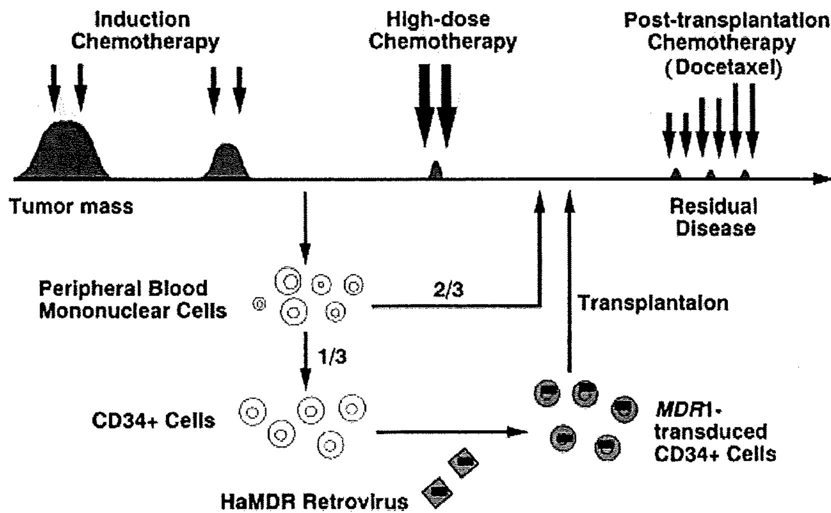

Fig. 1. The outline of multidrug resistance 1 (*MDR1*) gene therapy. After remission induction chemotherapy for metastatic breast cancer, peripheral blood stem cells (PBSC) were harvested from patients in remission, and one-third were transduced with retroviral *MDR1* vector. The patients then received high-dose chemotherapy with transplantation of both *MDR1*-transduced and unprocessed PBSC. After bone marrow reconstitution, the patients received docetaxel chemotherapy.

Patient selection. The protocol outline is shown in Fig. 1. Histologically confirmed, metastatic breast cancer patients who achieved good PR or CR to a precedent conventional dose chemotherapy regimen (using anthracycline and/or taxane) were selected. Other eligibility criteria were patients: (1) who had signed informed consent; (2) who had no serious active disease other than breast cancer; (3) whose age was 20–60 years; (4) whose Eastern Cooperative Oncology Group performance status was 0 or 1; (5) patients whose life expectancy was 3 months; (6) who had adequate physiological functions (white blood cells [WBC] = 3 500/μL, hemoglobin [Hb] = 9.0 g/dL, platelets = 100 000/μL, creatinine clearance = 60 mL/min [24 h], total serum bilirubin = 1.0 mg/dL, serum glutamic oxaloacetic transaminase [GOT], glutamic pyruvic transaminase [GPT] = 100 U/L, left ventricular ejection fraction = 60%, electrocardiogram [ECG] within normal limits); (7) whose bone marrow aspirate and biopsy showed no cancer cells; and (8) who had neither a past history of serious allergic reaction, bone metastases, brain metastases, irradiation for pelvis, were not pregnant or lactating, or had active infection.

Hematopoietic stem cell harvest, CD34-positive cell selection and gene transfer. The HaMDR retrovirus used in the present study carries wild-type (Gly-185) *MDR1* cDNA isolated from a human placenta cDNA library[19] in the Harvey sarcoma virus backbone. The HaMDR producer clone 3P26, with a retrovirus titer of 10^7 c.f.u./mL, was established in our laboratory. The clinical-grade HaMDR retrovirus was prepared by MAGENTA (BioReliance, Rockville, MD, USA) under a contract with our foundation. GMP-grade human cytokines, stem cell factor (SCF), Flk2/Flt3-ligand (FL-ligand), interleukin (IL)-6 soluble receptor (sIL-6R) and thrombopoietin (TPO), were obtained from R&D Systems (Minneapolis, MN, USA). GMP-grade human IL-6 was a generous gift of Serono Japan (Tokyo, Japan).

Patients were treated with 2 g/m^2 cyclophosphamide, and then 300 μg/day granulocyte colony-stimulating factor [G-CSF] at days 10–15. Peripheral blood stem cells (PBSC) were harvested at days 13–15 using the mononuclear cell collection procedure of the COBE Spectra Cell Separator (Gambro BCT, Lakewood, CO, USA). In this protocol, one-third of the total cells were transduced with the *MDR1* gene, and two-thirds were returned to the patients without genetic modification. To do this, cells harvested at day 14 were used for CD34 selection and gene transfer, and cells harvested at days 13 and 15 were frozen directly in liquid nitrogen. Harvest goals were a minimum of 2×10^6 CD34-positive cells/kg for non-transduced cells. CD34-positive cells were isolated from freshly harvested PBSC of the patient at day 14 by magnetic microbead selection using Isolex 50 Stem Cell Reagent kit (Nexell Therapeutics, Irvine, CA, USA) in the cell-processing center of our institution. The CD34-positive cells were subsequently cultured in a medium containing 50 ng/mL SCF, 100 ng/mL FL-ligand, 300 ng/mL sIL-6R, 20 ng/mL TPO and 100 ng/mL IL-6. At 36 h after the initiation of the *ex vivo* cultures, cells were cultured with HaMDR retrovirus on plates coated with CH-296 (Takara Bio, Otsu, Japan) for 4 h, then without the virus for 8 h. The culture was repeated three times over 36 h. The cells were then washed and cryopreserved. The expression of human P-gp protein in the transduced cells was examined by fluorescence-activated cell sorter [FACS] analysis. Contamination of breast cancer cells was also checked by cytocentrifugation of 10^6 PBSC and immunohistochemical examination using an EPIMET epithelial cell detection kit (Baxter, Unterschleissheim, Germany) after CD34 selection (day 14) and after *MDR1* gene transfer (day 17).

Transplantation and post-transplantation chemotherapy. One to two months after the final PBSC harvest, the patients received HDCT with 2 g/m^2 cyclophosphamide, 67 mg/m^2 thiotepa and 533 mg/m^2 carboplatin per day for 3 days. Three additional days after the completion of HDCT, the *MDR1*-transduced CD34-positive cells (approximately one-third of the transplanted CD34-positive cells) and unmodified cells (approximately two-thirds of the total transplanted CD34-positive cells) were reinfused to the patient simultaneously.

After bone marrow was reconstituted, patients were treated with 30 mg/m^2 docetacel (50% of the standard dose in Japan) per 3 weeks, and then the docetaxel dose was planned to be increased to 45 mg/m^2, then to 60 mg/m^2, if grade 4 neutropenia was not observed.

P-glycoprotein expression in the peripheral mononuclear cells of the patient. After transplantation, peripheral blood of patients was obtained at each time point. Mononuclear cells (MNC) were separated by Ficoll-Hypaque density gradient centrifugation. A portion of the MNC were used for P-gp FACS and *MDR1* polymerase chain reaction (PCR) analyses. The rest were cryopreserved for other experiments. To examine the expression levels of human P-gp on the surface of the *MDR1*-transduced hematopoietic cells, FACS analysis was carried out. Cells were incubated with the F(ab′)$_2$ fragment of MRK16 (100 μg/mL),[20] then washed and incubated with R-phycoerythrin-conjugated streptavidin (Becton Dickinson Biosciences, San Jose, CA, USA). The fluorescence staining level was analyzed using FACScan (Becton-Dickinson Biosciences).

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Table 1. Patient characteristics

Patient no.	Date of recurrence	Site of disease	Protocol	Response	Date of transplantaion	Response	Comments
1	March 2000	Lung	FAC	PR	4/01	CR	PD April 2007
2	March 1999	Lymph node	FAC	PR	10/01	CR	PD July 2005
3	October 2001	Lymph node, liver	DOX, DTX	Near CR	8/04	Near CR	PD March 2005

CR, complete response; DOX, doxorubicin; DTX, docetaxel; FAC, fluorouracil, doxorubicin, cyclophosphamide; NED, no evidence of disease; PD, progressive disease; PR, partial response.

Polymerase chain reaction from the peripheral MNC of the patient. Genomic DNA was extracted using a DNA Blood Mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. HaMDR provirus DNA was amplified with the following PCR primers: forward 5′-TGGGGGTTGGGGA-TTTAG-3′ and reverse 5′-GCACCAAAATGAAACCTG-3′. The PCR conditions were as follows: 95°C for 9 min; then 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; and final extension of 15 min at 72°C. Then the second PCR was carried out with the same procedure, using 10% of the first PCR product as template DNA. PCR products were analyzed on agarose gels.

Linear amplification-mediated PCR. Possible clonal expansion of the *MDR1*-tranduced cells was examined by linear amplification-mediated (LAM)-PCR.[21] The genomic–proviral junction sequence was preamplified by 50 cycles of primer extension using 0.25 pmol of vector-specific, 5′-biotinylated primer LTR1 (5′-AGCTGTTCTATCTGTTCTTGGCCCT-3′) with AmpliTaq Gold DNA Polymerase (2.5 U; Applied Biosystems, Foster, CA, USA) from 100 ng of each sample DNA. Selection of biotinylated extension products was carried out with a μMACS Streptavidin Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The samples were incubated with Klenow polymerase (4.4 U; Invitrogen, Carlsbad, CA, USA), dNTPs (300 μM; Applied Biosystems), and random hexanucleotide mixture (Roche Diagnostics, Mannheim, Germany) in a volume of 50 μL for 1 h at 37°C. Samples were washed on the magnetic particle concentrator and incubated with *Mae*II endonuclease (16 U in 100 μL; Roche Diagnostics) for 1 h at 50°C. After an additional wash step, 100 pmol of a double-stranded asymmetric linker cassette and T4 DNA Ligase (5 U; Invitrogen) were incubated with the beads in a volume of 40 μL at 4°C overnight. Then the ligase was purified with the magnetic beads. Each ligation product was amplified with AmpliTaq Gold DNA Polymerase, 25 pmol of vector-specific primer LTR2 (5′-GACCTTGATCTGAACTTCTC-3′), and linker cassette primer LC1 (5′-GACCCGGGAGATCTGAATTC-3′), using the same conditions as the PCR of the *MDR1* gene. Each PCR product served as a template for a second, nested PCR with internal primers LTR3 (5′-TCCATGCCTTGTAAAATGGC-3′; QIAGEN) and LC2 (5′-GATCTGAATTCAGTGGCACAG-3′; QIAGEN) under the same conditions. PCR products were analyzed on agarose gels.

Results

Clinical courses. Three patients have were treated under the protocol. The first and second patients received transplantation in 2001, and the third in 2004. The characteristics of the patients who received *MDR1*-transduced CD34-positive cells are shown in Table 1.

Patient 1. This patient was diagnosed with breast cancer and underwent surgery on 14 May 1990. Pathological diagnosis was scirrhous adenocarcinoma. In March 2000, multiple lung nodules were detected by computed tomography (CT) scan, and transbronchial biopsy revealed lung metastases of breast cancer. From April 2000 until September 2000, the patient received fluorouracil, doxorubicin, cyclophosphamide (FAC) chemotherapy and entered into PR. After informed consent, the first and the second PBSC harvest and *MDR1* gene transfer were done in November 2000 and February 2001, respectively. In April 2001, the patient received HDCT and the transplantation of *MDR1*-transduced CD34-positive cells. From June 2001 until February 2002, the patient received 10 cycles of docetaxel chemotherapy. The patient entered into CR after five cycles of docetaxel in October 2001. In April 2007, pleural nodules appeared at CT scan, and positron emission tomography (PET) scan confirmed breast cancer relapse. The patient showed no sign of secondary malignancies.

Patient 2. This patient was diagnosed with breast cancer and underwent surgery on 29 August 1990. Pathological diagnosis was solid tubular adenocarcinoma. In May 1999, left supraclavicular and parasternal lymph nodes were detected by ultrasonography and CT scan, and aspiration biopsy revealed metastases of breast cancer. From July 1999 to November 1999, the patient received six cycles of FAC chemotherapy and entered into PR. The patient then received six cycles of docetaxel and irradiation to supraclavicular lymph nodes. After informed consent, the first, second and third PBSC harvests and *MDR1* gene transfer were done in August 2000, January 2001 and April 2001, respectively. In October 2001, the patient received HDCT with transplantation of *MDR1*-transduced hematopoietic stem cells. The patient entered into CR after HDCT in November 2001. From May 2002 until August 2002 the patient received five cycles of docetaxel chemotherapy. In July 2005, the patient showed parasternal lymph node swelling at ultrasound and fine needle aspiration biopsy showed breast cancer relapse.

The third patient received transplantation of the *MDR1*-transduced cells in 2004, 18 months after the harvest, due to the adverse event of the X-linked severe combined immune deficiency (X-SCID) gene therapy in France. The patient then began receiving post-transplantation chemotherapy of vinorelbine. During chemotherapy, the patient developed liver metastases in March 2005. The patient was treated with some chemotherapeutic and endocrine therapies since then.

CD34 selection and *MDR1* gene transduction of PBSC. CD34-positive cells from the three patients were transduced with clinical-grade HaMDR retrovirus. After HDCT, the *MDR1*-transduced cells were reinfused to the patients. Results of CD34 selection and transduction efficiency in the three patients are summarized in Table 2. In this protocol, the total number of untransduced CD34-positive cells should be more than 2×10^6/kg. The number of untransduced cells in all of the patients was large enough to satisfy the requirement. Transduction efficiency of *in vitro MDR1* gene transfer to PBSC was 8–17% by flow cytometry (Fig. 2). Transplanted P-gp-expressing cells were $1.1–3.7 \times 10^5$/kg, which was approximately 3–7% of the transplanted CD34-positive cells. Safety examinations of the transduced cells (including the sarcoma-positive/leukemia-negative [S^+L^-] test) were carried out at SRL (Tokyo, Japan). The S^+L^- tests of the *MDR1*-transduced cells of each patient were all negative. Contamination of breast cancer cells into the transduced cells was also ruled out by cytokeratin immunohistochemistry (data not shown).

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Table 2. Reinfused cells

Patient no.	Total no. CD34⁺ cells reinfused (×10⁶/kg)	Untreated CD34⁺ cells reinfused (×10⁶/kg)	HaMDR-transduced CD34⁺ cells (× 10⁶/kg)	Transduction efficiency (% P-gp⁺)	Estimated no. of HaMDR-transduced cells reinfused (×10⁵/kg)	Ratio of P-gp⁺ cells in total reinfused cells (% P-gp⁺)
1	5.5	3.5	2.0	17	3.7	6.7
2	3.0	2.2	0.8	14	1.1	3.7
3	9.4	6.0	3.4	8	2.6	2.8

P-gp, P-glycoprotein.

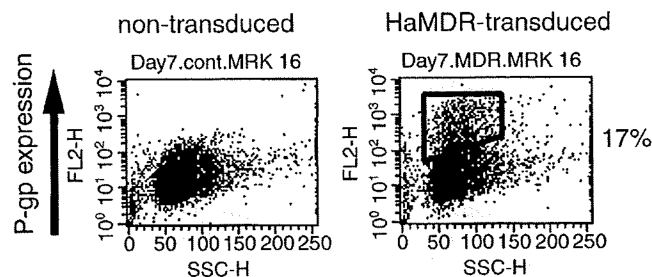

Fig. 2. *Ex vivo* multidrug resistance 1 (*MDR1*) transduction of peripheral blood stem cells (PBSC) in patient 1. CD34-positive cells were isolated from freshly harvested PBSC of the patients using an Isolex 50 Stem Cell Reagent kit. The CD34-enriched cells were then stimulated with granulocyte colony-stimulating factor (G-CSF), thrombopoietin (TPO), interleukin (IL)-6, sIL-6R and Flk2/Flt3-ligand (FL-ligand), and transduced with HaMDR viral supernatant. Non-transduced or HaMDR-transduced cells were incubated with the F(ab')$_2$ fragment of MRK16, and were washed and incubated with R-phycoerythrin-conjugated streptavidin. The fluorescence staining level was analyzed using FACScan. The analysis was carried out after 2 days of stem cell amplification, 2 days of viral transfer, and then 2 days of further culture. P-gp, P-glycoprotein.

Increase in MDR1-transduced leukocytes during docetaxel chemotherapy. Patients received HDCT with transplantation of the *MDR1*-transduced cells. After the recovery of bone marrow function, the patients were treated with docetaxel. Expression of P-gp in the peripheral leukocytes was investigated by FACS analysis. In patient 1 (upper panel of Fig. 3B), P-gp-positive cells were not detected until day 5, but started to increase at day 7, culminated near 5% at day 12, then decreased. The ratio of P-gp-positive cells decreased to 1% in 2 months. Docetaxel treatment was started at a dose of 30 mg/m^2 and increased to 45 mg/m^2, but further dose increases were not done because of grade 4 neutropenia during the second dose. In the first or second cycles of docetaxel treatment at 45 mg/m^2, the ratios of P-gp-positive cells in the peripheral blood of the patients at day 4 were approximately 1%. They increased transiently to 2–3% at day 8, and then decreased again within 2 weeks (upper panel of Fig. 3B). In the fourth to tenth cycles of docetaxel, the ratios of P-gp-positive cells prior to the chemotherapy were 3–4%, increasing to 6–10% after docetaxel treatment, and then fell to 3–4% within 2 weeks. This basal level remained unchanged over the next half year following completion of the treatment. In patient 2 (middle panel of Fig. 3B), P-gp-positive cells in the peripheral blood appeared at day 10 and increased to 4%, then decreased. In accordance with the patient's wishes, docetaxel chemotherapy was delayed until 200 days after transplantation. In the meantime, P-gp-positive cells continued to decrease and became undetectable. However, during five cycles of docetaxel chemotherapy (30–45 mg/m^2), P-gp-positive cells in the peripheral blood became detectable. They then became undetectable again after treatment with five cycles of docetaxel. These data clearly show that *MDR1*-transduced leukocytes were enriched by docetaxel chemotherapy *in vivo*. In patient 3, we observed 3% of P-gp-positive cells in the peripheral blood 7–12 days after the transplantation. They then decreased to less than 1% at day 19 and became very low in 2 months, and did not seem to increase with vinorelbine therapy (lower panel of Fig. 3B).

Semiquantitative PCR of HaMDR DNA in the peripheral blood cells also showed *in vivo* enrichment of *MDR1*-transduced leukocytes following docetaxel chemotherapy (Fig. 3C). In patient 1, first PCR showed a relative decrease of HaMDR DNA from days 12 to 40, but the PCR band widened during docetaxel chemotherapy. In patient 2, the PCR band decreased after transplantation, and became undetectable even with the second PCR at day 170. However, during docetaxel chemotherapy, HaMDR DNA became detectable at the first PCR. In patient 3, vinorelbine chemotherapy did not seem to increase HaMDR DNA.

Changes in peripheral neutrophil counts after docetaxel chemotherapy. When we compared neutrophil recovery after docetaxel chemotherapy in patient 1, who had a higher ratio of *MDR1*-transduced peripheral blood leukocytes, and patient 2, who had a lower ratio, neutrophil recovery in patient 2 was delayed even with G-CSF administration (Fig. 4).

Follow-up of the three patients. After cessation of docetaxel chemotherapy, two patients were in CR and were followed without chemotherapy. They received annual systemic follow-up (CT, bone scintigraphy, ultrasonography and tumor markers) and showed no sign of relapse 3 years after transplantation. Peripheral blood counts and blood chemistry showed no serious adverse events. The first patient showed relapse at pleura in April 2007, and started aromatase inhibitor. In July 2005, the second patient showed relapse in the parasternal lymph node, and started capecitabine monotherapy. She has had stable disease since then. The third patient was treated with capecitabine, mitomycin C and methotrexate, then anastrozole after vinorelbine, but liver metastases has progressed gradually. She complained of dyspnea at July 2006, and showed massive pleural metastases. She died from respiratory failure due to pleural metastases in December 2006. All three patients showed no sign of secondary malignancy.

Clonality analysis of HaMDR-transduced leukocytes. The *MDR1*-transduced cells of the first and second patients decreased gradually and almost disappeared by the end of 2004. Using LAM-PCR, we identified several *MDR1*-tranduced peripheral blood leukocyte clones at various time points after transplantation (Fig. 5). However, there was no indication of the amplification of specific clones. Analysis of insertion sites of *MDR1* retrovirus are ongoing in our laboratory (Mitsuhashi *et al*., unpublished data).

Discussion

A group at the M. D. Anderson Cancer Center (Houston, TX, USA) first reported results of a clinical trial of retroviral *MDR1* gene therapy.[15] They carried out retroviral gene transfer without the use of cytokines, either with PBSC alone or PBSC with autologous stromal cells. The *in vitro* transduction efficiency was 2.8% with the solution method and 5.6% with the stromal

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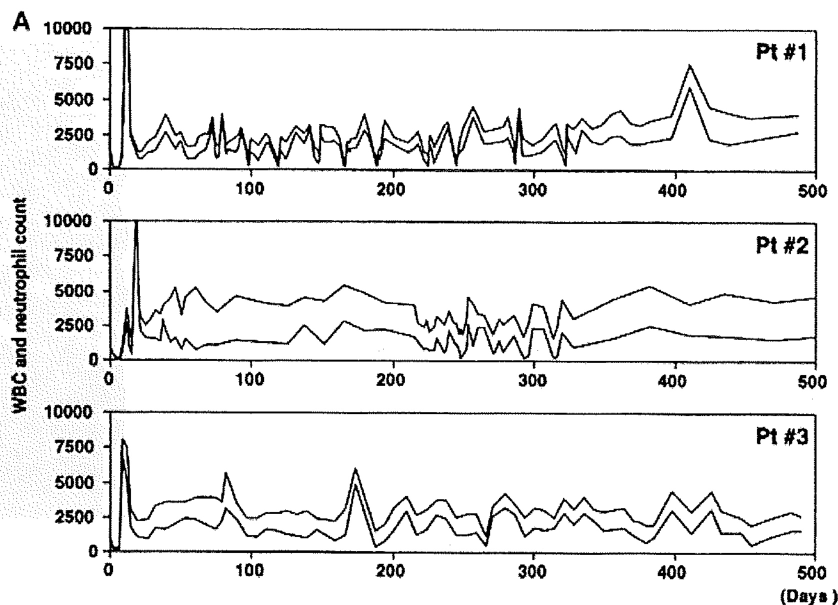

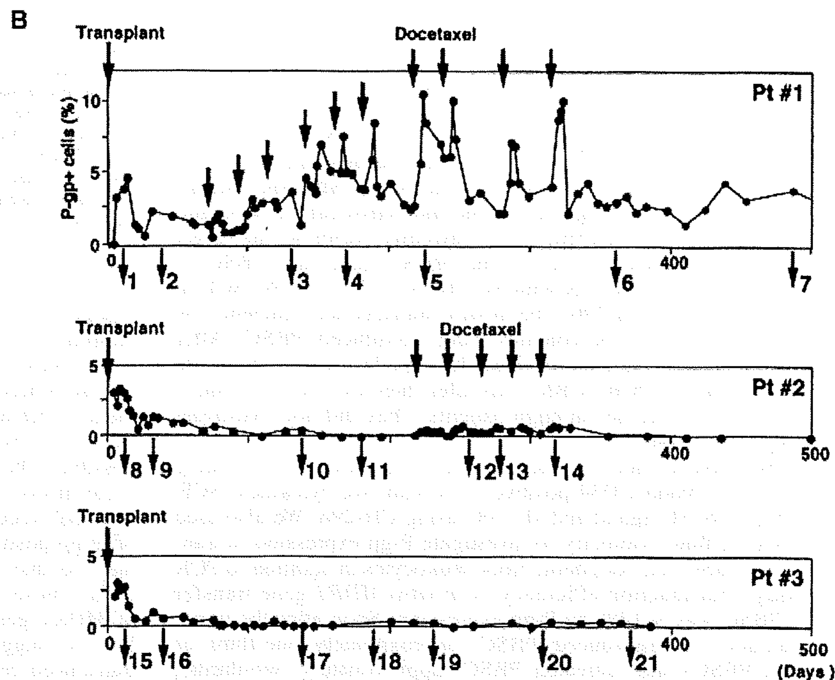

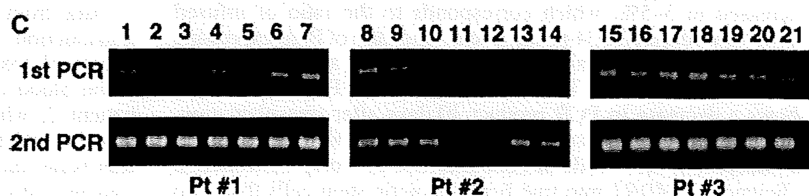

Fig. 3. Changes in (A) white blood cells (WBC) and neutrophils, and (B) P-glycoprotein (P-gp)-positive cells after peripheral blood stem cell transplantation (PBSCT) and (C) semiquantitative polymerase chain reaction (PCR) of HaMDR DNA in peripheral blood cells of the three patients. Docetaxel chemotherapy was started 60 days after PBSCT in patient 1 and 208 days after PBSCT in patient 2. The patients were then treated with 30 mg/m^2 (first course) or 45 mg/m^2 (second course and later) docetaxel (black arrow). (A) Plots of total white blood cells (upper line) and neutrophils (lower line). (B) Mononuclear cells (MNC) were separated from peripheral blood by Ficoll-Hypaque density gradient centrifugation, incubated with the F(ab′)$_2$ fragment of MRK16, and washed and incubated with R-phycoerythrin-conjugated streptavidin. The fluorescence staining level was analyzed using FACScan. (C) Genomic DNA was extracted from peripheral blood of the three patients at various time points. Multidrug resistance 1 (*MDR1*) genes were amplified with the PCR primers for 40 cycles. Then the second PCR was carried out with the same primers. PCR products were analyzed on agarose gels. Patient 1: 1, day 12 after PBSCT; 2, day 40; 3, day 131 (after third docetaxel chemotherapy); 4, day 170 (after fifth docetaxel); 5, day 226 (after seventh docetaxel); 6, day 362 (after 10th docetaxel); 7, day 488. Patient 2: 8, day 12 after PBSCT; 9, day 33; 10, day 138; 11, day 170; 12, day 247 (after second docetaxel chemotherapy); 13, day 268 (after third docetaxel); 14, day 310 (after fifth docetaxel). Patient 3: 15, day 12 after PBSCT; 16, day 40; 17, day 139; 18, day 188; 19, day 231; 20, day 308; 21, day 371.

method, detected by *in situ* PCR. However, 3–4 weeks after transplantation, direct PCR assay of peripheral blood leukocytes in the patients showed positive results in 0/10 with the solution method, and 5/8 with the stromal method. These data show insufficient transduction efficiency without using cytokines. The National Cancer Institute (NCI) also reported the results of a clinical trial of retroviral *MDR1* gene therapy.[16] They transferred the *MDR1* gene into bone marrow mononuclear cells or PBSC stimulated by IL-3, IL-6 and SCF. The *ex vivo* transduction efficiency was 0.2–0.5%. They treated transplanted patients with 175 mg/m^2 paclitaxel, but they could not show any enrichment of *MDR1*-positive white blood cells by PCR. A group at Columbia University also transferred the *MDR1* gene into bone marrow mononuclear cells or peripheral blood stem

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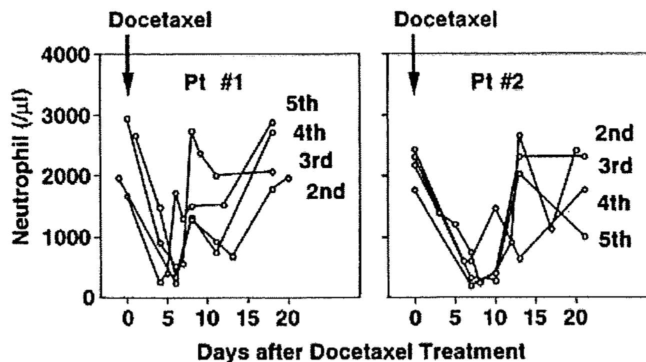

Fig. 4. Comparison of neutrophil count after docetaxel chemotherapy in patients 1 and 2. The changes in neutrophil count after the second to fifth docetaxel chemotherapy (45 mg/m²) in patients 1 and 2 are shown.

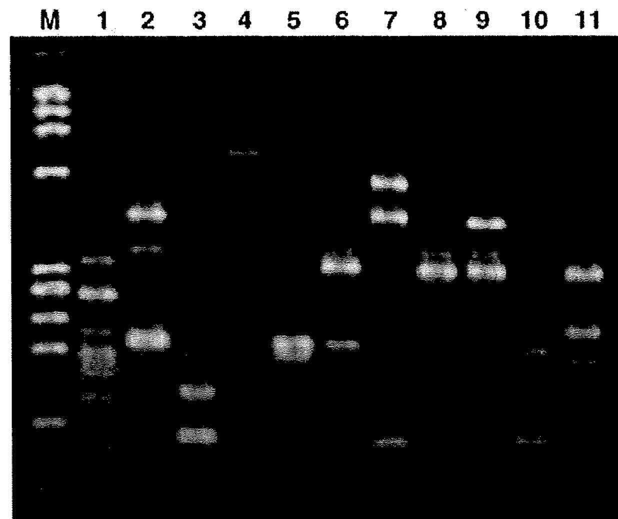

Fig. 5. Linear amplification-mediated (LAM)-polymerase chain reaction (PCR) analysis of HaMDR integration sites in the peripheral blood leukocytes of patient 1. Genomic DNA was extracted from peripheral blood of the first patient at various time points. The genomic–proviral junction sequence was preamplified by repeated primer. Biotinylated extension products were selected, treated with Klenow fragment and incubated with *Mae*II. The product was then treated with T4 DNA Ligase and amplified with AmpliTaq Gold DNA polymerase with vector-specific primer and linker cassette primer, then further amplified with the same primer set. PCR products were analyzed on agarose gels. 1, day 12 after PBSCT; 2, day 40; 3, day 112 (after second docetaxel chemotherapy); 4, day 152 (after fourth docetaxel); 5, day 196 (after sixth docetaxel); 6, day 257 (after eighth docetaxel); 7, day 327 (after 10th docetaxel); 8, day 586; 9, day 727; 10, day 762; 11, day 790.

cells stimulated by IL-3, IL-6 and SCF.[17] They showed that 20–70% of burst-forming unit-erythroid (BFU-E) and colony-forming unit–granulocyte macrophage (CFU-GM) colonies from *MDR1*-transferred CD34-positive cells were positive for *MDR1* by PCR. Bone marrow (BM) from patients 3–12 weeks after transplantation was shown to be *MDR1*-positive by PCR in 2/5 patients. They also analyzed P-gp expression in bone marrow cells using flow cytometry, but they could not show any expression.

A group at Indiana University (Indianapolis, IN, USA) tried to increase transduction efficiency by using recombinant fibronectin fragment (CH-296).[18] CH-296 contains specific adhesion domains for hematopoietic progenitor cells and retroviral vectors, and can increase the attachment of retrovirus vector to the target CD34 cells. They transferred the *MDR1* gene into PBSC of germ cell tumor patients with SCF/IL-6 or G-CSF/TPO/SCF in the presence of CH-296. The *in vitro* transduction efficiency by PCR was 4–52% of colonies from transduced PBSC. After transplantation, patients received three cycles of oral etoposide therapy. *MDR1* PCR of BM cells after three cycles was positive in 3–10% of colonies in eight patients. They did not investigate P-gp expression.

We tried to increase transduction efficiency by stimulating peripheral blood CD34-positive cells with five cytokines (SCF, TPO, IL-6, FL-ligand and sIL-6R) using CH-296. We also used MRK-16 flow cytometry to investigate P-gp expression in tranduced PBSC and peripheral blood leukocytes in addition to PCR assay. Transduction efficiency of *in vitro MDR1* gene transfer to PBSC was 8–17% by P-gp expression. Soon after the transplantation of transduced PBSC (approximately one-third of total PBSC) and untreated PBSC (approximately two-thirds), the ratio of P-gp-positive cells in peripheral white blood cells increased to 3–5%, which corresponds to the ratio of infused *MDR1*-transduced CD34-positive cells. The ratio of P-gp-expressing cells in the peripheral blood decreased to an undetectable level within 6 months. This corresponds to the PCR data, which shows a decrease in PCR product 30 days after transplantation. This might be due to a survival disadvantage of *MDR1*-transduced hematopoietic stem cells in bone marrow, or worse transduction efficiency of *MDR1* into true hematopoietic stem cells than into more differentiated stem cells.

Docetaxel chemotherapy increased the ratio of P-gp-positive white blood cells in patients 1 and 2. This shows the following two things. The first is that *MDR1*-transduced leukocytes can be maintained for a long time. In patient 1, *MDR1* gene-transduced white blood cells can be detected even 3 years after transplantation or 2.5 years after cessation of docetaxel chemotherapy (data not shown). *MDR1* gene transfer into 'true' long-term repopulating hematopoietic stem cells may be possible, but multilineage engraftment of *MDR1*-transduced blood cells has not be proven, because PCR of HaMDR DNA of each cell lineage from peripheral blood is difficult. The second is that enrichment of *MDR1* gene-transduced blood cells and hematopoietic stem cells by P-gp-correlated chemotherapeutic drugs may be possible. The transient increase of P-gp-positive cells in every cycle of docetaxel might be due to selection of resistant differentiated precursor cells (e.g. CFU-GM), but the basal increase of P-gp-positive cells during several cycles of chemotherapy may be due to selection of P-gp-positive hematopoietic stem cells. These results not only demonstrate the possible utility of *MDR1* gene therapy for chemotherapy of various cancers, but also suggest the utility of the *MDR1* gene for selection of transduced cells for various gene therapies, as has been shown in several animal models.[22]

Data from only two patients cannot show whether *MDR1* transduction of PBSC protected hematopoietic cells from docetaxel toxicity. However, comparison of patient 1, whose white blood cells had a higher ratio of P-gp-positive cells, and patient 2, whose white blood cells had a lower ratio, showed that the higher ratio of P-gp-positive cells might correlate with less bone marrow toxicity. It is possible that even *MDR1* gene transfer into one-third of PBSC protects hematopoietic stem cells from chemotherapeutic toxicity. We could not increase the dose of docetaxel to the standard dose because of grade 4 neutropenia at 75% dose. *MDR1* gene transfer into total PBSC might protect hematopoietic stem cells more efficiently than transfer into one-third, and might make standard-dose chemotherapy after autologous stem cell transplantation easy to carry out.

Those two patients had been in CR for more than 3 years after transplantation of *MDR1*-transduced PBSC and docetaxel

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chemotherapy. The first patient relapsed 6 years after transplantation, and the second patient relapsed 3 years and 9 months after transplantation, but both are presenting in good physical condition. So far there has been no severe side-effects with *MDR1*-transduced PBSC other than those typically accompanying HDCT and peripheral blood stem cell transplantation (PBSCT).

At the end of 2002, occurrence of T-cell leukemia in two patients after gene therapy of X-SCID was reported. A genetic defect in the γC gene, which is a common domain of multiple IL receptors (e.g. IL-2R, IL-4R and IL-7R), causes severe defects of T cells and natural killer cells and severe immune deficiency in X-SCID patients. Retroviral γC gene transfer using autologous CD34-positive hematopoietic cells in X-SCID patients restored the immune system in 9 of 11 patients.[23] But T-cell leukemia occurred in two of those nine patients. In the leukemic cells, retroviral vector was inserted into the *LMO2* gene, which causes T-cell leukemogenesis.[24] The third leukemic French X-SCID patient was reported at the beginning of 2005. Those adverse events have warned of the risk of carcinogenicity in retrovirus gene therapy. After thorough investigation of retroviral gene therapy trials for hematopoietic stem cells all over the world, no leukemic event has been found in clinical gene therapy trials other than the French X-SCID trial.[25] Screening of the Mouse Retroviral Cancer Gene database showed that retroviral insertion into the γC and *LMO2* genes was found in two leukemic mice, and insertion into both genes was found in one mouse. This fact suggests that both genes are oncogenes, and that the two genes can collaborate.[26] In X-SCID gene therapy, a double hit with retroviral activation of *LMO2* and exogenous activated γC might be necessary for leukemogenesis. Retroviral gene therapy with non-oncogenic genes may have a low risk of cancer.[27]

In a previous study, Bunting *et al.* showed that transduction of murine bone marrow cells with an *MDR1* vector may cause a myeloproliferative syndrome in transplanted mice.[28] They suggested that P-gp overexpression in bone marrow cells might cause leukemogenesis, because transduction of the dihydrofolate reductase gene into bone marrow cells did not cause myeloproliferative syndrome. However, this suggestion was lately denied by the same group. They showed that leukemias following retroviral transfer of *MDR1* were driven by combinatorial insertional mutagenesis due to usage of high titer of retrovirus, not by P-gp overexpression.[29]

We have also started to analyze clonal amplification of each transduced clone. So far, we have not seen any abnormal proliferation. Integration site analyses are ongoing in our laboratory.

Chemotherapy for cancer increases the risk of secondary leukemia. In breast cancer, the use of anthracyclines or cyclophosphamide might cause secondary leukemia or myelodysplastic syndrome, although the risk is not so high (0.2–1.7% in 10 years).[30,31] HDCT with autologous hematopoietic stem cell transplantation is reported to increase the risk of leukemia, especially in lymphoma patients (2.5–18% in 5–6 years).[32] However, HDCT for breast cancer is not accompanied by a high risk of secondary leukemia (0.3–0.9%).[33] It is possible that a combination of conventional chemotherapy, HDCT and retroviral integration into special genes such as *LMO-2* causes leukemogenesis, but the probability of that event is thought to be low. So far, an increase of specific clones, *MDR1* gene-positive cells or P-gp-positive cells in peripheral blood has not been detected using PCR or flow cytometry in our three patients.

In conclusion, we carried out *MDR1* gene therapy for three recurrent breast cancer patients. After HDCT with transplantation of peripheral blood hematopoietic cells transduced with the *MDR1* gene, the *MDR1*-transduced leukocytes were increased by docetaxel chemotherapy at the level of both DNA PCR and P-gp protein expression. Two patients reached CR after HDCT or docetaxel treatment. No side-effects associated with the transplantation of the *MDR1*-transduced cells were observed. No evidence of abnormal proliferation of specific clones was obtained. The two patients have been disease-free for more than 3 years, and have been performance status 0. These results suggest the safety and feasibility of our *MDR1* gene therapy.

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References

1 Gregory WM, Smith P, Richards MA, Twelves CJ, Knight RK, Rubens RD. Chemotherapy of advanced breast cancer: outcome and prognostic factors. *Br J Cancer* 1993; **68**: 988–95.
2 Inoue K, Ogawa M, Horikoshi N *et al.* Evaluation of prognostic factors for 233 patients with recurrent advanced breast cancer. *Jpn J Clin Oncol* 1991; **21**: 334–9.
3 Philip T, Guglielmi C, Hagenbeek A *et al.* Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma. *N Engl J Med* 1995; **333**: 1540–5.
4 Attal M, Harousseau JL, Stoppa AM *et al.* A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais du Myelome. *N Engl J Med* 1996; **335**: 91–7.
5 Ito Y, Mukaiyama T, Ogawa M *et al.* Epirubicin-containing high-dose chemotherapy followed by autologous hematopoietic progenitor cell transfusion for patients with chemotherapy-sensitive metastatic breast cancer: results of 5-year follow-up. *Cancer Chemother Pharmacol* 1999; **43**: 8–12.
6 Peters WP, Dansey RD, Klein JL, Baynes RD. High-dose chemotherapy and peripheral blood progenitor cell transplantation in the treatment of breast cancer. *Oncologist* 2000; **5**: 1–13.
7 Dunphy FR, Spitzer G, Fornoff JE *et al.* Factors predicting long-term survival for metastatic breast cancer patients treated with high-dose chemotherapy and bone marrow support. *Cancer* 1994; **73**: 2157–67.
8 Berry DA, Broadwater G, Klein JP *et al.* High-dose versus standard chemotherapy in metastatic breast cancer: comparison of Cancer and Leukemia Group B trials with data from the Autologous Blood and Marrow Transplant Registry. *J Clin Oncol* 2002; **20**: 743–50.
9 Crump M, Gluck S, Stewart D *et al.* A randomized trial of high-dose chemotherapy (HDC) with autologous peripheral blood stem cell support (ASCT) compared to standard therapy in women with metastatic breast cancer: a national cancer institute of Canada (NCIC) clinical trials group study. *Proc Am Soc Clin Oncol* 2001; **20**: 21a, #82.
10 Lotz JP, Cure H, Janvier M *et al.* High-dose chemotherapy with haematopoietic stem cell transplantation for metastatic breast cancer patients: final results of the French multicentric randomised CMA/PEGASE 04 protocol. *Eur J Cancer* 2005; **41**: 71–80.
11 Attal M, Harousseau JL, Facon T *et al.* Single versus double autologous stem-cell transplantation for multiple myeloma. *N Engl J Med* 2003; **349**: 2495–502.
12 Schmid P, Schippinger W, Nitsch T *et al.* Up-front tandem high-dose chemotherapy compared with standard chemotherapy with doxorubicin and paclitaxel in metastatic breast cancer: results of a randomized trial. *J Clin Oncol* 2005; **23**: 432–40.
13 Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, Gottesman MM. P-glycoprotein: from genomics to mechanism. *Oncogene* 2003; **22**: 7468–85.
14 Sorrentino BP, Brandt SJ, Bodine D *et al.* Selection of drug-resistant bone marrow cells *in vivo* after retroviral transfer of human MDR1. *Science* 1992; **257**: 99–103.
15 Hanania EG, Giles RE, Kavanagh J *et al.* Results of MDR-1 vector modification trial indicate that granulocyte/macrophage colony-forming unit cells do not contribute to posttransplant hematopoietic recovery following intensive systemic therapy. *Proc Natl Acad Sci USA* 1996; **93**: 15 346–51.
16 Cowan KH, Moscow JA, Huang H *et al.* Paclitaxel chemotherapy after autologous stem-cell transplantation and engraftment of hematopoietic cells transduced with a retrovirus containing the multidrug resistance complementary DNA (MDR1) in metastatic breast cancer patients. *Clin Cancer Res* 1999; **5**: 1619–28.

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17 Hesdorffer C, Ayello J, Ward M *et al.* Phase I trial of retroviral-mediated transfer of the human *MDR1* gene as marrow chemoprotection in patients undergoing high-dose chemotherapy and autologous stem-cell transplantation. *J Clin Oncol* 1998; **16**: 165–72.
18 Abonour R, Williams DA, Einhorn L *et al.* Efficient retrovirus-mediated transfer of the multidrug resistance 1 gene into autologous human long-term repopulating hematopoietic stem cells. *Nat Med* 2000; **6**: 652–8.
19 Kioka N, Tsubota J, Kakehi Y *et al.* P-glycoprotein gene (*MDR1*) cDNA from human adrenal: normal P-glycoprotein carries Gly185 with an altered pattern of multidrug resistance. *Biochem Biophys Res Commun* 1989; **162**: 224–31.
20 Hamada H, Tsuruo T. Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. *Proc Natl Acad Sci USA* 1986; **83**: 7785–9.
21 Schmidt M, Carbonaro DA, Speckmann C *et al.* Clonality analysis after retroviral-mediated gene transfer to CD34+ cells from the cord blood of ADA-deficient SCID neonates. *Nat Med* 2003; **9**: 463–8.
22 Sugimoto Y, Tsukahara S, Sato S *et al.* Drug-selected co-expression of P-glycoprotein and gp91 *in vivo* from an MDR1-bicistronic retrovirus vector Ha-MDR-IRES-gp91. *J Gene Med* 2003; **5**: 366–76.
23 Hacein-Bey-Abina S, Le Deist F, Carlier F *et al.* Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N Engl J Med* 2002; **346**: 1185–93.
24 McCormack MP, Rabbitts TH. Activation of the T-cell oncogene *LMO2* after gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 2004; **350**: 913–22.
25 Kohn DB, Sadelain M, Dunbar C *et al.* American Society of Gene Therapy (ASGT) ad hoc subcommittee on retroviral-mediated gene transfer to hematopoietic stem cells. *Mol Ther* 2003; **8**: 180–7.
26 Dave UP, Jenkins NA, Copeland NG. Gene therapy insertional mutagenesis insights. *Science* 2004; **303**: 333.
27 Berns A. Good news for gene therapy. *N Engl J Med* 2004; **350**: 1679–80.
28 Bunting KD, Galipeau J, Topham D, Benaim E, Sorrentino BP. Transduction of murine bone marrow cells with an MDR1 vector enables *ex vivo* stem cell expansion, but these expanded grafts cause a myeloproliferative syndrome in transplanted mice. *Blood* 1998; **92**: 2269–79.
29 Modlich U, Kustikova OS, Schmidt M *et al.* Leukemias following retroviral transfer of multidrug resistance 1 (MDR1) are driven by combinatorial insertional mutagenesis. *Blood* 2005; **105**: 4235–46.
30 Fisher B, Rockette H, Fisher ER, Wickerham DL, Redmond C, Brown A. Leukemia in breast cancer patients following adjuvant chemotherapy or postoperative radiation: the NSABP experience. *J Clin Oncol* 1985; **3**: 1640–58.
31 Curtis RE, Boice JD Jr, Moloney WC, Ries LG, Flannery JT. Leukemia following chemotherapy for breast cancer. *Cancer Res* 1990; **50**: 2741–6.
32 Milligan DW, Ruiz De Elvira MC, Kolb HJ *et al.* Secondary leukaemia and myelodysplasia after autografting for lymphoma: results from the EBMT. EBMT lymphoma and late effects working parties. European group for blood and marrow transplantation. *Br J Haematol* 1999; **106**: 1020–6.
33 Kroger N, Zander AR, Martinelli G *et al.* Low incidence of secondary myelodysplasia and acute myeloid leukemia after high-dose chemotherapy as adjuvant therapy for breast cancer patients: a study by the Solid Tumors Working Party of the European Group for Blood and Marrow Transplantation. *Ann Oncol* 2003; **14**: 554–8.

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Retroviral Integration Site Analysis and the Fate of Transduced Clones in an *MDR1* Gene Therapy Protocol Targeting Metastatic Breast Cancer

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ABSTRACT

A clinical study of an *MDR1* gene therapy protocol targeting metastatic breast cancer has been conducted in which the patients received high-dose chemotherapy, a transplant of *MDR1*-transduced autologous CD34^{+} cells, and docetaxel. We herein report the molecular results of a 6-year follow-up of an individual in this study (patient 1). HaMDR-transduced cells, which had been initially detected in the peripheral blood of this individual, were found to have gradually decreased. After 10 cycles of docetaxel (days 71–316), *MDR1* transgene levels were found to have increased, and then decreased to undetectable levels by day 1461. Thirty-eight *MDR1*-transduced clones were identified in patient 1, of which 11 showed a retroviral integration in close proximity to genes listed in the Retrovirus Tagged Cancer Gene Database (RTCGD). Four short-life clones in this group were found to harbor retroviral integrations close to the *ZFHX1B*, *NOTCH1*, *BMI1*, or *HHEX* gene; these genes have been frequently reported in the RTCGD. In addition, a long-lived RTCGD-hit clone, L-34, had a retroviral integration at a position 179 kb upstream of the *EVI1* gene. L-34 was detectable on days 327–1154, but became undetectable 3 years after the docetaxel treatments had ceased. An additional three docetaxel-induced long-life clones showed comparable polymerase chain reaction profiles, which were also similar to that of the total *MDR1*-transduced cells. Our results thus show that docetaxel may have been effective in promoting the expansion of several *MDR1*-transduced clones in patient 1, but that they persist in the peripheral blood for only a few years.

INTRODUCTION

The human multidrug resistance gene (*MDR1*) encodes the plasma membrane P-glycoprotein (ABCB1), which acts as an ATP-dependent efflux pump for various structurally unrelated natural product antitumor agents. Expression of the *MDR1* gene confers resistance to drugs such as anthracyclines, *Vinca* alkaloids, and taxanes (Gottesman and Ling, 2006). The ability of exogenous P-glycoprotein to protect hematopoietic cells from myelosuppression during cancer chemotherapy was first demonstrated in transgenic mice in which the *MDR1* cDNA was constitutively expressed in hematopoietic cells (Galski *et al.*, 1989; Mickisch *et al.*, 1991). Paclitaxel treatment of mice that had been transplanted with *MDR1* retrovirus-transduced bone marrow cells also resulted in a substantial enrichment of *MDR1*-positive cells (Podda *et al.*, 1992; Sorrentino *et al.*, 1992).

On the basis of these findings, clinical trials involving retroviral *MDR1* gene transfer have been conducted in which drug-sensitive hematopoietic cells derived from cancer patients are converted to drug-resistant cells to protect normal cells from intensive cancer chemotherapy (Hanania *et al.*, 1996; Hesdorffer *et al.*, 1998; Cowan *et al.*, 1999; Moscow *et al.*, 1999; Abonour *et al.*, 2000). In most cases, the patients received high-dose chemotherapy (HDCT) in conjunction with the transplantation of *MDR1*-transduced hematopoietic stem cells (HSCs),

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and then received additional chemotherapy with *MDR1*-related anticancer agents. The *in vivo* amplification of *MDR1*-transduced leukocytes has indeed been demonstrated in some of these clinical trials (Cowan *et al.*, 1999; Moscow *et al.*, 1999; Abonour *et al.*, 2000), but the ratios of the *MDR1*-positive cells in the peripheral blood were found to be low. Hence, the clinical benefits of using *MDR1* gene therapy to avoid myelosuppression have not yet been fully demonstrated.

We have also initiated a clinical study of the possible use of *MDR1* gene therapy to improve the efficacy and safety of chemotherapeutic drugs in the treatment of breast cancer (Takahashi *et al.*, 2007). Two patients (patients 1 and 2) received a transplantation of HaMDR retrovirus-transduced cells in 2001 and subsequently underwent posttransplantation docetaxel chemotherapy. A third patient also received this transplantation, but was then excluded from the protocol because of a progressive disease and thus no docetaxel chemotherapy was subsequently performed (Takahashi *et al.*, 2007).

Studies of retrovirus-mediated HSC gene therapy have been conducted for X-linked severe combined immunodeficiency (X-SCID), adenosine deaminase (ADA) deficiency, X-linked chronic granulomatous disease (X-CGD), and others, and clinical benefits have been demonstrated in the treatment of X-SCID and ADA-SCID (Blaese *et al.*, 1993; Malech *et al.*, 1997; Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002; Hacein-Bey-Abina *et al.*, 2002; Gaspar *et al.*, 2004; Ott *et al.*, 2006). X-SCID is an inherited immunodeficiency caused by the lack of common γ chain receptor (IL2RG). In earlier clinical trials, 9 of 10 infants who had been born with X-SCID were successfully treated with autologous HSCs transduced with the retrovirus carrying *IL2RG* cDNA. Unfortunately, however, 2 to 5 years after these transplantations, four of these children developed T cell leukemia. The leukemic cells of the first three patients harbored retroviral integrations in the *LMO2* locus. The *LMO2* gene encodes an LIM-only protein and is a target of chromosomal translocations in human T cell leukemia. Hence, the coexpression of retroviral *IL2RG* with activated *LMO2* is believed to be the underlying cause of the leukemia in these infants (Hacein-Bey-Abina *et al.*, 2003; Kohn *et al.*, 2003; McCormack and Rabbitts, 2004). After the reports of these adverse events, retroviral insertional mutagenesis has become a serious concern regarding the safety of HSC gene therapy, and many integration site analyses have been performed. In our current study, we show the results of our retroviral integration site analysis of the peripheral blood leukocytes of patient 1, and the associated persistence of each transduced clone *in vivo*. This is the first report of the clonal analysis of *MDR1*-transduced leukocytes.

MATERIALS AND METHODS

Clinical procedures

The HaMDR retrovirus used in this study carries a wild-type (Gly-185) *MDR1* cDNA insert in the Harvey sarcoma virus backbone (Kioka *et al.*, 1989; Hibino *et al.*, 1999; Sugimoto *et al.*, 2003). Our clinical protocol for *MDR1* gene therapy was approved by the Japanese government in February 2000. Briefly, advanced or recurrent breast cancer patients who responded well to conventional induction chemotherapy were enrolled in this protocol. After obtaining informed consent, peripheral blood mononuclear cells were harvested for three successive days from these patients. Cells harvested on two of the days were cryopreserved without any modification until transplantation. $CD34^+$ cells were separated from the cells harvested on the remaining day and then cultured in medium containing stem cell factor (50 ng/ml), Flk2/Flt3 ligand (100 ng/ml), soluble interleukin-6 receptor (300 ng/ml), thrombopoietin (20 ng/ml), and interleukin 6 (100 ng/ml). Thirty-six hours after initiation of the *ex vivo* cultures, the cells were transduced for 4 hr with HaMDR retrovirus on plates coated with CH-296, on three occasions over a 36-hr period. The cells were then washed and cryopreserved. After the biosafety tests of the transduced cells had been completed, the patients received HDCT consisting of cyclophosphamide (2 g/m^2), thiotepa (67 mg/m^2), and carboplatin (533 mg/m^2) daily for 3 days. Three days after the completion of HDCT, *MDR1*-transduced $CD34^+$ cells were reinfused together with unmodified cells. After their bone marrow function had been recovered, the patients received posttransplantation chemotherapy with docetaxel every 3–4 weeks. The docetaxel dose at the first treatment was 30 mg/m^2, which is 50% of the standard dose recommended in Japan, and the dose used from the second to tenth treatments was 45 mg/m^2.

MDR1 *polymerase chain reaction*

Genomic DNA extracted from peripheral blood mononuclear cells was preamplified with a GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences, Piscataway, NJ). HaMDR proviral DNA (100 ng/reaction) was amplified with 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 200 μM dNTPs (Applied Biosystems), and 25 pmol each of forward primer 5′-ATAGGGGTTTTACAAGAAT-3′ and reverse primer 5′-AGCATAGGAAAAATACATCA-3′. The polymerase chain reaction (PCR) conditions were as follows: 95°C for 9 min; then 30, 35, or 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and a final extension for 15 min at 72°C. This PCR amplifies a 657-bp fragment corresponding to region 2206–2862 from the first ATG of the *MDR1* cDNA.

Linear amplification-mediated polymerase chain reaction

The retroviral integration loci of the *MDR1*-transduced cells were amplified by linear amplification-mediated (LAM)-PCR (Schmidt *et al.*, 2002). First, the genomic–proviral junction sequence was preamplified by 50 cycles of primer extension, using 0.25 pmol of the HaMDR vector-specific, 5′-biotinylated primer LTR11 (5′-AGCTGTTCTATCTGTTCTTGGCCCT-3′), 2.5 U of AmpliTaq Gold DNA polymerase, and 100 ng of each DNA sample. The resulting products were then purified with a μMACS streptavidin kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The products linked to the magnetic beads were next incubated with a random hexanucleotide mixture (Roche Diagnostics, Mannheim, Germany) and 4.4 U of Klenow polymerase (Invitrogen, Carlsbad, CA) at 37°C for 1

hr, washed with a magnetic particle concentrator, and then digested with 16 U of *Mae*II endonuclease (Roche Diagnostics) at 50°C for 1 hr. After an additional washing step, the LAM-PCR products were ligated with 100 pmol of a double-stranded asymmetric linker cassette consisting of Linker4+ (5′-GACCCGGGAGATCTGAATTCAGTGGCACAGCA-3′) and Linker4– (5′-CGTGCTGTGCCACTGAATTCAGATCT-CCCGGGTC-3′). The ligation products were purified with the magnetic particle concentrator and then amplified by PCR with 25 pmol each of vector-specific primer LTR2 (5′-GACCT-TGATCTGAACTTCTC-3′) and linker cassette primer LC11 (5′-GACCCGGGAGATCTGAATTC-3′), and 2.5 U of AmpliTaq Gold DNA polymerase. The PCR conditions were as follows: 95°C for 9 min; then 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and a final extension for 15 min at 72°C. The PCR products then served as templates for a second, nested PCR using the internal primers LTR31 (5′-TCCATGCCTTGTAAAATGGC-3′) and LC12 (5′-GATCT-GAATTCAGTGGCACAG-3′) with the same cycling conditions. LAM-PCR products were subcloned into the TOPO TA cloning vector (Invitrogen) and then sequenced. The HaMDR proviral integration sites were then identified using the National Center for Biotechnology Information (NCBI, Bethesda, MD) BLASTN database (NCBI November 2006 assembly, http://ncbi.nih.gov/BLAST/). Genes located less than 500 kb from the proviral integration site were identified for each clone. We then examined whether these genes were homologous to any of the murine retroviral common integration sites (CISs) defined in the Retrovirus Tagged Cancer Gene Database (RTCGD; http://rtcgd.abcc.ncifcrf.gov/).

Clone-specific PCR

Clone-specific PCR was performed with long terminal repeat (LTR)-specific primer LTR3354 (5′-AAGGCACAGGG-TCATTTCAG-3′) and the appropriate clone-specific primers. The vector–genome junction for L-34 was amplified with primer P34-1 (5′-CTGGGAGGTGGGTATTATTG-3′). PCR was carried out with AccuPrime *Taq* DNA polymerase (Invitrogen) as follows: 94°C for 2 min; and then 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 68°C for 2 min. The vector–genome junctions of L-35 and L-36 were amplified with primers P35-1 (5′-ATTTGAGGATTAGACATACA-3′) and P36-1 (5′-GCTGAGGAGGAACAAAGATT-3′), respectively. PCR was carried out with AmpliTaq Gold DNA polymerase at 95°C for 9 min; followed by 50 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and a final extension for 15 min at 72°C. The vector–genome junctions of clones L-37 and L-38 were amplified by nested PCR. The first PCR for L-37 was carried out with primers P37-1 (5′-CTCAAGGGACAG-CCAGAAGG-3′) and LTR3354, and the second PCR was performed with primers P37-2 (5′-GTTTGAGGAAGGGACA-GATT-3′) and LTR3354. The first PCR for L-38 was carried out with primers P38-1 (5′-GACACAGGCTCACACCAAGG-3′) and LTR3354, and the second with primers P38-2 (5′-CA-CACCCACACCATAGACCT-3′) and LTR3354. These first and nested PCRs were performed with AccuPrime *Taq* DNA polymerase at 94°C for 2 min; followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 68°C for 2 min.

RESULTS

PCR analysis of MDR1 *transgene-positive cells in peripheral blood*

Patient 1 received a transplantation of *MDR1*-transduced, P-glycoprotein-positive cells (3.7×10^5/kg), which was 6.7% of the total reinfused CD34$^+$ cells (5.5×10^6/kg), in April 2001. This patient then received 10 cycles of consolidation chemotherapy with docetaxel on days 71, 92, 113, 141, 162, 183, 218, 239, 281, and 316 posttransplantation. The docetaxel dose on the first treatment (day 71) was 30 mg/m^2, and in treatments 2–10 was 45 mg/m^2, in accordance with the protocol. Serial peripheral blood samples were then obtained and tested for the presence of the *MDR1* transgene by PCR amplification of DNA samples collected between days 12 and 1832 (Fig. 1). The *MDR1* transgene was found to be present in the peripheral blood mononuclear cells obtained at the initial recovery phase after transplantation (days 12 and 40). These transgene levels were then observed to gradually decrease, however, with a nadir on day 257. On day 327, 11 days after the tenth round of docetaxel chemotherapy, the *MDR1* transgene levels were observed to have increased again, with the highest levels detectable on day 439.

In the period between days 327 and 727, the percentages of *MDR1*-transduced cells in the peripheral blood of patient 1 were estimated to be between 0.1 and 1.0. Further studies, including a direct comparison of docetaxel-treated and untreated patients, may be required, however, to definitively prove the effectiveness of docetaxel in the expansion of *MDR1*-transduced cells. As shown in Fig. 1, we could not detect the *MDR1* transgene in any of the blood samples taken from patient 1 after day 1461 posttransplantation. However, our monthly (or sometimes bimonthly) clinical examinations using two-cycle PCR revealed the presence of *MDR1*-transduced cells at low levels, even in the peripheral blood sample taken on day 2230, 6 years after the cell transplantation (data not shown).

Patient 2 also received a transplantation of *MDR1*-transduced, P-glycoprotein-positive cells (1.1×10^5/kg), which was 3.7% of the total reinfused CD34$^+$ cells (3.0×10^6/kg). This patient then received five cycles of consolidation chemotherapy with docetaxel on days 218, 240, 265, 286, and 307 posttransplantation. The docetaxel dose given at the first treatment (day 218) was 30 mg/m^2, and in treatments 2–5 this was raised to 45 mg/m^2. A delay in the start of docetaxel chemotherapy occurred because of the poor bone marrow function of patient 2 and the consolidation chemotherapy was eventually terminated after five cycles because this individual was already disease free by that time. By PCR analysis of the peripheral blood DNA samples of patient 2, *MDR1*-transduced cells were detectable in the blood samples taken on days 12, 590, 800, 898, 1185, 1414, and 1930 posttransplantation, but were negative on 16 other occasions (Fig. 1). The ratios of *MDR1*-transduced cells in the peripheral blood of this patient after docetaxel seemed to be much lower than those of patient 1.

Patient 3 also received a transplantation, but was excluded from this protocol because of the onset of a progressive disease. No docetaxel chemotherapy was therefore performed and patient 3 was consequently not included in our current study.

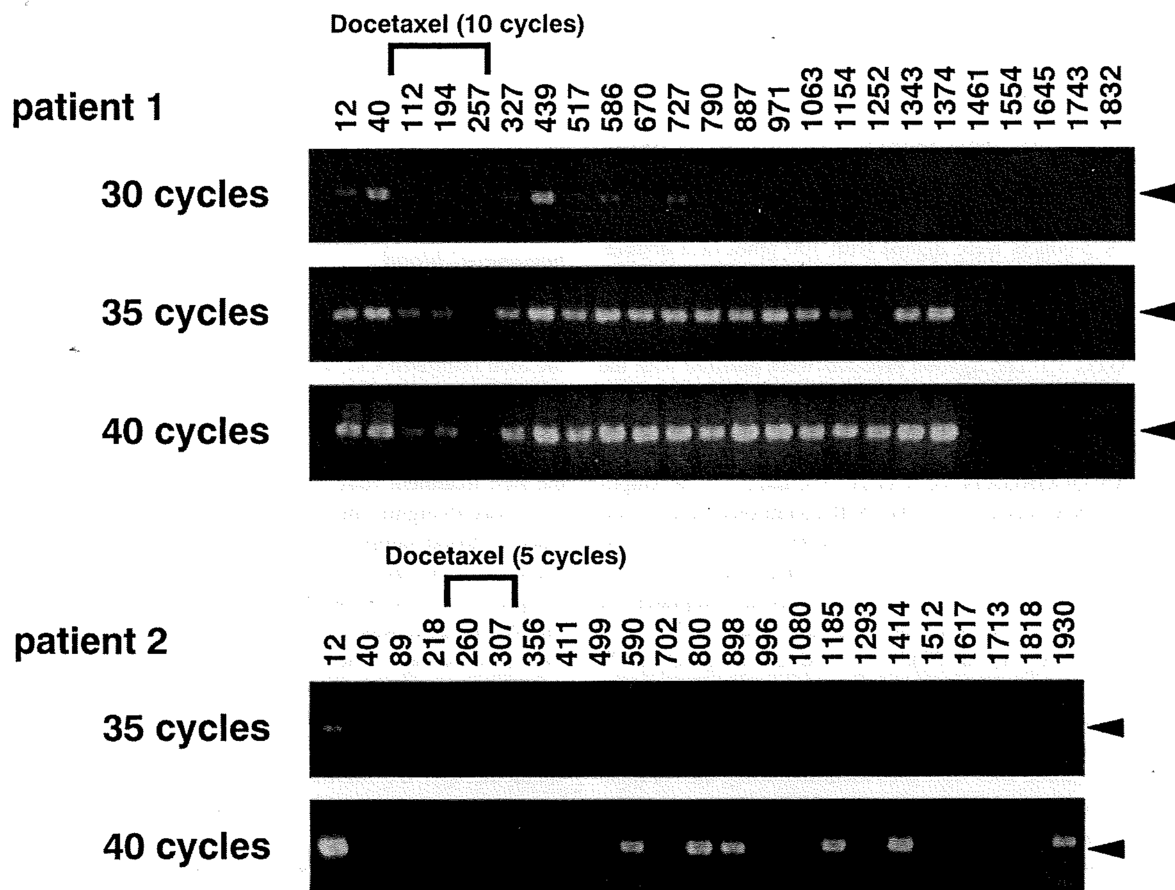

FIG. 1. PCR analysis of the *MDR1* transgene in the peripheral mononuclear cells of patients 1 and 2. Peripheral blood samples were obtained on the indicated days posttransplantation and genomic DNA extracted from these preparations was preamplified with ϕ29 DNA polymerase. HaMDR proviral DNA (100 ng/reaction) was amplified by PCR with AmpliTaq Gold DNA polymerase, using 30, 35, or 40 cycles. These PCR conditions amplify a 657-bp fragment corresponding to region 2206–2862 from the first ATG of the *MDR1* cDNA. PCR products were analyzed on 2.5% agarose gels.

Identification of HaMDR integration sites by LAM-PCR

LAM-PCR was performed to isolate the vector–genome junctions of the *MDR1*-transduced circulating leukocyte clones of patient 1. Nine peripheral leukocyte DNA samples were selected for this purpose. DNA samples obtained on days 12 and 40 were selected as the predocetaxel samples, and those on days 194, 257, and 327 were selected as the postdocetaxel samples. DNA samples on days 586, 727, 762, and 790 were also selected to isolate long-term repopulating clones. The HaMDR proviral integration sites of these clones were then identified using the NCBI database. We subsequently identified 38 *MDR1*-transduced peripheral leukocyte clones from patient 1, designated L-1 to L-38. Some of these clones were identified from different blood samples. We also attempted to isolate vector–genome junctions from the peripheral blood leukocytes DNA of patient 2, but could not obtain clones from DNA samples obtained later than day 12 posttransplantation, probably because of the low ratios of the *MDR1*-transduced cells in this patient.

The RTCGD lists murine retroviral common integration sites (CISs), defined as >2 independent reports of a murine retroviral integration in hematopoietic malignancies. Among the 38 *MDR1*-transduced peripheral leukocyte clones of patient 1, 11 were found to have proviral integrations within 500 kb of the RTCG and are therefore denoted as RTCGD-hit clones. Table 1 lists these RTCGD-hit clones, the blood samples from which they were identified, the integration sites involved, the gene loci at which the provirus integrated, the RTCGs, and the distance and orientation of the provirus with respect to the RTCGs.

Among the 38 clones isolated from patient 1, 30 were identified from the blood sample taken on day 12, whereas the remaining clones were isolated from DNA samples obtained on day 327 or later. We hereafter refer to the former as short-life clones, and the latter as long-life clones. We have summarized the long-life clones in Table 2. Ten RTCGD-hit clones of 11 are short-life, and the additional clone, L-34, is a long-life clone identified from blood samples taken on days 257, 586, 727, and 790.

HaMDR integration sites in RTCGD-hit clones

The positions and orientation of the RTCGs, together with the genes present within a 500-kb region from the retroviral integration sites of the 11 RTCGD-hit clones, are represented

Table 1. HaMDR Integration Sites Near RTCGs

		Integration site		Gene of interest		Provirus		
Clone	Days after transplantation	Locus	Chromosome	Comment	Gene name[a]	Position[b]	Distance (kb) from TSS[c]	Orientation[d]
L-2	12	206,166,060	1q32	RTCG	***CR2*** (4)	U	472	Opposite
L-10	12	144,845,972	2q22	3′-nearest and RTCG	***ZFHX1B*** (13)	D	148	Opposite
L-16	12	136,657,000	9q34	3′-nearest and RTCG	***NOTCH1*** (16)	U	97	Same
L-17	12	22,586,281	10p12	RTCG	***BMI1*** (17)	U	64	Opposite
L-19	12	94,442,327	10q23	In gene and RTCG	***HHEX*** (44)	Intron 2	3	Same
L-21	12	298,646	11p15	RTCG	***HRAS1*** (3)	D	227	Opposite
L-22	12	67,219,886	12q15	RTCG	***RAP1B*** (5)	U	71	Same
L-23	12	29,846,143	13q12	5′-nearest and RTCG	***HMGB1*** (3)	D	92	Same
L-24	12	45,793,077	13q1	RTCG	***LCP1*** (2)	U	139	Opposite
L-26	12	2,243,601	17p13	RTCG	***H1C1*** (6[e])	D	339	Same
				RTCG	***DPH1-OVCA2*** (6[e])	D	363	Same
L-34	257, 586, 727, 790	170,525,313	3q24	5′-nearest and RTCG	***EVI1*** (7)	U	179	Opposite

Abbreviation: RTCG, retrovirus tagged cancer gene.

[a]Genes listed in the RTCGD are shown in boldface, and their frequency in this database is indicated in parentheses.

[b]Position of the provirus with respect to the gene: U; provirus integrated upstream of the gene, D; provirus integrated downstream of the gene.

[c]TSS, transcriptional start site.

[d]Orientation of the inserted provirus in relation to the gene: Same, provirus in same orientation as the gene; Opposite, provirus in opposite orientation to the gene.

[e]Six murine lymphoma/leukemias with retroviral insertions in this region have been reported. The RTCGD does not specify the responsive gene.

Table 2. HaMDR Integration Sites of Long-Life Clones Present in Patient 1

		Integration site		*Gene of interest*		*Provirus*		
Clone	*Days after transplantation*	*Locus*	*Chromosome*	*Comment*	*Gene name*[a]	*Position*[b]	*Distance (kb) from TSS*[c]	*Orientation*[d]
L-31	12, 194, 790	32,777,308	1p35	5′-nearest	*ZBTB8*	U	0.1	Opposite
				3′-nearest	*LOC728116*	D	74	Opposite
L-32	40, 194, 762, 790	18,394,016	7p21	5′-nearest	*HDAC9*	U	108	Opposite
				3′-nearest	*PRPSIL1*	U	360	Same
L-33	40, 727	47,088,411	20q13	5′-nearest	*CSE1L*	U	8	Opposite
				3′-nearest	*ARFGEF2*	D	117	Opposite
L-34	257, 586, 727, 790	170,525,313	3q24	In gene	*MDS1*	Intron 2	176	Opposite
				5′-nearest and RTCG	***EVI1*** (7)	U	179	Opposite
				3′-nearest	*LOC730004*	U	159	Opposite
L-35	327	19,973,778	10p12	In gene	*C10orf112*	Intron 19	410	Same
				5′-nearest	None	—	—	—
				3′-nearest	*PLXDC2*	U	172	Same
L-36	327	17,603,226	17p11	In gene	*RAI1*	Intron 2	78	Same
				5′-nearest	*LOC644931*	U	77	Opposite
				3′-nearest	*SREBF1*	D	78	Opposite
L-37	586	72,857,949	15q23	5′-nearest	*CSK*	U	4	Opposite
				3′-nearest	*CYP1A2*	D	30	Opposite
L-38	790	11,498,273	16p13	5′-nearest	*LITAF*	D	89	Same
				3′-nearest	*LOC400499*	U	5	Same

[a]Genes listed in the RTCGD are shown in boldface, and their frequency in this database is indicated in parentheses.
[b]Position of the provirus with respect to the gene: U, provirus integrated upstream of the gene; D, provirus integrated downstream of the gene.
[c]TSS, transcriptional start site.
[d]Orientation of the inserted provirus in relation to the gene: Same, provirus in same orientation as the gene; Opposite, provirus in opposite orientation to the gene.

schematically in Fig. 2. In Fig. 2 the RTCGs are represented by closed bars, and non-RTCGs are denoted by open bars. We identified an RTCGD-hit clone, L-19, in which HaMDR was inserted in the 5′ portion of the target RTCG, and further six RTCGD-hit clones in which the HaMDR retrovirus was inserted upstream of the RTCGs. L-19 had an integration in the second intron of *HHEX* (hematopoietically expressed homeobox) which has been reported 44 times in the database. L-16 had an HaMDR integration 97 kb upstream of the transcription start site (TSS) of *NOTCH1* (Notch homolog 1), which has been reported 16 times in the RTCGD. No gene was found to be present between the provirus of L-16 and the target RTCG, *NOTCH1*. The L-34 clone mentioned previously had an HaMDR integration in the second intron of the *MDS1* gene, at a site 179 kb upstream of *EVI1*, which has been reported seven times. Two clones, L-22 and L-24, also had HaMDR integrations upstream of the target RTCG, where one gene was present between the provirus and the RTCG. L-22 had an integration 71 kb upstream of *RAP1B* (a member of the *RAS* oncogene family), which has been reported five times, and L-24 showed an integration 139 kb upstream of *LCP1* (lymphocyte cytosolic protein 1), which has been reported twice. Clone L-17 also had an HaMDR integration 64 kb upstream of a target RTCG that has been reported 17 times, *BMI1* (B lymphoma Mo-MLV insertion region), also known as *PCGF4* (polycomb group ring finger 4), and two genes were found in this instance to be present between the provirus and *BMI1*. L-21 had an integration at a distant site from which 10 genes were present between the provirus and the RTCG. This was 253 kb upstream of the TSS of *HRAS1* (v-Ha-*ras* Harvey rat sarcoma viral oncogene homolog), which has been reported three times.

The remaining four RTCGD-hit clones had HaMDR retroviral integrations downstream of established RTCGs. Two clones, L-10 and L-23, had HaMDR insertions at the 3′ portion of RTCGs. L-10 showed an integration 148 kb downstream of *ZFHX1B* (zinc finger homeobox 1b), which has been reported 13 times. L-23 had an integration 92 kb downstream of *HMGB1* (high-mobility group box 1), which has been reported three times. The other clones, L-2 and L-26, had proviral insertions that were separated from the RTCGs by five genes. Clone L-2 had an integration 472 kb downstream of the TSS of *CR2*, complement component (3d/Epstein Barr virus) receptor 2, which

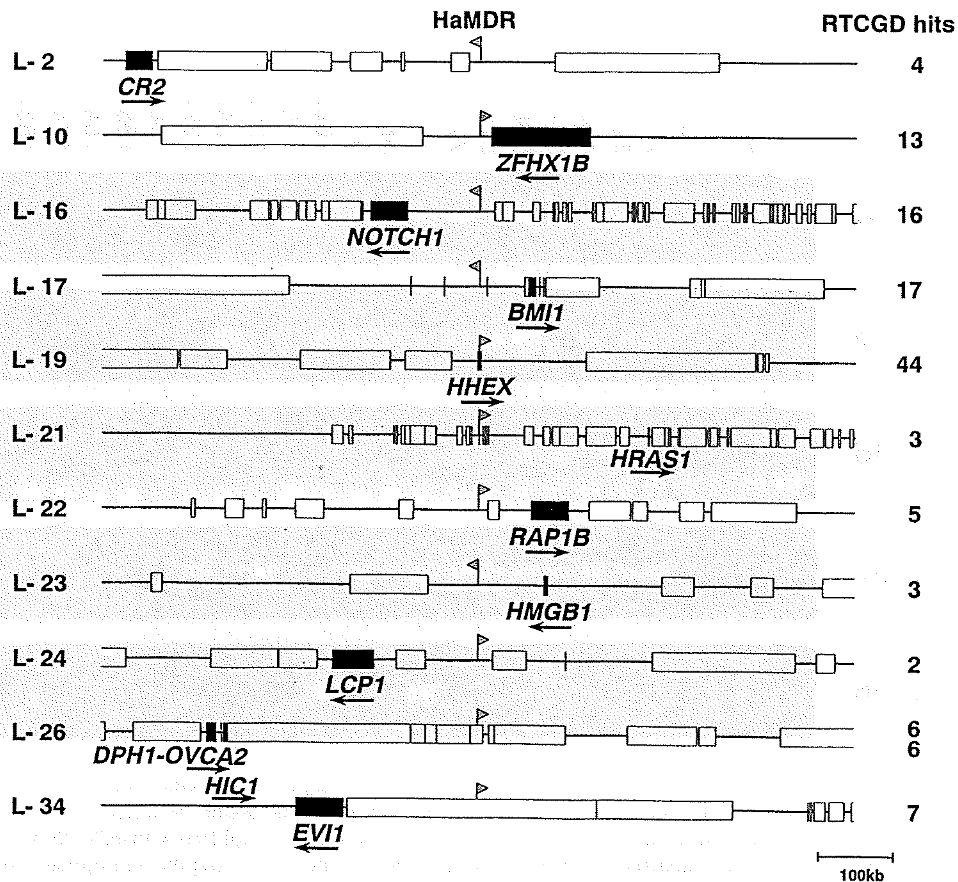

FIG. 2. Schematic representation of the HaMDR integration sites and their neighboring RTCGs, together with their orientations in the RTCGD-hit clones. RTCGs within the 500-kb region around the retroviral integration sites are shown. The HaMDR proviral insertion site is indicated by the triangular flags. The direction of the flag indicates the orientation of the provirus. RTCGs are indicated by the closed bars, and other genes are denoted using open bars. The orientation of each RTCG is indicated by an arrow.

has been reported four times. L-26 had an integration at a position that is 337 kb downstream of *HIC1* (hypermethylated in cancer 1) and 363 kb downstream of *DPH1-OVCA2* (region containing *DPH1* and *OVCA2*), which has been reported six times.

Fate of long-life clones

We identified eight long-life clones from the blood samples of patient 1 on day 327 or later (Table 2). To examine the persistence of these clones, clone-specific PCR was performed. For this purpose, two or three clone-specific primers per clone were designed to examine the possible amplification of vector–genome junctions, using the DNA samples from which the clone had been originally detected by LAM-PCR. Among these primers, only P34-1, P35-1, and P36-1 successfully amplified the vector–genome junctions of clones L-34, L-35, and L-36, respectively, in PCRs with the LTR-specific primer LTR3354. The vector–genome junctions of clones L-37 and L-38 were amplified by nested PCR. The vector–genome junctions of clones L-31, L-32, and L-33 could not be amplified.

Twenty-four peripheral leukocyte DNA samples from patient 1 were selected and subjected to clone-specific PCR. Two DNA isolates taken on days 12 and 40 were selected as the predocetaxel samples. Four DNA samples taken on days 112 to 327 were chosen to examine the effects of docetaxel. DNA samples from days 439 to 1832 were selected to examine the fate of the long-life clones. The results of our clone-specific PCR for clones L-34 to L-38 are shown in Fig. 3. L-34, the only RTCGD-hit clone of this group, was detectable in the blood samples taken on days 327 to 1154, but was undetectable before day 257 and after day 1252, suggesting that the use of docetaxel had efficiently expanded this clone. L-35 was detected in the blood samples taken on days 257 to 439, 586 to 1063, and on day 1343. Weak signals for this clone were also detectable in the blood samples taken on days 1374 and 1743. L-36 was detectable in the blood samples taken on days 327 to 790, and on day 1374. Weak signals for this clone were also observed in the blood samples taken on days 1252 and 1832. L-37 could be detected on days 257 to 971, 1252, and 1374. Because nested PCR was performed in this case, however, the signal intensities did not reflect the relative ratio of this clone in the peripheral blood. L-38 was identified on only two occasions, in the blood samples taken on days 790 and 1063, and was also detected by nested PCR. These results suggest that docetaxel treatment is effective in the enrichment of some long-life peripheral blood leukocyte clones, and that such clones could be detected for 3 to 5 years. We have not so far detected any sign of abnormal proliferation of specific clones in the peripheral blood of patient 1.

DISCUSSION

Breast cancer is typically responsive to chemotherapy and endocrine therapies, but the current prognosis for advanced or recurrent cases of HER2-negative breast cancer is still quite poor, with the complete response (CR) rates of most combination chemotherapies being 10–30%. Various agents have been introduced into clinics, but improvements in the curability rates have not yet been demonstrated for advanced cases of this dis-

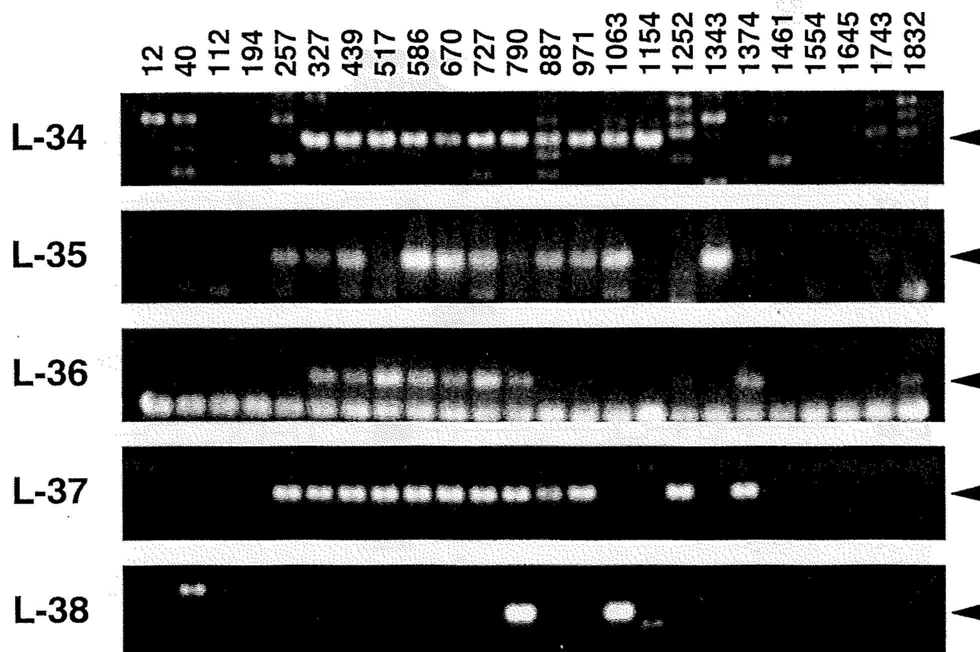

FIG. 3. Clone-specific PCR amplification of vector–genome junctions in mononuclear cells obtained from the peripheral blood of patient 1. Peripheral blood was harvested from patient 1 at the indicated time points between 12 and 1832 days posttransplantation. Genomic DNA samples extracted from these mononuclear cells were preamplified with $\phi29$ DNA polymerase. HaMDR proviral DNA (100 ng/reaction) was amplified by PCR, using an LTR-specific primer and the appropriate clone-specific primer, as described in Materials and Methods. PCR products were analyzed on 2.5% agarose gels.

ease. HDCT with autologous hematopoietic stem cell transplantations has resulted in longer survival times for patients compared with conventional chemotherapy in relapsed non-Hodgkin lymphoma (Philip *et al.*, 1995) and myeloma (Attal *et al.*, 1996). In trials of HDCT against advanced breast cancer, high CR rates of up to 50% and long durations of progression-free survival have been reported, but improvements in overall survival have not been evident (Ito *et al.*, 1999; Peters *et al.*, 2000; Lotz *et al.*, 2005). To improve the efficacy of HDCT, several clinical studies of *MDR1* gene therapy have been conducted (Hanania *et al.*, 1996; Hesdorffer *et al.*, 1998; Cowan *et al.*, 1999; Moscow *et al.*, 1999; Abonour *et al.*, 2000). In our current clinical study of such an *MDR1* gene therapy protocol, we evaluated three recurrent breast cancer patients who had developed metastases: to the lung for patient 1, the lymph nodes for patient 2, and the liver for patient 3. The overall survival times after HDCT for patients 1, 2, and 3 were >77, >71, and 28 months, respectively.

There are three existing reports in which the effects of anticancer agents were evaluated as the underlying cause of the expansion of *MDR1*-transduced cells in the peripheral blood of the respective patients. In a study at the National Cancer Institute (NCI, Bethesda, MD) by Cowan *et al.* (1999) a persistence of *MDR1*-transduced cells could be demonstrated for 6–12 months in the patients, but an expansion of *MDR1*-transduced cells in their peripheral blood during paclitaxel therapy was not observed. Another study at the NCI, reported by Moscow *et al.* (1999), showed a successful enrichment of *MDR1*-transdued hematopoietic cells after chemotherapy. In three of the six patients enrolled in that study, the *MDR1* transgene was initially undetectable, but became apparent in granulocytes after treatment with paclitaxel or doxorubicin. A third study from Indiana University (Bloomington, IN) has demonstrated the etoposide-dependent enrichment of *MDR1*-transdued hematopoietic cells in at least 4 patients of 12. Transgene-containing nucleated cells were detectable in seven of these patients up to 12 months after transplantation (Abonour *et al.*, 2000). We have shown in our present study that the HaMDR-containing cells had been present in the peripheral blood of patient 1 for more than 3 years, but subsequently decreased to undetectable levels. We have also shown from our current analyses that there was a docetaxel-induced expansion of *MDR1*-transduced peripheral leukocytes in patient 1. However, patient 1 also experienced severe neutropenia after each docetaxel treatment (data not shown). Therefore, similar to the three previous studies described above, the clinical benefit of using *MDR1* gene therapy to avoid myelosuppression remains unclear.

In a gene therapy trial in France for the treatment of X-SCID, 4 children of the 10 who were treated developed T cell malignancies within 2.5–5 years (Hacein-Bey-Abina *et al.*, 2003; Kohn *et al.*, 2003; McCormack and Rabbitts, 2004; Thrasher *et al.*, 2006). In contrast, there have been no malignancies reported in a similar X-SCID gene therapy clinical trial that was conducted in the United Kingdom. There may therefore have been subtle but fundamental differences in the protocols used in these trials, although the underlying mechanism is still not fully clear (Pike-Overzet *et al.*, 2006; Thrasher *et al.*, 2006). In our present study, patients 1 and 2 had been monitored for 6 years, and no sign of abnormal proliferation of their transduced cells was observed. We identified 38 clones in the peripheral blood of patient 1 but none had an HaMDR insertion at the *LMO2* locus. This is consistent with the results of other clinical studies (Kohn *et al.*, 2003; Schmidt *et al.*, 2003; Ott *et al.*, 2006).

We have identified an RTCGD-hit clone, L-34, that had an HaMDR integration in the *MDS1* gene, at a site located 179 kb upstream of *EVI1*. The long-term survival of clones that have retroviral integrations in this locus has been reported in a variety of human/nonhuman gene therapy studies. In a gene therapy trial for X-CGD, 42 and 49 hematopoietic clones from 2 of the patients involved were found to have retroviral insertions in the same *MDS1/EVI1* locus (Ott *et al.*, 2006). In another HSC gene therapy study using rhesus macaques, 14 clones with insertions at the *Mds1/Evi1* locus were identified in 9 animals and these clones were observed to be stable for the long term, primarily in myeloid cells. All of these animals were also found to be hematologically normal without evidence of leukemia (Calmels *et al.*, 2005). Kustikova *et al.* (2005) have also reported five long-term repopulating clones that had integration of a retroviral vector in the *evi1* locus of transplanted mice without malignant transformation. Taken together, these data suggest that retroviral insertion at the *EVI1* locus and the resulting overexpression of EVI1 might play a role in the long-term survival of hematopoietic progenitors. It is still unclear, however, whether the hematopoietic clones with retroviral insertions at the *EVI1* locus would eventually cause leukemia in humans. In this regard, our observation that the *MDS1/EVI1* clone, L-34, had been lost by 5 years posttransplantation may hold promise for HSC gene therapy.

We identified 11 RTCGD-hit clones in the peripheral blood of patient 1. Among these, four clones, L-10, L-16, L-17, and L-19, had retroviral integrations in RTCGs that have been reported 13, 16, 17, and 44 times in the RTCGD, respectively. In the whole RTCGD, 468 RTCGs are listed (as of June 2007), but only 32 have been reported more than 10 times (7%). In contrast, in our present analyses 4 of the 11 RTCGs identified have been reported more than 10 times (36%). This was found to be statistically significant ($p = 0.006$) via a Fisher's exact probability test. This suggests that clones that have a retroviral insertion near an RTCG that has been frequently reported in the database may have a higher proliferative capacity in the peripheral blood. In addition, all of these four clones had retroviral integrations in regions for which two genes or fewer were present between the provirus and the target RTCG, suggesting the possible activation of the adjacent genes. However, all of these four clones were isolated from blood samples taken on day 12 only, and these retroviral integrations did not seem to be associated with the long-term survival of the clones.

We identified eight long-life clones that were detectable in the blood samples of patient 1 on day 327 posttransplantation and later. Among these, we examined the presence of five long-life clones by clone-specific PCR (Fig. 3). None of these five clones were detectable in the peripheral blood samples of patient 1 taken on days 12 to 194 posttransplantation. Two clones, L-35 and L-37, initially appeared on day 257, after the eighth round of docetaxel treatment, and another two clones, L-34 and L-36, first appeared on day 327 after the tenth treatment. As shown in Fig. 1, the *MDR1* transgene was detected in the period between days 327 and 1374, but became undetectable in the blood samples after day 1461. This amplification pattern

was almost identical to the profile of these four clones. Because the percentages of total *MDR1*-transduced cells in peripheral blood were estimated to be at levels between 0.1 and 1.0 in the period between days 327 and 727, the proportion of each individual clone would necessarily be lower than this. Because some of the results of our clone-specific PCR were obtained with nested primers, the results shown in Fig. 3 are not quantitative, and we therefore cannot use these data to estimate the ratios of the individual clones in the peripheral blood mononuclear cells.

In conclusion, we have identified 38 *MDR1*-transduced clones in the peripheral blood of a breast cancer patient who has undergone an *MDR1* gene therapy protocol. Among these, 11 harbored a retroviral integration within a 500-kb range of genes listed in the RTCGD. Four short-life clones had proviral insertions near the *ZFHX1B*, *NOTCH1*, *BMI1*, or *HHEX* locus; these loci have been reported more than 10 times in the RTCGD. We also identified a long-life, RTCGD-hit clone, L-34, containing an HaMDR integration at a site located 179 kb upstream of the *EVI1* locus. L-34 was detectable in the blood samples of this patient taken on days 327 to 1154, suggesting that this clone was amplified by docetaxel, but became undetectable 3 years after the end of the docetaxel treatment. No indication of abnormally proliferating hematopoietic clones was observed.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

ABONOUR, R., WILLIAMS, D.A., EINHORN, L., HALL, K.M., CHEN, J., COFFMAN, J., TRAYCOFF, C.M., BANK, A., KATO, I., WARD, M., WILLIAMS, S.D., HROMAS, R., ROBERTSON, M.J., SMITH, F.O., WOO, D., MILLS, B., SROUR, E.F., and CORNETTA, K. (2000). Efficient retrovirus-mediated transfer of the multidrug resistance 1 gene into autologous human long-term repopulating hematopoietic stem cells. Nat. Med. **6**, 652–658.

AIUTI, A., SLAVIN, S., AKER, M., FICARA, F., DEOLA, S., MORTELLARO, A., MORECKI, S., ANDOLFI, G., TABUCCHI, A., CARLUCCI, F., MARINELLO, E., CATTANEO, F., VAI, S., SERVIDA, P., MINIERO, R., RONCAROLO, M.G., and BORDIGNON, C. (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science **296,** 2410–2413.

ATTAL, M., HAROUSSEAU, J.L., STOPPA, A.M., SOTTO, J.J., FUZIBET, J.G., ROSSI, J.F., CASASSUS, P., MAISONNEUVE, H., FACON, T., IFRAH, N., PAYEN, C., and BATAILLE, R. (1996). A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Français du Myelome. N. Engl. J. Med. **335,** 91–97.

BLAESE, R.M, CULVER, K.W., CHANG, L., ANDERSON, W.F., MULLEN, C., NIENHUIS, A., CARTER, C., DUNBAR, C., LEITMAN, S., BERGER, M., MILLER, A.D., MORGAN, R., FLEISHER, T., KOBAYASHI, R., KOBAYASHI, A.L., KLEIN, H., SHEARER, G., CLERICI, M., McGARRITY, G., and CASSELL, A. (1993). Treatment of severe combined immunodeficiency disease (SCID) due to adenosine deaminase deficiency with $CD34^+$ selected autologous peripheral blood cells transduced with a human ADA gene: Amendment to clinical research project. Project 90-C-195, January 10, 1992. Hum. Gene Ther. **4,** 521–527.

CALMELS, B., FERGUSON, C., LAUKKANEN, M.O., ADLER, R., FAULHABER, M., KIM, H.J., SELLERS, S., HEMATTI, P., SCHMIDT, M., VON KALLE, C., AKAGI, K., DONAHUE, R.E., and DUNBAR, C.E. (2005). Recurrent retroviral vector integration at the *Mds1/Evi1* locus in nonhuman primate hematopoietic cells. Blood **106,** 2530–2533.

CAVAZZANA-CALVO, M., HACEIN-BEY, S., DE SAINT BASILE, G., GROSS, F., YVON, E., NUSBAUM, P., SELZ, F., HUE, C., CERTAIN, S., CASANOVA, J.L., BOUSSO, P., DEIST, F.L., and FISCHER, A. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science **288,** 669–672.

COWAN, K.H., MOSCOW, J.A., HUANG, H., ZUJEWSKI, J.A., O'SHAUGHNESSY, J., SORRENTINO, B., HINES, K., CARTER, C., SCHNEIDER, E., CUSACK, G., NOONE, M., DUNBAR, C., STEINBERG, S., WILSON, W., GOLDSPIEL, B., READ, E.J., LEITMAN, S.F., McDONAGH, K., CHOW, C., ABATI, A., CHIANG, Y., CHANG, Y.N., GOTTESMAN, M.M., PASTAN, I., and NIENHUIS, A. (1999). Paclitaxel chemotherapy after autologous stem-cell transplantation and engraftment of hematopoietic cells transduced with a retrovirus containing the multidrug resistance complementary DNA (*MDR1*) in metastatic breast cancer patients. Clin. Cancer Res. **5**, 1619–1628.

GALSKI, H., SULLIVAN, M., WILLINGHAM, M.C., CHIN, K.V., GOTTESMAN, M.M., PASTAN, I., and MERLINO, G.T. (1989). Expression of a human multidrug resistance cDNA (*MDR1*) in the bone marrow of transgenic mice: Resistance to daunomycin-induced leukopenia. Mol. Cell Biol. **9**, 4357–4363.

GASPAR, H.B., PARSLEY, K.L., HOWE, S., KING, D., GILMORUR, K.C., SINCLAIR, J., BROUNS, G., SCHMIDT, M., VON KALLE, C., BARINGTON, T., JAKOBSEN, M.A., CHRISTENSEN, H.O., AL GHONAIUM, A., WHITE, H.N., SMITH, J.L., LEVINSKY, R.J., ALI, R.R., KINNON, C., and THRASHER, A.J. (2004). Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. Lancet **364,** 2181–2187.

GOTTESMAN, M.M., and LING, V. (2006). The molecular basis of multidrug resistance in cancer: The early years of P-glycoprotein research. FEBS Lett. **580,** 998–1009.

HACEIN-BEY-ABINA, S., LE DEIST, F., CARLIER, F., BOUNEAUD, C., HUE, C., DE VILLARTAY, J.P., THRASHER, A.J., WULFFRAAT, N., SORENSEN, R., DUPUIS-GIROD, S., FISCHER, A., DAVIES, E.G., KUIS, W., LEIVA, L., and CAVAZZANA-CALVO, M. (2002). Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. N. Engl. J. Med. **346,** 1185–1193.

HACEIN-BEY-ABINA, S., VON KALLE, C., SCHMIDT, M., McCORMACK, M.P., WULFFRAAT, N., LEBOULCH, P., LIM, A., OSBORNE, C.S., PAWLIUK, R., MORILLON, E., SORENSEN, R., FORSTER, A., FRASER, P., COHEN, J.I., DE SAINT BASILE, G., ALEXANDER, I., WINTERGERST, U., FREBOURG, T., AURIAS, A., STOPPA-LYONNET, D., ROMANA, S., RADFORD-WEISS, I., GROSS, F., VALENSI, F., DELABESSE, E., MACINTYRE, E., SIGAUX, F., SOULIER, J., LEIVA, L.E., WISSLER, M.,

PRINZ, C., RABBITTS, T.H., LE DEIST, F., FISCHER, A., and CAVAZZANA-CALVO, M. (2003). *LMO2*-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science **302,** 415–419.

HANANIA, E.G., GILES, R.E., KAVANAGH, J., ELLERSON, D., ZU, Z., WANG, T., SU, Y., KUDELKA, A., RAHMAN, Z., HOLMES, F., HORTOBAGYI, G., CLAXTON, D., BACHIER, C., THALL, P., CHENG, S., HESTER, J., OSTROVE, J.M., BIRD, R.E., CHANG, A., KORBLING, M., SEONG, D., COTE, R., HOLZMAYER, T., MECHETNER, E., HEIMFELD, S., BERENSON, R., BURTNESS, B., EDWARDS, C., BAST, R., ANDREEFF, M., CHAMPLIN, R., and DEISSEROTH, A.B. (1996). Results of MDR-1 vector modification trial indicate that granulocyte/macrophage colony-forming unit cells do not contribute to posttransplant hematopoietic recovery following intensive systemic therapy. Proc. Natl. Acad. Sci. U.S.A. **93,** 15346–15351.

HESDORFFER, C., AYELLO, J., WARD, M., KAUBISCH, A., VAHDAT, L., BALMACEDA, C., GARRETT, T., FETELL, M., REISS, R., BANK, A., and ANTMAN, K. (1998). Phase I trial of retroviral-mediated transfer of the human *MDR1* gene as marrow chemoprotection in patients undergoing high-dose chemotherapy and autologous stem-cell transplantation. J. Clin. Oncol. **16,** 165–172.

HIBINO, H., TANI, K., IKEBUCHI, K., WU, M.S., SUGIYAMA, H., NAKAZAKI, Y., TANABE, T., TAKAHASHI, S., TOJO, A., SUZUKI, S., TANIOKA, Y., SUGIMOTO, Y., NAKAHATA, T., and ASANO, S. (1999). The common marmoset as a target preclinical primate model for cytokine and gene therapy studies. Blood **93,** 2839–2848.

ITO, Y., MUKAIYAMA, T., OGAWA, M., MIZUNUMA, N., TAKAHASHI, S., AIBA, K., and HORIKOSHI, N. (1999). Epirubicin-containing high-dose chemotherapy followed by autologous hematopoietic progenitor cell transfusion for patients with chemotherapy-sensitive metastatic breast cancer: Results of 5-year follow-up. Cancer Chemother. Pharmacol. **43,** 8–12.

KIOKA, N., TSUBOTA, J., KAKEHI, Y., KOMANO, T., GOTTESMAN, M.M., PASTAN, I., and UEDA, K. (1989). P-glycoprotein gene (MDR1) cDNA from human adrenal: Normal P-glycoprotein carries Gly185 with an altered pattern of multidrug resistance. Biochem. Biophys. Res. Commun. **162,** 224–231.

KOHN, D.B., SADELAIN, M., DUNBAR, C., BODINE, D., KIEM, H.P., CANDOTTI, F., TISDALE, J., RIVIERE, I., BLAU, C.A., RICHARD, R.E., SORRENTINO, B., NOLTA, J., MALECH, H., BRENNER, M., CORNETTA, K., CAVAGNARO, J., HIGH, K., and GLORIOSO, J. (2003). American Society of Gene Therapy (ASGT) ad hoc subcommittee on retroviral-mediated gene transfer to hematopoietic stem cells. Mol. Ther. **8,** 180–187.

KUSTIKOVA, O., FEHSE, B., MODLICH, U., YANG, M., DÜLLMANN, J., KAMINO, K., VON NEUHOFF, N., SCHLEGELBERGER, B., LI, Z., and BAUM, C. (2005). Clonal dominance of hematopoietic stem cells triggered by retroviral gene marking. Science **308,** 1171–1174.

LOTZ, J.P., CURE, H., JANVIER, M., ASSELAIN, B., MORVAN, F., LEGROS, M., AUDHUY, B., BIRON, P., GUILLEMOT, M., GOUBET, J., LAADEM, A., CAILLIOT, C., MAIGNAN, C.L., DELOZIER, T., GLAISNER, S., MARANINCHI, D., ROCHE, H., and GISSELBRECHT, C. (2005). High-dose chemotherapy with haematopoietic stem cell transplantation for metastatic breast cancer patients: Final results of the French multicentric randomised CMA/PEGASE 04 protocol. Eur. J. Cancer **41,** 71–80.

MALECH, H.L., MAPLES, P.B., WHITING-THEOBALD, N., LINTON, G.F., SEKHSARIA, S., VOWELLS, S.J., LI, F., MILLER, J.A., DECARLO, E., HOLLAND, S.M., LEITMAN, S.F., CARTER, C.S., BUTZ, R.E., READ, E.J., FLEISHER, T.A., SCHNEIDERMAN, R.D., VAN EPPS, D.E., SPRATT, S.K., MAACK, C.A., ROKOVICH, J.A., COHEN, L.K., and GALLIN, J.I. (1997). Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease. Proc. Natl. Acad. Sci. U.S.A. **94,** 12133–12138.

McCORMACK, M.P., and RABBITTS, T.H. (2004). Activation of the T-cell oncogene *LMO2* after gene therapy for X-linked severe combined immunodeficiency. N. Engl. J. Med. **350,** 913–922.

MICKISCH, G.H., LICHT, T., MERLINO, G.T., GOTTESMAN, M.M., and PASTAN, I. (1991). Chemotherapy and chemosensitization of transgenic mice which express the human multidrug resistance gene in bone marrow: Efficacy, potency, and toxicity. Cancer Res. **51,** 5417–5424.

MOSCOW, J.A., HUANG, H., CARTER, C., HINES, K., ZUJEWSKI, J., CUSACK, G., CHOW, C., VENZON, D., SORRENTINO, B., CHIANG, Y., GOLDSPIEL, B., LEITMAN, S., READ, E.J., ABATI, A., GOTTESMAN, M.M., PASTAN, I., SELLERS, S., DUNBAR, C., and COWAN, K.H. (1999). Engraftment of *MDR1* and Neo^R gene-transduced hematopoietic cells after breast cancer chemotherapy. Blood **94,** 52–61.

OTT, M.G., SCHMIDT, M., SCHWARZWAELDER, K., STEIN, S., SILER, U., KOEHL, U., GLIMM, H., KUHLCKE, K., SCHILZ, A., KUNKEL, H., NAUNDORF, S., BRINKMANN, A., DEICHMANN, A., FISCHER, M., BALL, C., PILZ, I., DUNBAR, C., DU, Y., JENKINS, N.A., COPELAND, N.G., LUTHI, U., HASSAN, M., THRASHER, A.J., HOELZER, D., VON KALLE, C., SEGER, R., and GREZ, M. (2006). Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of *MDS1-EVI1, PRDM16* or *SETBP1*. Nat. Med. **12,** 401–409.

PETERS, W.P., DANSEY, R.D., KLEIN, J.L., and BAYNES, R.D. (2000). High-dose chemotherapy and peripheral blood progenitor cell transplantation in the treatment of breast cancer. Oncologist **5,** 1–13.

PHILIP, T., GUGLIELMI, C., HAGENBEEK, A., SOMERS, R., VAN DER LELIE, H., BRON, D., SONNEVELD, P., GISSELBRECHT, C., CAHN, J.-Y., HAROUSSEAU, J.-L., COIFFIER, B., BIRON, P., MANDELLI, F., and CHAUVIN, F. (1995). Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma. N. Engl. J. Med. **333,** 1540–1545.

PIKE-OVERZET, K., DE RIDDER, D., WEERKAMP, F., BAERT, M.R., VERSTEGEN, M.M., BRUGMAN, M.H., HOWE, S.J., REINDERS, M.J., THRASHER, A.J., WAGEMAKER, G., VAN DONGEN, J.J., and STAAL, F.J. (2006). Is *IL2RG* oncogenic in T-cell development? Nature **443,** E5

PODDA, S., WARD, M., HIMELSTEIN, A., RICHARDSON, C., DE LA FLOR-WEISS, E., SMITH, L., GOTTESMAN, M., PASTAN, I., and BANK, A. (1992). Transfer and expression of the human multiple drug resistance gene into live mice. Proc. Natl. Acad. Sci. U.S.A **89,** 9676–9680.

SCHMIDT, M., ZICKLER, P., HOFFMANN, G., HAAS, S., WISSLER, M., MUESSIG, A., TISDALE, J.F., KURAMOTO, K., ANDREWS, R.G., WU, T., KIEM, H.P., DUNBAR, C.E., and VON KALLE, C. (2002). Polyclonal long-term repopulating stem cell clones in a primate model. Blood **100,** 2737–2743.

SCHMIDT, M., CARBONARO, D.A., SPECKMANN, C., WISSLER, M., BOHNSACK, J., ELDER, M., ARONOW, B.J., NOLTA, J.A., KOHN, D.B., and VON KALLE, C. (2003). Clonality analysis after retroviral-mediated gene transfer to CD34$^+$ cells from the cord blood of ADA-deficient SCID neonates. Nat. Med. **9,** 463–468.

SORRENTINO, B.P., BRANDT, S.J., BODINE, D., GOTTESMAN. M., PASTAN. I., CLINE. A., NIENHUIS, A.W. (1992). Selection of drug-resistant bone marrow cells *in vivo* after retroviral transfer of human MDR1. Science **257,** 99–103.

SUGIMOTO, Y., TSUKAHARA, S., SATO, S., SUZUKI, M., NUNOI, H., MALECH, H.L., GOTTESMAN, M.M., and TSURUO, T. (2003). Drug-selected co-expression of P-glycoprotein and gp91 *in*

vivo from an *MDR*1-bicistronic retrovirus vector Ha-MDR-IRES-gp91. J. Gene Med. **5,** 366–376.

TAKAHASHI, S., AIBA, K., ITO, Y., HATAKE, K., NAKANE, M., KOBAYASHI, T., MINOWA, S., SHIBATA, H., MITSUHASHI, J., TSUKAHARA, S., ISHIKAWA, E., SUZUKI, R., TSURUO, T., and SUGIMOTO, Y. (2007). *MDR1* gene transfer into hematopoietic stem cells and maintenance of transduced leukocytes by docetaxel chemotherapy in metastatic breast cancer patients. Cancer Sci. **98,** 1609–1616.

THRASHER, A.J., GASPAR, H.B., BAUM, C., MODLICH, U., SCHAMBACH, A., CANDOTTI, F., OTSU, M., SORRENTINO, B., SCOBIE, L., CAMERON, E., BLYTH, K., NEIL, J., ABINA, S.H., CAVAZZANA-CALVO, M., and FISCHER, A. (2006). X-SCID transgene leukaemogenicity. Nature **443,** E5–E6.

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Inhibition of the mitogen-activated protein kinase pathway results in the down-regulation of P-glycoprotein

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Abstract

The *multidrug resistance gene 1* (*MDR1*) product, P-glycoprotein (P-gp), pumps out a variety of anticancer agents from the cell, including anthracyclines, *Vinca* alkaloids, and taxanes. The expression of P-gp therefore confers resistance to these anticancer agents. In our present study, we found that FTI-277 (a farnesyltransferase inhibitor), U0126 [an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)], and 17-allylamino-17-demethoxygeldanamycin (an inhibitor of heat shock protein 90) reduced the endogenous expression levels of P-gp in the human colorectal cancer cells, HCT-15 and SW620-14. In contrast, inhibitors of phosphatidylinositol 3-OH kinase, mammalian target of rapamycin, p38 mitogen-activated protein kinase, and c-Jun NH_2-terminal kinase did not affect P-gp expression in these cells. We further found that U0126 down-regulated exogenous P-gp expression in the *MDR1*-transduced human breast cancer cells, MCF-7/MDR and MDA-MB-231/MDR. However, the *MDR1* mRNA levels in these cells were unaffected by this treatment. PD98059 (a MEK inhibitor), *ERK* small interfering RNA, and *p90 ribosomal S6 kinase* (*RSK*) small interfering RNA also suppressed P-gp expression. Conversely, epidermal growth factor and basic fibroblast growth factor enhanced P-gp expression, but the *MDR1* mRNA levels were unchanged in epidermal growth factor–stimulated cells. Pulse-chase analysis revealed that U0126 promoted P-gp degradation but did not affect the biosynthesis of this gene product. The pretreatment of cells with U0126 enhanced the paclitaxel-induced cleavage of poly(ADP-ribose) polymerase and paclitaxel sensitivity. Furthermore, U0126-treated cells showed high levels of rhodamine123 uptake. Hence, our present data show that inhibition of the MEK-ERK-RSK pathway down-regulates P-gp expression levels and diminishes the cellular multidrug resistance. [Mol Cancer Ther 2007;6(7):2092–2102]

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Introduction

The acquisition of multidrug resistance to anticancer agents by tumor cells is characterized by a cross-resistance to structurally unrelated agents (1). Such multidrug-resistant cells express ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp)/ABCB1, breast cancer resistance protein (BCRP)/ABCG2, and multidrug resistance-related protein 1/ABCC1. These gene products pump out a variety of anticancer agents from the cell in energy-dependent manners. Human P-gp is a 170 to 180 kDa plasma membrane glycoprotein encoded by *multidrug resistance gene 1* (*MDR1*), and contains two ATP-binding sites and two transmembrane domains. P-gp functions as an efflux pump for different anticancer agents such as anthracyclines, *Vinca* alkaloids, and taxanes (1–4). Thus, P-gp–expressing cells display low intracellular concentrations of these agents and are resistant to their cytotoxic effects. P-gp is widely expressed in human tissues, including the adrenal gland, colon, kidney, liver, angioendothelial cells, and hematopoietic precursor cells (5–7), and its expression has been shown to be significantly elevated in drug-resistant tumors of the colon, kidney, and liver (8). P-gp expression levels have also been reported to be augmented in some cancers following a recurrence after chemotherapy (8). Hence, the P-gp expression status can significantly affect the sensitivity of cancer cells to its substrate anticancer agents.

The mitogen-activated protein kinase (MAPK) pathways are activated by various kinds of stimuli, including those provided by growth factors, different types of stress, or inflammatory cytokines, and this can result in cell proliferation, differentiation, development, inflammation, or apoptosis (9). The principal components of the MAPK comprise three subfamilies: extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase (SAPK), and p38MAPK. The activities of the MAPKs are regulated by two events, phosphorylation and dephosphorylation. The MAPK-associated pathways are composed of a growth factor–responsive pathway, including ERK1/2, and two stress-responsive pathways, including JNK/SAPK and p38MAPK. The former comprises Ras, Raf-1, MAPK/ERK kinase (MEK) 1/2, ERK1/2, and p90 ribosomal S6 kinase (RSK) 1/2/3/4. In cells stimulated by growth factors [e.g., epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF)], the Ras proto-oncogene is activated