but also sALCAM are involved in the regulation of glioblastoma cell invasion.

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Recognition of a Natural WT1 Epitope by a Modified WT1 Peptide-specific T-Cell Receptor

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Abstract. *Wilms' tumor gene WT1 is highly expressed in leukemia and in various types of solid tumors and exerts an oncogenic function. Thus, WT1 protein is a most promising tumor-associated antigen. We have been successfully performing WT1 vaccination with a 9-mer modified $WT1_{235}$ peptide, which has one amino acid substitution (M→Y) at position 2 of 9-mer natural $WT1_{235}$ peptide (235-243 a.a.), for close to 700 HLA-A*24:02-positive patients with leukemia or solid tumors. Although vaccination of modified $WT1_{235}$ peptide induced natural $WT1_{235}$ peptide-recognizing cytotoxic T-lymphocytes (CTLs) and exerted cytotoxic activity towards leukemia and solid tumor cells that expressed the natural $WT1_{235}$ peptide (epitope) but not the vaccinated modified $WT1_{235}$ peptide (epitope), the molecular basis has remained unclear. In this study, we established a modified $WT1_{235}$ peptide-specific CTL clone, we isolated T-cell receptor (TCR) genes from it and transduced the TCR genes into $CD8^+$ T-cells. The TCR-transduced $CD8^+$ T-cells produced interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) in response to stimulation not only with the modified $WT1_{235}$ peptide but also with the natural $WT1_{235}$ peptide and lysed modified or natural $WT1_{235}$ peptide-pulsed target cells and endogenously WT1-expressing leukemia cells in a HLA-A*24:02-restriction manner. These results provided us, for the first time at molecular basis, with a proof-of-concept of modified $WT1_{235}$ peptide-based immunotherapy for natural $WT1_{235}$ peptide-expressing malignancies.*

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Key Words: Wilms' tumor gene (*WT1*), cytotoxic T-lymphocytes (CTLs), peptide vaccine, cancer immunotherapy.

It is evident that T-cell-mediated immunity plays a crucial role in tumor regression and eradication, and the main effector cells that attack tumor cells are $CD8^+$ cytotoxic T-lymphocytes (CTLs) (1, 2). These CTLs recognize tumor-associated antigen (TAA)-derived peptides presented on the surface of target cells in association with major histocompatibility complex (MHC) class I molecules. To enhance the activity of the TAA-specific CTLs, various types of immunotherapies, including cancer vaccines, are being performed (3, 4).

WT1, which was originally identified as a gene responsible for the pediatric neoplasm Wilms' tumor, encodes a zinc finger transcription factor involved in the regulation of cell proliferation and differentiation (5-8). Although the *WT1* gene was first categorized as a tumor suppressor gene, we showed that it had an oncogenic function and the WT1 protein was highly expressed in various kinds of malignant neoplasms, including hematopoietic malignancies and solid tumors, indicating that the WT1 protein is a most promising TAA (9-21).

Our group and others have identified WT1 protein-derived CTL epitope peptides with the restriction of several HLA class I types. Clinical trials using WT1 CTL epitopes, including HLA-A*0201-restricted $WT1_{126}$ and HLA-A*24:02-restricted $WT1_{235}$ peptides, were performed and showed successful results with clinical response (22, 23). However, we identified a modified $WT1_{235}$ peptide with much higher affinity for HLA-A*24:02 than the natural $WT1_{235}$ peptide. The modified $WT1_{235}$ peptide was found to have the ability to elicit robust induction of the peptide-

specific CTLs that also recognized the natural $WT1_{235}$ peptide (epitope) presented on the tumor cell surface (24). In fact, vaccination of the modified $WT1_{235}$ peptide, which was mainly conducted by our group, showed favorable clinical response, including tumor shrinkage and leukemia cell reduction, in association with immunological response, such as an increase in the frequency of natural $WT1_{235}$ peptide-specific $CD8^+$ T-cells in the peripheral blood (PB) of patients with various kinds of malignancies (3, 25-36). However, why the vaccination of modified $WT1_{235}$ peptide exerted clinical effect and killed tumor cells that expressed the natural $WT1_{235}$ peptide (epitope) but not the modified $WT1_{235}$ peptide (epitope) has not yet been explained on a molecular basis.

In the present study, we describe the establishment of a modified $WT1_{235}$ peptide-specific CTL clone, the isolation of the T-cell receptor (TCR) genes from it, and the molecular basis of clinical findings that the vaccination of modified $WT1_{235}$ peptides is effective for eradication of natural $WT1_{235}$ peptide (epitope)-expressing tumor cells.

Materials and Methods

Cells. Peripheral blood mononuclear cells (PBMCs) were obtained from a healthy donor with HLA-A*24:02 by density gradient using a lymphocyte separation solution (Nacalai Tesque, Kyoto, Japan), and $CD8^+$ T-cells were isolated from the PBMCs using the Human CD8 T-Lymphocyte Enrichment Set-DM (BD Biosicences, San Jose, CA, USA).

K562 is a cell line derived from a blast crisis of chronic myeloid leukemia (CML). K562 endogenously expresses *WT1*, but does not express HLA molecules on the cell surface. K562/24:02 is an HLA-A*24:02-expressing K562 cell line, which was generated by the transduction of HLA-A*24:02 cDNA into K562 (37). T2 is a cell line deficient in transporter-associated with antigen processing (TAP) protein that is essential for the transportation and presentation of peptides generated from endogenous proteins. T2/24:02 was made by the transduction of HLA-A*24:02 cDNA into T2 cells (38). K562, K562/A24:02, and T2/A24:02 cells were cultured in RPMI-1640 (Nacalai Tesque), supplemented with 10% fetal bovine serum (FBS; EuroClone, Pero, Italy).

Induction of the modified $WT1_{235}$ peptide-specific $CD8^+$ T-cell clones. Modified $WT1_{235}$ peptide (CYTWNQMNL)-specific $CD8^+$ T-cell clones were generated by a mixed lymphocyte peptide culture (MLPC) in a modification of the method described by Karanikas *et al.* (39). PBMCs from an HLA-A*24:02+ healthy donor were cultured in X-VIVO 15 medium (Lonza, Walkersville, MD, USA), supplemented with 10% human AB type serum (GEMINI Bio-Products, West Sacramento, CA, USA) in the presence of the modified $WT1_{235}$ peptide (1 μg/ml) and recombinant interleukin-2 (IL-2) (40 U/ml, kindly donated by Shionogi & Co., Ltd., Osaka, Japan) in a 96-well U-bottom plate at a density of 2×10^5 cells/well so that cell expansion occurred in fewer than 10 wells among 96 wells (39).

After two weeks of culture, the expanded cloned cells were screened for positivity for the phycoerythrin (PE)-conjugated modified $WT1_{235}$ peptide tetramer (MBL, Nagoya, Japan) and positive clones were confirmed for the peptide specificity by peptide-specific interferon-γ (IFNγ) production.

Cloning of TCR cDNA and construction of a lentivirus vector. cDNA was obtained by reverse-transcription of total mRNA of the modified $WT1_{235}$ peptide-specific $CD8^+$ T-cell clone B10. cDNAs of TCR-α and -β chains were cloned, amplified by 5'RACE PCR using SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) with gene-specific primers of *TRAC* (CTGTCTTACAATCTTGCAGATC) for TCR-α chain, and *TRBC1* (CACTTCCAGGGCTGCCTTC) and *TRBC2* (TGACCTGGGATGGTTTTGGAGCTA) for TCR-β chain, and sequenced.

To construct a vector that simultaneously expressed both the TCR-α and -β chains, cDNAs of the TCR-α and -β chains were linked *via* a viral P2A sequence (40), followed by cloning into a lentiviral SIN vector (CSII-EF-MCS-IRES2-Venus), with the Venus gene that expressed yellow fluorescent proteins (YFPs) (41).

Transduction of TCR construct into $CD8^+$ T-cells. HEK293T packaging cells were transfected with the TCR construct vector, pCAG-HIVgp and pCMV-VSV-G-RSV-Rev using linear polyethyleneimines (Polysciences, Inc., Warrington, UK) in low-serum media (Gibco, Grand Island, NY, USA). The original CSII-EF-MCS-IRES2-Venus mock vector (mock vector) was used as a negative control. After 12 h of incubation, the HEK293T cells were cultured for virus production in DMEM, containing 4.5 g/l glucose (Nacalai Tesque) supplemented with 10% FBS for 48 h. The virus particles were concentrated by precipitating the culture supernatant using polyethylene glycol (SBI, Mountain View, CA, USA).

The TCR genes were transduced into $CD8^+$ T-cells. In brief, $CD8^+$ T-cells were isolated from PBMCs of an HLA-A*24:02+ healthy donor and activated in X-VIVO 15 medium containing a monoclonal antibody (mAb) against CD28 (eBiosicence Inc., San Diego, CA, USA) and 10% human AB type serum in a CD3 mAb (eBiosicence Inc.)-coated culture plate. After 3 days of activation, the cells were infected with the TCR-containing lentivirus vector using 8 μg/ml of polybrene in RetroNectin (TaKaRa, Tokyo, Japan)-coated plate for 12 h, washed, and cultured in X-VIVO 15 medium, supplemented with 10% human AB type serum.

Flow cytometric analysis. For multicolor staining of cells with tetramer and mAbs, the cells were suspended in phosphate-buffered saline (PBS) containing 2% FBS, followed by staining with the PE-conjugated natural or modified WT_{235} tetramer according to the manufacturer's protocol. The cells were then stained with mAbs on ice for 20 min, washed twice with PBS, containing 2% of FBS, and analyzed with a FACSAria instrument (BD Biosciences). mAbs used were Pacific Blue-conjugated anti-CD3 (BD BioScience), allophycocyanin (APC)-conjugated anti-CD8 (BD BioScience), and PE-conjugated anti-Vβ1 (TRBV9 in another family nomenclature) mAbs (Beckman Coulter Inc., Bera, CA, USA).

Cytokine production assay. For cytokine production assay, 2.5×10^4 of responder cells were stimulated by the appropriate stimulator cells pulsed with 10 μg/ml of a natural $WT1_{235}$ peptide (CMTWNQMNL), the modified $WT1_{235}$ peptide (CYTWNQMNL), or an irrelevant CMV pp65 peptide (QYDPVAALF) in culture medium containing anti-CD28/49d (BD Bioscience) and 10 μg/ml

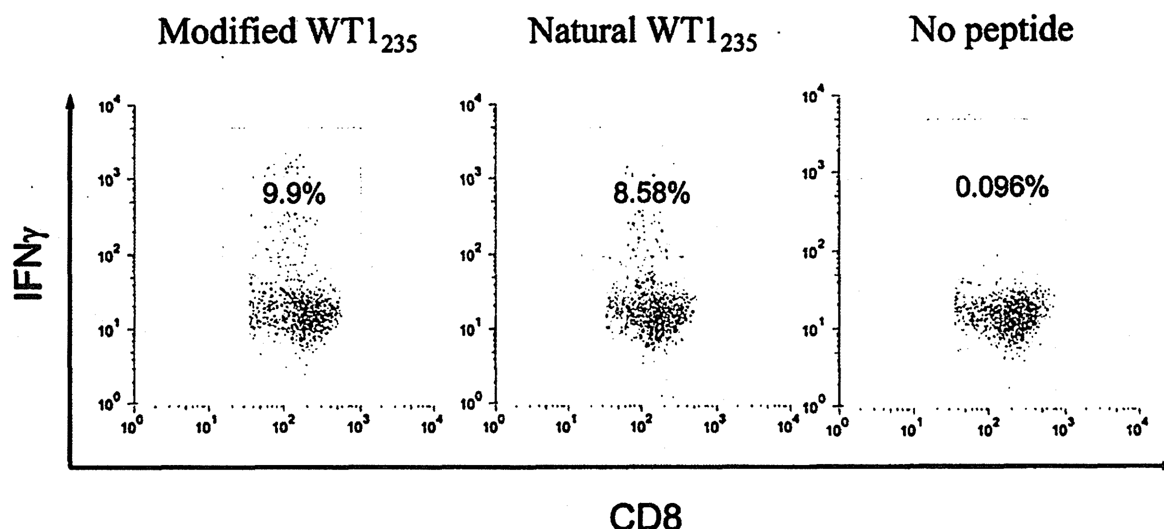

Figure 1. *Establishment of a modified $WT1_{235}$ peptide-specific $CD8^+$ T-cell clone, B10. B10 cells were stimulated by the modified $WT1_{235}$ peptide, the natural $WT1_{235}$ peptide, or not stimulated. Flow cytometory of interferon-γ (IFNγ) production by B10 cells is shown.*

Brefeldin A for 5 h. After the stimulation, the responder cells were stained with APC-Cy7-conjugated anti-CD8 mAb, washed twice, fixed, and permeabilized with Cytofix/Cytoperm (BD Bioscience). The cells were then stained by a PE-conjugated anti-IFNγ and APC-conjugated anti-TNFa mAbs (BD Bioscience), and analyzed using a FACSAria instrument.

For HLA blocking assay, an appropriately titrated blocking mAb for HLA class I (clone wb/32) or HLA-DR (clone L243) was added to cell culture for cytokine production assay.

Cytotoxicity assay. Target cells for cytotoxicity assay were labeled with ^{51}Cr in X-VIVO 15 medium, supplemented with 1% human AB type serum for 2 h, and washed with PBS. The target cells were incubated with appropriate concentrations of antigen peptides, if needed. TCR-transfected $CD8^+$ T-cells were co-cultured with the ^{51}Cr-labeled target cells in X-VIVO 15 medium supplemented with 1% human AB type serum for 4 h. The supernatant was collected, and the radioactivity was counted using a MicroBeta2 plate counter. The percentage-specific lysis was calculated by the equation: (cpm experimental release – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release).

Results

Establishment of a modified $WT1_{235}$ peptide-specific $CD8^+$ T-cell clone. PBMCs of an HLA-A*24:02$^+$ healthy donor were stimulated with modified $WT1_{235}$ peptide seeded at concentrations of $2{\times}10^5$ cells/well in a 96-well plate and then cultured in the presence of modified $WT1_{235}$ peptide (1 μg/ml) and IL-2 (40 IU/ml) for two weeks. Cell expansion was observed in only two of a total of 192 wells and finally only one clone, designated B10, was established. B10 cloned cells were positive for staining with HLA-A*24:02/modified $WT1_{235}$ tetramer and produced IFNγ on stimulation with not only modified $WT1_{235}$ but also natural $WT1_{235}$ peptides (Figure 1). These results show that B10 was a modified $WT1_{235}$ and natural $WT1_{235}$ peptide-specific $CD8^+$ T-cell clone.

Isolation of the TCRs from B10 and establishment of the TCR-transfected $CD8^+$ T-cells. cDNA of TCR-α and -β chains was made from mRNA of the B10 cells using each gene-specific primer, cloned, and sequenced. V- and J- regions of Vα were TRAV27*01 and TRAJ28*01, respectively, while V-, D-, and J-regions of Vβ were TRBV9*01, TRBD2*01, and TRBJ2-3*01, respectively. The TCRs isolated from B10 cells are referred to as B10-TCRs in the following text.

Next, the TCR-α and -β chain genes were linked *via* a viral P2A sequence for dual gene expression (40) and inserted into a lentiviral vector for transfection. Activated $CD8^+$ T-cells were transfected with a B10-TCR-containing lentiviral vector, stimulated by irradiated autologous PBMCs loaded with modified the $WT1_{235}$ peptide three days after transfection, cultured for two weeks, and stained with mAbs to CD3, CD8 and either of the anti-Vβ1 family mAb and the modified $WT1_{235}$-tetramer (Figure 2).

A considerable proportion (18.3%, 9.6/(9.6+42.9)) of YFP-positive cells in B-10-TCR-transfected $CD8^+$ T-cells were positive for staining with mAb to Vβ1 (=TRBV9), whereas 4.3% (2.9/(2.9+65.3)) of YFP-positive cells in mock-transfected $CD8^+$ T-cells were positive for staining with the mAb against Vβ1 mAb (Figure 2a). On the other hand, 4.1% (4.1/(4.1+95.9)) of the untransfected $CD8^+$ T-cells were stained with mAb to Vβ1, which suggested

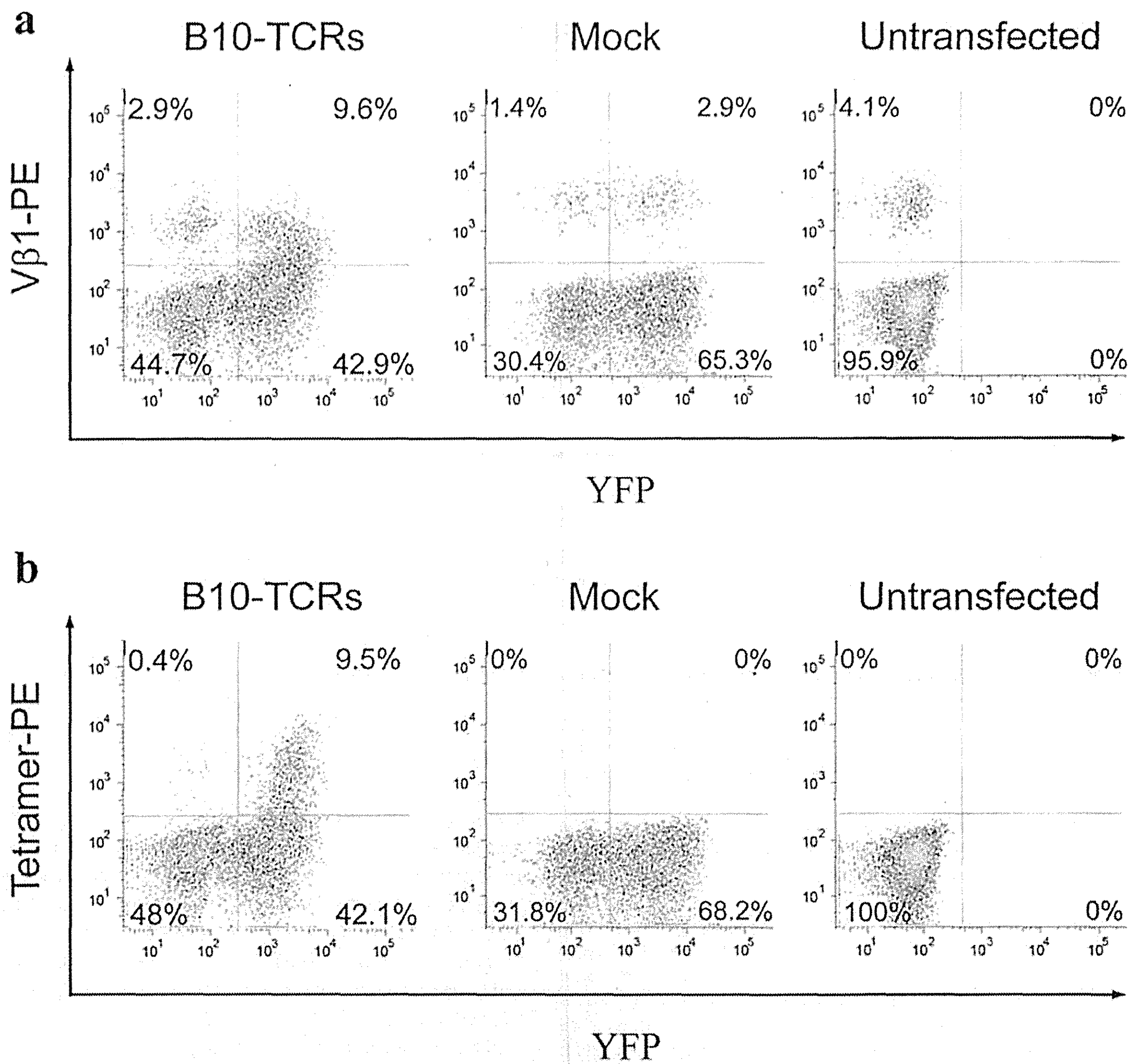

Figure 2. *Functional expression of B10-TCR genes in CD8⁺ T-cells. Activated CD8⁺ T-cells were transfected with B10- TCR-containing a lentivirus vector or a mock vector, and then stained with a monoclonal antibody to Vβ1 family (a) or modified $WT1_{235}$-tetramer (b). Representative data of three experiments are shown.*

endogenous expression of Vβ1 and/or artificial staining with mAb to Vβ1. Furthermore, importantly, modified $WT1_{235}$ tetramer-positive cells were detected only in B10-TCR-transfected $CD8^+$ T-cells at frequencies of 18.4% (9.5/(9.5+42.1)) in YFP-positive cells (Figure 2b). These results indicate that the TCRs from B10 were successfully transduced into $CD8^+$ T-cells and were functional.

To assess the function of B10-TCRs, the antigen-specific cytokine production from the $CD8^+$ T-cells transfected with B10-TCRs was examined (Figure 3a and b). B10-TCR-transfected $CD8^+$ T-cells were stimulated by irradiated autologous PBMCs loaded with the modified $WT1_{235}$ peptide for two weeks and then stimulated again with modified, natural $WT1_{235}$ peptide, or irrelevant CMV pp65 peptide for 5 h and examined for production of IFNγ and TNFα. Cells stimulated with the modified or natural $WT1_{235}$ peptide produced IFNγ and TNFα, whereas cells stimulated with the irrelevant peptide (CMV pp65) did not.

Next, HLA class I restriction of B10-TCR-transfected $CD8^+$ T-cells was examined (Figure 3c). The B10-TCR-

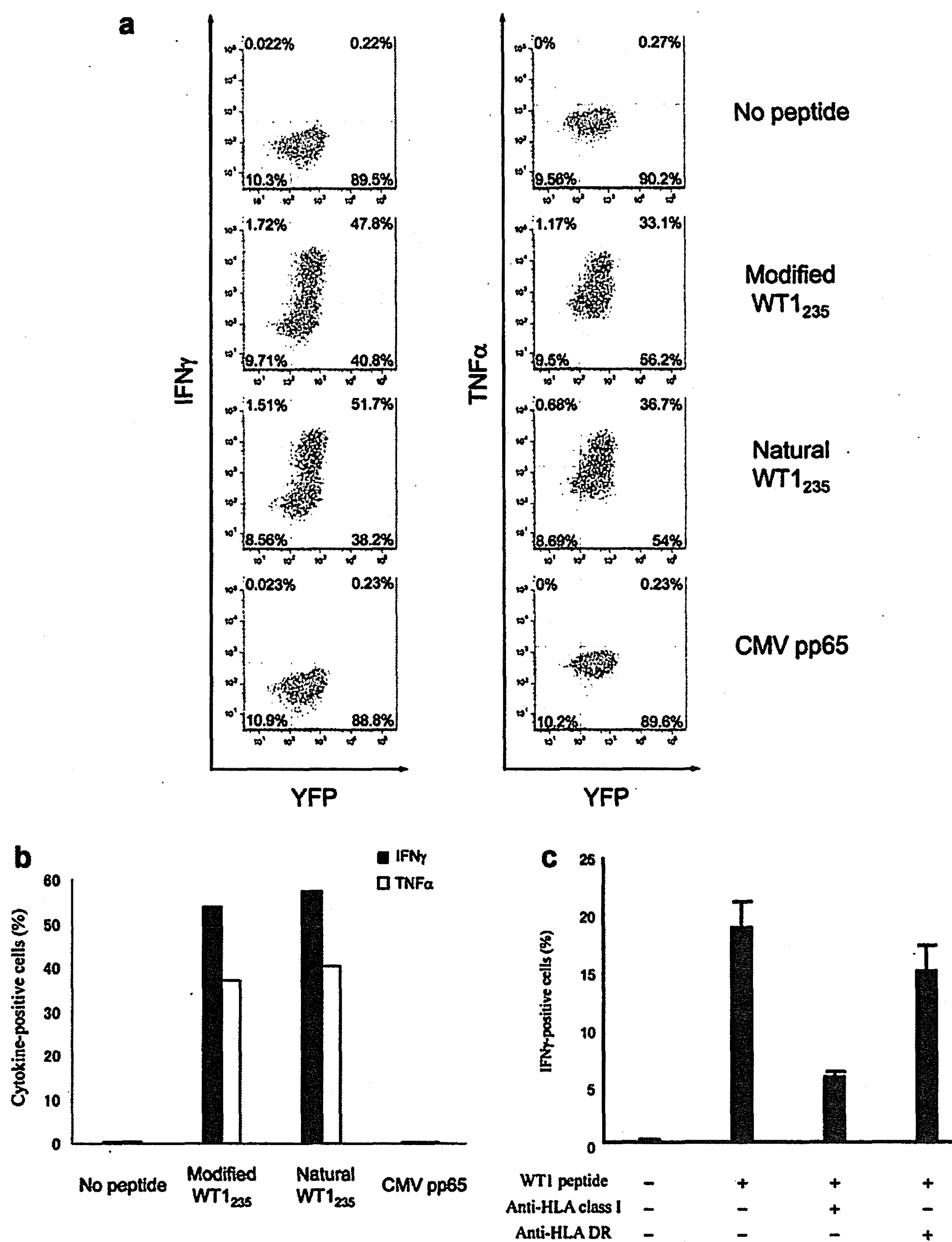

Figure 3. *Cytokine production by the stimulation of B10-TCR-transfected CD8⁺ T-cells. a: B10-TCR-transfected CD8⁺ T-cells were stimulated with the indicated antigen peptides and examined for IFNγ and TNFα production. Representative data of two experiments is shown. b: Frequencies of intracellular IFNγ- and TNFα-positive cells among YFP-positive cells in B10-TCR-transfected CD8⁺ T-cells, stimulated with the indicated antigen peptides. c: CD8⁺ T-cells transfected with the B10-TCRs were stimulated with the modified $WT1_{235}$ peptide-loaded T2/24:02 cells, and were assayed for IFNγ production in the presence of HLA class I- or HLA DR-blocking monoclonal antibody. Representative data of two experiments are shown. T2/24:02 cells, HLA-A*24:02-positive T2 cells.*

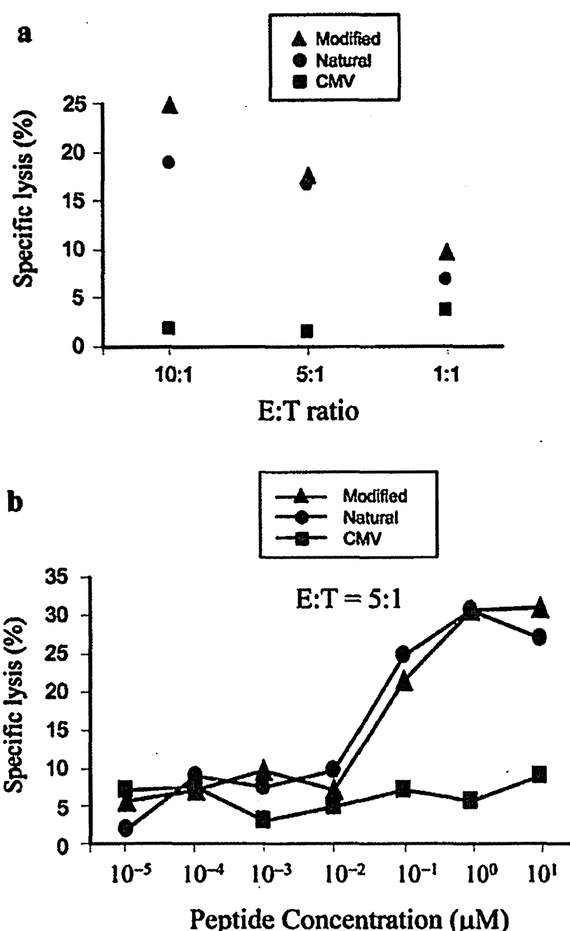

Figure 4. *$WT1_{235}$ peptide-specific cytotoxic activity of B10-TCR-transfected $CD8^+$ T-cells. $CD8^+$ T-cells transfected with B10-TCRs were assayed for their cytotoxic activity towards T2/24:02 cells loaded with modified, natural $WT1_{235}$ peptide, or with CMV pp65 peptide, at a concentration of 20 μM (a), or at different concentrations (b). Representative data of two experiments are shown. E:T, effector/target ratio.*

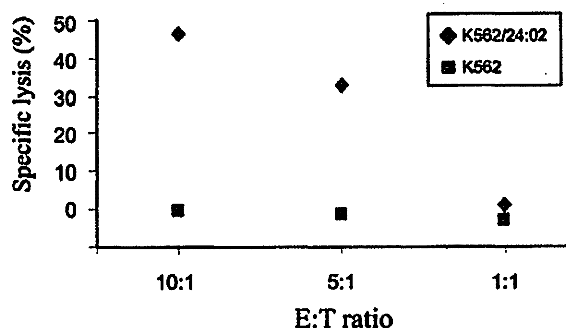

Figure 5. *Cytotoxic activity of B10-TCR-transfected $CD8^+$ T-cells towards endogenously WT1-expressing leukemia cells. Cytotoxic activity of B10-TCR-transduced $CD8^+$ T-cells towards endogenously WT1-expressing K562 leukemia cells with or without HLA-A*24:02 expression was examined. Representative data of three experiments are shown.*

transfected $CD8^+$ T-cells were stimulated by T2/24:02 cells loaded with the modified $WT1_{235}$ peptide in the presence of an HLA class I or HLA DR blocking mAb and stained for intracellular IFNγ. The production of IFNγ was inhibited by anti-HLA class I mAb, but not by anti-HLA DR blocking mAb. These results indicate that the cytokine production of B10-TCR-transfected $CD8^+$ T-cells by antigenic stimulation was restricted to HLA class I.

$WT1_{235}$ peptide-specific cytotoxic activity of B10-TCR-transfected $CD8^+$ T-cells. To test the antigen-specific cytotoxicity of B10-TCR-transfected $CD8^+$ T-cells, they were co-cultured with irradiated autologous PBMCs loaded with modified $WT1_{235}$ peptide for two weeks and assayed for cytotoxicity towards ^{51}Cr-treated HLA-A*24:02-transfected T2 (T2/24:02) cells, loaded with modified or natural $WT1_{235}$ peptide (Figure 4a). The B10-TCR-transfected $CD8^+$ T-cells exhibited cytotoxicity towards the T2/24:02 cells loaded with modified or natural $WT1_{235}$ peptide in an effector/target (E/T) ratio-dependent manner, but not towards those loaded with an irrelevant peptide (CMV pp65 peptide). These results indicated that B10-TCRs recognized not only the modified $WT1_{235}$ peptide/HLA-A*24:02 complex but also the natural $WT1_{235}$ peptide/HLA-A*24:02 complex.

Next, specific lysis by B10-TCR-transfected $CD8^+$ T-cells was assayed for the T2/24:02 target cells pulsed with different concentrations of modified or natural $WT1_{235}$ peptide (Figure 4b). The specific lysis increased in parallel with an increase in the peptide concentrations and reached a plateau at an E/T ratio of 5:1, at a concentration of 1 μM in both peptides. The half-maximal lysis for modified and natural $WT1_{235}$ peptide was obtained at a concentration of about 0.06 μM and 0.04 μM, respectively. These results indicate that the affinity of B10-TCRs for natural $WT1_{235}$ peptide/HLA-A*24:02 complex was high enough to expect that B10-TCRs would be able to recognize the endogenous WT1 protein-derived (natural) $WT1_{235}$ peptide that was presented on the cell surface in association with HLA-A*24:02 molecules.

*Lysis of endogenously WT1-expressing leukemia cells by B10-TCR-transfected $CD8^+$ T-cells with an HLA-A*24:02 restriction.* Whether or not B10-TCR-transfected $CD8^+$ T-cells had the ability to lyse endogenously WT1-expressing leukemia cells with a restriction of HLA-A*24:02 was

examined. The B10-TCR-transfected $CD8^+$ T-cells were stimulated by irradiated autologous PBMCs loaded with the modified $WT1_{235}$ peptide. After two weeks of the stimulation, the B10-TCR-transfected $CD8^+$ T-cells were assayed for the lysis of HLA-A*24:02-transfected K562 leukemia cells (K562/24:02) that endogenously expressed WT1. The B10-TCR-transfected $CD8^+$ T-cells were cytotoxic towards the K562/24:02 cells, but not towards K562 cells without an HLA-A*24:02 expression (Figure 5). These results indicate that B10-TCR-transfected $CD8^+$ T-cells were able to kill endogenously WT1-expressing leukemia cells in an HLA-A*24:02 restriction manner.

Discussion

In the present study, a modified $WT1_{235}$ peptide-specific CTL clone (B10) was established and its TCRs (B10-TCRs) were cloned. B10-TCR-transfected $CD8^+$ T-cells were able to kill both modified $WT1_{235}$ peptide-pulsed and natural $WT1_{235}$ peptide-pulsed target cells and endogenously WT1-expressing leukemia cells.

An important finding presented here was that B10-TCRs, isolated from a modified $WT1_{235}$ peptide-specific CTL clone, was able to recognize and kill both natural $WT1_{235}$ peptide-pulsed target cells and endogenously WT1-expressing leukemia cells that were possibly expressing natural $WT1_{235}$ peptide (epitope) on their cell surface in complexes with HLA-A*24:02 molecules. The evidence, at the molecular level, showing that a modified $WT1_{235}$ peptide-specific TCR recognizes both its own modified and other natural $WT1_{235}$ peptides (epitopes) has been demonstrated here for the first time due to our successful cloning a modified $WT1_{235}$ peptide-specific TCR gene. This evidence provided us with a strong proof-of-concept of modified $WT1_{235}$ peptide-based immunotherapy, in which the modified (not natural) $WT1_{235}$ peptides were effectively vaccinated for the eradication of tumor cells that were possibly expressing natural (not modified) $WT1_{235}$ peptides in complexes with HLA-A*24:02 molecules. In fact, there are some clinical findings showing that vaccination with modified $WT1_{235}$ peptides induced modified $WT1_{235}$ peptide-specific CTLs and other CTLs that were able to recognize both the modified and natural $WT1_{235}$ peptides (epitopes). For example, Narita *et al.* successfully vaccinated a patient with CML with the modified $WT1_{235}$ peptides and showed that some $CD8^+$ T-cells in PBMCs that were obtained after repeated WT1 vaccination were dually stained with the modified $WT1_{235}$ peptide-specific and natural $WT1_{235}$ peptide-specific tetramers. They also showed that the modified $WT1_{235}$ peptide-specific CTL clones established, exerted cytotoxic activity towards both the modified $WT1_{235}$ peptide-pulsed and natural $WT1_{235}$ peptide-pulsed target cells (42). However, since the cloning of TCRs from the modified $WT1_{235}$-specific CTLs was not done, it was not demonstrated, at the molecular level, that the TCRs of the modified $WT1_{235}$-specific CTLs recognized both the modified and natural $WT1_{235}$ peptides (epitopes). On the other hand, it was demonstrated that a natural $WT1_{235}$ peptide-specific CTL clone, TAK-1, recognized both the natural and modified $WT1_{235}$ peptides (24). However, the molecular basis of this finding has not yet been reported. Thus, detailed analysis at the molecular level for explaining how $WT1_{235}$ peptide-specific CTLs are able to recognize both natural and modified $WT1_{235}$ peptides (epitopes) has been reported here for the first time.

Results presented here suggest the possibility for adoptive transfer therapy of $CD8^+$ T-cells transfected with the modified $WT1_{235}$ peptide-specific TCR genes. Half-maximal lysis by the $CD8^+$ T-cells that were transfected with the TCRs from the modified $WT1_{235}$ peptide-specific CTLs was obtained against the natural $WT1_{235}$ peptide-pulsed target cells at concentrations of as low as 0.04 μM. This indicates the high affinity of the TCRs for the natural $WT1_{235}$ epitope on tumor cells. These results should allow us to expect a good clinical effect of adoptive cell therapy using the TCR genes isolated here.

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III. 臨床応用の進歩と展望 免疫療法(免疫細胞療法)

WT1分子を標的にしたワクチン療法

杉山治夫

WT1-targeting cancer vaccine

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Abstract

Wilms' tumor gene WT1 encodes a transcription factor and functions as an oncogene.
WT1 gene product WT1 protein is a promising par-tumor-associated antigen.
WT1 peptide-based immunotherapy has been performing for more than six hundred patients with leukemias and various types of solid tumors.
This immunotherapy is safe and has clinical benefit especially for leukemia, glioblastoma multiforme, advanced pancreatic cancer, and ovarian cancer.
As a new strategy for cancer treatment, it should be recommended to initiate immunotherapy that had a potential of eradication of cancer stem cells before surgery, chemo- and radio-therapy.

Key words: WT1, cancer immunotherapy, Wilms' tumor gene solid tumor

はじめに

ウィルムス腫瘍遺伝子(WT1)は，小児の腎癌の原因遺伝子として単離され，がん抑制遺伝子と定義されているが，著者らは一連の研究から，WT1は根源的ながん遺伝子である可能性が高いことを提唱している[1]．著者らは，まず1994年にWT1mRNAが白血病の微小残存病変を検出するための有用なマーカーであることを見いだし，白血病治療に必須なWT1mRNA定量検査を開発し，国内外に広めた．このWT1mRNA定量検査は，2007年11月に急性骨髄性白血病(AML)に対し，また2011年8月には骨髄異形成症候群(MDS)に対し，保険適用された．更にWT1タンパクは，白血病などの血液悪性疾患および，ほとんどすべての種類の固形がんに発現する汎腫瘍抗原であることを見いだし，WT1タンパクを標的にしたがんの免疫療法を開発した．

WT1タンパクは，白血病やほとんどすべての種類の固形がんで高発現する汎腫瘍抗原である(図1)[2]．既存の76種類の腫瘍抗原の有用性について，米国立がん研究所が9項目について評価を行い，WT1を第1位にランクした(図2)[3]．

1. 第I相臨床研究

2001年，WT1ペプチド免疫療法の第I相臨床試験を開始した(表1)[4]．HLA-A*2402の

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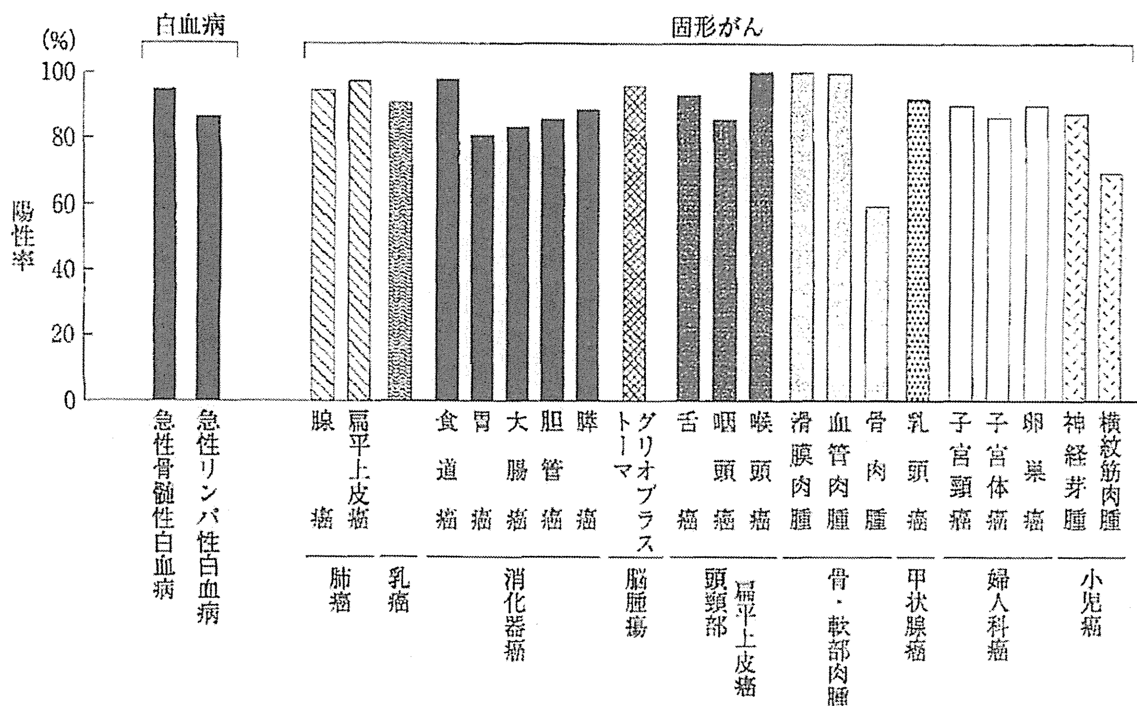

図1 白血病や固形がんにおけるWT1陽性率
WT1タンパクは，白血病やほとんどの種類の固形がんで高発現する汎腫瘍がん抗原．

患者(日本人の約60%)に，天然型(natural)9-mer WT1ペプチド(CMTWNQMNL)あるいは改変型(modified)9-mer WT1ペプチド(CYTWNQMNL)[9]とモンタナイドアジュバントのエマルジョンを2週間ごとに1回，計3回皮内注射した．0.3mg/body→1.0mg/body→3.0mg/bodyと3人ずつdose-upした．計26人にWT1ペプチドを1回以上投与した．MDS 2人に1回だけWT1ペプチドを投与したところ，両症例とも白血球(白血病細胞)が著減し，MDSには著効を呈することを示した．正常造血能を保ったMDS以外の24人の患者には，WT1ワクチン投与部位の発赤，腫脹以外，特記すべき副作用はみられなかった．規定の3回のWT1ワクチン投与を完遂した18人の臨床効果を表1にまとめた．

症例No.12の乳癌患者は，右乳房の広範囲切除後，化学療法を行ったが，リンパ節再発→化学療法→右肺門リンパ節転移→化学療法→小脳転移をきたし，他の治療法がないことから，WT1ワクチンの投与を開始したところ，5回の投与で肺転移巣が縮小し，部分寛解(PR)となった(図3)．また，症例No.16の乳癌患者は，両側乳房の部分切除後，リンパ節転移，骨移転がみられ，化学療法を行うも全く無効で，がん性腹膜炎を起こし，腸壁にがんが増殖し腸閉塞をきたし，ほかに治療法がないことからWT1ワクチンを投与した(図4)．2回投与後，がん性腹膜炎が軽快し，腸閉塞も消失．以後，骨転移の再発で死亡するまで，3年1カ月生存した．

分子再発したAMLの3症例(表1のNo.21，23，24の患者)は，WT1ワクチンを現在まで最長9年2カ月以上継続投与されているが，白血球数の減少は全く起こらず，白血病細胞のみが減少し，完全寛解が持続しており，WT1ワクチンの投与部位の発赤・腫脹以外，重篤な副作用はみられず，WT1ワクチンの長期投与の安全性が明らかになってきた．

2. 新臨床研究

第I相臨床研究の結果から，正常造血が十分に残存している疾患では，WT1免疫能を強める臨床研究が安全に行えることが，そして正常造血がほとんどないMDSや慢性骨髄性白血病では，WT1免疫能を弱める臨床研究が必要であることが明らかになった[5]．

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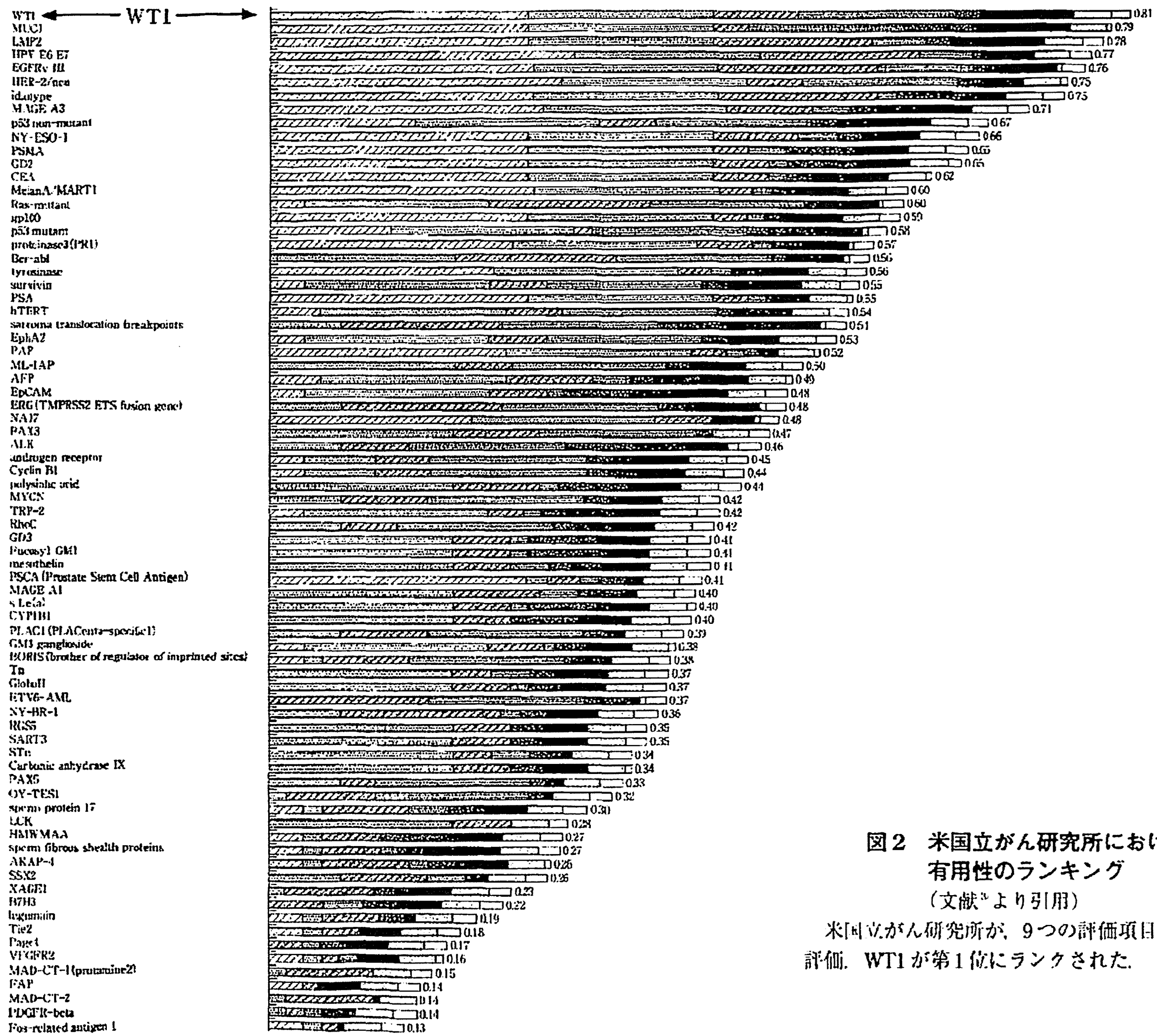

9項目で評価

- 治療効果
- 免疫原生
- 特異性
- がん遺伝子性
- 発現レベル・陽性率
- がん幹細胞での発現
- 抗原陽性患者数
- エピトープ数
- 抗原発現部位

図2 米国立がん研究所におけるがん抗原76種類の有用性のランキング

（文献[3]より引用）

米国立がん研究所が，9つの評価項目に応じて，76種類のがん抗原を評価．WT1が第1位にランクされた．

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表1 第I相臨床研究 完遂症例まとめ(2012年8月現在)

No.	WT1ペプチド (mg/body)	病名	病期	年齢/性	副作用 局所/全身	臨床効果
1	0.3	LC	IV	46/F	+/−	+(CEA ↓)
2	0.3	LC	III B	52/M	+/−	SD(stable disease)
4	0.3	LC	IV	70/M	+/−	2年1カ月生存
10	M0.3	LC	IV	58/M	+/−	PD
11	M0.3	LC	IV	68/M	+/−	PD
⑫	M0.3	BC	III A	56/F	+/−	PR(部分寛解)
13	1.0	AML	CR	54/F	+/−	評価病変なし
15	1.0	AML	CR	54/M	+/−	評価病変なし
⑯	1.0	BC	IV	46/F	+/−	3年1カ月生存
17	M1.0	LC	III A	50/M	+/−	+(SCC ↓)
18	M1.0	AML	CR	56/M	+/−	評価病変なし
19	M1.0	AML	CR	45/M	+/−	評価病変なし
20	3.0	AML	CR	42/F	+/−	+(WT1 ↓)
㉑	3.0	AML	分子再発	32/M	+/−	完全寛解持続 8年投与 9年4カ月経過
22	3.0	AML	CR	40/F	+/−	+(WT1 ↓)
㉓	M3.0	AML	分子再発	49/F	+/−	完全寛解持続 9年2カ月投与 9年2カ月経過
㉔	M3.0	AML	分子再発	60/F	+/−	完全寛解持続 7年10カ月投与 9年4カ月経過
26	M3.0	AML	CR	56/F	+/−	SD

臨床効果 12/14

LC: 肺癌, BC: 乳癌, AML: 急性骨髄性白血病.

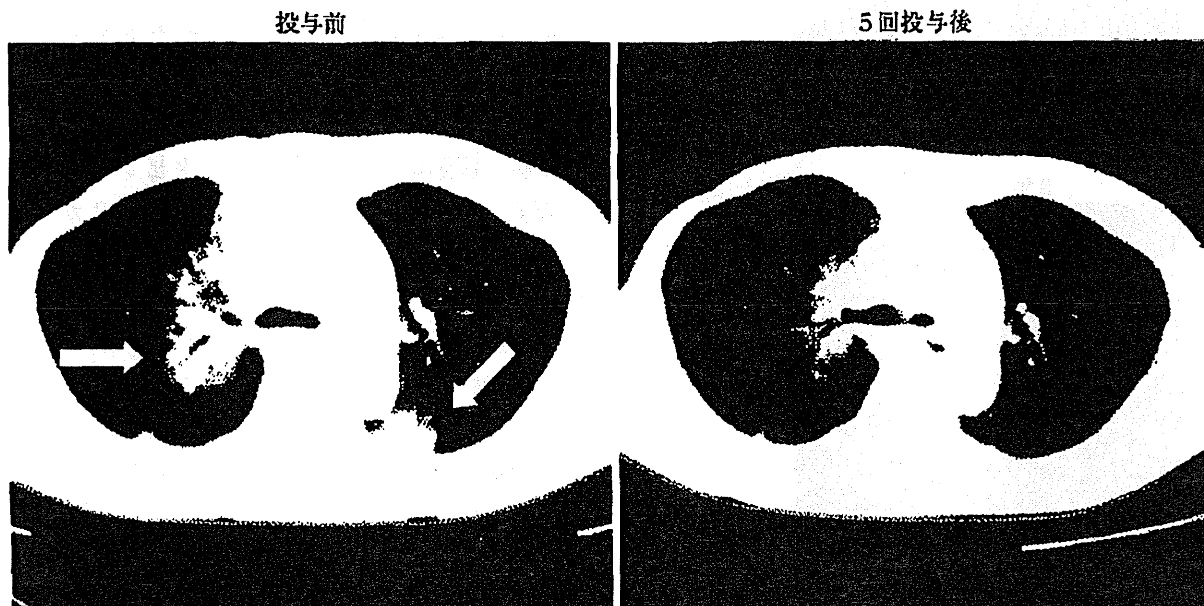

図3 乳癌—WT1ワクチン投与による肺転移巣の縮小—
表1の症例No.12. WT1ワクチンを2週間ごとに5回投与したところ, 部分寛解となった.

1) WT1免疫能を強めるWT1ワクチン療法

WT1免疫能を強化したWT1ペプチド免疫療法として, 改変型WT1ペプチド3.0mg/bodyを毎週, 計12回投与した.

多発性骨髄腫では, 4例中3例で臨床効果がみられ, MRの一例では, 骨髄腫細胞と, 尿中Mタンパクの減少と, 肋骨病変の改善がみられた[6]. 再発神経膠芽腫では[7], 65例中CR 1例, PR 2例, SD 28例, PD 34例で, SDのうち3人は, 4年1カ月〜7年5カ月間無増悪生存して

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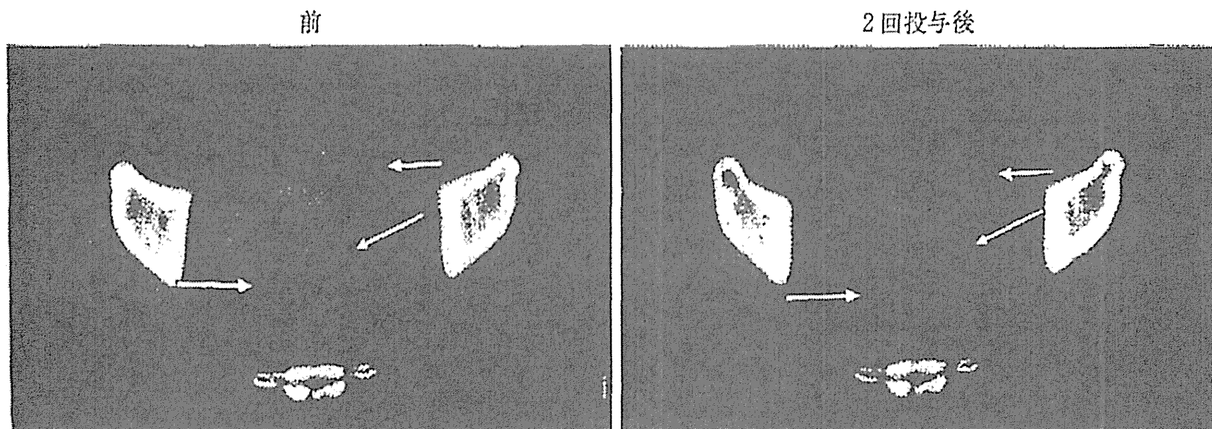

図4 乳癌—WT1ワクチン投与による大腸転移巣の縮小—
表1の症例No.16. WT1ワクチンを2週間ごとに2回投与したところ，がん性腹膜炎が軽快し，転移がんで肥厚した腸壁(矢印)がほぼ正常化し，腸閉塞が軽快した.

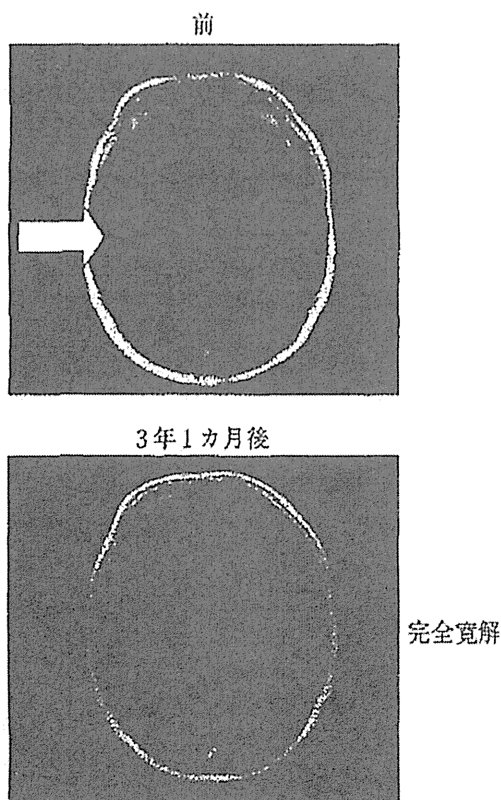

図5 再発悪性神経膠芽腫(グリオブラストーマ)
41歳 男性 現在6年6カ月生存.
WT1ワクチンの投与によりSDとなる．その後，ゆっくり腫瘍が縮小し，3年1カ月後にはCRとなり，現在まで6年6カ月生存し，社会復帰している.

おり，臨床的には完全寛解と考えられる(図5).

婦人科癌も，WT1ワクチンの効果が期待されるがん腫の一つである．末期の卵巣癌患者にWT1ワクチンが投与されたところ，評価できた4人のうち2人はSDで2人はPDであった．また子宮頸癌患者では，評価できた3人のうち2人がSDで1人がPDであった[8,9].

腎癌は，一般的に免疫療法の有効性が高いがん腫であるが，WT1ワクチンも有効性が期待される．腎細胞癌の一例(67歳，男性)では，手術後，IFN-α→IL-2と治療されたが増悪してきたので，WT1ワクチンを投与したところ，SDとなった(図6)[10].

膵癌，胆道癌に対してゲムシタビンとの併用でWT1ワクチンが投与された．投与2カ月目での病勢コントロール率は，膵癌で89%，胆道癌で50%であり，安全性が明らかになった[11]．更に，胆道癌に対するゲムシタビン＋シスプラチン対ゲムシタビン＋シスプラチン＋WT1ワクチンのランダマイズド臨床研究が進行中である[12]．そのほか，唾液腺癌でも有効症例がみられた[13].

小児癌でもWT1タンパクが高発現しているものが多くあり，WT1ワクチンが期待されている．橋井らは，末期の横紋筋肉腫患者(7歳，女児)にWT1ワクチンを投与したところ，3カ月で骨転移が消失し，完全寛解し，現在まで6年4カ月完全寛解が持続しており，完治の可能性がある(図7)[14,15]．ほかにも類似の症例が複数あり，本疾患はWT1ワクチン感受性が高い可能性がある.

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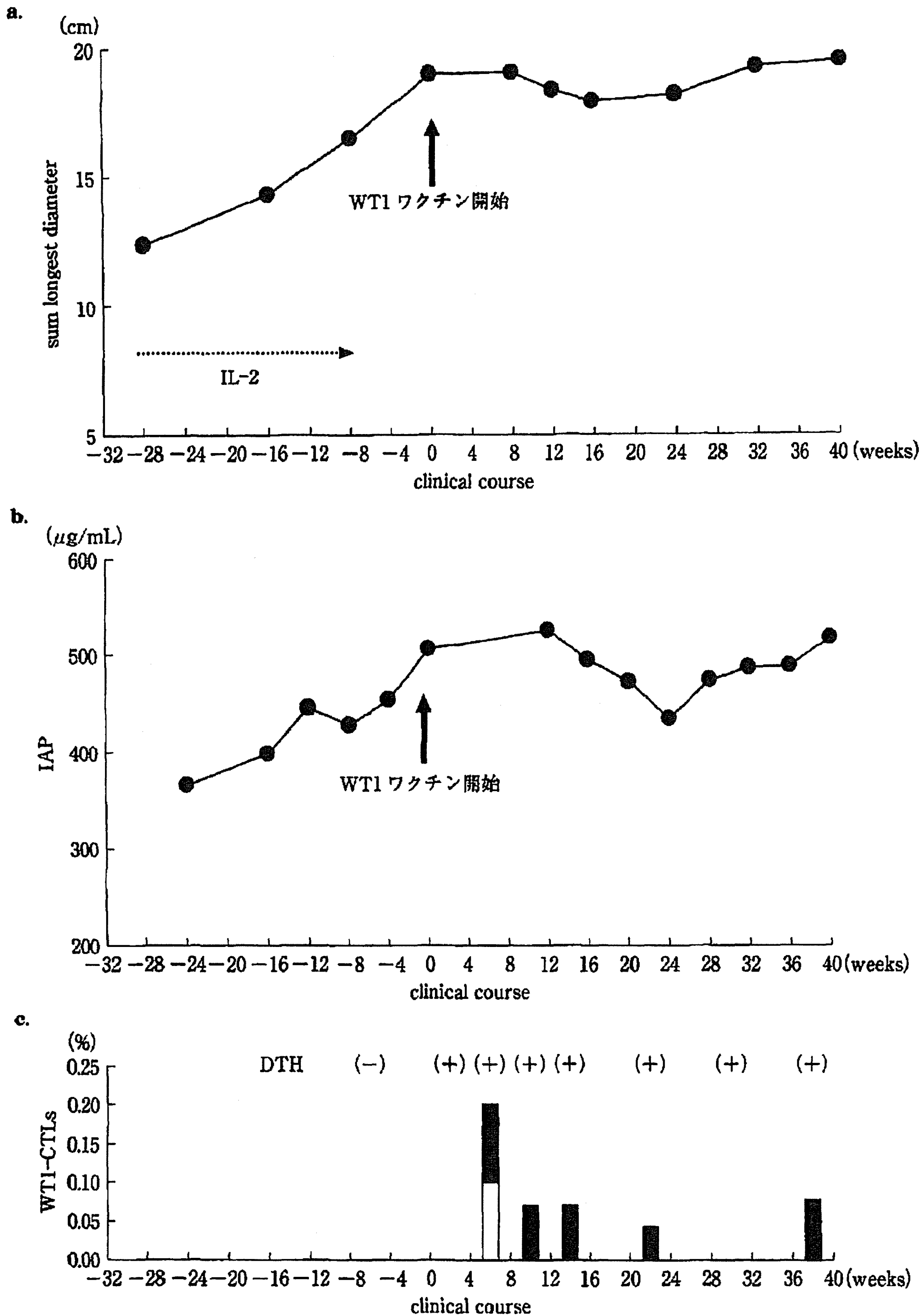

図6 腎細胞癌

WT1 ワクチン投与後，sum longest diameter(a)，腫瘍マーカー IAP(b)が stable になり，WT1 特異的 CTLs も上昇し，WT1 ペプチドに対する遅延型アレルギー(DTH)も出現した(c).

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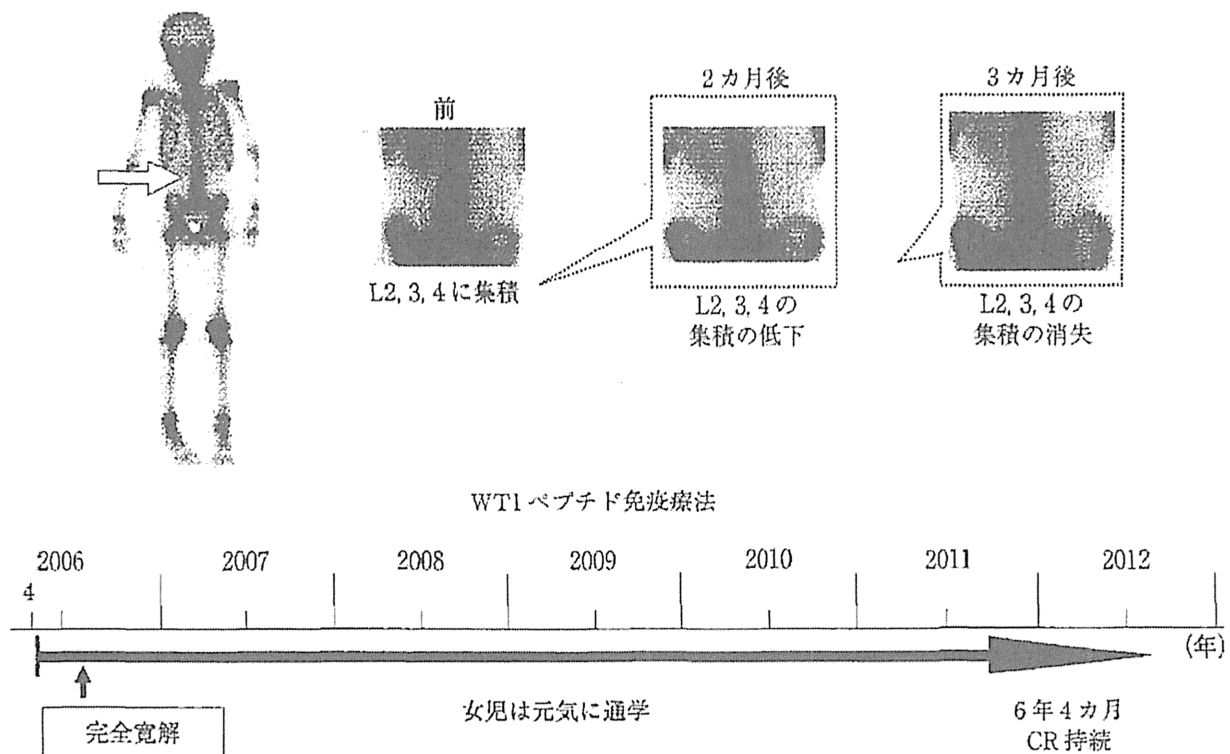

図7 横紋筋肉腫

骨病変の消失—完全寛解を達成．手術，化学療法，放射線療法後，骨転移が残存し，予後が極めて不良と判断され，WT1ワクチンを投与．3カ月で骨転移が消失し，現在まで完全寛解が続く．

2) モンタナイドアジュバント以外のアジュバントを用いたWT1ワクチン療法

BCG-CWS(cell wall skeleton)は，結核菌の菌体成分であり，非常に強い自然免疫を活性化する．そこで種村らは，BCG-CWSを皮内投与し，24時間後，同一部位にWT1ペプチド水溶液(アジュバントを含まず)を皮内投与した．肺転移したメラノーマの一例ではSDとなり，約6カ月間新転移巣が出現しなかったので，肺転移巣の切除術が可能となった[16]．大野らは，アジュバントとしてGM-CSFやCpGを使って，各種の固形がんにWT1ワクチンを投与した[17]．モンタナイドアジュバント，GM-CSF，CpGを使った際の病勢コントロール率は，それぞれ20%，25%，60%であり，CpGで有効率が高かった．

3. ま と め

免疫療法は，免疫能を十分に温存している患者には，予想をはるかに超えたポテンシャルをもっていると考えられる．今後のがんの治療戦略としては，がんの診断がつき次第，静止期にあるがん幹細胞を死滅させうる能力をもった免疫療法を開始する．免疫療法をベースラインとして継続させながら，手術，抗がん剤，放射線療法を的確に行い，免疫療法によって，がん幹細胞の撲滅をはかり，治癒につなげるのがよいと考える(図8)．WT1ペプチド免疫療法は，この治療戦略において重要な役割を果たすものと考えられる．造血幹細胞移植(HSCT)後の早期再発患者や早期再発ハイリスクのAMLや急性リンパ性白血病(ALL)患者に対して，移植後早期のまだ免疫抑制剤が使われている時期でのWT1ワクチンが非常に有効であることが次第に明らかになってきた．予期に反し，強力な免疫抑制療法中にもかかわらず，WT1ワクチンの投与により，WT1特異的キラーT細胞が誘導され，白血病細胞が傷害されることが明らかになってきた．最初は，橋井らによって小児癌に対するHSCT後のWT1ワクチンの有用性が

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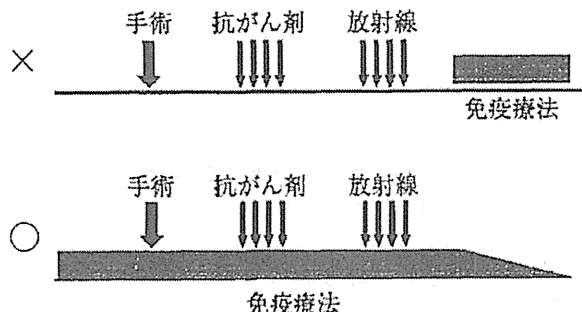

図8 がんの治療戦略

がんと診断されたならば，すぐに免疫療法を開始し，その後，手術，化学療法や放射線などを併用することにより，免疫療法のみがもつがん幹細胞を死滅させうる能力などが十分に発揮できる．

示されたが[15]，その後，成人白血病に対するHSCT後のWT1ワクチンの有用性も明らかになってきた．このことは，人為的に作り出された免疫抑制状態が，WT1ワクチンのプライミングに適している可能性を示しているものと考えられる．このことを更に演繹すると，化学療法後の免疫抑制状態(体力的には全免疫能は温存されており，一時的な免疫抑制状態を意味し，化学療法のために免疫能が完全に消失している状態ではない)は，WT1ワクチンのプライミングに適している可能性があり，このことを検証したい．現在，WT1ワクチンは，中外製薬，大日本住友製薬および大塚製薬によって治験中である．

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