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Feasibility and Immune Response of WT1 Peptide Vaccination in Combination with OK-432 for Paediatric Solid Tumors

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Abstract. *Background/Aim: Wilms' tumor 1 (WT1) peptide-based vaccination has been reported for its potential usefulness in targeting several cancers. The adjuvant drug OK-432 is known to have potent immunomodulation and therapeutic properties when applied in cancer treatment and may, thus, be important to trigger the appropriate immunological response in paediatric patients with a solid tumor that are vaccinated with a WT1 peptide. Patients and Methods: Paediatric patients with a solid tumor were vaccinated with a WT1 peptide and OK-432 once every 2 weeks, for a total of seven times. Results: Of the 24 patients, 18 completed the scheduled vaccinations. Sixteen patients had local skin symptoms and/or fever. In 1 patient, anaphylactic symptoms emerged at the time of the final injection, but these quickly subsided after the treatment. WT1-specific immunological responses were observed in 4 patients (22.2%). WT1 and HLA class I expression were confirmed in 100% and 85% of primary tumors, respectively. Conclusion: WT1 peptide vaccine therapy combined with OK-432 appears to be relatively safe for children. However further studies in a larger number of patients are necessary to confirm its safety and efficacy.*

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While the use of multidisciplinary approaches including surgery, chemotherapy and/or radiotherapy has resulted in improved cure rates in paediatric patients with solid tumors (1), many of these patients suffer from a relapse and/or treatment-related complications (2). Cancer immunotherapies, developed to improve survival rates and reduce treatment-related complications, have drawn a lot of attention within the past few years.

Wilms' tumor 1 (WT1) protein is a zinc finger transcription factor essential in urogenital embryogenesis. Although, at first, WT1 was identified as a tumor suppressor gene responsible for Wilms' tumors of the kidney (3-5), it possesses an oncogenic, rather than a tumor-suppressive function (6, 7). Wild-type WT1 is expressed in many types of haematological and solid tumors, which indicates that WT1-targeting immunotherapy can be used for a variety of malignancies (8, 9). Some clinical studies using WT1 peptide vaccine have been reported; however, there are few studies reporting the same in paediatric patients with solid tumors (10).

Despite the usefulness of adoptive cancer immunotherapies using the patient's blood cells such as T cells and natural killer cells and acquired immunotherapy with dendritic cells (DCs), there are some problems associated with paediatric patients with solid tumors, such as difficulties in harvesting specific materials from blood cells. Therefore, peptide cancer vaccination targeting specific cancer-associated antigens such as WT1 peptide may be one of the important options for such patients. However, because tumor antigens are self-antigens, it is likely that high-avidity T-cell receptors will be deleted from the repertoire (11). Adjuvants play an important role in combination with cancer peptide vaccine to enhance antigen

immunogenicity and elicit the desired immune response. Montanide ISA-51, a water-in-oil emulsion composed of a mineral oil and a surfactant from the mannide monooleate family, is an adjuvant carrier with immunostimulatory effect that is widely used in WT1 peptide vaccine studies. The immune-enhancing effect of ISA-51 is suggested to be associated with depot formation (slowing down the release of antigens at the immunization site), inflammation (stimulating the recruitment of antigen-presenting cells) and lymphocyte trapping (stimulating the accumulation of lymphocytes in draining lymph nodes) (12, 13). However, Hailemichael *et al*. reported that persisting vaccine depots can induce specific T-cell sequestration, dysfunction and deletion at the vaccination sites (14). OK-432, a lyophilized biological preparation containing cells of *Streptococcus pyogenes* Su strain treated with benzylpenicillin, has potent immunomodulation and therapeutic properties when applied in cancer treatment as a biological response modifier (15). Although little data are available on WT1 peptide vaccination therapy combined with OK-432 as an adjuvant, OK-432 was recently used as an adjuvant in WT1 peptide-pulsed DC vaccine for pancreatic cancer in Japan (16, 17). These trials suggested that some patients achieved survival benefits without serious adverse effects. Thus, OK-432 might be a potential candidate as an adjuvant for immunotherapy (17). Therefore, we investigated the safety of WT1 peptide vaccination therapy with OK-432 as an adjuvant in paediatric patients with solid tumors.

Materials and Methods

Trial design and patient eligibility. This clinical trial was approved by the Ethical Committee of Shinshu University School of Medicine on 8 May 2013 (approval number: 2297) and was registered in the University Hospital Medical Information Network Clinical Trial Registry (UMIN-CTR) as a Clinical Trial (Unique trial number: UMIN000011030) on 1 July 2013. Written informed consent to participate was obtained from each patient and/or guardian. The trial was conducted in accordance with the Declaration of Helsinki and International Conference Harmonization guidelines for Good Clinical Practice.

Eligibility criteria were as follows: age <20 years, patients having human leukocyte antigen (HLA)-A*24:02, HLA-A*02:01 or HLA-A*02:06 allele, pathologically proven cancers, having been treated in accordance with standard clinical guidelines, patients having neither allergies to OK-432 nor to penicillin, with a Karnofsky performance status over 60% (18), an estimated survival duration of over 3 months and with maintained function of major organs.

WT1 vaccination. We used HLA-A*24:02-restricted mutant WT1 peptide (residues 235–243: CYTWNQMNL) and/or A*02:01/A*02:06-restricted wild-type WT1 peptide (residues 126-134: RMFPNAPYL), both of which could be prepared at practice grade level and were derived from NeoMPS Inc (19, 20). Seven doses of HLA-A*24:02- or A*02:01/*02:06-restricted WT1 peptide (1-3 mg) mixed with 0.25-2 KE of OK-432 were injected intradermally in the bilateral axillary and inguinal areas once every 2 weeks following the protocol of DC vaccination (21). Doses of WT1 peptide were adjusted according to the patient's body weight (BW) (1 mg of WT1 peptide for BW <10 kg, 2 mg for 10 kg <BW <20 kg, 3 mg for BW >20 kg). Initial doses of OK-432 were adjusted according to the patient's BW (0.25 KE of OK-432 for BW <10 kg, 0.5 KE for 10 kg <BW <20 kg, 1 KE for BW >20 kg). The doses of OK-432 after second vaccination were adjusted based on vaccine-related toxicities. If both WT1 peptides were applicable to patients according to the HLA typing, one peptide was injected into the axillary and inguinal area at one side and the other one was injected at the opposite side.

Evaluation of WT1 peptide vaccine-related toxicities. Adverse effects including fevers and allergic reactions were monitored and graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0. The skin reaction at the injection site was examined after each vaccination in all patients. Redness and induration were assessed after 24, 48 and 72 hours. Any reaction at the injection site was measured as the maximum diameter of redness and induration. A positive skin reaction was defined as an erythema of more than 3 mm.

Evaluation of the immunological response. Peripheral blood mononuclear cells were obtained before initiating the first vaccination and at the completion of the seventh vaccination. The phenotypes of circulating T-cell populations were determined with fluorescence-activated cell sorting by measuring the total $CD3^+$ population, the $CD4^+$ subpopulation, the $CD8^+$ subpopulation and the activation markers HLA-DR on $CD3^+$ cells and other, $CD19^+$ and $CD56^+$ populations. We determined $CD4^+CD25^{int/high}CD127^{low}$ cells as the regulatory T-cell population (22, 23). The WT1 tetramer assay was performed only in patients who received HLA-A*24:02-restricted mutant WT1 peptide in the CD3/CD8 double-positive population (19, 24). Enzyme-linked immunosorbent spot (ELISpot) assays were performed to examine WT1-specific interferon (IFN)-γ production by $CD8^+$ T cells called cytotoxic T lymphocytes (CTLs) (19, 24).

Immunohistochemical analyses of WT1 and antigen presentation-related molecules. We analysed WT1, HLA class I and HLA-DR protein expression in primary tumor samples by the previously described method (25). In addition to this panel, we analysed the transporter associated with the antigen processing 1 (TAP1) using rabbit polyclonal anti-TAP1 antibody (ADI-CSA-620-E, Enzo Life Sciences, Inc., Farmingdale, NY, USA).

Statistical analyses. While we assessed the changes in lymphocyte-related phenotypes before and after vaccination with the Wilcoxon signed-rank test, differences in lymphocyte-related phenotypes between patients with/without WT1-specific CTL were analyzed with the Mann–Whitney *U*-test. We considered $p<0.05$ statistically significant. In this study, statistical analyses were performed using the EZR (26).

Results

Patients and disease characteristics. In total, 24 paediatric patients with solid tumors were enrolled. The diagnoses were brain tumors (n=14), rhabdomyosarcomas (n=5), neuroblastomas (n=3), osteosarcomas (n=1) and clear cell sarcoma of the kidney (n=1). The median age of the patients was 7.5

years (range=2-19 years). The ratio of male to female was 17:7. Of the 24 patients, 6 dropped out of this trial: 4 for aggravation of their general condition due to the affecting cancer, 1 because of a relapse of the original cancer and 1 because of the development of a second malignancy (myeloid leukaemia). The number of patients who received only HLA-A*24:02-restricted mutant or A*02:01/*02:06-restricted WT1 peptide was 11 and 10, respectively. Of the total, 3 patients received both WT1 peptides. Nobody dropped out due to WT1 vaccine-related toxicity. Table I shows the clinical characteristics and the immunological outcomes of patients who completed one course of WT1 peptide vaccination therapy.

Toxicity of the WT1 vaccination. The total frequency of vaccination was 126 times among the 18 patients who completed the trial. An adverse effect attributable to WT1 peptide vaccine was observed 77 times (61%) in 16 patients (89%): Grade-1 and -2 fevers were observed 20 times (16%) in 10 patients and 3 times (2%) in 3 patients, respectively, a Grade-1 reaction at the injection site was observed 53 times (42%) in 13 patients. An anaphylaxis CTCAE Grade-3 was observed in a single case after the last vaccine was administered. The patient who developed anaphylactic shock was a nine-year-old male who had suffered from asthma, atopic dermatitis and hay fever. He developed anaphylactic shock immediately following the seventh injection of WT1 peptide vaccine. His allergic symptoms disappeared quickly after antihistamine and glucocorticoid were administered.

WT1-specific immune response after the vaccination. Exploratory analyses of the immune response consisted of assessment of the ELISpot assay to WT1 peptide and WT1 peptide/HLA-A*24:02-tetramer$^+$CD3$^+$CD8$^+$ T-lymphocytes in peripheral blood of 18 patients who had completed this trial. The ELISpot and tetramer assays were performed in 18 and 10 patients, respectively, before and at the end of the trial. Of the 18, 4 patients (22%) were observed to demonstrate a WT1-specific immune response in at least one assay at the end of one course of vaccination (Table I). When comparing the difference in lymphocyte-related phenotypes, including CD3$^+$, CD4$^+$, CD8$^+$, HLA-DR on CD3$^+$, CD19$^+$, CD56$^+$ and regulatory T-cell, before and at the end of one course of vaccination, no significant change was observed. In addition, we could not observe significant differences between 4 patients with WT1-specific immunological responses and other negative groups. Of 4 patients who were diagnosed with WT1-specific CTL, 1 patient (no. 8) demonstrated stable disease for 14 months under chemotherapy and the remaining 3 patients (no. 25, 39 and 44) presented with a progressive disease. Furthermore, the disease in patient no. 39 and 44 was uncontrollable irrespective of chemotherapy and/or radiotherapy before vaccination.

Expression of WT1 and antigen-presentation-related molecules in paediatric patients with solid tumors. We analysed 20 patient samples (brain tumor, 11; rhabdomyosarcoma, 5; neuroblastoma, 2; osteosarcoma, 1 and clear-cell sarcoma of the kidney, 1). All samples were WT1-positive. HLA class I also expressed highly (85%); however, the expression rate of HLA-DR was low (55%). The expression rate of TAP1 was also high (95%) (Table II).

Discussion

Only one report has been published on WT1 vaccination therapy, targeting a relatively large number of paediatric patients with solid tumors (10). Sawada *et al*. reported that vaccination with HLA-A*24:02-restricted mutant WT1 peptide in Montanide ISA-51, in a clinical trial of 26 paediatric patients with haematologic or solid tumours, was well tolerated and safe. In our study, we used either HLA-A*24:02-restricted mutant WT1 peptide and/or A*02:01/A*02:06-restricted wild-type WT1 peptide in combination with a mixture of group A streptococcus OK-432. As in the previous report with WT1 peptide vaccines, most patients had only mild (Grade-1 or -2) toxicities during the course of vaccination, although 1 patient developed Grade-3 anaphylactic shock at the final administration of the vaccine. OK-432 is reported as an allergic-reaction-inducing agent (27). Furthermore, anaphylactic shock in this patient could be attributed to the presence of allergies such as asthma, atopic dermatitis and hay fever. Although 4 of 24 patients had allergies in this study, only this patient developed an allergic reaction. Perhaps, the differences between this patient and others could be the number of allergies and the presence allergy symptoms at the initiation of WT1 vaccination (the other 3 patients had only asthma and did not have any allergic symptoms at the beginning of vaccination). Therefore, this therapy should only be implemented after conducting a detailed interview for the patient's past history and family history. Patients with a history of allergic reaction should be closely monitored after vaccination. Furthermore, the patient who has multiplex and/or active allergic symptoms at the beginning of vaccination may have higher risk for allergic reaction.

The primary immunological objective of this trial was the assessment of the WT1-specific CTL responses following vaccination and the potency of the WT1 vaccine using the OK-432 as an adjuvant. In accordance with the recommendations of the Society for Biological Therapy (28), tetramer-based quantitative assays and ELISpot assays were used to determine T-cell responses. In this study, 4 of the 18 patients (22%) were observed to have a WT1-specific immune response in at least one assay with rather low potency. Van Driessche *et al*. summarized 21 clinical

Table I. *Clinical characteristics and immunological outcomes in patients who completed one course of WT1 peptide vaccination therapy.*

No.	Age	Gender	Disease	Disease status	Pathology				HLA (A loci)	WT1		Total amount of OK-432 (KE/kg)	Combination therapy (*intravenous; $oral)	Use of immunosuppressive agents	Side effects based on CTCAE ver.4.0 Grade (number of times)			WT1-specific immunologic assessment	
					WT1	HLA class I	HLA-DR	TAP1		Type of peptide	Dose (mg)				Fever	Injection site reaction	Allergy	ELISpot	Tetramer
8	3	F	Glioma (Ependymoma)	Non CR	2+	1+	1+	1+	02:01/02:06	02:01	2	0.18	TMZ*	-	-	1 (5)	-	Pos	NA
10	6	M	Glioma (Glioblastoma)	Non CR	1+	-	1+	1+	02:06/31:01	02:01	3	0.27	TMZ*	Prednisolone	-	-	-	Neg	NA
23	8	M	Neuroblastoma	Non CR	NA	NA	NA	NA	02:01/24:02	02:01/24:02	3	0.09	-	Tacrolimus	1 (2)	1 (4)	-	Neg	Neg
24	3	M	Glioma (Ependymoma)	Relapse	1+	1+	-	1+	02:01/11:01	02:01	2	0.10	CPT-11*	Dexamethasone	-	-	-	Neg	NA
25	9	M	Glioma (Ependymoma)	Relapse	2+	1+	-	1+	24:02/33:03	24:02	3	0.12	IFN-β*	-	1 (2)	-	3 (1)	Pos	Pos
29	19	M	Glioma (Medulloblastoma)	Relapse	NA	NA	NA	NA	01:01/02:01	02:01	3	0.17	-	-	1 (1), 2 (1)	1 (6)	-	Neg	NA
30	8	M	Glioma (Glioblastoma)	Relapse	NA	NA	NA	NA	02:01/31:01	02:01	3	0.27	TMZ*, IFN-β*, Bevacizumab*	-	1 (1)	-	-	Neg	NA
32	4	F	Neuroblastoma	CR	1+	1+	1+	1+	24:02/31:01	24:02	2	0.12	Isotretinoin$	-	1 (4)	1 (5)	-	Neg	Neg
33	2	M	Pineoblastoma	Non CR	1+	1+	-	1+	24:02/-	24:02	2	0.27	-	-	-	1 (4)	-	Neg	Neg
35	10	F	Rhabdomyosarcoma	CR	3+	1+	1+	1+	02:06/-	02:01	3	0.21	-	-	1 (2)	1 (2)	-	Neg	NA
36	4	M	Neuroblastoma	Relapse	2+	1+	1+	1+	24:02/11:01	24:02	2	0.21	VP-16*	-	-	1 (5)	-	Neg	Neg
37	11	M	Glioma (Ependymoma)	Relapse	2+	1+	-	1+	24:02/26:01	24:02	3	0.21	-	-	1 (2)	1 (2)	-	Neg	Neg
39	17	F	Osteosarcoma	Relapse	3+	2+	2+	2+	24:02/02:01	02:01/24:02	3	0.11	VP-16*	-	2 (1), 1 (3)	1 (5)	-	Neg	Pos
42	2	F	Glioma (PNET)	CR	3+	2+	1+	1+	24:02/02:06	02:01/24:02	2	0.27	CY$	-	-	1 (4)	-	Neg	Neg
44	5	M	Clear cell sarcoma of the kidney	Relapse	2+	-	-	1+	24:02/26:03	24:02	2	0.17	CPT-11*	-	-	1 (4)	-	Pos	Pos
45	12	F	Rhabdomosarcoma	Non CR	3+	1+	-	1+	24:02/31:01	24:02	3	0.15	CY$	-	2 (1)	-	-	Neg	Neg
46	10	M	Rhabdomyosarcoma	CR	3+	1+	1+	1+	02:01/31:01	02:01	3	0.22	CY$	-	1 (1)	1 (5)	-	Neg	NA
47	2	M	Rhabdomyosarcoma	Non CR	3+	-	-	-	02:06/33:03	02:01	2	0.28	CY$	-	1 (2)	1 (2)	-	Neg	NA

WT1: Wilms' tumor 1; HLA: human leukocyte antigen; TAP1: transporter associated with antigen processing 1; CTCAE: common terminology criteria for adverse events; ELISpot: enzyme-linked immunospot assay; PNET: primitive neuroectodermal tumor; CR: complete remission; NA: not assessed; TMZ: temozolomide; INF-β: interferon-beta; VP-16: etoposide; CPT-11: irinotecan; CY: low dose cyclophosphamide; Pos: positive; Neg: negative.

Table II. *Immunohistochemical analyses of WT1 and antigen presentation-related molecules.*

Disease	No. of patients	WT1	HLA class I	HLA-DR	TAP1
Brain tumor	14	11/11 (100%)	10/11 (91%)	6/11 (55%)	10/10 (100%)
Ependymoma	5	5/5	5/5	2/5	5/5
Glioblastoma	3	1/1	0/1	1/1	1/1
Medulloblastoma	2	1/1	1/1	0/1	1/1
Diffuse intrinsic pontine glioma	1	1/1	1/1	1/1	NA
Pineoblastoma	1	1/1	1/1	1/1	1/1
Malignant transformation of craniopharyngioma	1	1/1	1/1	1/1	1/1
Primitive neuroectodermal tumor	1	1/1	1/1	0/1	1/1
Rhabdomyosarcoma	5	5/5 (100%)	4/5 (80%)	2/5 (40%)	4/5 (80%)
Neuroblastoma	3	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
Osteosarcoma	1	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)
Clear cell sarcoma of the kidney	1	1/1 (100%)	0/1 (0%)	0/1 (0%)	1/1 (100%)
Total	24	20/20 (100%)	17/20 (85%)	11/20 (55%)	18/19 (95%)

WT1: Wilms' tumor 1; HLA: human leukocyte antigen; TAP1: transporter associated with antigen processing 1.

trials including both adults and children that administered with WT1 peptide. Of the 125 patients, 69 (55%) were observed to have a WT1-specific immune response after vaccination. However, response rates were significantly different between haematological malignancies and solid tumors: Of 77 patients, 52 (68%) were positive for haematological malignancies, and of 48 patients, 17 (35%) were positive for solid tumors (29). This indicates that the acquisition of a WT1-specific immune response is considerably influenced by the type of disease. Although our acquisition rate of WT1-specific CTL seems to be still insufficient, this may be attributed to the specific immune system of the paediatric patients with solid tumors, inadequate vaccine frequency (in other reports, 64% of patients were administered vaccine 12 times compared to only 7 times in our study), differences in the WT1 peptide (A*24:02-restricted mutant WT1 peptide was administered in 83% of patients in other reports), the limited availability of solid tumor cases in this study, the heterogeneity in the disease status and the combination of chemotherapy and immunosuppressant therapy. In fact, no patient achieved WT1-specific immunological responses under treatment comprising immunosuppressive agents; for instance, steroid agents to prevent cerebral edema because of a brain tumor in 2 patients and tacrolimus for graft-versus-host disease after stem cell transplantation in 1 patient. Conversely, all 4 patients who were diagnosed with WT1-specific CTL were undergoing chemotherapy. However, the type of chemotherapy, including the degree of myelosuppression, varied in each patient. This result might indicate that while the combination of chemotherapy on the acquisition of WT1-specific CTL could be insignificant, the use of immunosuppressive agents could have adverse effects. The potency of vaccine might be also influenced by different adjuvant setting using OK-432 rather than the conventional Montanide ISA51. It has been reported that OK-432 strongly induces the maturation of DCs and that OK-432-stimulated DCs can induce tumor antigen-specific CTLs (15, 30-33). Because our major aim was to assess the safety of WT1-peptide vaccine with OK-432, the acquisition rate of WT1-specific CTL and the clinical response should be evaluated in prospective trials. Although we could not conclude positive effect in our 4 patients who had a WT1-specific immunological response in this study, the achievement of disease control before the induction of anti-tumor effect from WT1-specific CTLs seems imperative.

The prerequisite for a successful tumor-specific CTL response is HLA class I expression on the surface of cancer cells because the absence or down-regulation of HLA class I leaves the T cells incapable of recognizing the cancer cell (34). Loss or down-regulation of HLA class I has been described in human tumors of different origins at frequencies that range from 60% to 90% (35-37). In this study, the limitation of immunohistochemical analyses was the inability to assess tissue specimens from all patients. However, in our analysis of the expression of WT1 and antigen-presentation-related molecules, all samples were WT1-positive (15/15) and highly expressed HLA class I (12/15). Moreover, TAP1, which is involved in the transport of antigens from the cytoplasm to the endoplasmic reticulum for association with major histocompatibility complex class I molecules, was positive in most samples (14/15). These results indicate that WT1-specific CTL may be able to recognize and kill tumor cells if paediatric patients with solid tumors obtain a WT1-specific immune response.

In addition to its easy administration, peptide vaccine therapy has the advantage that multiple tumor-specific immune responses can be acquired at the same time by using a peptide cocktail vaccine that contains different cancer antigens (38, 39). Tumor-specific CTL therapy including peptide vaccine and DC-based therapy can also target intracellular antigens, unlike monoclonal antibody and chimeric antigen-receptor T-cell therapies. Nowadays, a new class of treatment, immune-checkpoint-inhibitor antibodies, which targets the immune system, has shown promising results in clinical trials (40). Immune-checkpoint-inhibitor antibodies such as nivolumab, atezolizumab, pembrolizumab inhibit programmed death-1 (PD-1)-mediated signalling by blocking PD-ligand 1 from binding to PD-1, allowing T-cell activation and immune-system recognition. These antibodies assist in restoring antitumor activity. In several mice models, the combination of various cancer peptide vaccines and anti-cytotoxic T-lymphocyte-associated protein (CTLA)-4 or anti-PD-1 monoclonal antibody demonstrated additive and/or synergistic effects in specific T-cell induction and tumor growth control (41, 42). If the efficiency of the tumor-specific CTL response by cancer peptide vaccine is improved dramatically, combination therapy with immune-checkpoint-inhibitor antibodies may become a promising therapeutic strategy in the future.

In conclusion, we report that almost all WT1-peptide vaccinations were safe, but because an acute allergic reaction can always happen, it should be used with care. Because the immunological response rates of WT1-peptide vaccine still seemed to be insufficient in our small heterogeneous population, we recommend considering increasing times and/or the amount of WT1 peptide administered and narrowing the target population under the establishment of the appropriate eligibility based on the patient's disease, status, concomitant treatment and other clinical information in a future clinical study. WT1 is highly expressed together with antigen presentation-related molecules in paediatric patients with solid tumors. Improvement of the immunological induction efficiency and combination with other treatments may change the position of paediatric tumor-specific CTL therapy in the future.

Conflicts of Interest

The Authors declare no competing financial interests. S.H. is the inventor of patents for WT1 peptides (PCT/JP02/02794 and PCT/JP04/16336). This work was supported by JSPS KAKENHI Grant Number 16K10021.

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