

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

Ichiya Honma · Hiroshi Kitamura · Toshihiko Torigoe · Akari Takahashi · Toshiaki Tanaka · Eiji Sato · Yoshihiko Hirohashi · Naoya Masumori · Taiji Tsukamoto · Noriyuki Sato

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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.

**Keywords** Immunotherapy · Survivin · Peptide vaccination · Urothelial cancer

### 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

I. Honma (✉) · H. Kitamura · T. Tanaka · E. Sato · N. Masumori · T. Tsukamoto  
Department of Urology, Sapporo Medical University  
School of Medicine, Sapporo, Japan  
e-mail: ichiya@sapmed.ac.jp

T. Torigoe · Y. Hirohashi · N. Sato  
Department of Pathology,  
Sapporo Medical University, Sapporo, Japan

A. Takahashi  
Innovation Plaza Hokkaido,  
Japan Science and Technology Corporation,  
Sapporo, Japan

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 <sup>a</sup>
2	51	F	Bladder Ca.	Cervical LN <sup>a</sup>
3	67	M	Bladder Ca.	Lung
4	65	F	Bladder Ca.	Pelvic soft tissue <sup>a</sup>
5	65	M	Bladder Ca.	Lung Bone
6	64	M	Bladder Ca.	Lung
7	38	F	Bladder Ca.	Inguinal LN <sup>a</sup> Mesenchymal LN
8	59	F	Bladder Ca.	Pelvic soft tissue <sup>a</sup>
9	57	M	Bladder Ca.	Lung Liver

LN lymph node

<sup>a</sup> histologically 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).

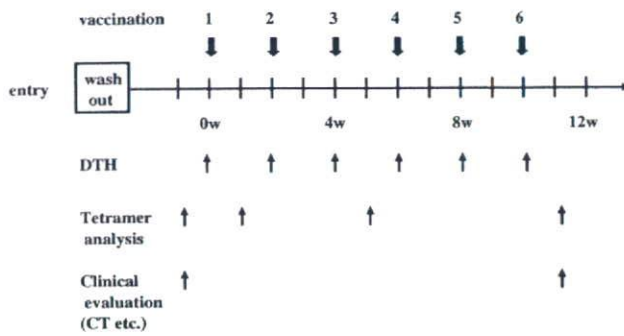
### Peptide preparation

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  $-80^{\circ}\text{C}$  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 β<sub>2</sub>-microglobulin 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 ≥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) <sup>a</sup>	DTH skin test	Tetramer staining assay % of HLA-A24/survivin-2B80-88 peptide tetramer-positive 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) <sup>b</sup>	NA
8	1.0 mg × 6	Induration at the injection site (Grade 1)	(-)	Increased (0.78/0.07) <sup>b</sup>	-87.6% (PD)
9	1.0 mg × 6	None	(-)	Increased (1.42/0.13) <sup>b</sup>	-81.4% (PD)

NA not available

<sup>a</sup> Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria (NCI-CTC)

<sup>b</sup> 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).

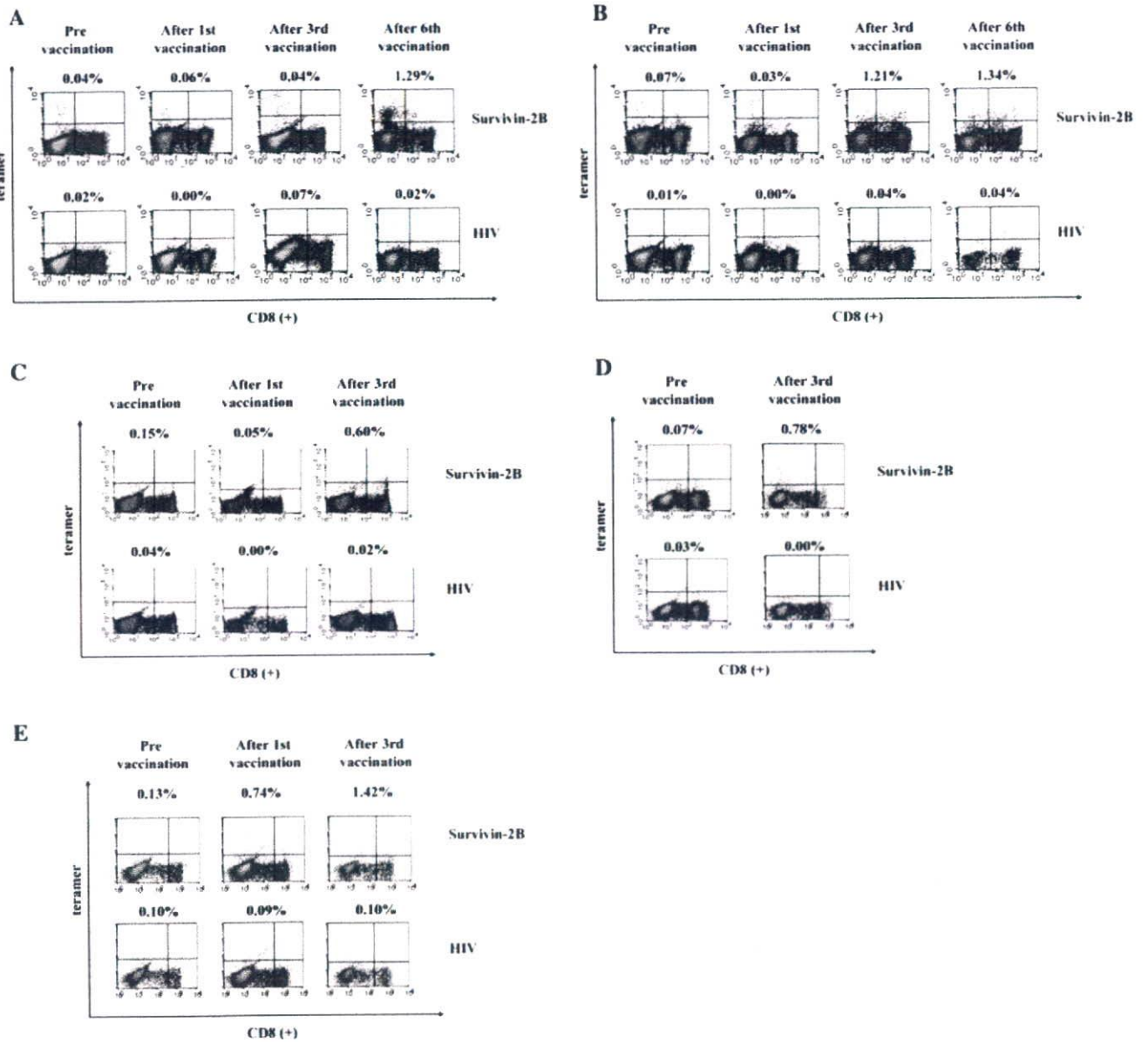
In case 7 who could not receive the complete regimen due to disease progression, HLA-A24/survivin-2B80-88 peptide tetramer analysis after the third vaccination revealed an increase in the peptide-specific CTL frequency from 0.15 to 0.60% (Fig. 2c). In cases 8 and 9, peptide-specific CTL frequency increased 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)

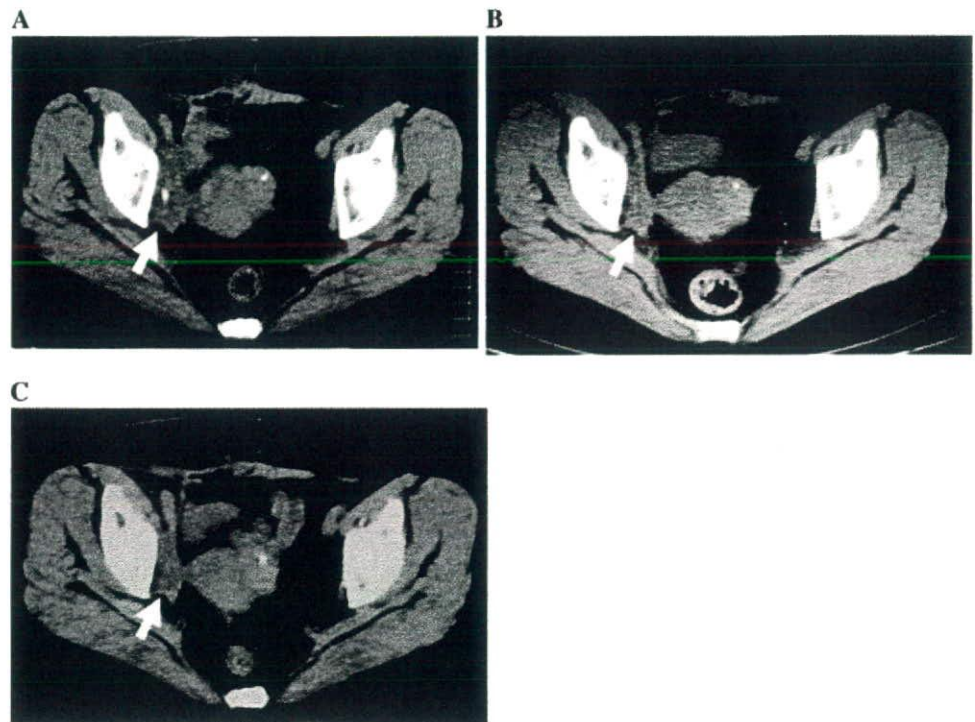
An increasing number of T-cell epitopes derived from these various tumor-associated antigens have been reported and proved to play significant roles in CTL-based immunotherapy. Survivin was originally identified as a member of the inhibitor of apoptosis protein family. It has the capability to inhibit 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, over-expression 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 reac-

tion 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<sup>±</sup>/tetramer<sup>±</sup> cells represent the survivin-2B80-88-specific CTL, CD8<sup>±</sup>/tetramer<sup>±</sup> 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 survivin-2B80-88 tetramer showed the peptide-specific killing activity, indicating that CD8<sup>±</sup>/tetramer<sup>±</sup> cells represent the survivin-2B80-88-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- $\alpha$  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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## Wild-type AIRE cooperates with p63 in HLA class II expression of medullary thymic stromal cells<sup>☆</sup>

Akiko Tonooka<sup>a</sup>, Terufumi Kubo<sup>a</sup>, Shingo Ichimiya<sup>a,\*</sup>, Yutaka Tamura<sup>b</sup>, Tanja Ilmarinen<sup>c</sup>, Ismo Ulmanen<sup>c</sup>, Sachiko Kimura<sup>d</sup>, Shigeaki Yokoyama<sup>d</sup>, Yoshihide Takano<sup>a</sup>, Tomoki Kikuchi<sup>a</sup>, Noriyuki Sato<sup>a</sup>

<sup>a</sup> Department of Pathology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan

<sup>b</sup> Department of Bioinformatics, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chiba 260-8670, Japan

<sup>c</sup> Department of Molecular Medicine, National Public Health Institute, Biomedicum Helsinki, Haartmaninkatu 8, 00290 Helsinki, Finland

<sup>d</sup> Division of Pathology and Laboratory Medicine, Hokkaido Medical Center for Child Health and Rehabilitation, Kanayama 1-1, Teine-ku, Sapporo 060-0041, Japan

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### ABSTRACT

During T cell development in the thymus, autoreactive T cells are deleted through a mechanism that is actively supported by medullary epithelial cells. These epithelial cells possess particular transcription factors including autoimmune regulator (AIRE), which is responsible for regulating expression of self-antigens, as well as p63, a p53-like molecule. Here we present evidence suggesting interaction of AIRE with p63 through a SAND domain and a transactivation domain, respectively. Interestingly an AIRE molecule with a mutated SAND domain of G228W, whose genetic alteration is inherited in an autosomal dominant manner, could not establish a complex with p63 as indicated by immunoprecipitation and molecular modeling analyses. Further *in vitro* study indicated that the G228W mutation led to downregulation of the transcription levels of CIITA and, accordingly, the cell surface expression of HLA class II molecules in thymic epithelial cells with p63. This indicates novel involvement of AIRE and p63 in the regulation of HLA class II, and suggests that defects in the AIRE–p63 interaction may lead to malfunction of HLA-based selection of self-reactive helper CD4<sup>+</sup> T cells in the thymus.

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To establish immunosurveillance, which is largely conducted by effector T cells, the thymus generates fully functional T cell repertoires equipped with immunological self-tolerance [1,2]. This central process of the thymus is mainly achieved by major histocompatibility complex (MHC)-based positive and negative selection of developing T cells in the cortical and medullary regions, respectively. Since helper CD4<sup>+</sup> T cells constituting major peripheral T cells control most immune cells, including cytotoxic CD8<sup>+</sup> T cells, B cells, NK cells, macrophages and dendritic cells, dysregulation of the selection event of helper T cells would cause an unfavorable shift into systemic autoimmune-prone conditions [3,4]. Medullary thymic epithelial cells mainly take part in negative selection of immature T cells after rearrangement of T cell receptor genes and, as a result, are believed to prevent the escape of autoreactive T cells from the thymus [5].

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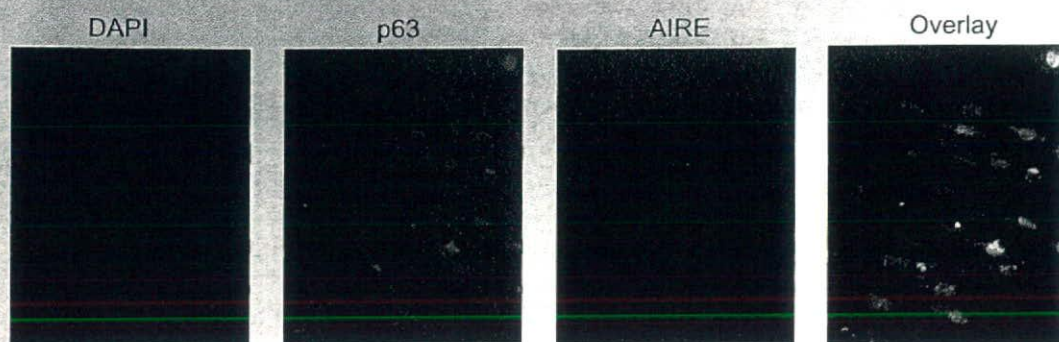
\* Corresponding author. Fax: +81 11 643 2310.

E-mail address: [ichimiya@sapmed.ac.jp](mailto:ichimiya@sapmed.ac.jp) (S. Ichimiya).

The gene encoding human AIRE (autoimmune regulator), a 545 amino acid molecule, was reported to be behind the non-HLA-linked disorder autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) [6,7]. APECED exhibits a broad spectrum of autoimmune phenotypes affecting many organs such as the parathyroid and adrenal glands, pancreas, epidermis, liver and bone marrow. This phenotypic feature has led to a hypothesis of the functional properties of AIRE in terms of thymic selection. Consequently, analyses of AIRE-deficient mice and other studies have revealed that medullary thymic epithelial cells express AIRE, which helps to purge immature self-reactive T cells at the stage of negative selection during T cell development [8–11]. Accordingly, the discovery of AIRE has had a decisive impact on the understanding of the regulatory mechanism of medullary thymic epithelial cells in terms of intrathymic T cell development and autoimmune-related disorders [12]. Thus far, the functional significance of AIRE in medullary thymic epithelial cells has been highlighted with regard to T cell selection and antigen presentation.

We have previously reported the expression of a p53-related transcription factor, p63, in human medullary thymic epithelial cells [13,14], which evidence prompted us to examine whether the function of AIRE might be controlled by p63 because transcriptional regulators occasionally constitute an oligo-molecular





**Fig. 1.** Colocalization of AIRE and p63 of a p53-related transcription factor in medullary thymic epithelial cells of primary culture. Images were obtained from immunofluorescence microscopy after double-staining with p63 (green) and AIRE (red) and subsequent nuclear staining with DAPI (blue). Original magnification: x400.

complex. Our experimental evidence suggested that, when AIRE was introduced into cells together with p63 mimicking the native status of medullary thymic epithelial cells, the two molecules could interact through the association of a SAND domain of AIRE and a transactivation (TA) domain of p63. Interestingly, a naturally occurring mutation with autosomal dominant inheritance in the SAND domain of AIRE (G228W-AIRE) could not bind to the TA domain of p63, probably due to protrusion of the tryptophan residue from the original structure of the SAND domain. Furthermore, the surface expression of HLA-DR was affected by combinatory expression of p63 and G228W-AIRE in thymic epithelial cells due to the downregulation of CIITA in that state. These findings illustrate a cooperative role of p63 with AIRE in the regulation of HLA class II of medullary thymic epithelial cells and will help elucidate the mechanism of the genetic defect causing G228W-AIRE mutation.

## Experimental procedures

**Tissues and cell culture.** Normal human thymuses were obtained from patients <3 years of age undergoing cardiovascular surgery for congenital cardiac disease in the Hokkaido Medical Center for Child Health and Rehabilitation in Japan. The procedures used for primary thymic epithelial cell culture were previously [14]. Human P1.4 thymic epithelial cells and HEK293 cells were maintained in MEM with 10 mM HEPES, and DMEM, respectively, both of which were supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 50  $\mu$ g/ml streptomycin. All cells were cultured at 37 °C in a humidified atmosphere in 5% CO<sub>2</sub>.

**Antibodies and immunohistochemistry.** A 4A4 mouse anti-p63 mAb and H137 rabbit anti-p63 pAb were purchased from Lab Vision and Santa Cruz Biotechnology, respectively. Mouse anti-HLA class II (L243), anti-HA (HA-7), anti-GFP (JL-8), and anti-myc (9E10) mAbs were also used in this study. A rabbit anti-AIRE pAb was produced by immunization of polypeptides of the SAND domain. The procedures conducted for single- or double-staining analysis were as previously described [13].

**Plasmid DNAs.** Expression plasmids of pEGFPC2 carrying wild-type AIRE (EGFP-AIRE) or G228W-AIRE (EGFP-G228W) were used in this study [15]. We newly constructed six EGFP-tagged deleted forms based on EGFP-AIRE by PCR techniques. Expression plasmids of pcDNA3.1 carrying HA-tagged p63 and  $\Delta$ Np63 cDNA were also employed. To establish stable transformants, expression plasmids of pIRESHyg2 carrying p63 (pIRESHyg2-p63) and pIRESpuromycin carrying AIRE (pIRESpuromycin-AIRE) and G228W-AIRE (pIRESpuromycin-G228W-AIRE) were constructed.

**Flow cytometry.** For detecting surface HLA-DR molecules, cells were detached from the tissue culture bottles using PBS containing EDTA. Then the cells were stained with L243 as the primary antibody. After washing with PBS three times, the cells were subse-

quently stained with a rabbit anti-mouse pAb conjugated with FITC. After washing, these cells were subjected to analysis with FACS Calibur (BD Biosciences). All data shown were obtained with gates set on living cells and analyzed with CellQuest software.

**Calculation of interaction energy and electrostatic complementarity.** The three-dimensional structures of the SAND domain of AIRE (aa 194–279) and the TA domain of p63 (aa 1–55) were constructed with MOE (CCG, Montreal, Canada) based on the Brookhaven Protein Databank 1O1Z and 2OJQ, respectively. The structure and molecular mechanics of these complexes were analyzed by using the Amber99 force field in MOE as well as ZDOCK (Boston University, MA). To calculate interaction energy and electrostatic complementarities, we employed MOE and MolFeat-EC (FiatLux, Tokyo, Japan), as previously described with some modification [16,17].

## Results

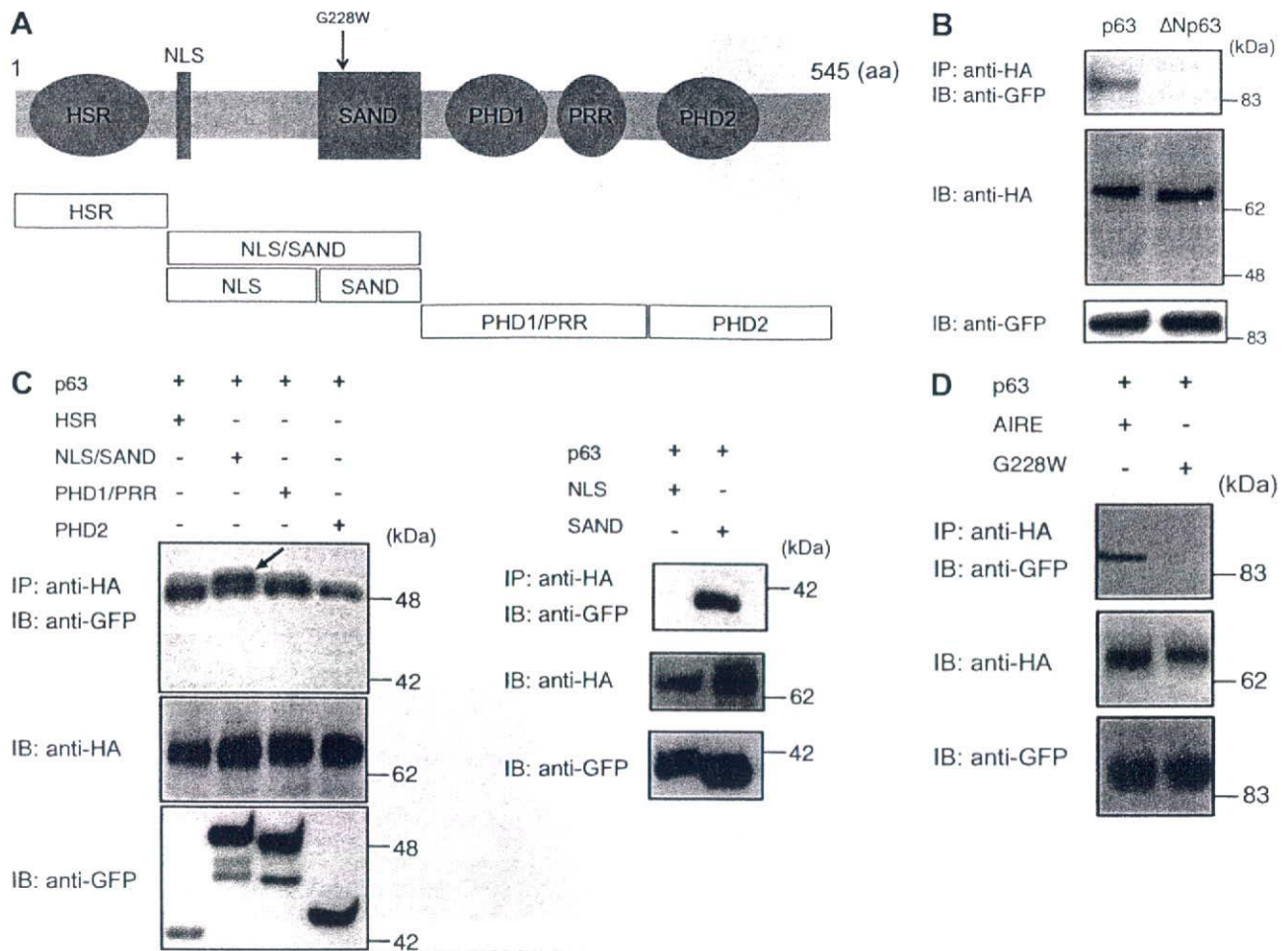
### Colocalization of AIRE and p63 in human thymic epithelial cells

First we studied the expression of AIRE and p63 in medullary thymic epithelial cells of primary culture to reconcile the evidence observed in thymic tissue sections [13,14]. Medullary thymic epithelial cells of primary culture showed typical flat shapes with round nuclei forming small cell clusters (Fig. 1). Indeed, we could find a cell population expressing AIRE and p63 in their nuclei of medullary thymic epithelial cells of primary culture. Thus, AIRE was certainly colocalized with p63 in the nuclei of these human thymic epithelial cells.

### Association of AIRE with p63

To examine whether AIRE had the capacity to associate with p63, we next performed immunoprecipitation experiments. The AIRE protein is constituted of multiple structural domains, including a homogeneously staining region (HSR) domain at the N-terminus, a nuclear localization signal (NLS), an Sp100, AIRE, NucP41/75 and DEAF-1 (SAND) domain and two plant homeodomain-type zinc-finger (PHD) domains at the C-terminus (Fig. 2A). By using EGFP-tagged deletion constructs covering the whole domain structures of such AIRE proteins, results from immunoprecipitation experiments interestingly indicated that p63 could bind to the NLS/SAND domain of AIRE, whereas  $\Delta$ Np63, an alternative spliced form of p63 lacking its TA domain, did not associate with any type of domain of AIRE (Fig. 2B and C). These results implied that the TA domain of p63 was responsible for the interaction with the NLS/SAND domain of AIRE. Further analysis suggested that an EGFP-tagged construct containing only the SAND domain would bind to p63 (Fig. 2C). Thus, it was suggested that the SAND domain of





**Fig. 2.** Interaction of p63 with AIRE. (A) Functional domains of an AIRE protein and the respective fragments employed to transfect cells. To cover the whole domain structure of AIRE, six different constructs with an EGFP tag, EGFP-HSR, EGFP-NLS/SAND, EGFP-NLS, EGFP-SAND, EGFP-PHD1/PRR and EGFP-PHD2, were made. (B) Immunoprecipitation analysis of HEK293 cells transiently transfected with HA-p63 or HA- $\Delta$ Np63 expression plasmids and EGFP-AIRE. (C) Interaction of p63 with the NLS/SAND domain of AIRE was suggested by immunoprecipitation in a left panel (the arrow indicating EGFP-NLS/SAND), whereas no association with p63 was indicated for the other constructs. Bands due to immunoglobulin heavy chains are detected in each lane. In a right panel, the p63 protein was indicated to interact with the SAND domain, but not the NLS domain of AIRE. (D) The p63 protein was indicated to interact with wild-type AIRE, whereas p63 did not associate with AIRE carrying G228W-mutation in the SAND domain.

wild-type AIRE (wt-AIRE) could have a capacity to directly bind to the TA domain of p63.

This evidence prompted us to perform further experiments to determine whether naturally occurring mutant AIRE carrying the missense mutation G228W in its SAND domain (G228W-AIRE) could bind to p63. This type of missense mutation of AIRE is inherited in an autosomal dominant manner, whereas most mutant types of AIRE are recessively transmitted [18,19]. Compared to the binding capacity of wt-AIRE, G228W-AIRE seemed to abolish the binding of AIRE to p63 (Fig. 2D).

#### Effects of a G228W moiety of mutant AIRE on AIRE-p63 interaction

Given that the physical association of wt-AIRE with p63 was inhibited by G228W mutation of AIRE, we employed molecular modeling to determine how the G228W portion of AIRE abrogated the interaction of AIRE with p63. The interaction energies of the SAND domains of wt-AIRE and G228W-AIRE with the TA domain of p63 were calculated to be  $-340.7$  kcal/mol and  $-254.1$  kcal/mol, respectively, implying that the wt-AIRE/p63 complex constituted a more stable form than the G228W-AIRE/p63 complex (Table 1). Following this analysis, we further modeled how G228W-AIRE inhibited the robust interaction with the

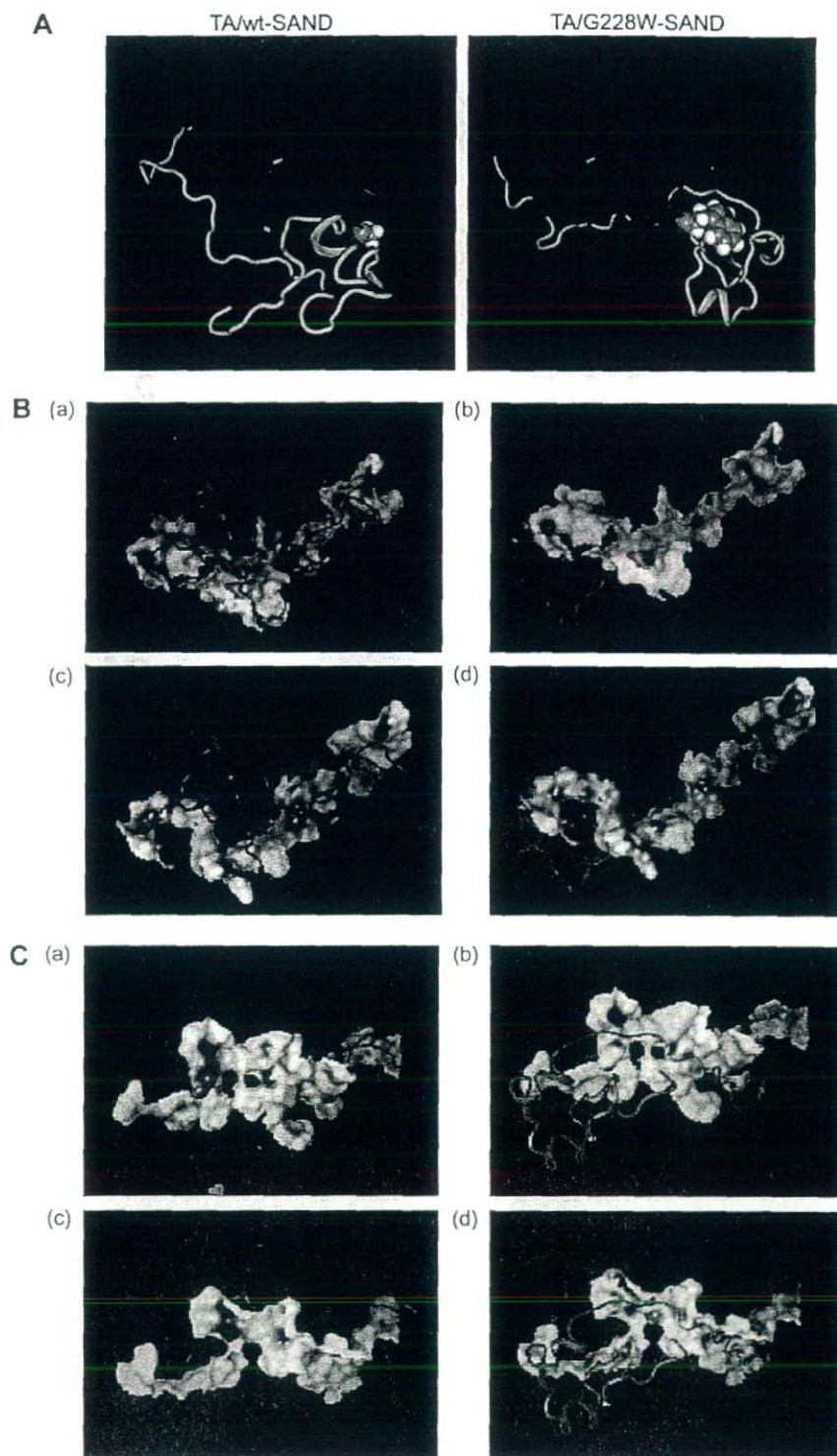
**Table 1**

Interaction energy (IE) and electrostatic complementarity (EC) between two portions of the SAND domains of wt-AIRE and G228W-AIRE and the TA domain of p63 as depicted in wt-AIRE/p63 and G228W-AIRE/p63, respectively. IE and EC were calculated by parse and Amber analyses, respectively.

	p63/wt-AIRE	p63/G228W-AIRE
IE (kcal/mol)	-340.7	-254.1
EC (Person's)	0.34	-0.05

TA domain of p63 (Fig. 3A). The manner of binding of the SAND domain of wt-AIRE to the TA domain of p63 was proposed to be the sole feasible binding pattern. In contrast, due to the hydrophobic contact repulsion of tryptophan at position 228, the SAND domain of G228W-AIRE prevented active interaction of wt-AIRE with the TA domain of p63. In addition to such a docking simulation, we investigated the electrostatic complementarity (EC) for quantitative analysis of the protein-protein interaction between the SAND domain of wt-AIRE and the TA domain of p63. This was in accord with our observation that the value of EC of wt-AIRE/p63 (0.34) was much higher than that of G228W-AIRE/p63 (EC =  $-0.05$ ) (Table 1). Indeed, the protein-protein interfaces of the SAND domain of wt-AIRE and the





**Fig. 3.** Molecular interaction models of the SAND domains of wild-type AIRE and G228W-AIRE with the TA domain of p63. (A) The atomic structures of glycine<sup>228</sup> of the wild-type AIRE and tryptophan<sup>228</sup> of G228W-AIRE within the predicted SAND domain are shown in the left and right panels, respectively. Due to the bulging of tryptophan<sup>228</sup>, the surface interactions of the G228W-SAND domain and the TA domain seem to be interrupted. The putative domain structure of the SAND domain is demonstrated in blue and that of the TA domain is in yellow. (B) The electrostatic potential on the molecular surfaces buried in the interface between the wild-type SAND domain (green) and the TA domain (blue). (a) EC on the buried molecular surface of the TA domain generated by its own charges. (b) EC of the TA domain generated by charges on the wild-type SAND domain. (c) EC of the wild-type SAND domain generated by charges on the TA domain. (d) EC of the wild-type SAND domain generated by its own charges. (C) Electrostatic potential on the molecular surfaces buried in the interface between the G228W-SAND domain (green) and the TA domain of p63 (blue). (a) EC on the buried molecular surface of the TA domain generated by its own charges. (b) EC of the TA domain generated by charges on the G228W-SAND domain, (c) EC of the G228W-SAND domain generated by charges on the TA domain. (d) EC of the G228W-SAND domain generated by its own charges.

TA domain of p63 had anticorrelated and complementary surface electrostatic potentials when compared to those of the SAND domain of G228W-AIRE and TA domain of p63 (Fig. 3B and C).

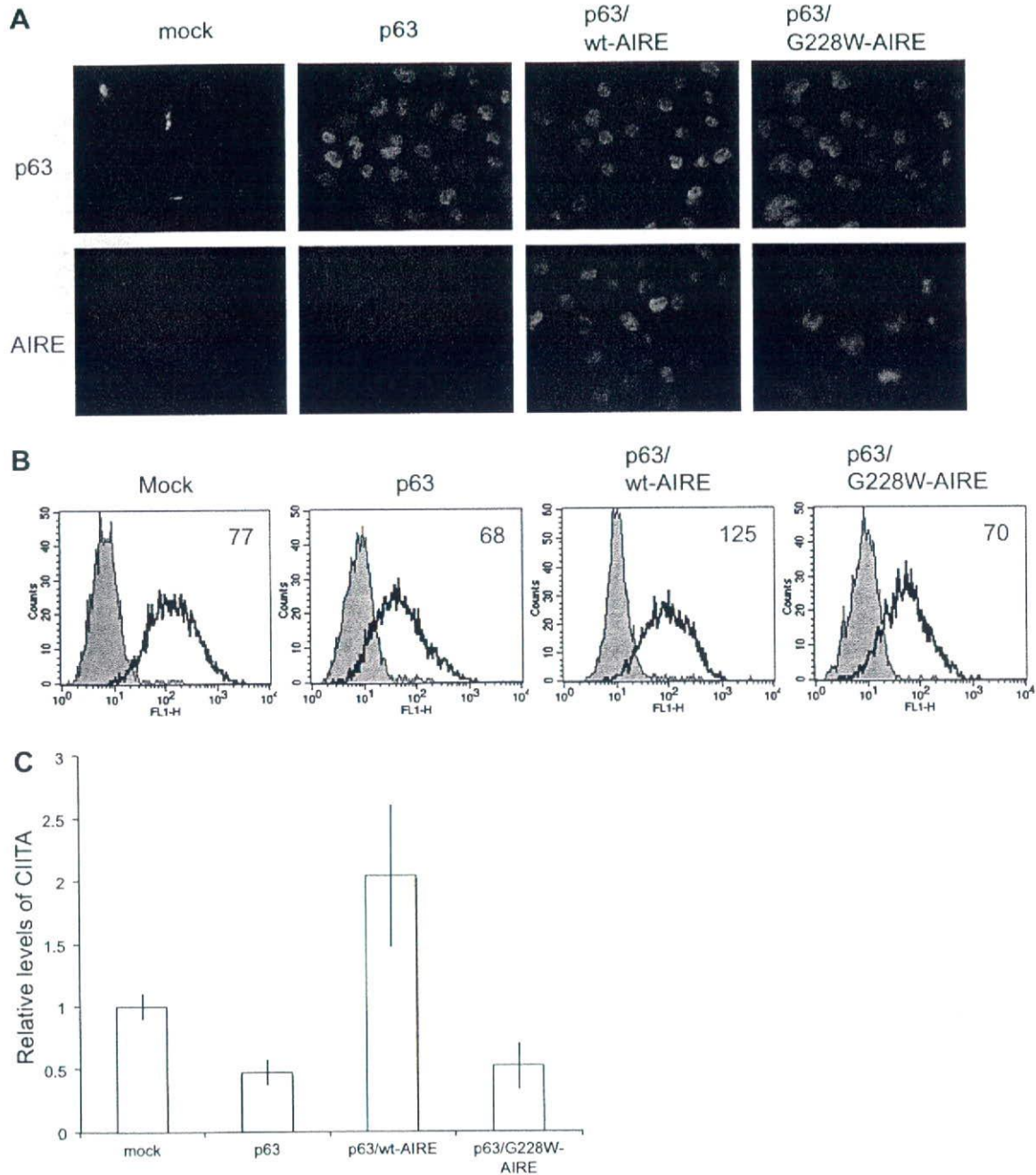
These results further supported our finding that the G228W residue might be unfavorable when the SAND domain of AIRE accesses the TA domain of p63.



### Involvement of AIRE/p63 complex in the regulation of HLA class II expression

To further address the question of functional consequences of the G228W mutation of AIRE, we established P1.4 cells stably expressing wt-AIRE or the G228W-AIRE mutant together with p63 as well as p63 alone (Fig. 4A). Especially focusing on CD4<sup>+</sup> T cell selection, flow cytometry analysis of the cell surface expression of HLA class II was conducted to these cells. Results revealed that the levels of HLA class II were slightly downregulated in P1.4 cells with p63 alone (mean fluorescence intensity, MFI = 68),

when compared to mock transfectants (MFI = 77) as shown in Fig. 4B. More interestingly the levels of HLA class II of P1.4 cells with p63 and wt-AIRE were relatively increased (MFI = 125), while the levels of HLA class II of P1.4 cells expressing p63 and G228W-AIRE (MFI = 70) were found to be similar to those of P1.4 cells of mock controls. These suggest that, in the presence of p63, wt-AIRE but not G228W-AIRE could have a capacity to maintain the levels of HLA class II. It is well investigated that the expression of HLA class II is chiefly regulated by a class II master regulator, CIITA, in antigen-presenting cells [20]. Quantitative PCR analysis of these P1.4 cells expressing p63 suggested that G228W-AIRE suppressed



**Fig. 4.** The roles of AIRE and p63 in the regulation of HLA class II expression. (A) Immunohistochemical analysis was performed using anti-p63 and anti-myc antibodies to confirm expression phenotypes of P1.4 stable transformants as indicated in mock, p63 (alone), p63/wild-type-AIRE and p63/G228W-AIRE. (B) FACS analysis of cell surface expression of HLA class II on P1.4 transformants. The expression of HLA class II is depicted by the solid line with isotype control as indicated by shaded areas. The mean fluorescence intensity (MFI) is also noted in the upper right corner of each figure. x-axis: signal intensity in arbitrary units, y-axis: number of cells. A representative data is shown from three independent experiments. (C) Quantitative PCR analysis of CIITA transcripts in P1.4 transformants. To evaluate the levels of CIITA in cells, the  $\Delta\Delta\text{CT}$  method was employed to analyze triplicate specimens. Values are expressed at the mean  $\pm$  SD for each group.



the levels of transcripts of CIITA (Fig. 4C). When compared to the endogenous levels of CIITA transcripts of P1.4 cells, the levels were 2.0-fold in P1.4 cells expressing wt-AIRE and p63, whereas P1.4 cells expressing G228W-AIRE and p63 showed significantly decreased amounts of CIITA transcripts (0.5-fold).

## Discussion

Comparative studies of the molecular structure of the SAND domain with those of Sp100b and GMEB1/2 suggest that, in addition to the structural similarity, AIRE also shares their functions and that the SAND domain of AIRE might contribute to protein–protein interaction as well as DNA binding [21,22]. Based on this feature, the mutated SAND domain of G228W-AIRE would be predicted to disturb intermolecular interactions with other molecules. Furthermore, the G228W mutant has a putative dominant negative effect by interfering with the transactivation capacity of the AIRE protein oligomer [15,19]. Since a broad range of tissue-restricted antigens encompassing a large set of tissue-specific molecules in medullary thymic epithelial cells is presumably dependent on promiscuous gene expression by AIRE, the G228W mutant is expected to cause alteration of expression profiles of tissue-restricted antigens in these cells [23]. This might support the abnormal selection of immature T cells in patients with the G228W mutation. In addition to this putative mechanism, our hypothesis focusing on altered expression of HLA class II molecules of medullary thymic epithelial cells in concert with p63 would also account for the reason why the G228W mutation promotes the emergence of self-reactive T cells. Thus, it might be possible that, in addition to AIRE-related functions, the G228W mutant could affect AIRE-independent functions via HLA class II modulation. This may reflect the phenotype of patients with the G228W mutation, which is not typical for APECED.

The physiological relevance of p53-related factors of the human thymus has been suggested by previous reports. For example, mutations of the p63 gene cause ectrodactyly-ectodermal dysplasia cleft lip/palate (EEC) and EEC-related syndromes [24]. Some of those syndromes exhibit histological abnormalities of the thymus and prominent depletion of cells in T-zones of lymph nodes [25]. Since the nuclear factor or factors responsible for the function of medullary thymic epithelial cells have been incompletely studied, the discovery of AIRE as well as a p53 family member localized in these cells allows us to investigate the regulatory networks in them, which may contribute to thymic selection. The fact that AIRE and p63 might mutually regulate each other's functions would suggest a complex mechanism in the modulation of medullary thymic epithelial cell function and provides an opportunity to further understand how promiscuous gene transcription is achieved within the cells. From a broader point of view, p63 is thought to be a potential determinant of the stem cell properties of epithelia by regulating a wide range of target genes [26]. With regard to gene expression of medullary thymic epithelial cells, it poses the question of whether p63 is associated with the fundamental role of AIRE. In another study, we found that p63 might take part in the regulation of claudin-1, which widely join medullary cells, including epithelial cells and dendritic cells [14]. Besides cell surface expression of HLA class II, AIRE in conjunction with p63 might also have a role in the instruction of cell surface molecules required for establishing the medullary cell network.

In summary, we described a potential complex formed of AIRE and p63 and the regulatory role of the complex in CIITA expression of medullary thymic epithelial cells. It remains to be seen whether the putative role of AIRE/p63 in the regulation of HLA class II is relevant for explaining the molecular phenotypes of other mutations

of AIRE. However, AIRE function seems, at least in part, to be more understandable by adding p63 in the context of HLA class II expression.

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## Clonal diversity of cytotoxic T lymphocytes that recognize autologous oral squamous cell carcinoma

Jun-Ichi Kobayashi <sup>a,b</sup>, Yoshihiko Hirohashi <sup>a,\*</sup>, Toshihiko Torigoe <sup>a</sup>, Yoshitaka Michifuri <sup>a,b</sup>, Takashi Yamamoto <sup>a,b</sup>, Yasuaki Tamura <sup>a</sup>, Kenjiro Kamiguchi <sup>a</sup>, Akihiro Miyazaki <sup>b</sup>, Akira Yamaguchi <sup>b</sup>, Hiroyuki Hariu <sup>a</sup>, Hiroyoshi Hiratsuka <sup>b</sup>, Noriyuki Sato <sup>a</sup>

<sup>a</sup> Department of Pathology, Sapporo Medical University, School of Medicine, Sapporo, Japan

<sup>b</sup> Department of Oral Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan

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## ABSTRACT

Cytotoxic T lymphocytes (CTLs) play an essential role in immunologic responses for tumor rejection. In the past decade, various melanoma tumor-associated antigens (TAAs) have been identified, and several clinical trials of vaccination immunotherapy and adoptive immunotherapy using such antigens with or without adjuvants have had fascinating results. However, this has not been the case with oral squamous cell carcinoma (OSCC) because of the difficulty of establishing oral cancer cell lines and CTLs against autologous oral cancer cells. Therefore, few oral cancer antigens have been identified with such CTLs.

We herein present the successful establishment of an oral squamous cell carcinoma cell line, POT-1, and an HLA-A24-restricted CTL line (TcPOT-1) from a patient's autologous peripheral blood lymphocytes. TcPOT-1 recognized autologous POT-1 cells in an HLA-A24-restricted manner, and also allogeneic HLA-A24 (+) OSCC cell lines OSC-70 and HSC-2. We also succeeded in isolating two distinct CTL clones from TcPOT-1, HLA-A24-restricted CTL clone 4F11 and HLA-A33-restricted clone 4A11. Both of these clones recognized autologous POT-1 but not allogeneic OSCC cell lines. These data imply that the TcPOT-1 CTL line may include several CTL subpopulations with distinct antigen specificities, such as an HLA-A24-restricted POT-1-specific clone, HLA-A33-restricted POT-1-specific clone, and HLA-A24-restricted allogeneic OSCC-recognizing clone. Therefore, precise analysis of TcPOT-1-recognizing antigens may provide us with important information on as-yet-unknown tumor rejection antigens in OSCC.

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### 1. Introduction

Recently, huge numbers of tumor-associated antigens (TAAs) have been identified and characterized. For most of them, this has been done through autologous cytotoxic T lymphocyte (CTL) clones isolated from peripheral blood mononuclear cells (PBMCs) of melanoma patients [1,2]. Even now, however, little is known about TAAs recognized by autologous CTLs in oral squamous cell carcinoma (OSCC) because of the difficulties in the generation of autologous pairs of CTL clones and OSCC cell lines. Recent advances in reverse immunogenetic strategies have facilitated the identification of antigenic peptides, but such analysis harbors the risk that *in vitro* CTL reactions to allogeneic tumor cells do not always reflect *in vivo* immune reactions against autologous tumor cells. Hence, it is very important to analyze CTLs that recognize autologous OSCC cells for precise identification of tumor-rejection antigens. Thus far, great efforts have been made to establish such autologous pairs of

CTLs and tumor cell lines, but notable achievements still remain very rare for OSCC.

Previously, we reported the establishment of OSCC cell line OSC-20 and HLA-DR-restricted autologous CD4+ T cells [3,4]. In a subsequent report, we also showed that a human enolase- $\alpha$ -derived antigenic peptide could be presented by HLA-DR molecules and might be a target antigen of CD4 T cells [5]. This was the first report concerning the characterization of an OSCC antigenic peptide. However, because enolase- $\alpha$  is a ubiquitous self-antigen, further analysis is required to explain the tumor specificity of the CD4 T cells. Taking into consideration that CD8 T cells are an essential component for tumor rejection, identification of HLA class I-restricted antigenic peptides has great importance in the development of cancer immunotherapy. In this context, we have attempted to establish more pairs of OSCC cell lines and CTLs. Herein, we report the establishment of a novel autologous pair of OSCC cell lines, POT-1 and CTL line TcPOT-1, which recognized POT-1 cells in an HLA-A24-restricted manner. We isolated two CTL clones, 4F11 and 4A11, from the TcPOT-1 CTL line. Clone 4F11 recognized POT-1 cells in an HLA-A24-restricted manner, whereas clone 4A11 recog-

\* Corresponding author.

E-mail address: [hirohash@sapmed.ac.jp](mailto:hirohash@sapmed.ac.jp) (Y. Hirohashi).



nized POT-1 cells in an HLA-A33–restricted manner. Although parental TcPOT-1 recognized allogeneic HLA-A24 (+) OSCC cell lines as well as POT-1, CTL clones 4F11 and 4A11 recognized only autologous POT-1 cells. These data indicate that the TcPOT-1 CTL line is composed of several CTL clones with distinct antigen specificities, which may recognize shared OSCC antigens and POT-1–specific antigens.

## 2. Subjects and methods

### 2.1. Cell lines

The oral squamous cell carcinoma cell line POT-1 was established from a surgically resected specimen of a 60-years-old female patient with moderately differentiated oral squamous cell carcinoma, and was cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Palo Alto, CA). The HLA genotype of POT-1 was HLA-A\*2402/3303, B\*4403/5201, Cw\*1202/1403, DRB1\*1302/1502, DQA1\*0102/0103, DQB1\*0601/0604, DPB1\*0401/0901. An Epstein-Barr (EB) virus-transformed B-cell line (POT-1 EBV) was established from autologous PBMCs using B95-8 culture supernatant. OSC-19 (HLA-A2), OSC-20 (HLA-A2/A11, B\*4601, Cw\*0102), and OSC-70 (HLA-A24) oral squamous cell carcinoma cell lines were also established in our laboratory. Erythroleukemia cell line K562 was purchased from ATCC. HSC-2 (HLA-A\*2402/3302, B\*44031/5401, Cw\*0102/1403), and HSC-3 (HLA-A\*0201/2402, B\*4801/5201, Cw\*0803/1202) oral cell carcinoma cell lines were purchased from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). All of these cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS. Autologous PBMCs from the patient were density-gradient centrifuged with Lymphoprep (Axis Shied, Oslo, Norway) and frozen at  $-80^{\circ}\text{C}$  for storage.

### 2.2. FACSscan analysis and monoclonal antibodies

Cells were incubated with murine monoclonal antibodies (mAbs) at saturating concentration for 30 minutes on ice, washed with PBS, and stained with a polyclonal goat anti-mouse antibody coupled with FITC for 30 minutes. Samples were analyzed using a FACSscan analyzer (Becton Dickinson, Mountain View, CA). Anti-HLA class I (W6/32), anti-HLA-DR (L243), and anti-HLA-A24 (C7709A2.6) mAbs, as well as anti-HLA-B,C (B1.23.2), were prepared from hybridomas. An anti-A33 monoclonal antibody (Cat. no. 0612HA) was purchased from One Lambda (Canoga Park, CA). Anti-A24 and anti-HLA-B,C hybridomas were kind gifts from Dr. P. G. Coulie (Ludwig Institute for Cancer Research, Brussels, Belgium). To increase the protein expression of HLA molecules, cell lines were treated with 100 U/ml interferon (IFN)- $\gamma$  for 48–72 hours.

### 2.3. CTL induction and establishment of CTL clones

Mixed lymphocyte tumor cell culture was performed as described previously [6]. Briefly,  $\text{CD8}^{+}$  T cells were positively separated from an OSCC patient's PBMCs using immunomagnetic beads coupled with an anti-CD8 mAb (Miltenyi Biotech GmbH, Germany) following the manufacturer's protocol. Then  $2 \times 10^6$   $\text{CD8}^{+}$  T cells were cocultured in a 24-well plate with  $2 \times 10^5$  irradiated POT-1 cells as antigen-presenting cells (APCs) in 2 ml of AIM-V medium supplemented with 10% human serum. Three days later, human recombinant interleukin-2 (IL-2; a kind gift from Takeda Pharmaceutical Co., Osaka, Japan) was added at a final concentration 20 IU/ml. The same mixed lymphocyte tumor cell culture procedure was performed every 7 days. After stimulation four times, cytotoxic activity and IFN- $\gamma$  secretion were measured with the standard  $^{51}\text{Cr}$  release assay and enzyme-linked immunosorbent assay (ELISA), respectively.

To obtain the CTL clones, standard limiting dilution was performed as described previously [7]. Briefly, series of diluted CTLs

were incubated with  $1 \times 10^4$  100 Gy-irradiated LG2-EBV (a kind gift from Dr. B. J. Eynde, Ludwig Institute for Cancer Research, Brussels, Belgium) and  $5 \times 10^4$  allogeneic PBMCs as feeder cells in 200  $\mu\text{l}$  of AIM-V medium supplemented with 10% HS, the anti-CD3 Ab (40 ng/ml) (BD), and 100U/ml rIL-2 in 96-well round plates. On day 7, the culture medium was half exchanged for AIM-V supplemented with 10% HS and 100 U/ml rIL-2. On day 14, growing wells were transferred to 24-well culture plates and the specific cytotoxic activity was examined by  $^{51}\text{Cr}$  release assay.

### 2.4. Cytotoxicity assay and ELISA

The lytic activity of CTLs was tested by  $^{51}\text{Cr}$  release assay as described previously [6]. Briefly, 2000 target cells labeled with 100  $\mu\text{Ci}$  of chromium were incubated in 96-well microtiter plates with CTLs at different E/T ratios in 200  $\mu\text{l}$  of AIM-V medium.  $^{51}\text{Cr}$  release was measured after 6 hours' incubation in a  $37^{\circ}$  incubator. For the inhibition assay with monoclonal antibodies (mAbs),  $^{51}\text{Cr}$ -labeled target cells were incubated with appropriate concentrations of mAbs at room temperature for 60 min, before  $^{51}\text{Cr}$  release assay. Percent cytotoxicity was calculated as: % Cytotoxicity = (experimental release – spontaneous release)/(maximum release – spontaneous release)  $\times$  100. All target cells were treated with 100 U/ml IFN- $\gamma$  for 48–72 hours before assay.

For the detection of IFN- $\gamma$  in the culture supernatant, standard IFN- $\gamma$  ELISA (R&D Systems, Minneapolis, MN) was performed following the manufacturer's instructions.

For the specific inhibition with antibodies, an anti-HLA-class I mAb (W6/32), anti-HLA-B,C mAb (B1.23.2), anti-HLA-A24 mAb (C7709A2.6), and anti-HLA-DR mAb (L243) were added at half the volume of the hybridoma culture supernatant.

### 2.5. Immunoselection

For the immune selection of cancer cells,  $1 \times 10^6$  POT-1 cells were cocultured with  $1 \times 10^8$  TcPOT-1 clones for 6 hours, and then the CTLs were removed. The same coculture procedure was performed weekly. After immunoselection four times, POT-1 variant cells were established.

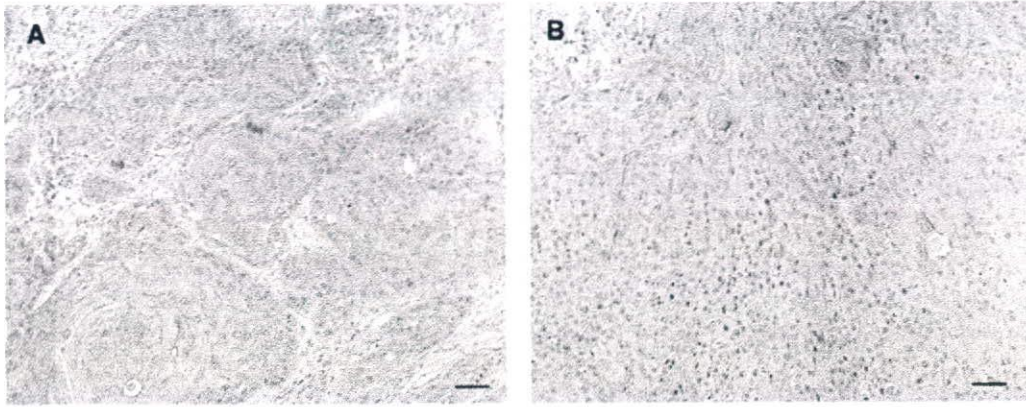
## 3. Results

### 3.1. Establishment of the oral squamous cell carcinoma cell line POT-1

For successful identification of TAAs, it is essential to analyze an autologous tumor cell line and CTL pair. Therefore, we attempted to establish an oral squamous cell carcinoma cell line. A surgically resected specimen of oral squamous cell carcinoma from a 60-year-old female patient was cultured in RPMI-1640 supplemented with 10% FBS as described in Subjects and methods, and we successfully established oral squamous cell carcinoma cell line POT-1. The primary lesion of the carcinoma specimen was well-differentiated squamous cell carcinoma (Fig. 1A). This cell line had a standard doubling time of 48–72 hours, and grew stably through more than 50 passages. Moreover, this cell line exhibited tumor-forming ability in immune-compromised host mice, and the tumors were typical squamous cell carcinoma resembling the primary lesion (Fig. 1B).

For immunologic evaluation of the POT-1 cell line, the surface MHC molecules were then examined by FACSscan analysis. As shown in Fig. 2, POT-1 expressed a pan-HLA class I molecule (W6/32), HLA-B,C (B1.23.2), HLA-A24 (C7709A2.6), HLA-A33, and a relatively low level of HLA-DR (L243). The expression levels of HLA-DR molecules were enhanced by IFN- $\gamma$  treatment for 48 hours, but those of HLA-class I molecules were not. These HLA expression profiles might indicate that immune cells can interact with POT-1 through HLA-class I molecules.





**Fig. 1.** Pathologic findings of the primary oral squamous cell carcinoma lesion of the POT-1 patient (A), and the tumor tissue in immune-deficient mice (B). Hematoxylin and eosin stain; magnification  $\times 100$  (scale bar, 100  $\mu\text{m}$ ).

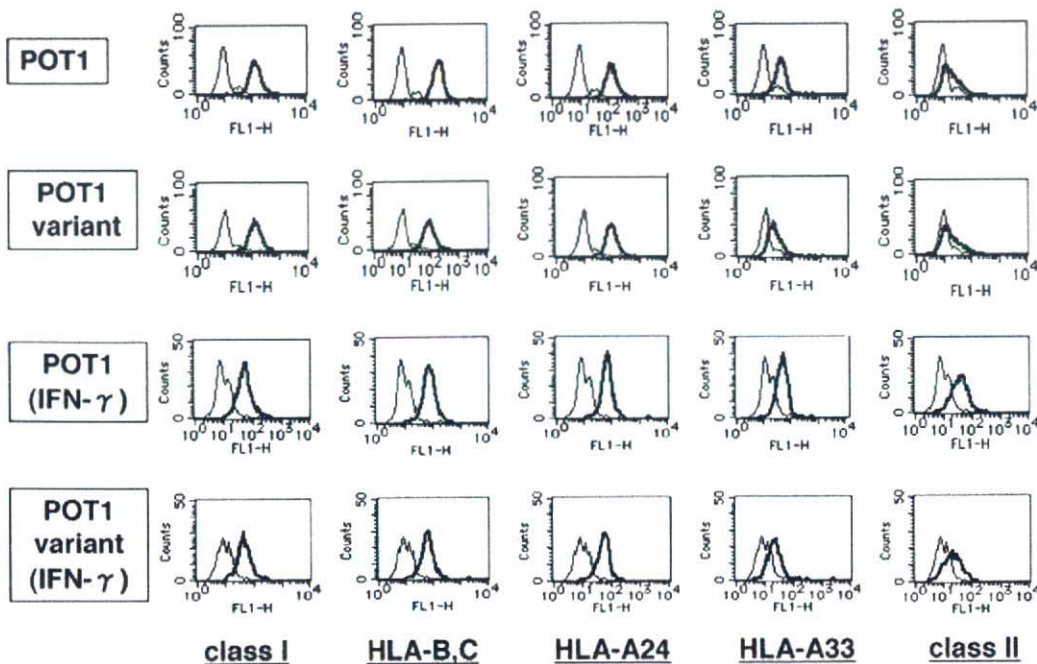
**3.2. Induction and analysis of CTLs**

To establish CTLs that recognized the POT-1 cell line, we stimulated PBMCs with autologous POT-1 several times. After stimulation four times, the specific reactivity was evaluated by  $^{51}\text{Cr}$  release assay (Fig. 3A). The CTLs (TcPOT-1) recognized autologous POT-1 specifically, but not the autologous EBV-virus-transformed B cells (POT-1 EBV) or erythroleukemia cell line K562 used as negative controls. For further analysis of TcPOT-1, cytotoxic activity was measured against the allogeneic OSCC cell line by  $^{51}\text{Cr}$  release assay (Fig. 3A). TcPOT-1 line recognized allogeneic OSCC cell line HSC-2 (HLA-A\*2402/3302, B\*44031/5401, Cw\*0102/1403) cell line, but did not OSC-19 and OSC-70 cell lines. To confirm the HLA restriction of TcPOT-1, blocking assays with HLA-specific antibodies were performed. As shown in Fig. 3B, the cytotoxic activity was blocked by an anti-HLA-class I antibody (W6/32). The cytotoxic reactivity was also likely to be inhibited with anti-HLA-A24 antibody (C7709A2.6) but not inhibited by an anti-HLA-B,C antibody (B1.23.2) or anti-HLA-DR antibody (L243). Inasmuch as we could observe independent experiments, some populations of TcPOT-1 line might be

restricted HLA-A24 molecule, and this suggests the possibility that TcPOT-1 recognized HSC-2 on the context of HLA-A24 molecule.

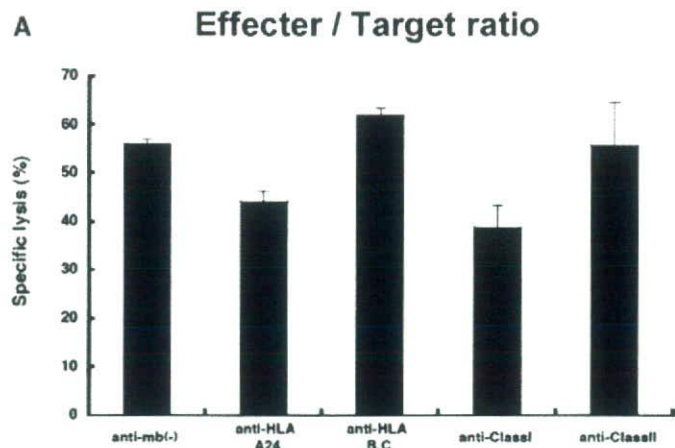
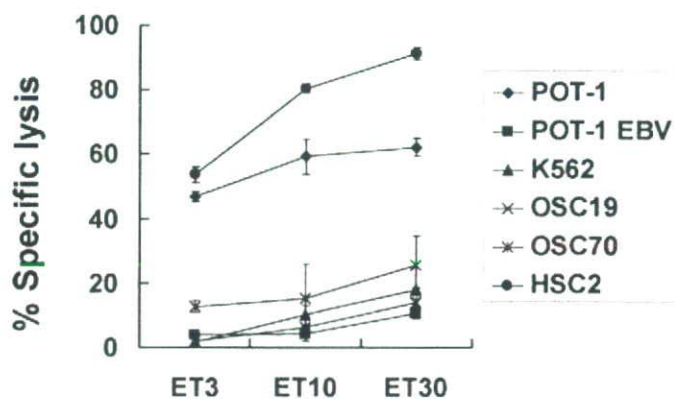
**3.3. Establishment of TcPOT-1 clones**

For detailed analysis of TcPOT-1, CTL TcPOT-1 clone lines were generated by the standard limiting dilution method as described in Subjects and methods. We successfully established two CTL clones, designated 4F11 and 4A11. As shown in Fig. 4A, clones 4F11 and 4A11 both recognized the autologous POT-1 cell line specifically compared with POT-1 EBV cells used as a negative control. Next, blocking assays using mAbs were performed to identify the HLA-restriction elements. As shown in Fig. 4B, the IFN- $\gamma$ -secreting ability of clone 4F11 was blocked by anti-HLA-class I and anti-HLA-A24 antibodies, indicating that 4F11 was an HLA-A24-restricted CTL. On the other hand, the IFN- $\gamma$  secretion of clone 4A11 was inhibited by an anti-HLA-class I antibody, but not other antibodies, including anti-HLA-A24 and HLA-B,C (Fig. 4C). This highly suggested that clone 4A11 is restricted to another allele of the HLA-A locus, HLA-A33. To analyze clone 4F11 and 4A11 in detail, the



**Fig. 2.** FACSscan analysis of the surface HLA molecules. Several cell lines were evaluated with FACSscan analysis. Anti-pan-HLA-class I (W6/32), anti-HLA-B,C (B1.23.2), anti-HLA-A24 (C7709A2.6), anti-HLA-A33 (0612HA) and anti-HLA-DR (L243) monoclonal antibodies were used for specific detection.





**Fig. 3.** Cytotoxic activity of TcPOT-1 CTL line. (A) TcPOT-1 CTL cells were examined for cytotoxic activity against autologous oral squamous cell carcinoma cell line POT-1 by <sup>51</sup>Cr release assay as described in Subjects and methods. POT-1 EBV is the autologous EBV-B cell line. (B) Cytotoxic activity against allogeneic oral squamous cell carcinoma was examined. Allogeneic oral squamous cell lines OSC-19 (HLA-A2), OSC-70 (HLA-A24) and HSC-2 (HLA-A\*2402/3302, B\*44031/5401, Cw\*0102/1403) were used as target cells. (C) Blocking assays with HLA-specific mAbs was performed as described in Subjects and methods. Anti-pan-HLA-class I (W6/32), anti-HLA-B,C (B1.23.2), anti-HLA-A24 (C7709A2.6), and anti-HLA-DR (L243) monoclonal antibodies were used. Similar results could be obtained with independent experiments three times.

reactivity against allogeneic OSCC cell lines was examined (Fig. 4B and 4C). Clones 4F11 and 4A11 recognized the autologous POT-1 cell line but not allogeneic OSCC cell lines including OSC-19, OSC-20, HSC-2, and HSC-3. These data indicated that 4F11 and 4A11 recognized the POT-1 specific antigen in an HLA-A24- and HLA-A33-restricted manner, respectively.

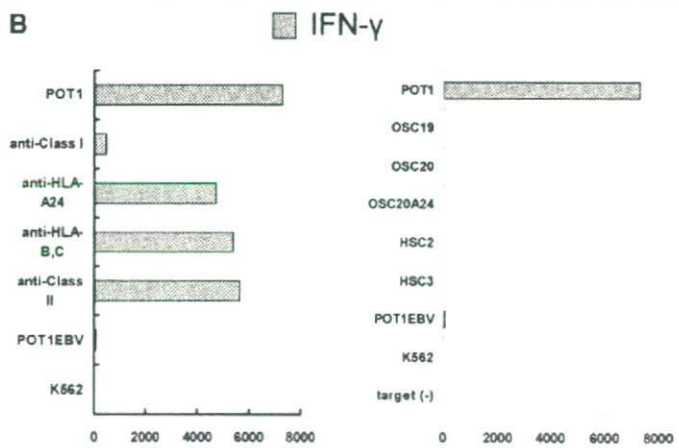
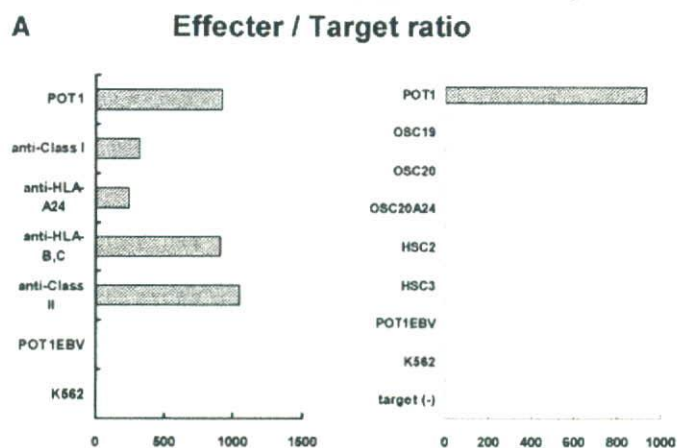
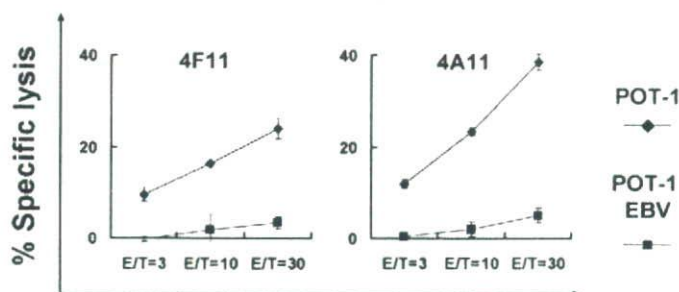
**3.4. Immune selection of POT-1 and establishment of immune-tolerant subline POT-1 variant**

For further analysis of TAAs of POT-1, we established a POT-1 subline that was immunoselected by coculture with both CTL clones 4F11 and 4A11. The established subline was termed as a POT-1 variant. As shown in Fig. 5, CTL clones 4F11 and 4A11 recognized POT-1, but not POT-1 variant or the negative controls POT-1 EBV and K562. However, the TcPOT-1 line did recognize POT-1 variant as well as POT-1, suggesting TcPOT-1 line mainly recognized different antigens as CTL clones 4F11 and 4A11. For immunologic evaluation of POT-1 variant, cell surface HLA expression was also examined by FACScan analysis (Fig. 2). The expression levels of HLA-class I, HLA-B,C, and HLA-A24 showed no significant difference compared with POT-1. The expression of HLA-A33 was relatively decreased compared with POT-1, but the expression of HLA-A33 was restored with IFN- $\gamma$  treatment. Thus, the expression

levels of HLA molecules might not be critical for the immunoevasion of POT-1 variant cells, and POT-1 variant might lose some antigen gene expression or antigen-presenting machinery. Therefore, OSCC cell line POT-1 seems to express at least three different TAAs, and TcPOT-1, 4F11, and 4A11 recognize independent antigens.

**4. Discussion**

Oral squamous cell carcinoma is a major malignancy all over the world, with an annual mortality rate 5–10 per 100,000 in industri-



**Fig. 4.** Establishment and characterization of CTL clones 4-F-11 and 4-A-11. (A) Cytotoxic activities of CTL clones 4-F-11 and 4-A-11 for autologous OSCC cell line POT-1 and autologous EBV-B cell line POT-1 EBV were examined by <sup>51</sup>Cr release assay. (B) Reactivities of CTL clone 4F11. Blocking assays with mAbs were performed with CTL clones using IFN- $\gamma$  ELISA. Anti-pan-HLA-class I (W6/32), anti-HLA-B,C (B1.23.2), anti-HLA-A24 (C7709A2.6), and anti-HLA-DR (L243) monoclonal antibodies were used. 4F11 reactivity for allogeneic OSCC cell lines were evaluated. Allogeneic oral squamous cell lines OSC-19 (HLA-A2), OSC-20 (HLA-A2/A11, B\*4601, Cw\*0102), OSC-20-A24 HLA-A24 transfected OSC-20 cell line, OSC-70 (HLA-A24) and HSC-2 (HLA-A\*2402/3302, B\*44031/5401, Cw\*0102/1403) were used as target cells. (C) Reactivities of CTL clone 4A11.



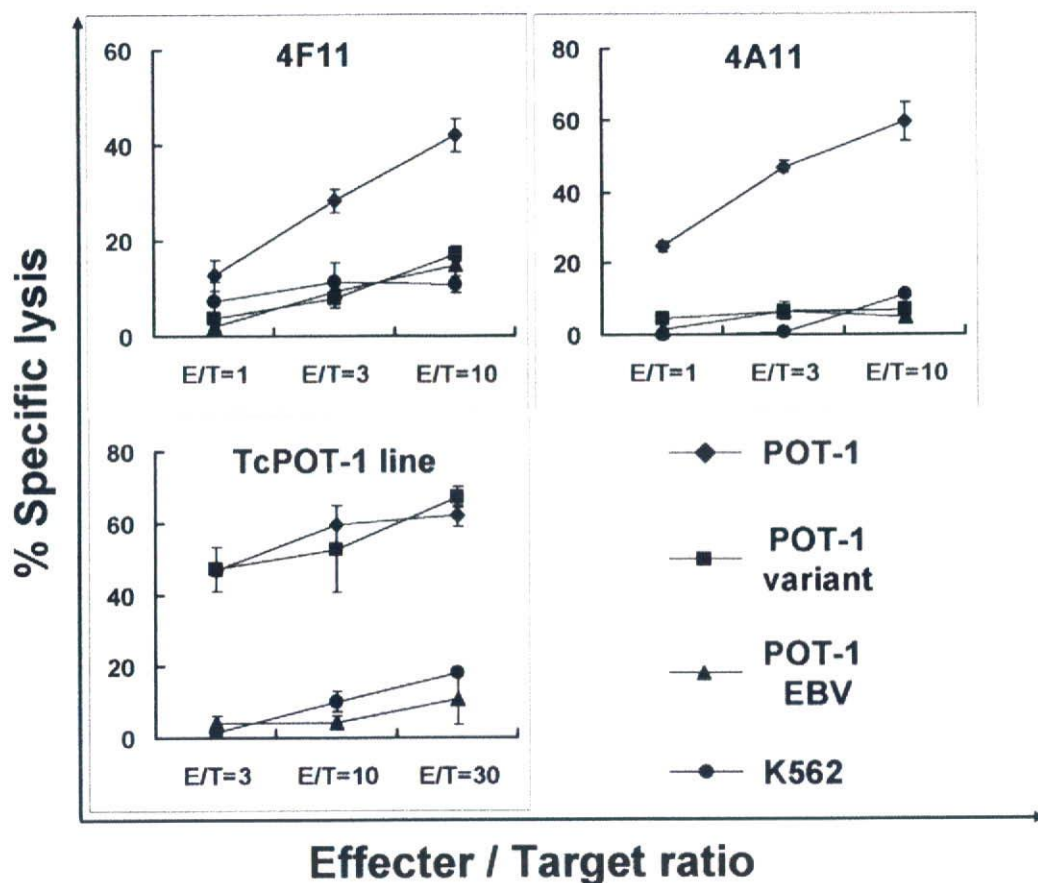


Fig. 5. Immunologic escape of POT-1 variant. Cytotoxic activity of CTL clones 4F11 and 4A11 and TcPOT-1 CTL line were assessed the reactivity for POT-1 and POT-1 variant cell line.

alized countries. Surgical resection, chemotherapy and radiation therapy are the major treatments for OSCC. With recent progress, the outcome of the disease has greatly improved. However, even now, the prognosis for advanced cases (*i.e.* with neck lymph node metastasis or distant metastasis), is still not favorable. Therefore, another breakthrough in OSCC therapy is desired.

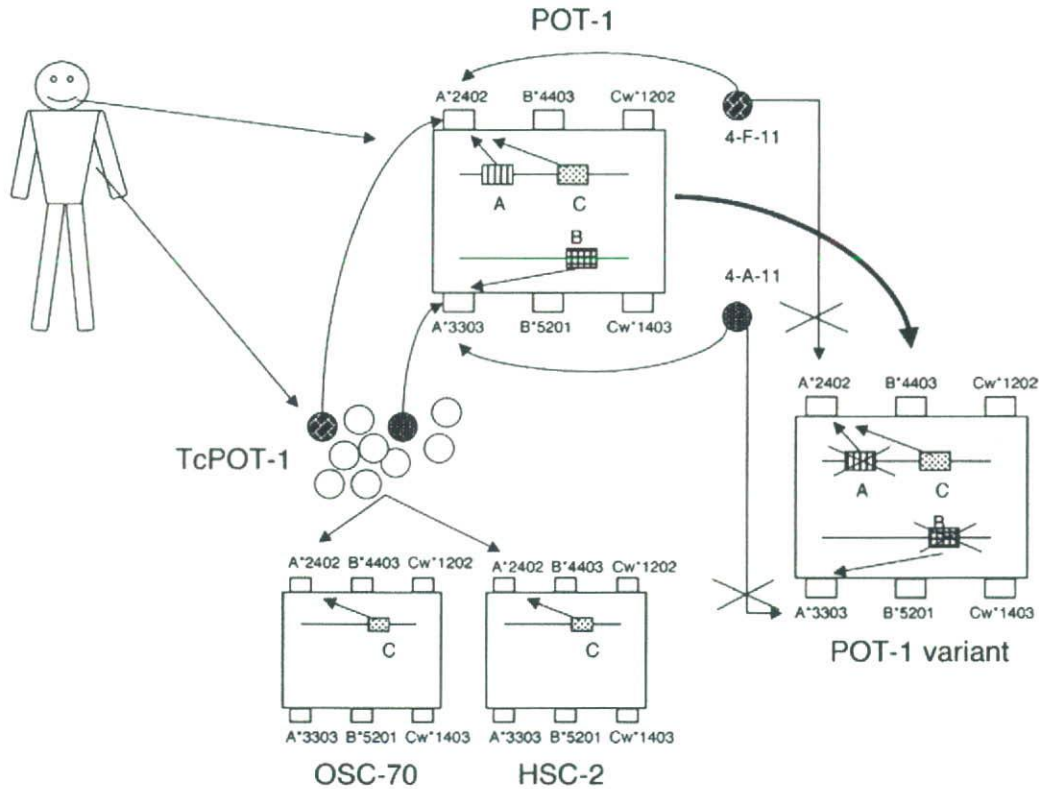
With recent innovative advances in cancer immunity initiated from the identification of the MAGE gene family and other melanoma antigens [8,9], immune therapy can be one of the representative therapies for malignancies. Using such melanoma antigens, several groups reported the efficacy of cancer immunotherapy for advanced melanoma patients [10,11]. There are three major approaches to identifying TAAs. These reports prompted the further analysis of other epithelial malignancies. Previously, we reported autologous cancer cell line and CTL pairs from several epithelial malignancies [3,12–15]. By analysis of those CTLs, we succeeded in isolating novel cancer-associated antigens [5,16], as well as antigenic peptides by reverse immunogenetic analysis [7,17–19]. With this progress, we started a clinical trial for cancer vaccine therapy with an antigenic peptide derived from one of the cancer-associated antigens, survivin [20]. Some patients showed decreases in tumor markers and remarkable tumor regression. These findings suggested that cancer vaccine therapy might also be effective for OSCC patients.

In this study, we successfully established OSCC cell line POT-1 and autologous CTL line Tc-POT-1. TcPOT-1 did recognized allogeneic OSCC cell line HSC-2. This suggests TcPOT-1 line recognized POT-1 and HSC-2 shared TAA (antigen C in Fig. 6). Inasmuch as POT-1 and HSC-2 shared several HLA-class I molecules, we cannot definitely conclude which HLA is the restriction element of TcPOT-1. However, the cytotoxic activity of TcPOT-1 tended to be inhibited with anti-HLA-A24

mAb, HLA-A24 might be one candidate. Because antigen C was shared with allogeneic cell line, this is presumed to be overexpressed antigen. As previously reported, cancer-testis antigens and Wilms' tumor 1 (WT1) antigens express in OSCC [21,22]. There is possibility that antigen C is one of the cancer-testis antigen or WT1. Both 4F11 and 4A11 recognized only autologous cell line, highly suggesting these CTL clones recognized POT-1-specific antigens (antigens A and B in Fig. 6). Autologous cell-specific antigens are usually coded by mutated genes [23]. Thus, antigens A and B seem to be coded with some mutated genes. However, at this moment, we cannot conclude that antigen A and antigen B are coded in same genes or different genes. The gene cloning will resolve all of these questions.

Some analyses of OSCC cancer immunity have been reported, including *in vivo* analysis of immunologic parameters. Cruz *et al.* showed that  $\beta_2$  microglobulin expression in cancer tissues was down-regulated in 17 of 47 OSCC cases [24]. The decreased level of  $\beta_2$  microglobulin correlated well with decreased expression of HLA class I and decreased infiltration of immune cells such as CD8<sup>+</sup> T cells. Matsui *et al.* reported that antigen-presenting machinery such as TAP1/2, LMP7/tapasin, and PA28  $\alpha/\beta$  were downregulated in OSCC cell lines [25]. We also examined the HLA-class I expression level of OSCC in surgically resected specimens using the novel anti-HLA class I antibody EMR8-5. A decrease in HLA-class I was observed in 22 of 81 cases at several levels (data not shown). These findings indicated that the immunologic surveillance *in vivo* was potent enough to reject cancer cells. Therefore, vaccination therapy with antigenic peptides of OSCC patients might have enough potential to remove cancer cells. Recently we reported that HLA-class I downregulation was one of the factors for poor prognoses in bladder cancer and osteosarcoma [26,27]. These findings also sup-





**Fig. 6.** POT-1 was established from a 60-year-old female OSCC patient. TcPOT-1 contains several clone populations of CTLs specific for more than 3 TAAs. One was the highly likely HLA-A24-restricted clone specific for OSCC shared TAA (C). The others were an HLA-A24-restricted clone specific for POT-1-specific antigen (A) and an HLA-A33-restricted clone specific for POT-1-specific antigen (B).

port the idea that immunoreaction against cancer cells has sufficient competency for cancer rejection.

Recently, Morgan *et al.* reported remarkable results for adoptive cancer immune therapy [28]. After transduction of anti-MART-1, gp100, NY-ESO-1, and p53 TCRs into autologous T lymphocytes and expansion of CTLs *in vitro*, more than 1 billion CTLs were transferred into advanced melanoma patients with 720,000 IU/kg IL-2 every 8 hours. One month later, two of 17 patients showed partial responses (PR) of solid tumors, and the tumor regression lasted more than 10 months. This report showed the CTLs were powerful for tumor regression in advanced melanoma patients. This was also the case with the POT-1 patient. The CTL cytotoxicity of TcPOT-1 was ~60% at an E/T ratio of 30 and was quite high compared with the autologous CTLs we previously reported. After surgical resection of the oral squamous cell carcinoma, the patient is still disease free and has enjoyed good quality of life for more than 4 years. One of the reasons for the good prognosis was the high CTL reactivity. Furthermore, the CTL precursor frequency seemed to be quite high because we succeeded in inducing CTLs from the POT-1 patient's PBMCs three times over 3 years. These findings also support the idea that CTL is quite potent and plays an essential role in cancer immunosurveillance.

As shown in Fig. 6, the TcPOT-1 CTL line and CTL clones 4F11 and 4A11 recognized more than 3 TAAs. Because HLA-A24 and A33 loci are common throughout the world, further analysis of TcPOT-1 and clones 4-F-11 and 4-A-11 should be informative for the analysis of OSCC cancer immunity.

#### Acknowledgments

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