels of suppression of endogenous E6-E7 mRNA in HPV16+ cancer cells varied among them. Five of the 10 siRNAs suppressed the expression of target mRNA by more than 75%, while the others showed suppression of 35–65%. These results suggest that RISC loading is not a sole determinant of RNAi activity and other factors, such as accessibility of RISC to the target sequence,[38–40] target cleavage and release of cleaved RNA, might be involved in the RNAi activities of designed siRNAs. Target accessibility is assumed to be due to the RNA secondary structure and interaction of target mRNA with other molecules. Thus, structural prediction of target mRNA would be beneficial for designing siRNAs.

Despite initial reports showing a high specificity for siRNA-directed RNAi,[10,11] recent studies have revealed that siRNAs induce off-target effects by several mechanisms.[20,25,41] An siRNA can tolerate some mismatches with its target mRNA when undergoing directed target cleavage, though enzyme efficiency decreases with increases in the number of mismatches.[25] In fact, we observed that a single nucleotide mismatch in the central portion of an siRNA targeting mRNA caused a fivefold decrease in the value of IC_{50}. siRNA sequences selected by siDirect software have at least three base mismatches with nonredundant sequence sets of human genes.[27]