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ORIGINAL ARTICLE

Phase I clinical study of anti-apoptosis protein survivin-derived peptide vaccination for patients with advanced or recurrent urothelial cancer

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Abstract Survivin, a member of the inhibitor of apoptosis protein family, is expressed in many malignant tumors including urothelial cancer but is hardly detectable in normal, differentiated adult tissues. Previously we reported CD8-positive cytotoxic T-lymphocytes (CTLs) were successfully induced by stimulation with survivin-2B80-88 peptide in vitro. We started a phase I clinical study of survivin-2B80-88 peptide vaccination for advanced urothelial cancer patients to assess the safety and efficacy of this vaccination. Nine patients were received vaccination and were evaluated for immunological evaluation, adverse events, and clinical responses. A total of 46 vaccinations were carried out. There was no severe adverse event. HLA-A24/survivin-2B80-88 peptide tetramer analysis revealed a significant increase in the peptide-specific CTL frequency after the vaccination in five patients. Slight reduction of the tumor volume was observed in one patient. Survivin-2B80-88 peptide-based vaccination is safe and should be further considered for potential immune and clinical efficacy in urothelial cancer patients.

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Abbreviations

CR	Complete response
CT	Computed tomography
CTL	Cytotoxic T-lymphocyte
DTH	Delayed-type hypersensitivity
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSP	Heat shock protein
IFA	Incomplete Freund's adjuvant
IFN	Interferon
NC	No change
PBMC	Peripheral blood mononuclear cell
PD	Progressive disease
PR	Partial response

Introduction

Increasing numbers of T-lymphocyte epitopes derived from various cancer-associated antigens have been reported, and they have been proved to play significant roles in cytotoxic T-lymphocyte (CTL)-based immunotherapy [1]. Survivin, a member of the inhibitor of apoptosis protein family, is expressed in various malignant tumors but is undetectable in normal and differentiated adult tissues [2–4]. Because of its cancer-specific expression, survivin might be an attractive target for immunotherapy via CTL responses.

We previously reported that survivin and its splicing variant survivin-2B were expressed abundantly in various cancer tissues and cancer cell lines, including urothelial cancer, and were suitable as target antigens for active-specific anticancer immunization [5]. Subsequently, we identified the human leukocyte antigen (HLA)-A24-restricted antigenic peptide survivin-2B 80-88 (AYACNTSTL) derived from the exon 2B-encoded region and recognized by CTLs in the context of HLA-A24 molecules. In addition, we reported further evidence that the survivin-2B80-88 peptide might serve as a potent immunogenic cancer vaccine for various cancers, including those of the colon, lung and breast [6]. On the basis of these studies, we started a phase I clinical study using survivin-2B80-88 peptide vaccination for colorectal and breast cancers. These studies revealed that survivin-2B80-88 peptide vaccination was safe and well tolerated without severe side effects and could induce survivin-2B80-88 peptide-specific CTLs [7, 8].

With respect to urogenital cancers, we previously reported that survivin was selectively expressed in 87.5% of bladder cancers and survivin-specific CTLs were successfully induced from peripheral blood mononuclear cells of a bladder cancer patient [9]. On the basis of these studies, we therefore started a phase I clinical study assessing the safety and efficacy of survivin-2B80-88 peptide vaccination in patients with advanced or recurrent urothelial cancer expressing survivin. Herein we show that survivin-2B80-88 peptide-based vaccination is safe and should be further considered for potential immune and clinical efficacy in HLA-A24+ survivin-expressing patients with urothelial cancer.

Materials and methods

Patient selection

The study protocol was approved by the Clinical Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University, Japan. All the patients gave informed consent before being enrolled. Patients enrolled in this study were required to conform to the following criteria: (1) to have histologically proven urothelial cancer, (2) to be HLA-A*2402 positive, (3) to have survivin- and HLA class I-positive carcinomatous lesions on the primary site by immunohistochemistry, (4) to be between 20 and 85 years old, (5) to have received surgical excision of the primary tumor and (6) to have Eastern Cooperative Oncology Group (ECOG) performance status between 0 and 3. Exclusion criteria included (1) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy, or other immunotherapy within the previous 4 weeks, (2) the presence of other cancers that might influence the prognosis, (3) immunodeficiency or a history of splenectomy, (4) severe cardiac insufficiency, acute infection, or hematopoietic failure, and (5) unsuitability for the trial based on clinical judgment.

Table 1 Summary of clinical characteristics of patients enrolled in the study

	Age	Sex	Primary	Recurrence site
1	57	F	Rt ureteral Ca.	Pelvic LN[a]
2	51	F	Bladder Ca.	Cervival LN[a]
3	67	M	Bladder Ca.	Lung
4	65	F	Bladder Ca.	Pelvic soft tissue[a]
5	65	M	Bladder Ca.	Lung
				Bone
6	64	M	Bladder Ca.	Lung
7	38	F	Bladder Ca.	Inguinal LN[a]
				Mesenchymal LN
8	59	F	Bladder Ca.	Pelvic soft tissue[a]
9	57	M	Bladder Ca.	Lung
				Liver

LN lymph node

[a] hisologically survivin and HLA class I expression confirmed

Nine patients with refractory recurrent urothelial cancer were initially enrolled in this study (Table 1). Of the nine patients, eight (cases 2–9) had recurrent advanced bladder cancer and one (case 1) had ureteral cancer. Four patients (cases 3, 5, 6 and 9) had lung metastasis and three (cases 1, 2 and 7) had regional and/or distant lymph node metastasis. All patients had previously received systemic chemotherapy such as MVAC (methotrexate, vinblastine, adriamycin and cisplatin), GC (gemcitabine and cisplatin) and/or TIN (paclitaxel, ifosfamide and nedaplatin). Survivin and HLA class I expression on the metastatic sites were confirmed histologically in five patients (cases 1, 2, 4, 7 and 8).

Peptide preparation

The survivin-2B80-88 peptide with the sequence AYACNTSTL, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA). The identity of the peptide was confirmed by mass spectrometry analysis, and the purity was shown to be more than 98% as assessed by high pressure liquid chromatography analysis. The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 ml of physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and stored at −80°C until just before use.

Incomplete Freund's adjuvant preparation

Montanide ISA 51 (SEPPIC Inc., NJ, USA) was used as an incomplete Freund's adjuvant (IFA).

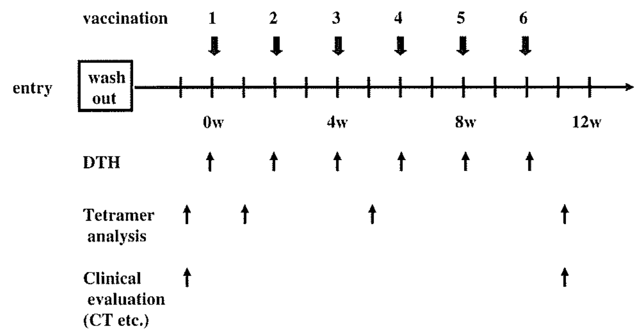

Fig. 1 Vaccination, immunological and clinical evaluation schedule. The vaccination with survivin-2B peptide (0.1 or 1.0 mg) vaccines with incomplete Freund's adjuvant (IFA) were carried out. Patients received vaccinations every 2 weeks, and A DTH skin test was performed at each vaccination. Patients were examined closely for signs of toxicity during and after vaccination. Before and 1-week after the third and sixth vaccination, HLA-A24/survivin-2B80-88 peptide tetramer analysis were evaluated. Tumor size was evaluated by computed tomography (CT) before treatment and at the end of the study period

Vaccinations

We previously showed that the peptide doses of 0.1 and 1 mg had efficacy with little adverse events [7]. On the basis of these data, we set two arms of which peptide dosage has 0.1 and 1.0 mg. Vaccinations with survivin-2B80-88 peptide (0.1 or 1.0 mg) vaccines with IFA serving as a carrier for water-in-oil emulsion [10] were given subcutaneously six times at 14-day intervals (Fig. 1).

Delayed-type hypersensitivity skin test

A delayed-type hypersensitivity (DTH) skin test was performed at each vaccination. The peptide (10 μg) solution in physiological saline (0.1 ml) or physiological saline alone (0.1 ml) was separately injected intradermally (i.d.) into the forearm. A positive reaction was defined as more than 4 mm diameter area of erythema and induration 48 h after the injection.

Toxicity evaluation

Patients were examined closely for signs of toxicity during and after vaccination. Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria (NCI-CTC).

Tetramer staining

HLA-A24/survivin-2B80-88 peptide tetramers were constructed according to the procedure described by Altman et al. [11]. Briefly, recombinant HLA-A24 heavy chain [12] and human β2-microgloblin were refolded with survivin-2B80-88 peptides as described [13]. The resulting HLA-A24-peptide monomer was biotinylated by incubation with the BirA enzyme (Avidity, Denver, CO) for 17 h at room temperature and purified using fast protein liquid chromatography. A tetrameric HLA-peptide complex was produced by incubating streptavidin-phycoerythrin (PE) (Vector Laboratories, Burlingame, CA, USA) with the biotinylated monomer at a molar ratio 1:4. For flow cytometric analysis, peripheral blood mononuclear cells were isolated from blood samples by Ficoll-Conray density gradient centrifugation. Then, they were incubated in the presence of 40 μl/ml survivin-2B peptide in AIM-V medium containing 10% human serum, followed by addition of interleukin-2 at a final concentration of 50 U/ml at 1 h, 2, 4 and 6 days after the addition of the peptide. On day 7 of culture, the Peripheral blood mononuclear cells (PBMCs) were stained with the PE-labeled tetramer at 37°C for 20 min, followed by staining with a fluorescein isothiocyanate (FITC)-conjugated anti-CD8 monoclonal antibody (mAb) (Beckton Dickinson Biosciences) at 4°C for 30 min. Cells were washed twice with phosphate-buffered saline (PBS) before fixation in 1% formaldehyde. Flow cytometric analysis was performed using FACSCalibur and Cell Quest software (BD Biosciences). As a negative control, a tetramer with an HLA-A*2402-restricted human immunodeficiency virus (HIV) peptide (RYLRDQQLL) was used. The frequency of CTL precursors was calculated as the number of tetramer-positive cells over the number of CD8-positive cells.

Clinical response evaluation

Physical examinations and hematological examinations were conducted before and after each vaccination. Tumor size was evaluated by computed tomography (CT) before treatment and at the end of the study period. A complete response (CR) was defined as complete disappearance of all measurable diseases. A partial response (PR) was defined as a $\geq$30% decrease from the baseline in the size of all measurable lesions (sum of products of maximal perpendicular diameters) lasting for a period of at least 4 weeks. Progressive disease (PD) was defined as an increase in the sum of the bidimensional measurements of all known disease sites by at least 20% or by the appearance of new lesions. No change (NC) was defined as the absence of matched criteria for CR, PR, or PD.

Results

Vaccinations

Altogether, 46 vaccinations were carried out in nine patients. Three patients (cases 3, 5 and 7) discontinued halfway

Table 2 Summary of clinical courses of patients administered survivin-2B80-88 peptide vaccine

	Peptide dose	Adverse events (Grade)[a]	DTH skin test	Tetramer staining assay % of HLA-A24/survivin-2B80-88 peptide tetramer-positiv e CTL (post-/pre-vaccination)	Reduction rate (judgment)
1	0.1 mg × 6	Induration at the injection site (Grade 1)	(–)	Increased (1.29/0.04)	19.4% (SD)
2	0.1 mg × 6	Induration at the injection site (Grade 1)	(–)	Increased (1.34/0.07)	17.6% (SD)
3	0.1 mg × 2	None	(–)	NA	NA
4	0.1 mg × 6	Induration at the injection site (Grade 1)	(–)	No change (0.28/0.06)	−52.5% (PD)
5	1.0 mg × 3	None	(–)	NA	NA
6	1.0 mg × 6	Induration at the injection site (Grade 1)	(–)	No change (0.06/0.10)	−68.0% (PD)
7	1.0 mg × 5	Induration at the injection site (Grade 1)	(–)	Increased (0.60/0.15)[b]	NA
8	1.0 mg × 6	Induration at the injection site (Grade 1)	(–)	Increased (0.78/0.07)[b]	−87.6% (PD)
9	1.0 mg × 6	None	(–)	Increased (1.42/0.13)[b]	−81.4% (PD)

NA not available

[a] Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria (NCI-CTC)

[b] Evaluation after third vaccination

through the protocol because of disease progression. Six patients (cases 1, 2, 4, 6, 8 and 9) received the complete regimen including six vaccinations and were evaluated. Table 2 summarizes the immunological and clinical outcomes of the nine patients.

Safety

Peptide vaccination was well tolerated in all nine patients. As shown in Table 2, no hematologic, cardiovascular, hepatic, or renal toxicity was observed. No other severe adverse events such as fever and fatigue were observed during or after vaccination in any patient. As minor side effect, six patients (cases 1, 2, 4, 6, 7 and 8) developed grade 1 local skin reactions with redness and swelling at the injection sites.

DTH skin test

A DTH skin test was performed at each vaccination and assessed 48 h later. No positive DTH reaction was observed in any patient.

Tetramer staining assay

Peripheral blood lymphocytes of patients using HLA-A24/survivin-2B80-88 peptide tetramers were available from seven patients (cases 1, 2, 4, 6, 7, 8 and 9). HLA-A24/survivin-2B80-88 peptide tetramer analysis revealed a significant increase in the peptide-specific CTL frequency of CD8-positive T cells after the vaccination in five patients (cases 1, 2, 7, 8 and 9) (Table 2). In cases 1 and 2, the frequency of HLA-A24/survivin-2B80-88 peptide tetramer-positive CTLs was increased from 0.04 to 1.29% and 0.07 to 1.34%, respectively, after the sixth vaccination (Fig. 2a, b). In case 7 who could not receive the complete regimen due to disease progression, HLA-A24/survivin-2B80-88 peptide tetramer analysis after the third vaccination revealed an increase in the peptide-specific CTL frequency from 0.15 to 0.60% (Fig. 2c). In cases 8 and 9, peptide-specific CTL frequency increased from 0.07 to 0.78% and 0.13 to 1.42%, respectively, after the third vaccination (Fig. 2d, e). In these five patients, these CTLs did not show any increases of frequency with the HLA-A24/HIV peptide tetramer.

Clinical responses

As indicated in Table 2, the two patients (cases 1 and 2) who showed increased frequencies of tetramer-positive CTLs showed slight reduction of the tumor volume after six-vaccination therapy. One responder (case 1) with right ureteral cancer who developed chemotherapy-refractory obturator lymph node metastasis chose to continue vaccination after finishing this phase I study because there were no severe adverse events. Before this study, as shown in Fig. 3a, pelvic CT showed that the recurrent right obturator node metastasis was 60 × 25 mm in size. The metastatic nodal disease was decreased to 46 × 15 mm after the sixth vaccination (arrow, Fig. 3b) and to 45 × 14 mm 21 months after first vaccination (arrow, Fig. 3c). In this patient with advanced urothelial cancer, slight reduction of the tumor volume was observed for 2 years, which was considered as minor response.

Discussion

High-throughput gene expression profiling of cancer has led to the discovery of many novel genes associated with it.

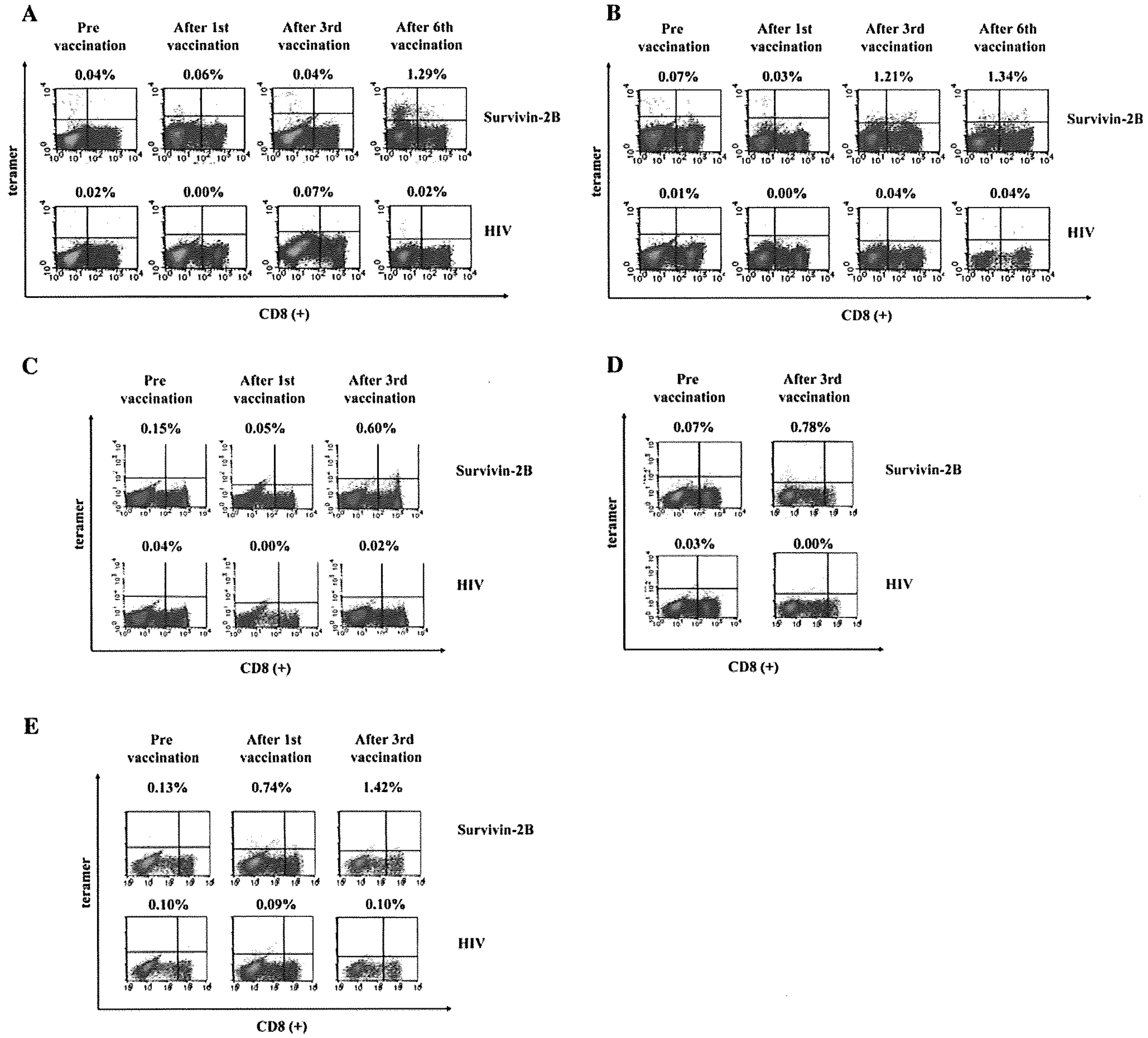

Fig. 2 Frequency of peptide-specific CTLs analyzed by HLA-A24/survivin-2B80-88 peptide tetramer analysis (**a** case 1, **b** case 2, **c** case 7, **d** case 8 and **e** case 9). Lymphocytes were collected from peripheral blood of the patients before and after the first, third and sixth vaccinations, stained with an FITC-labeled anti-CD8 mAb and PE-labeled HLA-A24/survivin-2B80-88 peptide tetramer, and analyzed by flow cytometry. As a negative control, a tetramer with an HIV peptide was used. The frequency of CTL precursors was calculated as the number of tetramer-positive cells over the number of CD8-positive cells. The frequencies of HLA-A24/survivin-2B80-88 peptide tetramer -positive CTLs were increased from 0.04 to 1.29%, and 0.07 to 1.34% after the sixth vaccination in cases 1 and 2, respectively (**a** and **b**). In case 7, 8 and 9 peptide-specific CTL frequency increased from 0.15 to 0.60%, 0.07 to 0.78% and 0.13 to 1.42%, respectively, after the third vaccination (**c**–**e**)

An increasing number of T-cell epitopes derived from these various tumor-associated antigens have been reported and proved to play significant roles in CTL-based immunotherapy. Survivin was originally identified as a member of the inhibitor of apoptosis protein family. It has the capability to inhibit caspase-3, -7, and -9 in cells receiving an apoptotic stimulus and may lead to tumor initiation, progression, and therapeutic resistance [4]. It is expressed in colorectal, breast, and urothelial cancers but is hardly detectable in normal, differentiated adult tissues [2–5]. Moreover, overexpression of survivin in cancer cells is associated with unfavorable clinicopathologic variables such as poor prognosis and shorter patient survival rates.

Because of its cancer-specific expression and function of protecting cancer cells from apoptotic stimuli, survivin might be an ideal target for CTL-based immunotherapy. We focused on a survivin-derived peptide carrying the HLA-A24 binding motif. This HLA-A24 genotype is

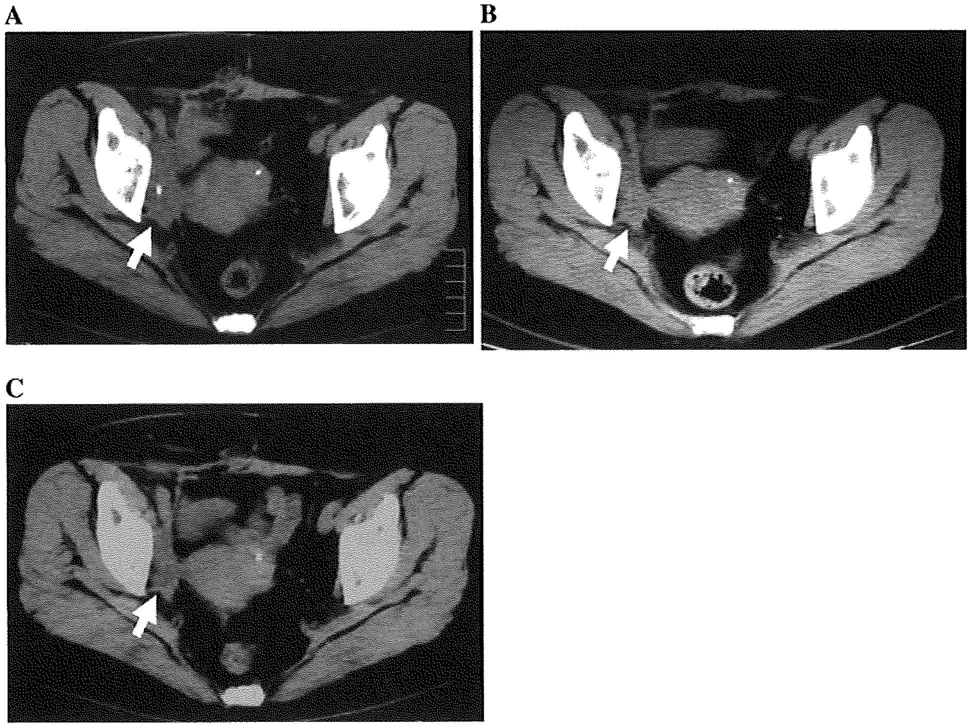

Fig. 3 Pelvic CT findings of case 1 patient. Pelvic CT shows slight reduction of recurrent right obturator node tumor size after the sixth vaccination. The vaccinations were continued and, after 21 months, the size of the recurrent right obturator node tumor was almost unchanged. Arrows, recurrent right obturator node masses and changes of the tumor size (mm) are shown as follows: pre (**a** 60 × 25), after sixth vaccination (**b** 46 × 15), and after 21 months (**c** 45 × 14)

predominant in Japanese (about 60%) and less abundant in Caucasians (17%), Blacks (9%), and Hispanics (27%) [14]. Previously we reported that survivin-2B80-88, a survivin-derived peptide carrying the HLA-A24 binding motif, was established and CD8-positive CTLs were successfully induced by stimulation with this peptide in vitro [5]. In addition, we demonstrated that survivin was expressed in a large proportion of bladder cancer specimens, and this survivin-2B-derived peptide could induce CTL responses in the context of HLA-A24 [9]. On the basis of these observations, we started a phase I clinical study of survivin-2B80-88 peptide vaccination for patients with advanced or recurrent urothelial cancer.

The primary end point in the current clinical trial was to evaluate the safety and toxicity of survivin-2B80-88 peptide vaccination. In this study, there was no severe adverse event during or after vaccination in either the 0.1 or 1.0 mg group. Thus, we concluded that the survivin-2B80-88 peptide vaccine was safe and could be repeatedly injected into patients without serious side effects.

The secondary aims of our study were to evaluate vaccine-induced specific immune reactions and clinical responses. With respect to the immunological response, by objective scientific HLA-A24/peptide tetramer analysis, we could confirm that the peptide-based vaccines activated peptide-specific CTLs in some patients. Under conditions in which HIV peptide-specific CTL frequencies in PBMCs remained at background levels (less than 0.1%), the frequencies of tetramer-positive CTLs were increased after the vaccination in five cases. However, no positive DTH reaction was observed in any vaccination of the nine patients. A previous study reported that there was a positive correlation between DTH and clinical responses [15]. The CTL response induced by the survivin-2B peptide vaccine might not be sufficient to induce cytotoxic activity against bulky recurrent masses and induce dramatic clinical regression in patients with an advanced urothelial cancer.

HLA-A24/survivin-2B80-88 peptide tetramer analysis revealed tetramer-positive cells were detected in CD8-positive population (Fig. 2a–e), however, they were considered to be non-specific binding since they could be eliminated after titration of the tetramer. In order to confirm if CD8±/tetramer± cells represent the survivin2B80-88-specific CTL, CD8±/tetramer± T-cells were cloned from PBMCs of vaccinated patients by single cell sorting and analyzed for tetramer reactivity and specific killing activity. The CTL clones that were approximately 98% positive for the survivin2B80-88 tetramer showed the peptide-specific killing activity, indicating that CD8±/tetramer± cells represent the survivin2B80-88-specific CTLs (data not shown).

This survivin-2B80-88 peptide vaccination therapy may have limitations to induce clinically relevant results because of using only single HLA class I restricted peptides. Recently, a number of survivin epitopes restricted to several additional HLA molecules have been identified [16], and several clinical trials of immunotherapy based on survivin-derived peptides have been initiated. It would be interesting to identify and use other HLA class I or HLA class II survivin epitopes to induce more survivin-specific CTLs. Furthermore, it is possible that vaccination with the peptide in combination with some cytokines may be able to lead to stronger immune responses both in the induction and effector phases [17, 18]. On the basis of the information obtained from this study, further studies are required to evaluate the efficacy of the survivin-2B peptide vaccine in combination with various adjuvant drugs such as granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-2 and interferon (IFN). Our preliminary clinical study suggested that survivin-2B80-88 peptide vaccination in conjunction with IFN-α was more effective than the peptide alone in colon and pancreas cancer patients. Moreover, heat shock protein (HSP)-peptide complexes elicited antitumor responses in studies on immunization protocols. In our laboratories, we have found that HSPs such as Hsp70 and Hsp90 could be subjected to receptor-mediated uptake by antigen-presenting cells with subsequent representation of the HSP-associated peptides to HLA class I molecules on antigen presenting cells, facilitating efficient cross-presentation [19]. Toll-like receptors (TLR) have an essential role in the innate immune recognition of antigens [20]. Thus, it should be effective to use TLR-mediated signaling pathways to induce more survivin-specific CTLs.

Although our study consisted of only a limited number of patients, these preliminary data seem to suggest that survivin-2B peptide vaccination is safe without serious adverse events. As the first step, this study revealed that survivin-2B peptide-based vaccines activated peptide-specific CTLs and may be considered for potential immune and clinical efficacy in HLA-A24 positive/survivin-expressing patients with urothelial cancer. In the future, if the efficacy and safety of this vaccination therapy are established, we might be able to use this vaccine as an adjuvant therapy for high-risk non-immune-suppressed patients before systemic chemotherapy.

Conclusion

This phase I clinical study indicates that survivin-2B80-88 peptide-based vaccination is safe and should be further considered for potential immune and clinical efficacy in HLA-A24+ survivin-expressing patients with urothelial cancer.

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Review Article

The functioning antigens: beyond just as the immunological targets

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Antigenic peptides derived from tumor-associated antigens (TAAs) facilitate peptide cancer vaccine therapies. With the recent progress in cancer immunity research, huge amounts of antigenic peptides have already been reported. Clinical trials using such peptides are underway now all over the world. Some reports have shown the efficacy of peptide vaccine therapies. However, others ended with unfavorable results, suggesting fundamental underlying problems. One major mechanism that negates the peptide vaccine therapy is tumor escape from immunological systems caused by loss of antigens. TAAs that are used in cancer vaccine therapies may be divided into two major groups: functioning antigens and non-functioning antigens. A 'functioning antigen' could be defined as a TAA that is essential for tumor growth, is expressed in several kinds of malignancies and shows homogenous expression in cancerous tissues. It is not difficult to imagine that antigen loss will occur easily with non-functioning antigens as a target of cancer vaccine therapy. Thus, it is essential to use functioning antigens for successful cancer vaccine therapy. In this review, we discuss the functioning antigens and their categorization in detail. (*Cancer Sci* 2009; 100: 798–806)

Immunotherapy is a very old concept that stems from the vaccination therapy established by Edward Jenner for treating smallpox. That novel therapeutic strategy has had a great impact, enabling complete elimination of the disease. This glittering triumph also raised a simple and significant question: 'Are malignant diseases treatable with vaccination?' Since then, a vast body of work on cancer immunity has been reported, and tumor immunity research have already reached the bedside.

There are two major approaches of tumor immunity: (i) tumor immunotherapy based on tumor-specific cytotoxic T lymphocytes (CTLs); and (ii) tumor immunotherapy based on tumor-specific antibodies (Fig. 1). In 1997, anti-CD20 monoclonal antibody (rituximab) has been approved by the US Food and Drug Administration (FDA) for treating CD20-positive B-cell malignancies, and antibody-based immunotherapy has become one of the standard therapies in several malignancies. However, antibody-based immunotherapy can target only cell surface proteins or secreted proteins like p185$^{HER2/neu}$ for breast carcinoma, CD20 for B-cell lymphoma, vascular endothelial growth factor (VEGF) for renal cell carcinoma, epidermal growth factor receptor (EGFR) for colorectal carcinoma and chemokine (C-C motif) receptor 4 (CCR4) for T cell lymphoma. So, antibody-based immunotherapy is very restricted for further application. On the other hand, CTLs recognize 9- to 14-mer antigenic peptides that are derived from endogenously expressed proteins digested by several proteases, including proteasomes and the endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP).[1,2] Thus, CTLs can recognize potentially all tumor-specific antigens (Fig. 1). Very recently, some lines of CTL-based immunotherapy reagents have been approved (Table 1). Heat shock proteins (HSPs) purified from cancer cells have the potency to induce CTL reactivity, and HSP-based reagent (Oncophage) was approved in Russia in April 2008. Further, other CTL-based immunotherapy reagents are now under Phase III studies, and part of these will be approved in a few years. The wave of CTL-based immunotherapy is coming to the bedside.

The progress of molecular biological techniques in the past several decades has brought us enormous knowledge about

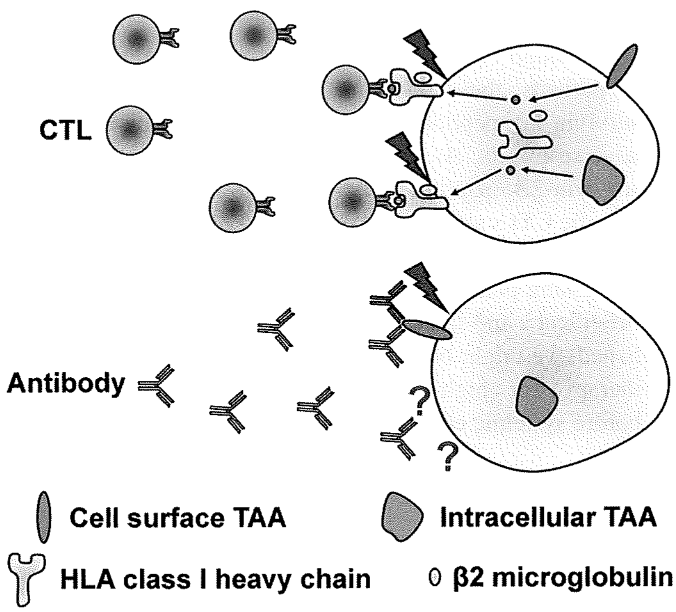

Fig. 1. Cancer-specific immunotherapy based on cytotoxic T lymphocytes (CTLs) and antibodies. In a variety of immuno systems, CTLs and B cell (antibody) show antigen-specific reactions through their antigen-specific receptors. CTL recognizes endogenously processed antigenic peptides presented with human leukocyte antigen (HLA) molecule on the cell surface. Thus, CTL can recognize all cell-distributed antigens. Antibody recognizes cell surface antigen with direct binding to antigen molecule with its fragment antigen binding (Fab) region. Antibody only recognizes cell surface antigens, but does not recognize intracellular antigens.

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The abbreviations used are:
TAA, tumor associated antigen; CTL, cytotoxic T lymphocyte; HLA, human leukocyte antigen.

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Table 1. Exploitation of cancer vaccine and related companies (October 2008)

Designation	Content	Company	Country	Organs	Approved
Oncophage	Protein/peptide	Antigenics	Russia	Kidney	April 2008
DCVax	Cell	Northwest Biotherpeutics	Switzerland	Brain	July 2007
BiovaxID	Protein	Biovest International	USA	B cell lymphoma	Phase III
MAGE-A3ASCI	Protein	GlaxoSmithKline	Belgium	Lung	Phase III
GV1001	Peptide	Pharmexa	UK	Pancreas	Phase III
GVAX	Cell	Cell Genesys	USA	Pancreas	Phase II
Stimuvax	Peptide	Oncothyreon	Several	Lung	Phase III
TroVax	DNA	Oxford Biomedica	UK	Kidney	Phase III

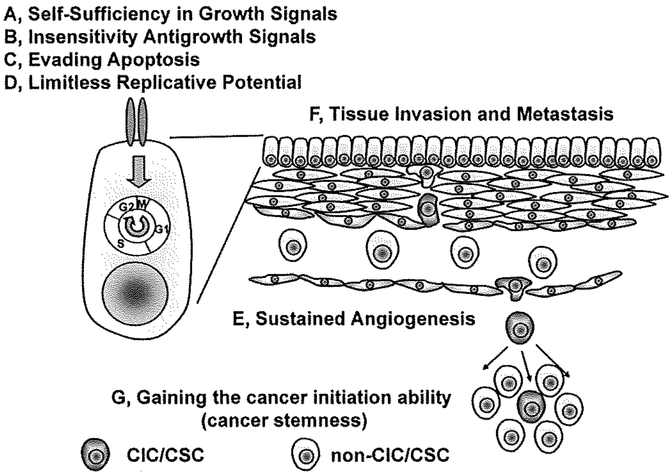

Fig. 2. The 'Functioning antigens'. The 'Functioning antigens' play several roles in carcinogenesis, tumor initiation, invasion and metastasis.

molecular aspects of oncogenesis and also cancer immunity. This enabled van der Bruggen and colleagues to publish their milestone work on the identification of melanoma antigen (*MAGE*) gene family as the first human TAA.[3] Initially, TAAs were screened with cDNA expression cloning using CTLs specific for melanomas. Later, serological analysis of recombinantly expressed clones (SEREX) and the reverse-immunogenetical approach were developed to identify novel TAAs and CTL epitopes. To summarize these TAAs, Boon *et al.* proposed categorizing TAAs based on the expression profiles of malignant and normal cell TAAs.[4] This categorization includes four groups: (A) tumor antigens resulting from mutations; (B) shared tumor-specific antigens; (C) differentiation antigens; and (D) antigens overexpressed in tumors (Cancer Immunity web site http://www.cancerimmunity.org/index.htm.). This provides us with very important information to establish cancer immunotherapy protocols. Malignant cells commonly have genomic instability and are genetically unstable, and often lose the expression of immunogenic antigens after cancer vaccine therapy, suggesting that non-functioning antigens might not be suitable for cancer vaccine therapies. The functioning antigens are usually non-mutated cancer-related antigens, and belong to group (D) antigens overexpressed in tumors. To discriminate functioning antigens from non-functioning antigens, several features of malignant phenotypes as follows are essential:[5] (A) self-sufficiency in growth signals; (B) insensitivity antigrowth signals; (C) evading apoptosis; (D) limitless replicative potential; (E) sustained angiogenesis; and (F) tissue invasion and metastasis. Furthermore, to understand the tumor initiation ability *in vivo*, we need to mention about the 'cancer stem cell' theory. Cancer initiating cells/cancer stem cells (CICs/CSCs) are described as small populations that have (i) high tumorigenic potency, (ii) self-renewal and (iii) differentiation ability. This concept is very important and intriguing, since CICs/CSCc have very high tumor generating ability resistance to treatment and high metastatic ability.[6] Therefore, we propose that (G) gaining cancer initiation ability (cancer stemness) should also be included as a seventh malignant phenotype (Fig. 2). In this review, we re-categorize the functioning antigens into the seven new categories (summarized in Table 2).

Self-Sufficiency in Growth Signals

One of the features of cancer cells that distinguish them from normal cells is their uncontrolled cell division. Usually, normal cells require mitogenic growth signals (GS) before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind several signaling molecules. Cancer cells are often overexpressed in the genes related to cell growth to mimic normal growth signaling. In consequence, cancer cells are overexpressed in cell-cycle-related molecules like normal cells. Thus, antigens of this category contain two subgroups: (i) antigens that code for receptors of growth factors, including p185$^{HER2/neu}$ and EGFR, and (ii) cell cycle-related antigens, including Cyclin B1, Cep55/c10orf3, survivin and Aurora-A kinase. These overexpressed molecules are also expressed in normal cells, whereas CTLs can be generated from cancer patients' lymphocytes, suggesting that CTLs specific for this category of antigens are not tolerated in cancer patients.

***p185$^{HER2/neu}$* and EGFR.** *p185$^{HER2/neu}$* belongs to the ErbB family, is one of the receptor tyrosine kinases (RTKs) and is overexpressed in several types of cancer cells playing several essential roles in oncogenesis, cancer progression and metastasis.[7] Amplification and overexpression of p185$^{HER-2/neu}$ have been reported in 20–40% of primary breast cancers and also in ovarian (20–25%), colorectal and pancreatic adenocarcinomas (80–85%). However, p185$^{HER2/neu}$ does express in several normal tissues at very low levels, so the CTLs might be tolerated in cancer patients. Fisk *et al.* reported the identification of a p185$^{HER/neu}$-coded HLA-A2-restricted antigenic peptide.[8] The authors found that 9-mer peptide E75 (HER-2, 369–377:KIFGSLAFL)-specific cytotoxic activity could be detected in malignant ascites of ovarian carcinoma. This report indicated that the p185$^{HER2/neu}$ derived antigenic peptide could be presented, and CTLs might be activated and clonally expanded *in vivo*. Thereafter, several reports on identification of HLA-class I restricted p185$^{HER2/neu}$ peptides have suggested that the CTLs specific for p185$^{HER2/neu}$ are not eliminated in the thymus and exist in the periphery.

Epidermal growth factor receptor *EGFR* is also in the ErbB family and is overexpressed in several types of malignancies. EGFR signaling is essential in some malignancies, thus targeting EGFR might be a reasonable treatment. Although EGFR is also expressed ubiquitously in normal tissues at a very low level, Shomura *et al.* have shown that EGFR can also be a target of

Table 2. **Summary of the tumor-associated antigens (TAAs) and antigenic peptides**

Groups	Antigen	HLA restriction	Peptide sequence	Position	Reference
A, Self-sufficiency in growth signals	p185$^{HER2/neu}$	A2	ALCRWGLLL	5–13	(87)
		A2	HLYQGCQVV	48–56	(88)
		A2	KIFGSLAFL	369–377	(8)
		A2	PLQPEQLQV	391–399	(89)
		A2	TLEEITGYL	402–410	(89)
		A2	ILHNGAYSL	435–443	(87)
		A2	ALIHHNTHL	466–474	(89)
		A2	PLTSIISAV	650–658	(89)
		A2	IISAVVGIL	654–662	(90)
		A2	VVLGVVFGI	665–673	(91)
		A2	RLLQETELV	689–697	(91)
		A2	YMIMVKCWMI	952–961	(91)
		A2	YLVPQQGFFC	1023–1032	(88)
		A3	VLRENTSPK	754–762	(92)
		A24	TYLPTNASL	63–71	(93)
	EGFR	A2	KLFGTSGQKT	479–488	(9)
		A2	YLNTVQPTCV	1138–1147	(9)
		A24	MFNNCEVVL	54–62	(10)
		A24	NYDANKTGL	124–132	(10)
		A24	DYVREHKDNI	800–809	(10)
	Cyclin B1	A2	AGYLMELCC*	323–341	(11)
	Aurora-A	A2, A24	YLILEYAPL	207–215	(16)
	Cep55/c10orf3	A24	VYVKGLLAKI	193–202	(14)
B, Insensitivity antigrowth signals	MDM2	A2	VLFYLGQYI	53–61	(18)
C, Evading apoptosis	Survivin	A1	QFEELTLGEF	92–101	(94)
		A2	TLPPAWQPFL	5–14	(95)
		A2	RISTFKNWPFL	18–28	(94)
		A2	ELTLGEFLKL	95–104	(95,96)
		A2	LTLGEFLKL	96–104	(96)
		A11	DLAQCFFCFK	53–62	(94)
		A24	STFKNWPFL	20–28	(31)
		A24	FFCFKELEGW	58–67	(33)
		B35	CPTENEPDL	46–54	(97)
		B35	EPDLAQCFF	51–59	(97)
	Survivin-2B	A24	AYACNTSTL	80–88	(28)
	ML-IAP/Livin	A2	SLGSPVLGL	34–42	(47)
		A2	RLASFYDWPL	90–99	(47)
		A2	RLQEERTCKV	245–254	(49)
		A2	QLCPICRAPV	280–289	(49)
		A3	RLQEERTCK	245–253	(48)
		A24	KWFPSCQFLL	146–155	(44)
	Bcl-2	A2	PLFDFSWLSL	208–217	(51)
	Bcl-xL	A2	YLNDHLEPWI	173–182	(53)
	Mcl-1	A1	RLLFFAPTR	95–103	(54)
		A1	QSLEIISRY	177–185	(55)
		A1	RTKRDWLVK	300–309	(55)
D, Limitless replicative potential	hTERT	A2	ILAKFLHWL	540–548	(58)
		A2	RLFFYRKSV	572–580	(62)
		A2	RLVDDFLLV	865–873	(63)
		A3	KLFGVLRLK	973–981	(64)
		A24	VYAETKHFL	324–332	(65)
		A24	VYHFVRACL	461–469	(65)
		B*0702	RPAEEATSL	277–285	(66)
		B*0702	RPSFLLSSL	342–350	(66)
		B*0702	RPSLTGARRL	351–360	(66)
E, Sustained angiogenesis	VEGF	B*2705	SRFGGAVVR	5′UTR	(75)
	VEGF-R1	A2	TLFWLLLTL	770–778	(74)
		A2	VLLWEIFSL	1087–1095	(74)
	VEGF-R2	A2	YMISYAGMV	190–198	(73)
		A2	VIAMFFWLL	773–781	(73)
F, Tissue invasion and metastasis	MMP2	A2	GLPPDVQRV	484–492	(79)
G, Gaining the cancer initiation ability (cancer stemness)	SOX2	A2	TLMKKDKYTL	118–127	(84)
	SOX10	A2	AWISKPPGV	332–340	(86)

*wild type sequence is AKYLMELTM; HLA, human leukocyte antigen; EGF-R, epidermal growth factor receptor; MDM2, murine double minute 2; ML-IAP, melanoma inhibitor apoptosis protein; hTERT, human telomerase reverse transcriptaseVEGF-R, vascular endothelial growth facotr-receptor; MMP2 matrix metalloprotease 2; SOX2, SRY (sex determining region Y)-box 2.

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CTLs, indicating that cellular immunity for EGFR is not tolerated.[9,10] The authors identified HLA-A2- and HLA-A24-restricted CTL epitopes.

Cyclin B1. Cyclin B1 is expressed predominantly in the G2/M phase of cell division and is essential for the initiation of chromosome condensation, destruction of the nuclear membrane, and assembly of the mitotic spindle. Kao *et al.* eluted antigenic peptides from an HLA-A2.1-positive breast carcinoma cell line, and isolated several peptides highly homologous to the Cyclin B1 amino acid sequence.[11] Interestingly, these 9-mer peptide sequences were changed at the second position lysine to glycine. In the C-terminal methionine was substituted methionine to valine, phenylalanine or cysteine. They confirmed that peptide4 (AGYLMRLCV) was immunogenic with an HLA-A2.1-positive cancer patient's blood. The natural sequence expected from the gene sequence was actually AKYLMELTM. The precise mechanisms of the amino-acid substitutions remain elusive. Several reports showed that overexpression of Cyclin B1 protein was related to poor prognosis and radiotherapy resistance, suggesting that Cyclin B1 had some role in cancer progression and resistance to therapy.

Cep55/c10orf3. Cep55/c10orf3 is one of the proteins localized to centrosomes and the midbody, and has an essential role in cytokinesis.[12] The centrosome is the principal microtubule organizing center of the mammalian cell, consisting of a pair of barrel-shaped microtubule assemblies that are non-identical and are referred to as the mother and daughter centrioles.[13] Defects in the number, structure or function of centrosomes can generate mono- or multipolar mitotic spindles and cytokinesis defects, resulting in aneuploidy and chromosome instability, which are common characteristics of tumor cells. Thus, abnormal centrosome constituents may be exploited as therapeutic targets for malignantly transformed or dysplastic cells. Survivin, Aurora-A kinase and part of Cyclin B1 are also centrosome-related antigens.

Recently, we found that Cep55/c10orf3 could be a target of CTLs from HLA-A24-positive breast cancer patients.[14] As Cep55/c10orf3 is one of the mitosis-related molecules, low level expression of Cep55/c10or3 mRNA can be detected in some normal tissues, including thymus and testis. On the other hand, we could not detect the Cep55/c10orf3 protein in normal tissues adjacent to Cep55/c10orf3-positive cancerous tissues. Furthermore, the Cep55/c10orf3 protein expression can be detected not only in mitotic cells but also in the cytosol of interphase cells. The accumulation of Cep55/c10orf3 protein might evoke immuno-reactivity.

Aurora-A kinase. Aurora-A kinase is a member of the serine/threonine kinase family, and the Aurora-A gene is located at chromosome 20q13, a region frequently amplified in breast cancer. Aurora-A kinase is mainly expressed in the G2/M phase of the cell cycle and regulates mitotic cell division in normal cells. Aurora-A kinase is overexpressed in several types of malignancies, and its overexpression causes transformation of rodent fibroblasts.[15] Recently, Ochi *et al.* reported that an HLA-A2- and A24-restricted Aurora-A kinase derived peptide could induce CTL.[16] The authors showed that an Aurora-A peptide-specific CTL clone could recognize Aurora-A-positive acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) cell lines in the context of HLA-A2, suggesting that the peptide was presented by HLA-A2 endogenously. As Aurora-A is expressed in several kinds of malignancies, this antigenic peptide might also be suitable for other types of malignancies.

Insensitivity Antigrowth Signals

In normal tissues, several anti-proliferative signals maintain the normal cell growth. The representative way to suppress growth signals is cyclin-dependent kinase inhibitors (CDKIs). CDKIs, including p15, p16, p21 and p27 directly bind a cyclin-cyclin-dependent kinase (CDK) complex and suppress the kinase activity. Tumor growth factor (TGF)-beta signals suppress tumor growth by up-regulating CDKIs. p53 suppresses tumor growth by up-regulating p21. One of the p53 regulators, murine double minute 2 (MDM2), was identified as a target of CTLs.

MDM2. MDM2 is overexpressed in several types of cancer cells[17] and has an essential role in oncogenesis by down-regulating the p53 tumor suppressor protein level via degradation. Thus MDM2 might be a reasonable target for cancer therapy; however, MDM2 is also expressed in normal tissues at a low level, thus MDM2-specific CTLs might be tolerated.

Asai *et al.* found that HLA-A2-restricted HDM2 (human MDM2 homolog) peptide-specific CTLs could be generated in human systems.[18] However, HLA-A2-restricted HDM2-specific CTLs could be established only from HLA-A2-positive healthy volunteers, not from HLA-A2-positive cancer patients. Stanislawski *et al.* and Ramirez *et al.* also showed that cellular immunity for MDM2 was tolerated, whereas high-affinity T-cell receptor (TCR) gene-transfer T-cells or multiple peptide vaccination could break the tolerance.[19,20]

Evading Apoptosis

In physiological conditions, the gross cell number is well controlled by programmed cell death, that is, apoptosis. Growth signal stimulation converts cells from the quiescent state to the proliferative state to recover and maintain the tissue. Then the excess cells will be eliminated by apoptosis. Malignant transformed cells growing in an uncontrolled fashion use several mechanisms to evade apoptosis and survive. One subgroup of this gene is inhibitor apoptosis proteins (IAPs), and the other is Bcl-2 family proteins. IAP family proteins inhibit the lower effector enzymes termed caspases, including caspase 9, caspase 8 and caspase 3. IAP family protein like survivin and Melanoma-IAP (ML-IAP)/Livin, are reported to be the targets of CTLs. Bcl-2 family proteins mainly inhibit the secretion of cytochrome *c* from mitochondria following apoptosis. This group contains Bcl-2, Bcl-$_{XL}$ and Mcl-1, which are already proved to be targets of CTLs. The functions of this group of proteins are well characterized and related to poor prognosis; thus this group of proteins is a reasonable target for cancer immunotherapy.

Survivin. Originally, survivin was isolated as one of the IAP family.[21] As described above, it was proved to have a critical role in cell cycle progression, especially mitosis. Survivin expression is up-regulated in a large proportion of malignancies, and is related to resistance to chemotherapy or radiotherapy, and its overexpression is linked to poor prognosis. Thus, survivin is thought to be promising target molecule.[22]

As survivin is overexpressed in several types of malignancies, it is thought to be one of the universal and ideal antigens.[23] From this point of view, several HLA-class I-restricted survivin peptides have been reported (summarized in Fig. 3). There are several splicing variants with different functions and subcellular localizations.[24] Survivin, survivin-Δ Ex3 and survivin-3B have anti-apoptotic potential. On the other hand, survivin-2α have pro-apoptotic potential. Survivin-2B is quite complicated, with three different kinds of reports. First, Ling *et al.* reported that high expression of survivin-2B was related to good prognosis and no relapse in non-small-cell lung cancer and that its overexpression caused apoptosis.[25] Second, Nakano *et al.* reported that Survivin-2B expression showed no relation to tumor progress.[26] Finally, in contrast, Wagner *et al.* reported that low-expression of survivin-2B was related to good prognosis in adult AML.[27] Since these three reports focused on different types of malignancies, the functions of survivin-2B are still elusive. Above all, survivin-2B is expressed in several types

Table 3. Phase I clinical trials of survivin-2B peptide vaccines with three protocols in colon cancers (Sept. 2008)

Protocol	Adverse effects	Tumor marker (not increased)	CT imaging (SD, PR)	CTL detection (tetramer)
(1) Peptide alone	Anemia, fever	20%	40%	20%
	General malaise	(2/10)	(4/10)	(2/10)
(2) Peptide + IFA	Induration	0%	20%	0%
	Itching, fever	(0/5)	(1/5)	(0/5)
(3) Peptide + IFA + IFNα	Induration	67%	67%	50%
	Leucopenia	(4/6)	(4/6)	(3/6)
	Itching, fever			

CTL, cytotoxic T lymphocyte; IFA, incomplete Freud's adjuvant; IFN, interferon.

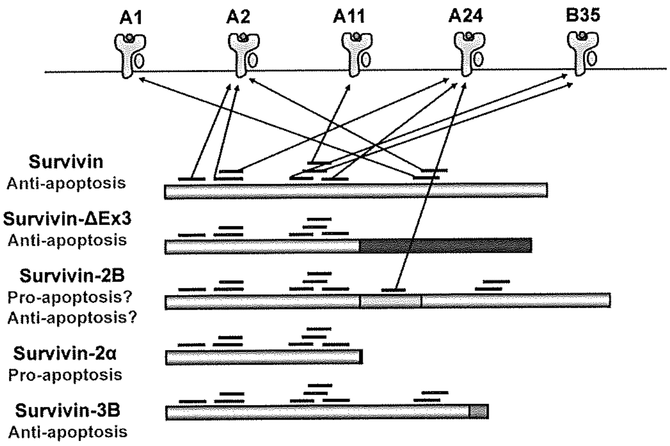

Fig. 3. Several antigenic peptides from survivin and variants. There are five survivin transcripts already reported. Wild-type survivin, survivin-ΔEx3 and survivin-3B have anti-apoptotic function. Survivin-2α has pro-apoptotic function. Survivin consists of four exons. Survivin-ΔEx3 skips exon 3 causing frame shift and code 64 different amino acids with exon 4 (red bar). Survivin-2B contains an additional exon 2B between exon 2 and exon 3. Exon 2B codes an additional 23 amino acids (yellow bar). Survivin-2α contains exon 2α following exon 2. Exon 2α codes only one amino acid (light blue) and ends with the stop codon. Survivin-3B contains an additional exon 3B between exon 3 and exon 4. Exon 3B codes seven additional amino acids (green bar) and ends with the stop codon. There are several antigenic peptides derived from survivin and its variants. Survivin-2B contains the human leukocyte antigen (HLA)-A24-restricted splicing variant-specific antigenic peptide.

of malignancies in very high proportion,[28–30] thus survivin-2B can also be an ideal target for cancer immunotherapy. HLA-A1-, A2-, A11- and B35-restricted survivin peptides are derived from wild type survivin and several splicing variants, including survivin ΔEx3, survivin-2B, survivin-2α and survivin-3B. These peptides target anti-apoptotic molecules and also pro-apoptotic molecules. One of the HLA-A24 restricted peptides is derived only from survivin-2B (survivin-$2B_{80–88}$).[28] Andersen *et al.* reported that different HLA-A24 restricted peptides from survivin and splicing variants shared the sequence (survivin$_{20–28}$).[31] However, the authors did not show survivin$_{20–28}$ peptide-specific CTLs could recognize HLA-A24-positive and survivin-positive cancer cells. Thus, it is unclear whether the survivin$_{20–28}$ peptide is presented by HLA-A24 molecules endogenously. Survivin-$2B_{80–88}$ peptide shows high immunogenic potency from HLA-A24 cancer patients,[32] and survivin-$2B_{80–88}$ peptide-specific CTLs do recognize HLA-A24-positive cancer cells, suggesting that survivin-$2B_{80–88}$ is presented endogenously with the HLA-A24 molecule. And, survivin-$2B_{80–88}$-specific CTL precursors were ~10-fold more frequent than wild-type HLA-A24 survivin-derived peptide-specific CTL precursors in HLA-A24-positive cancer patients in our recent study.[33] At this moment, we do not know why the survivin-2B-derived peptide is more immunogenic than the survivin-derived peptide. One of the reasons might be the different expression levels of these molecules. As survivin is expressed in several normal tissues at very low levels, cellular immunity for survivin might be partially tolerated. However, survivin-2B expression is several-fold less than that of survivin,[28,34] thus survivin-2B-specific immunity might not be tolerated and it retains high CTL cytotoxic activity. To summarize, we cannot conclude whether survivin or survivin-2B is the most suitable target for immunotherapy, but we can conclude that both of them have CTL-inducing potential and might be ideal targets.

CTLs kill target cells through secretion of perforin and Granzyme B, causing apoptosis on target cells. However, survivin-overexpressing cells are sensitive to CTLs, suggesting that survivin has no or little role in apoptosis caused by Granzyme B. Caldas *et al.* showed that fusion of the survivin gene promoter to the coding sequence of active Granzyme B led to increased expression of Granzyme B in tumor cells, resulting in a higher rate of apoptotic cell death.[35] This also supports the idea that survivin-overexpressing cells are sensitive to CTLs. Survivin is also immunogenic for humoral immunity, supporting its high immunogenicity in the immune system.[36–39] The significant survivin immunogenicity for the cellular and humoral immune systems suggests fascinating possibilies for its use as a molecular target of immunotherapy.

On the basis of the immunogenicity of survivin antigenic peptides, some clinical trials have already been launched and reported, using survivin-$2B_{80–88}$[40,41] and survivin$_{96–104}$.[42] Each report showed some clinical response with survivin-2B and survivin-derived peptides, indicating that survivin/survivin-2B immunotherapy is a promising modality for cancer therapy. In our recent studies, survivin-$2B_{80–88}$ peptide and interferon-α (IFN-α) vaccination therapy is under Phase I study (Table 3). With peptide alone, we could detect CTL precursor (CTLp) frequency elevation in only 20% of patients, and the clinical responses were also relatively low (tumor marker 20%, imaging 40%). However, peptide plus IFN-α vaccination improved the detection of CTLp up to 50%. And also we could observe 67% tumor marker response and tumor mass stabilization or regression in clinical imaging. These observations suggest that immunological response to survivin-$2B_{80–88}$ peptide in the periphery is associated with the clinical response of peptide vaccine therapy, and IFN-α improves the efficacy of peptide vaccination therapy.

ML-IAP/Livin. Melanoma-IAP (ML-IAP)/Livin was identified as a novel IAP family protein overexpressed in melanoma.[43] ML-IAP/Livin contains a single BIR and RING domain, and inhibits caspase 3 and caspase 9 activities, causing the inhibition of apoptosis. In the following studies, ML-IAP was proved to be overexpressed in lung cancer,[44] renal cell carcinoma[45] and childhood AML.[46] High-level expression of ML-IAP/Livin is related to poor prognosis.[46] These reports suggest that ML-IAP/Livin can be the target of immunotherapy for such malignancies.

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ML-IAP/Livin is also immunogenic for cellular and humoral immune systems.[44,47–49]

Bcl-2, Bcl-xL and Mcl-1. Bcl-2 was initially identified as the t(8;14) and t(14;18) translocations those are related to hematological malignancies. In subsequent reports, Bcl-2 protein was proved to be overexpressed in several types of malignancies including carcinomas. Bcl-2 is located in the inner membrane of the mitochondrion and regulates all major types of cell death, including apoptosis, necrosis, and autophagy; hence Bcl-2 is suitable for molecular targeting therapy, including cancer immunotherapy.[50] Andersen *et al.* found an HLA-A2 restricted low affinity Bcl-2 peptide (bcl208) could be a target of CTLs.[51] Interestingly, Bcl-2 peptides with high HLA-A2 affinity (bcl85, bcl124, bcl218, bcl220, bcl222 and bcl224) did not show any CTL response. These observations might indicate that CTLs specific for Bcl-2 peptides with high affinity to HLA-A2 are tolerated.

Bcl-x_L is one of the Bcl-2 protein families and has anti-apoptotic activity. Increased expression of Bcl-xL has been reported in a variety of different malignancies, including acute myeloid leukemia and multiple myeloma as well as solid cancers like bladder cancer, breast cancer, pancreatic cancer, and melanoma.[52] Andersen *et al.* detected immunological responses for the Bcl-$x_{L173-182}$ peptide in 9/18 breast cancer patients and 2/6 melanoma patients' PBMC with enzyme-linked immunospot (ELISpot) assays after stimulation *in vitro*.[53]

Mcl-1 is also a Bcl-2 family protein with anti-apoptotic potential. Andersen *et al.* reported that immune reactivity for Mcl-1-derived peptides could be detected in cancer patients' lymphocytes.[54,55] However, they did not find that Mcl-1 peptide-specific CTLs could recognize Mcl-1-positive cancer cells; thus it remains elusive whether Mcl-1-derived peptides are presented by HLA-A1 molecules endogenously.

Limitless Replicative Potential

Growth signal autonomy, insensitivity to antigrowth signals and evading apoptosis lead to cell growth independent from its environment. However, the resulting deregulated proliferation program does not guarantee the growth of a tumor. Mammalian cells carry an intrinsic, cell-autonomous program that limits their multiplication. One representative intrinsic mechanism that limits cell growth is the telomere, the cap of the chromosomal end. Each replication of the genomes shorten the telomere by 50–100 base pairs (bps), finally causing chromosomal instability and cell growth arrest termed 'crisis'.[56] Telomerase, a eukaryotic ribonucleoprotein (RNP) complex, helps to stabilize telomere length in human stem cells, reproductive cells and cancer cells by adding TTAGGG repeats onto the telomeres using its intrinsic RNA as a template for reverse transcription. The human telomerase reverse transcriptase catalytic subunit (hTERT) is overexpressed in 85–90% of several malignancies. This makes telomerase a target not only for cancer diagnosis but also for the development of novel anti-cancer therapeutic agents.[57]

hTERT. On the way to find universal TAAs that are expressed in various malignancies, Vonderhide *et al.* identified the HLA-A2-restricted hTERT-derived peptide (hTERT540).[58] The authors reported that hTERT peptide-specific CTLs could recognize HLA-A2-positive and hTERT-positive cancer cells in an HLA-restricted and peptide-specific fashion. Several clinical studies using hTERT-derived peptides have already been launched. hTERT-specific T lymphocytes were induced in four of seven patients with advanced breast or prostate carcinoma after vaccination with dendritic cells pulsed with hTERT peptides, resulting in partial tumor regression in one patient.[59] Another clinical trial used hTERT messenger RNA (mRNA)-transfected dendritic cells in patients with metastatic prostate cancer.[60] A trial investigating vaccination with hTERT peptides in patients with non-small-cell lung cancer showed immune responses in 12 of 24 evaluable patients during the primary regimen, with a complete tumor response observed in one patient.[61] These studies justify further clinical testing to evaluate the efficacy of hTERT-based vaccinations, and several hTERT-based clinical vaccination trials are currently ongoing. Several HLA-class I-restricted hTERT-derived peptides make the hTERT peptide more universal: HLA-A2,[62,63] HLA-A3,[64] A24[65] and B*0702.[66]

In contrast to the above studies, several groups reported that the hTERT540 peptide is not presented on the surface of tumor cells in the context of HLA-A2.[67–69] Above all, Wenandy *et al.* confirmed that hTERT540-specific CTL clones could recognize HLA-A2-positive and hTERT-positive cancer cell lines.[70] The discrepancies among these groups might depend on the conditions, including cell culture conditions, T-cell avidity, target cell condition and so on.

Sustained Angiogenesis

Oxygen and nutrients are essential for cell survival. After organogenesis, angiogenesis can be observed in only strictly limited situations such as wound healing and tumorigenesis. Targeting angiogenesis is believed to be an attractive approach for cancer therapy. This method has several merits compared to others. First, angiogenesis is one of the physiological reactions of the host, therefore therapy resistance caused by genomic instability will be unlikely to occur. Second, each capillary has the potential to feed hundreds of cancer cells; thus, targeting angiogenesis may have amplified effects.[71] Angiogenesis can be the target of immunotherapy, and several studies have demonstrated the effects of anti-angiogenesis immunotherapy *in vitro* and *in vivo*.

VEGF-R1, VEGF-R2, VEGF. Since, vascular endothelial growth factor (VEGF) is not expressed in normal blood vessels, but is expressed in pathologic angiogenic vessels, including tumor angiogenesis, and VEGF signaling plays a major role in angiogenesis, VEGF-receptors (VEGF-Rs) might be a major target for anti-angiogenesis immunotherapy. Niethammer *et al.* showed the efficacy of targeting VEGF-R2 with DNA vaccination in a mouse tumor model in which a significant anti-tumor effect was observed.[72] The authors reported that vaccination with VEGF-R2 DNA caused inhibition of tumor growth, prolongation of survival and a decrease of vessels in tumor tissues. They further showed that DNA vaccination did not affect wound healing, so this strategy might be safe and feasible for a clinical trial. HLA-A2-restricted VEGF-R2 and VEGF-R1 antigenic peptides have also been reported.[73,74] Weinzierl reported an HLA-B27-restricted antigenic peptide that is coded in the cryptic translated region of VEGF using differential mass spectrometry in primary renal cell carcinomas (RCCs), suggesting that this peptide is endogenously presented *in vivo*.[75] This antigenic peptide might be useful for RCCs.

Survivin. Survivin is related to angiogenesis and anti-sense targeting of survivin inhibits capillary formation by endothelial cells, suggesting that targeting survivin does target angiogenesis.[76] Xiang demonstrated the anti-angiogenesis effect of therapy targeting survivin with DNA vaccination.[77] These findings suggest that targeting survivin equals therapy targeting both cancer cells and angiogenesis as well. This makes survivin a unique and attractive antigen.

Tissue Invasion and Metastasis

In the process of malignant tumor cell growth, the cells need to invade the normal connective tissue barrier. Tissue invasion and metastasis are landmarks of malignant diseases that distinguish them from benign diseases. Several classes of proteins are involved in this mechanism. One classic mechanism for gaining invasive ability is the overexpression of several enzymes to break the connective tissue barrier. Matrix metalloproteases (MMPs) are representative molecules of tissue invasion. MMPs are known to be overexpressed in cancerous tissues, and further, to be related to poor prognosis, cancer progression, advanced stages and high risk of metastasis.[78] MMPs play an essential role in carcinogenesis and might be targets for immunotherapy. However, MMPs are also expressed in normal tissues so that immunity for MMPs might be tolerated. Cellular immunity for MMP-2 has been demonstrated in a very unique fashion, as described below.

MMP-2. Matrix metalloproteinase-2 plays an essential role in tumor progression; however it is expressed ubiquitously, and the peptides derived from MMP-2 might be tolerated. Godefroy *et al.* showed how an MMP-2-derived peptide could be presented by tumor cells specifically and recognized by CTLs.[79] They reported that an HLA-A*0201-restricted peptide from the MMP-2 (GLPPDVQRV) was cross-presented by $\alpha v\beta 3$-expressing tumor cells following clathrin-coated pit-mediated uptake of secreted extracellular MMP-2 and proteasome activity. The classic endogenous cytosolic pathway did not present this peptide. Hence, an MMP-2-derived peptide vaccine might be useful for MMP-2 secreting and $\alpha v\beta 3$-expressing melanoma cells.

Gaining Cancer Initiation Ability (Cancer Stemness)

The cancer-initiating cell (CIC)/cancer stem cells (CSC) hypothesis is both an old and a new concept. In the former studies it was discovered that a small population of cancer cells had growth potential in soft agar and were called 'tumor stem cells'. Recently, CICs/CSCs are described as small populations that have (i) tumor initiation ability, (ii) differentiation ability and (iii) self-renewal. Since CICs/CSCc have very high tumor-generating ability, resistance to treatment and high metastatic ability, the CICs/CSCs concept is very important.[6] The CICs/CSCs resistance for several treatments raise another question as to whether CICs/CSCs are also resistant to CTLs. Kawasaki *et al.* reviewed CD200, one of the immunosuppressive factors expressed in normal and cancer stem cells, and CD200-expressing cancer cells suppressed secretion of type 1 helper T-cell cytokines (IFN-gamma and interleukin [IL]-2),[80] suggesting that CICs/CSCs might suppress immunity against cancers. At this moment, CIC/CSC-related molecules SRY (sex determining region Y)-box 2 (SOX2) and SOX10 are known to be targets of CTLs as described below. Since these reports did not show cytotoxic activity for CICs/CSCs, we cannot conclude whether CICs/CSCs are susceptible to CTLs. The concept targeting CICs/CSCs with CTLs is intriguing and further reports are eagerly awaited.

SOX2. SRY (sex determining region Y)-box 2 is a transcription factor that is essential to maintain self-renewal of undifferentiated embryonic stem cells. SOX2 is one of the red-hot genes. In 2006, Takahashi and Yamanaka reported that induced pluripotent stem (iPS) cells could be generated from mouse fibroblasts by retrovirus-mediated introduction of four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4, in an epoch-making work of regenerative medicine.[81] This reports suggest that SOX2 is essential in the maintenance of embryonal stem (ES) cells and neuronal progenitor cells. It is also reported to be expressed in several malignancies.[82–84] Furthermore, Schmits *et al.* reported HLA-A2-restricted SOX2 peptides, and SOX2 could be the target of CTLs in glioma cells.[84] In addition, Ben-Porath *et al.* reported that an embryonic stem cell-like gene expression signature including Nanog, Oct3/4, Sox2 and c-Myc was preferentially overexpressed in poorly differentiated aggressive human tumors.[85] To summarize these reports, targeting ES phenotypic genes seems to be a reasonable strategy and SOX2 is representative of this approach.

SOX10. Khong *et al.* showed that SOX10 was recognized by tumor-infiltrating lymphocytes obtained from a patient who experienced a dramatic clinical response to immunotherapy.[86] It acts as a critical transactivator of tyrosinase-related protein-2 during melanoblast development and as a potent transactivator of micropthalmia-associated transcription factor (MITF), which is considered to be a master gene that controls the development and postnatal survival of melanocytes. Thus, SOX10 is a potential target for melanoma CICs/CSCs.

Conclusion

As described above, considerable numbers of TAAs have essential roles in carcinogenesis. Since, TAAs are defined as antigens that are expressed in cancer cells specifically, it is reasonable that various overexpressed carcinogenesis-related genes can also be TAAs. Thus, the functioning antigen concept is tightly connected to carcinogenesis and essential for both cancer immunotherapy and cancer biology. Finally, to accomplish effective cancer immunotherapy, we need to improve recent therapies. There are several possibilities: combination of several peptides, combination with another molecular targeting therapy, and combination with radiation therapy and combination with chemotherapy. The concept of functioning antigens is the blueprint of cancer therapy, including immunotherapy and points us in the direction to move ahead.

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