遺伝子解析の結果が外部に漏洩されないよう厳重な注意、処置が施行される。

レトロウイルスを用いたヒト末梢血単核球への腫瘍抗原特異的TCRの導入実験は三重大学の組換えDNA実験審査委員会及び三重大学医学部研究倫理委員会において承認されている。これらの実験は三重大学において承認を受けたP2レベルの研究室にて行なわれる。

実験動物を用いたT細胞輸注療法、遺伝子免疫療法の研究は三重大学の組換えDNA実験審査委員会、三重大学医学部研究倫理委員会、動物実験審査委員会においてすでに承認を受けており、三重大学において承認をうけた実験室、飼育室において実施される。

C. 研究結果

1. NY-ESO-1特異的TCR遺伝子導入ヒトリンパ球に関して、自家細胞を用いた治験製品概要書(2014年12月2日第1版完成)を作製した。2. NY-ESO-1-TCR遺伝子導入自家Tリンパ球を用いた場合の輸注療法プロトコルについて、9月30日にPMDAと薬事戦略相談の事前面談を行った。

D. 考察

自家細胞を用いた治験製品概要書を作成し、自家細胞を持ちいた場合の輸注療法プロトコルについて薬事戦略相談の事前面談を行った。今後は非自己細胞を用いた場合についてPMDAと相談していく予定である。

E. 結論

平成２６年度は自家細胞を用いた治験製品概要書を作成し、自家細胞を持ちいた場合の輸注療法プロトコルについて薬事戦略相談の事前面談を行った。平成２７年度には非自己細胞を用いた場合についてPMDAと相談していく予定であり、平成２８年度の治験開始を目指す。

F. 健康危険情報

該当無し。

G. 研究発表

1. 論文発表

21) Kageyama S, Ikeda H, Miyahara Y, Imai N, Ishihara M, Saito K, Sugino S, Ueda S, Ishikawa T, Kokura S, Naota H, Ohishi K, Shiraishi T, Inoue N, Tanabe M, Kidokoro T, Yoshioka H, Tomura D, Nukaya I, Mineno J, Takesako K, Katayama N, Shiku H. Adoptive transfer of MAGE-A4 T-cell receptor gene-transduced lymphocytes in patients with recurrent esophageal cancer. Clin. Cancer Res., 2015 in press.

22) Ishihara M, Seo N, Mitsui J, Muraoka D, Tanaka M, Mineno J, Ikeda H, Shiku H. Systemic CD8+ T Cell-Mediated Tumoricidal Effects by Intratumoral Treatment of Oncolytic Herpes Simplex Virus with the Agonistic Monoclonal Antibody for Murine Glucocorticoid-Induced Tumor Necrosis Factor Receptor. PLoS One, 9(8):e104669. 2014.

23) Asai H, Fujiwara H, Kitazawa S, Kobayashi N, Ochi T, Miyazaki Y, Ochi F, Akatsuka Y, Okamoto S, Mineno J,

Kuzushima K, Ikeda H, Shiku H, Yasukawa M. Adoptive transfer of genetically engineered WT1-specific cytotoxic T lymphocytes does not induce renal injury. J Hematol Oncol. 7:3, 2014.

24) O'Sullivan T, Saddawi-Konefka R, Gross E, Tran M, Mayfield SP, Ikeda H, Bui JD. Interleukin-17D mediates tumor rejection through recruitment of natural killer cells. Cell Rep., 7(4):989-998, 2014.

25) Hosoi H, Ikeda H, Imai N, Amaike C, Wang L, Orito Y, Yamane M, Ueno H, Ideno M, Nukaya I, Enoki T, Mineno J, Takesako K, Hirano S, Shiku H. Stimulation through very late antigen-4 and -5 improves the multifunctionality and memory formation of $CD8^+$ T cells. Eur J. Immunol., 44:1727-1758, 2014.

2. 学会発表

22) Hiroaki Ikeda, Hiroaki Ueno, Isao Tawara, Ayumi Kawamura, Naoko Imai, Sachiko Okamoto, Junichi Mineno, Kazutoh Takesako, Naoyuki Katayama[2], Hiroshi Shiku. Tumor-specific TCR-engineered donor lymphocyte infusion therapy with reduced GVHD induction utilizing novel retrovirus vector silencing endogenous TCR expression. American Society of Hematology 56th annual meeting, SF, USA, 2014.

23) Hiroaki Ikeda, Hiroaki Ueno, Ayumi Kawamura, Naoko Imai, Sachiko Okamoto, Junichi Mineno, Kazutoh Takesako, Hiroshi Shiku. Tumor-specific donor Lymphocyte infusion therapy with allogeneic T cells utilizing novel retrovirus vector silencing endogenous TCR expression. Society for immunotherapy of Cancer 29th Annual Meeting. National Harbor, MD, USA, 2014.

24) 池田裕明 TCR遺伝子改変T細胞輸注による新しいがん免疫療法の臨床開発 2014第６３回日本輸血・細胞治療学会東海支部例会　名古屋　2014.

25) Hiroaki Ikeda, Hiroaki Uneno, Ayumi Kawamura, Makiko Yamane, Naoko Imai, Sachiko Okamoto, Junichi Mineno, Kazutoh Takesako, Hiroshi Shiku. TCR gene therapy with allogeneic T cells. 第２０回日本遺伝子治療学会学術集会　東京　2014.

26) Yasushi Akahori, Motohiro Yoneyama, Hiroaki Ikeda, Yuki Orito, Yoshihiro Miyahara, Yasunori Amaishi, Sachiko Okamoto, Junichi Mineno, Kazutoh Takesako, Hiroshi Shiku. Development of chimeric antigen receptor immunotherapy targeting intracellular WT1 gene product. 第２０回日本遺伝子治療学会学術集会　東京　2014.

27) Hiroaki Uneno, Hiroaki Ikeda, Ayumi Kawamura, Makiko Yamane, Naoko Imai, Sachiko Okamoto, Junichi Mineno,

Kazutoh Takesako, Hiroshi Shiku. Development of TCR gene therapy with allogeneic T cells. 第１８回日本がん免疫学会総会　松山 2014.

28) 赤堀泰　米山元裕　池田裕明　宮原慶裕　織戸由貴　天石泰典 岡本幸子　峰野純一　竹迫一任　珠玖洋　WT1ペプチド-HLA-A24複合体を認識するヒト抗体の単離とそれを用いたCAR治療法の開発 第１８回日本がん免疫学会総会　松山 2014.

H. 知的所有権の出願・取得状況

1. 特許取得

該当無し

2. 実用新案登録

該当無し

3. その他

該当無し

Ⅲ. 学会等発表実績

様式第１９

学　会　等　発　表　実　績

委託業務題目「同種移植後再発の成人Ｔ細胞白血病リンパ腫に対する次世代型レトロウイルスベクターによる
Ｔ細胞レセプター遺伝子導入ドナーリンパ球輸注療法」

機関名　国立大学法人　三重大学

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
Tumor-specific TCR-engineered donor lymphocyte infusion therapy with reduced GVHD induction utilizing novel retrovirus vector silencing endogenous TCR expression.	Hiroaki Ikeda, Hiroaki Ueno, Isao Tawara, Ayumi Kawamura, Naoko Imai, Sachiko Okamoto, Junichi Mineno, Kazutoh Takesako, Naoyuki Katayama, Hiroshi Shiku.	American Society of Hematology 56th annual meeting	December, 2014	国外
Tumor-specific donor Lymphocyte infusion therapy with allogeneic T cells utilizing novel retrovirus vector silencing endogenous TCR expression.	Hiroaki Ikeda, Hiroaki Ueno, Ayumi Kawamura, Naoko Imai, Sachiko Okamoto, Junichi Mineno, Kazutoh Takesako, Hiroshi Shiku.	Society for immunotherapy of Cancer 29th Annual Meeting	November, 2014	国外
TCR遺伝子改変T細胞輸注による新しいがん免疫療法の臨床開発	池田裕明	第６３回日本輸血・細胞治療学会東海支部例会	2014年9月	国内
TCR gene therapy with allogeneic T cells.	Hiroaki Ikeda, Hiroaki Uneno, Ayumi Kawamura, Makiko Yamane, Naoko Imai, Sachiko Okamoto, Junichi Mineno, Kazutoh Takesako, Hiroshi Shiku.	第２０回日本遺伝子治療学会学術集会	2014年8月	国内
Development of chimeric antigen receptor immunotherapy targeting intracellular WT1 gene product.	Yasushi Akahori, Motohiro Yoneyama, Hiroaki Ikeda, Yuki Orito, Yoshihiro Miyahara, Yasunori Amaishi, Sachiko Okamoto, Junichi Mineno, Kazutoh Takesako, Hiroshi Shiku.	第２０回日本遺伝子治療学会学術集会	2014年8月	国内
Development of TCR gene therapy with allogeneic T cells.	Hiroaki Uneno, Hiroaki Ikeda, Ayumi Kawamura, Makiko Yamane, Naoko Imai, Sachiko Okamoto, Junichi Mineno, Kazutoh Takesako, Hiroshi Shiku.	第１８回日本がん免疫学会総会	2014年7月	国内
WT1ペプチド-HLA-A24複合体を認識するヒト抗体の単離とそれを用いたCAR治療法の開発	赤堀泰　米山元裕　池田裕明　宮原慶裕　織戸由貴　天石泰典　岡本幸子　峰野純一　竹迫一任　珠玖洋	第１８回日本がん免疫学会総会	2014年7月	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所 （学会誌・雑誌等名）	発表した時期	国内・外の別
Adoptive transfer of MAGE-A4 T-cell receptor gene-transduced lymphocytes in patients with recurrent esophageal cancer.	Kageyama S, Ikeda H, Miyahara Y, Imai N, Ishihara M, Saito K, Sugino S, Ueda S, Ishikawa T, Kokura S, Naota H, Ohishi K, Shiraishi T, Inoue N, Tanabe M, Kidokoro T, Yoshioka H, Tomura D, Nukaya I, Mineno J, Takesako K, Katayama N, Shiku H.	Clin. Cancer Res., in press	2015	国外
Histone deacetylase inhibition regulates inflammation and enhances Tregs after allogeneic hematopoietic cell transplantation in humans.	1) Sung Won Choi, Erin Gatza, Guoqing Hou, Yaping Sun, Joel Whitfield, Yeohan Song, Katherine Oravecz-Wilson, Isao Tawara, Charles A. Dinarello, Pavan Reddy.	Blood, 125(5), 815-819	2015	国外
Interleukin-17 Induces an Atypical M2-Like MacrophageSubpopulation That Regulates Intestinal Inflammation.	Kenichiro Nishikawa, Naohiro Seo, Mie Torii, Nei Ma, Daisuke Muraoka, Isao Tawara, Masahiro Masuya, Kyosuke Tanaka, Yoshiyuki Takei, Hiroshi Shiku, Naoyuki Katayama, Takuma Kato.	PLOS One, 9(9) e108494	2014	国外
Systemic CD8+ T Cell-Mediated Tumoricidal Effects by Intratumoral Treatment of Oncolytic Herpes Simplex Virus with the Agonistic Monoclonal Antibody for Murine Glucocorticoid-Induced Tumor Necrosis Factor Receptor.	Ishihara M, Seo N, Mitsui J, Muraoka D, Tanaka M, Mineno J, Ikeda H, Shiku H.	PLoS One, 9(8) e104669	2014	国外
Stimulation through very late antigen-4 and -5 improves the multifunctionality and memory formation of CD8+ T cells.	Hosoi H, Ikeda H, Imai N, Amaike C, Wang L, Orito Y, Yamane M, Ueno H, Ideno M, Nukaya I, Enoki T, Mineno J, Takesako K, Hirano S, Shiku H.	Eur J. Immunol., 44:1727-1758	2014	国外
Interleukin-17D mediates tumor rejection through recruitment of natural killer cells.	O'Sullivan T, Saddawi-Konefka R, Gross E, Tran M, Mayfield SP, Ikeda H, Bui JD.	Cell Rep., 7(4):989-998	2014	国外
Adoptive transfer of genetically engineered WT1-specific cytotoxic T lymphocytes does not induce renal injury.	Asai H, Fujiwara H, Kitazawa S, Kobayashi N, Ochi T, Miyazaki Y, Ochi F, Akatsuka Y, Okamoto S, Mineno J, Kuzushima K, Ikeda H, Shiku H, Yasukawa M.	J Hematol Oncol. 7:3	2014	国外

Ⅳ. 研究成果の刊行物・別刷

Article title:

Adoptive transfer of MAGE-A4 T-cell receptor gene–transduced lymphocytes in patients with recurrent esophageal cancer

Authors:

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Running title;

MAGE-A4 TCR transduced T cell transfer for esophageal cancer

Key words;

MAGE-A4 antigen, TCR-gene T cell therapy, esophageal cancer, immunotherapy, in vivo persistence

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Grant Support;

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Disclosure of Potential Conflicts of Interest;

This study was technologically supported by TakaraBio Inc. Naoki Inoue, Masashige Tanabe, Tomohide Kidokoro, Hirofumi Yoshioka, Daisuke Tomura and Ikuei Nukaya are employees of TakaraBio Inc., and Junichi Mineno and Kazutoh Takesako are employees/directors. TakaraBio Inc. provided the Department of Immuno-Gene Therapy at Mie University with funding resources. Shinichi Kageyama, Hiroaki Ikeda, Yoshihiro Miyahara, Naoko Imai and Hiroshi Shiku are members of this department. The other authors have no conflicting interests.

Word count; 4,618 Total number of figures and tables; 6

Translational Relevance

This article describes the first TCR-gene T cell therapy for esophageal cancer. The regimen did not include preparative lymphodepletion. Monitoring the in vivo kinetics of transferred cells by sensitive quantitative PCR and specific tetramers, we demonstrated that the transferred T cells persisted for more than 5 months, trafficked to tumor sites, and maintained tumor-specific reactivity in patients. None of the patients exhibited tumor shrinkage in the short term. However, 3 patients who had minimal disease at the time of cell transfer remained free from disease progression for more than a year without any treatment. These findings suggest that this sort of TCR-transduced T-cell therapy might be beneficial for patients bearing minimal tumor burdens. Also the discordance between T-cell survival and short-time tumor response suggests that multiple mechanisms underlie the benefits of lymphodepletive preconditioning in adoptive T cell therapy.

Abstract:

Purpose: Preparative lymphodepletion, the temporal ablation of the immune system, has been reported to promote persistence of transferred cells along with increased rates of tumor regression in patients treated with adoptive T cell therapy. However, it remains unclear whether lymphodepletion is indispensable for immunotherapy with T cell receptor (TCR) gene–engineered T cells.

Experimental Design: We conducted a first-in-man clinical trial of TCR-gene transduced T-cell transfer in patients with recurrent MAGE-A4–expressing esophageal cancer. The patients were given sequential MAGE-A4 peptide vaccinations. The regimen included neither lymphocyte-depleting conditioning nor administration of IL-2. Ten patients, divided into 3 dose cohorts, received T-cell transfer.

Results: TCR-transduced cells were detected in the peripheral blood for 1 month at levels proportional to the dose administered, and in 5 patients they persisted for more than 5 months. The persisting cells maintained ex vivo antigen-specific tumor reactivity. Despite the long persistence of the transferred T cells, 7 patients exhibited tumor progression within 2 months after the treatment. Three patients who had minimal tumor lesions at baseline survived for more than 27 months.

Conclusion: These results suggest that TCR-engineered T cells created by relatively short-duration in vitro culture of polyclonal lymphocytes in

peripheral blood retained the capacity to survive in a host. The discordance between T-cell survival and tumor regression suggests that multiple mechanisms underlie the benefits of preparative lymphodepletion in adoptive T cell therapy.

Introduction

Initial studies of adoptive T cell therapy for patients with malignancy reported a lack of prolonged persistence of the transferred cells and limited clinical responses (1, 2). In later studies, lymphodepletive preparative regimens using chemotherapy alone or in combination with total-body irradiation were reported to enhance persistence of the transferred cells, accompanied by an increased clinical response, e.g., in adoptive therapy of melanoma patients utilizing tumor infiltrating lymphocytes (TILs) (3, 4). In these trials, persistence of transferred T cells was correlated with tumor regression (5). Technology for the engineering of antigen receptor genes presents opportunities for novel T cell–based therapies. Such approaches could potentially expand the application of adoptive therapy with tumor-reactive T cells to patients with tumor types that are difficult to isolate, and for which it is therefore challenging to expand tumor-reactive T cells. Lymphodepleting pretreatments have also been incorporated into adoptive therapy with T cells genetically engineered to express tumor-specific T-cell receptors (TCRs) or tumor-reactive chimeric antigen receptors (CARs); these therapies have resulted in durable tumor regression in patients with metastatic melanoma, synovial cell sarcoma, and hematopoietic malignancy (6–12).

In contrast to TIL-based cells used for transfer, TCR gene–engineered T cells are created from polyclonal T cells in peripheral blood that have undergone

minimal in vivo exposure to chronic antigen stimulation and the tumor microenvironment. Such gene-engineered T cells are cultured in vitro for relatively short periods, e.g., 7–14 days before transfer. These differences between TILs and gene-engineered T cells may influence the degree to which these cells are prone to clonal exhaustion following transfer, and may therefore result in different requirements for lymphodepletive pretreatment in order to achieve in vivo persistence. However, the necessity of preparative lymphodepletion, both to ensure survival of gene-engineered T cells and to achieve durable tumor regression in cancer patients, has not yet been carefully investigated.

We previously reported establishment of a CTL clone that recognizes MAGE-A4$_{143\text{-}151}$ peptide in an HLA-A*24:02–restricted fashion (13). We constructed a retrovirus vector, MS-bPa, for transduction of T cells with TCR-α and -β chains derived from the MAGE-A4$_{143\text{–}151}$–specific T-cell clone. Retroviral transduction of MAGE-A4–specific TCR genes confers MAGE-A4 specificity on 28–52% of CD8+ T cells (14). These T cells exhibited HLA-A*24:02-restricted cytotoxicity against MAGE-A4–expressing tumor cells; moreover, they stably exerted antigen-specific functions for over 6 months in vitro (14).

In this study, we investigated whether the unique nature of TCR gene–engineered T cells preserved the capacity to survive in hosts. Given that 38–52% of tumor tissues from esophageal squamous cell carcinoma express the

MAGE-A antigen (15, 16), we performed a first-in-man clinical trial of TCR gene therapy for recurrent esophageal cancer patients, targeting MAGE-A4, without any preparative lymphodepleting regimen or administration of IL-2. In this study, we monitored the in vivo kinetics of transferred cells by sensitive quantitative PCR of inserted vectors as well as the specific tetramer. T cells engineered to express MAGE-A4–specific TCR were safe, persisted for long periods, trafficked to tumor sites, and maintained tumor-specific reactivity. Unexpectedly, we observed discordance between T-cell persistence and tumor response, suggesting that lymphodepleting pretreatment in humans contributes not only to T-cell survival but also other mechanisms beneficial to the clinical response.

Materials and Methods

Preparation of TCR gene–transduced lymphocytes

Lymphocytes were collected from each patient by processing 5 L of peripheral blood by apheresis. In the cell-processing facility, the lymphocytes were cultured with IL-2, anti-CD3 antibody, and RetroNectin™ (Takara Bio Inc.) under institutional GMP control. Proliferating lymphocytes were infected with the retroviral vector, MS-bPa, which was constructed from DNA encoding MAGE-A4$_{143\text{-}151}$/HLA-A*24:02–specific TCR-α and -β chains (14). After 7–10 days in culture, the lymphocytes were harvested, and then frozen until use. The IFN-γ responding cells were assayed as described

previously, with some modification (17). Briefly, the cells were stimulated with MAGE-A4 peptide. Brefeldin A was added, and they were then incubated with anti-CD8 monoclonal antibody (Becton Dickinson). After permeabilization and fixation, cells were stained intracellularly with anti-IFN-γ monoclonal antibody.

Study design

This study was a phase 1, cell dose–escalating clinical trial of MAGE-A4–specific TCR gene-transduced lymphocyte transfer for treatment of patients with recurrent esophageal tumors expressing the MAGE-A4 antigen. The primary objective was to determine clinical safety, and the secondary objective was to assess cell kinetics in peripheral blood and infiltration of the TCR-transduced lymphocytes into the tumor tissue. The other aims were to determine MAGE-A4–specific immune responses and clinical responses.

Patients were eligible for study entry if they met each of the following criteria: had recurrent or metastatic esophageal tumors that expressed the MAGE-A4 antigen; were positive for HLA-A*24:02; had a performance status (PS) of 0, 1, or 2; were between 20 and 75 years old; had a life expectancy of 4 months or more; and did not have impaired organ function.

The patients were divided into 3 cohorts of 3 patients each: Cohort 1, 2×10^8 cells (whole cells including TCR-transduced lymphocytes) per dose; Cohort 2, 1×10^9 cells per dose; and Cohort 3, 5×10^9 cells per dose. In case of impaired

PS due to disease progression, patients discontinued the clinical study. When a patient was withdrawn from the trial before safety evaluation before day 35, they were replaced with another patient. After withdrawing, they were followed up to assess clinical events and cell kinetics in the peripheral blood.

Clinical safety was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (NCI-CTCAE ver.3.0) (18). Tumor responses were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST ver1.0) (19).

The study was conducted in accordance with the current version of the Declaration of Helsinki. Written informed consent was obtained from all patients participating in this study. The protocol was approved by the institutional review board of Mie University Hospital and the Ministry of Health, Labor, and Welfare of Japan. This clinical trial was registered in the UMIN Clinical Trials Registry as ID: UMIN000002395.

Expression of MAGE-A4 antigen

MAGE-A4 expression was assessed by quantitative real-time PCR (qRT-PCR) using specific primers (20), or by immunohistochemistry (IHC) using the monoclonal antibodies 57B (21), MCV-1 and MCV-4. MCV-1 and -4 were produced from hybridomas generated in our laboratory by cell fusion of the mouse myeloma cell line SP2/0 and splenocytes harvested from C/B F1

mice (CLEA Japan, Inc.) immunized with recombinant MAGE-A4 protein. MCV-1 and -4 recognize amino acids 255–277 and 71–95 of MAGE-A4, respectively. MCV-1 reacted to MAGE-A2, -A4 and -A12, and MCV-4 covered MAGE-A1 and A4, respectively. MAGE-A4 expression in tumors was judged primarily by quantitative PCR. Immunohistochemical staining was alternatively used if the tumor sample was unavailable for PCR. The cut-off value of PCR-amplified copies for MAGE-A4 was 12.2, which was determined as the mean copy number +2 S.D. (standard deviation) of the amplified products in normal human tissues. Tumor samples expressing 12.2 or more PCR-amplified copies were judged as MAGE-A4 positive (22). The sensitivity was qualified by the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene amplification. For IHC, 57B monoclonal antibody was used first, since 57B covers the MAGE-A antigen family (23). Samples that were also positive for MCV-1 and MCV-4 were judged as MAGE-A4 positive. Tissue samples with 5% positive or more stained area were judged as MAGE-A4 positive. Focally stained samples were also positive.

Treatment protocol

After preparation of TCR-transduced lymphocytes, patients were given the lymphocytes intravenously without pre-conditioning treatment. On days 14 and 28, patients were subcutaneously given 300 μg of MAGE-A4 peptide (NYKRCFPVI) (PolyPeptide Laboratories) emulsified with incomplete

Freund's adjuvant (IFA; Montanide ISA-51VG) (SEPPIC). On days 35 and 63, safety and clinical responses were assessed. After completing the study, patients who wished to do so received continuous MAGE-A4 peptide vaccination on a monthly basis.

Cell kinetics and tumor infiltration of TCR-transduced lymphocytes

Heparinized peripheral blood was collected at baseline and at pre-determined time points during the 63-day period. On day 35, if the patients had tumors at esophageal lesions, esophageal tumors were endoscopically biopsied. After day 63, peripheral blood mononuclear cells (PBMCs) were collected on the days of patients' visits to the clinic.

PBMCs were isolated and cryopreserved. The PBMCs were thawed prior to qPCR assay, from which DNAs were isolated using the DNA-extraction kit. Primers for proviral DNA sequence (retroviral packaging signal region, existing in TCR-transduced cells) and human IFN-γ DNA (genes of whole T cells) from the Provirus Copy Number Detection Primer Set, Human (Product code 6167, Takara Bio Inc.) were used for quantitative PCR assay using the Cycleave PCR Core Kit (Product code CY501, Takara Bio Inc.). Ten microliters of 10 ng/μl solution of the isolated DNA samples were amplified by 50 cycles of 3-step PCR reactions. For standard curve generation, serially diluted (2,000 to 0.02 fg/μl) DNA Control Template for Provirus, Human (a component of the Provirus Copy Number Detection Primer Set, Human),