Institute of Bioregulation, Sapporo Medical University, Japan. All patients gave their written informed consent before entry into the study. Patients enrolled in this study were required to conform to the following criteria: (i) to have histologically proven oral cancer; (ii) to be HLA-A*2402 positive; (iii) to have survivin-positive cancerous lesions by immunohistochemistry; (iv) to have HLA class I-positive cancerous lesions by immunohistochemistry using the anti-pan HLA class I mAb EMR8-5; (v) to be 20–85 years old; (vi) to have an unresectable, locally advanced or recurrent tumor; and (vii) to have an Eastern Cooperative Oncology Group (ECOG) performance status of between 0 and 3. The exclusion criteria included: (i) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy or other immunotherapy within the previous 4 weeks; (ii) the presence of other cancers that might influence the prognosis; (iii) immunodeficiency or a history of splenectomy; (iv) severe cardiac insufficiency, acute infection or hematopoietic failure; (v) pregnancy or breast-feeding; and (vi) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Oral Surgery, Sapporo Medical University Primary Hospital from September 2003.

Peptide preparation. The survivin-2B80-88 peptide (amino acid sequence AYACNTSTL), which was derived from a splicing variant survivin-2B-specific exon 2B, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA). The identity of the peptide was confirmed by mass spectral 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.

Treatment protocol. Vaccinations with survivin-2B peptide were administered subcutaneously (s.c.) into the ipsilateral neck or intratumorally six times at 14-day intervals. Two incremental dose levels were planned for the peptide administration, with a starting dose of 0.1 mg. Six patients received 0.1 mg (group 1) and four patients received 1.0 mg (group 2), while each group was divided into the two different administration sites as stated above. Before proceeding to the next dose level, all previously administered patients had to have completed the trial period. Dose escalation for group 2 was allowed if no patients in group 1 experienced grade 3–4 toxicity.

If patients hoped for continuation of this peptide vaccine therapy, we conducted it in the same manner after the sixth administration.

Delayed-type hypersensitivity (DTH) skin test. The 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 area of erythema and induration with a diameter of more than 4 mm, 48 h after the injection.

Evaluation of toxicity and response. Patients were examined closely for signs of toxicity during and after the vaccination. The US National Cancer Institute Common Toxicity Criteria (NCI-CTC Version 2.0, Jan.30, 1998) were used to classify the toxicity grades.

Physical examinations and hematological examinations were conducted before and after each vaccination. The serum level of squamous cell carcinoma (SCC) antigen, which is the current standard tumor marker for head and neck cancer, was examined at 14-day intervals. A SCC antigen level of 1.5 ng/mL was generally taken as the upper limit of the normal range. The tumor size was evaluated by visual inspection, computed tomography (CT) and magnetic resonance imaging (MRI) before treatment, after three vaccinations and at the end of the study period. The tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines:[21] a complete response (CR) was defined as the disappearance of all target lesions; and a partial response (PR) was defined as at least a 30% decrease in the sum of the longest diameters of the target lesions for at least 4 weeks without the appearance of new lesions. Progressive disease (PD) was defined as at least a 20% increase in the sum of the longest diameters of the target lesions or the appearance of one or more new lesions. Stable disease (SD) was defined as neither sufficient shrinkage to qualify for a PR nor a sufficient increase to qualify for PD.

***In vitro* stimulation of PBMC.** The PBMC were isolated from blood samples by Ficoll–Conray density gradient centrifugation and then frozen and stored at −80°C. As needed, frozen PBMC were thawed and incubated in the presence of 30 μL/mL survivin-2B peptide in AIM-V medium containing 10% human serum at room temperature. Interleukin-2 (IL-2) was added at a final concentration of 50 U/mL for 1 h on days 0, 2, 4 and 6 of culture. On day 7, the PBMC were analyzed by tetramer staining.

Tetramer staining. HLA-A24/peptide tetramers were constructed according to the procedure described by Altman *et al.*[22] Briefly, recombinant HLA-A24 heavy chain[23] and human β-2-microglobulin were refolded with the survivin-2B80-88 peptide as described previously.[24] The resulting HLA-A24-peptide monomer was biotinylated by incubation with the enzyme BirA (Avidity, Denver, CO, USA) for 17 h at room temperature and purified using fast protein liquid chromatography. A tetromeric HLA-peptide complex was produced by incubating streptavidin-PE (Vector Laboratories, Burlingame, CA, USA) with the biotinylated monomer at a 1:4 molar ratio. For flow cytometric analysis, the PBMC, which were stimulated *in vitro* as above, were stained with the phycoerythrin (PE)-labeled tetramer at 37°C for 20 min, followed by staining with an FITC-conjugated anti-CD8 mAb (Becton Dickinson Biosciences, San Jose, CA, USA) at 4°C for 30 min. The cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using a FACSCalibur and the CellQuest software program (Becton Dickinson Biosciences). The frequency of the CTL precursors was calculated as the number of tetramer-positive cells over the number of CD8-positive cells. Moreover, the PBMC were stained with an FITC-labeled HLA-A*2402-restricted human immunodeficiency virus (HIV) peptide (RYLRDQQLL) tetramer and PE-labeled HLA-A*2402-survivin-2B80-88 peptide tetramer, which were purchased from MBL Co., Ltd. (Nagoya, Japan), at 37°C for 20 min, followed by staining with an FITC- or PerCP-conjugated anti-CD8 mAb (Becton Dickinson Biosciences) at 4°C for 30 min. The frequency of the CTL precursors was calculated in the same manner.

Results

Patient characteristics. Eleven patients (six males, five females) were eligible and agreed to participate in this phase I study. The patients' characteristics are summarized in Table 1. The patients' median age at enrolment was 66.5 years, with a range 38–84 years. Based on the ECOG classification, five patients were PS1, five were PS2, and one was PS3. The patients' primary tumor sites were: buccal mucosa, three; palate, two; upper or lower alveolus and gingiva, two; mandible, one; floor of mouth, one; submandibular gland, one; and tongue, one. The histological type was SCC in seven patients, adenoid cystic carcinoma (ACC) in three and alveolar soft part sarcoma (ASPS) in one. Table 2 summarize the clinical and immunological outcomes for the 11 patients. One patient discontinued the regimen after four vaccinations. She (case 8) had a growing locoregional recurrence and her general condition deteriorated. Subsequently she was removed from the study after four vaccinations because she refused to continue the protocol. None of

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Table 1. Summary of the characteristics of patients enrolled in the present study

Patient no.	Histology	Age/Sex	PS	Primary tumor site	Recurrent or metastatic sites
1	ASPA	38/M	1	Mandible	Local, brain, lung
2	ACC	60/M	1	Hard palate	Local, lung
3	SCC	84/F	3	Floor of mouth	Locoregional
4	ACC	50/F	2	Submandibular gland	Lung
5	SCC	83/F	2	Upper alveolus and gingiva	Locoregional
6	SCC	72/M	2	Buccal mucosa	Local
7	SCC	55/M	2	Tongue	Locoregional
8	SCC	82/F	2	Lower alveolus and gingiva	Neck
9	SCC	73/M	1	Hard palate	Lung, liver
10	SCC	82/F	1	Buccal mucosa	Neck
11	SCC	68/M	1	Buccal mucosa	Locoregional

ACC, adenoid cystic carcinoma; ASPS, alveolar soft part sarcoma; SCC, squamous cell carcinoma.

Table 2. Profiles of the enrolled patients and clinical responses to the survivin-2B peptide vaccination

Patient no.	Dose of peptide (mg)	Injection route	HLA class I expression	Prior therapy (washout time)	Adverse events	Tetramer staining† (pre-/post-)	Tumor marker	Clinical response	Follow up (months)	Progress
1	0.1	Intratumoral	+	S + C (1 month)	–	ND	ND	PD	43	AWD
2		Intratumoral	+	S + C (1 month)	–	121/103	ND	PD	25	DOD
3		Intratumoral	+	C + R (1 month)	–	ND	ND	PD	3	DOD
4		s.c.	+	S + C + R (6 years, 4 months)	–	1/100	ND	PD	15	DOD
5		s.c.	+	C (1 month)	–	6/16	INC	PD	6	DOD
6		s.c.	+	S + R + C (1 months)	–	65/244	INC	PD	3	DOD
7	1.0	Intratumoral	+	S + R + C (1 month)	–	96/528	ND	PD	6	DOD
8‡		Intratumoral	+	S + R (1 month)	–	ND	ND	ND	2	DOD
9		s.c.	+	S + C (2 months)	–	77/204	DEC	PD	5	DOD
10		Intratumoral	+	S + C (1 month)	–	5/20	DEC	PR	5	DOD
11		s.c.	+	S + R + C (5 months)	–	5/1	ND	PD	8	DOD

†Tetramer staining: Tetramer(+)CD8(+) in 10 000 CD8(+) cells. ‡Patient refused to continue the protocol (case 8). AWD, alive with disease; C, chemotherapy; DEC, decreased; DOD, dead of disease; HLA, human leukocyte antigen; INC, increased; ND, not determined; PD, progressive disease; post-, after the fourth vaccination; PR, partial response; pre-, before the first vaccination; R, radiotherapy; S, surgery.

the treatment interruptions were due to any adverse reactions to the vaccination. Ten patients received the complete regimen including six vaccinations and thereafter were evaluated.

Safety. The peptide vaccination was well tolerated in all 10 patients. No hematological, cardiovascular, hepatic or renal toxicity was observed during or after vaccination. Skin reactions such as induration, pain or rash were not observed in any case.

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.

Clinical responses. In two patients (cases 9 and 10) the tumor marker level (SCC antigen) transiently decreased. In two patients (cases 5 and 6) it increased and in the remainder it was not useful for monitoring. A PR was observed in one patient (case 10), who also demonstrated a remarkable decrease in the SCC antigen level (6.0 ng/mL → 0.7 ng/mL). The remaining nine patients experienced PD.

Case 9, who had multiple lung metastases, transiently showed a positive level of SCC antigen of 2.1 ng/mL that decreased after the second vaccination and was within the normal range just after the third vaccination. However, after the fourth vaccination it increased abruptly, which closely corresponded to his clinical progress. Until the fourth vaccination, CT imaging of the lung revealed virtually dormant disease, however, it revealed progressive disease after the sixth vaccination.

One responder (case 10) with PR developed multiple neck metastases and skin metastases in the left side of her neck at 3 months after surgery followed by treatment with tegafur/uracil (UFT) at a daily dose of 400 mg as oral adjuvant chemotherapy. She was judged to be impossible to treat radically because CT imaging showed that the recurrent tumor had metastasized to lymph nodes and the skin, including the parotid gland, submandibular region, posterior cervical region, occipital region of the head, posterior skull base and lower cervical region (Fig. 1A). The metastatic progressive tumor samples from her neck obtained by neck dissection previously were confirmed by immunohistochemical staining to markedly express survivin and HLA class I molecules. Survivin-2B peptide vaccine was administered intratumorally to the left side of her neck nine times at biweekly intervals. The SCC antigen level was 6.0 ng/mL before vaccination. Her skin metastatic tumor and pain disappeared transiently after the fifth vaccination, thus resulting in an improvement in her quality of life. A tumor regression rate of 70% was observed by CT imaging (Fig. 1B). The SCC antigen level decreased to 0.7 ng/mL after the sixth vaccination (Fig. 2). Nevertheless, these effects were maintained for 2 months only.

Tetramer staining assay. Peptide-specific immunological responses were evaluated in eight patients by HLA-A24/survivin-2B80-88 peptide tetramer analysis. The change of the tetramer-positive CTL frequency was evaluated by comparison with that before the first vaccination and that after each vaccination. The frequency of tetramer-positive CTL tended to increase after the vaccination in six patients (cases 4, 5, 6, 7, 9 and 10) (Table 2). In Figure 3, the peptide-specific CTL frequencies in cases 9 and 10 are indicated as the percentages of

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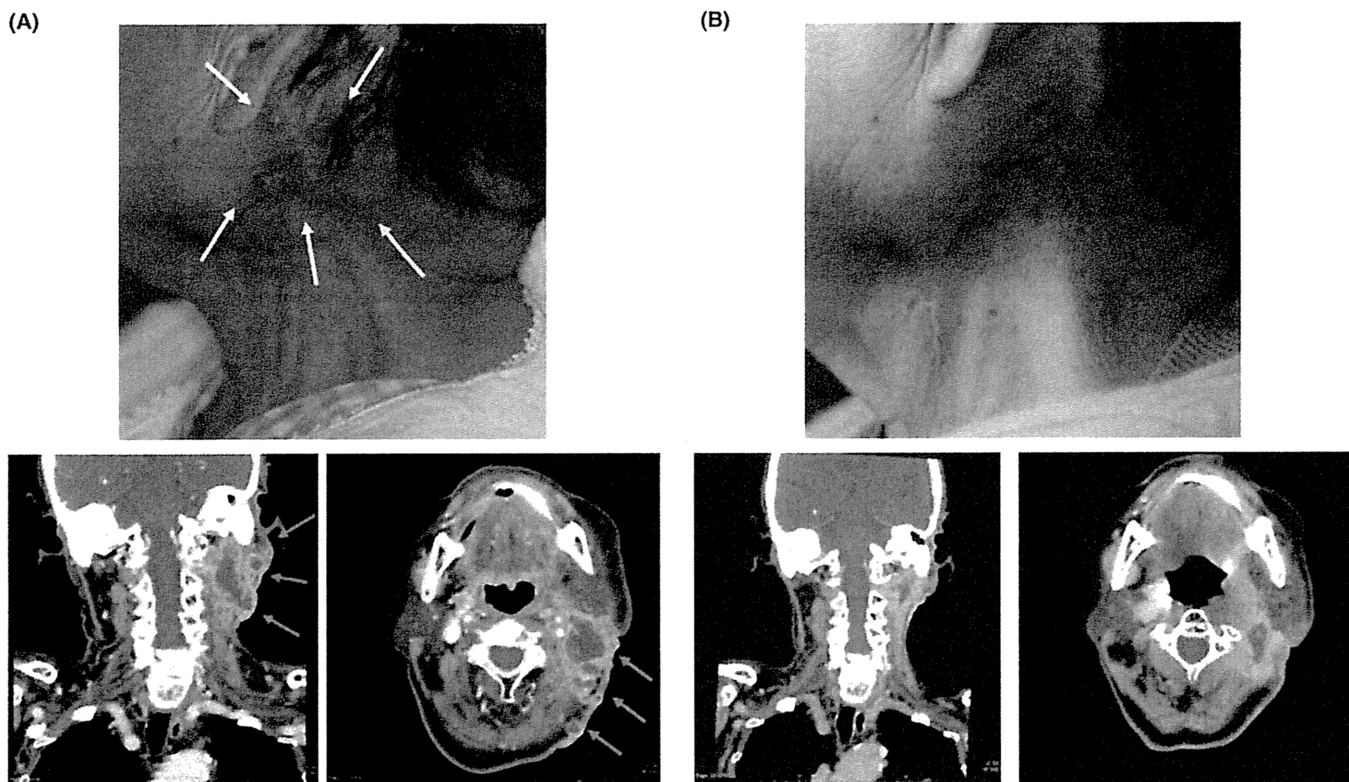

Fig. 1. Photograph of skin on the neck and computed tomography (CT) scan image of the neck showing metastatic tumors of case 10. (A) Photograph of skin on the neck and CT scan image of the neck before vaccination. Axial contrast-enhanced CT image shows multiple metastatic tumors (arrows). (B) Photograph of skin on the neck and CT scan image of the neck after the fifth vaccination. The metastatic tumors show significant remission after the fifth vaccination compared with before vaccination (70% reduction).

tetramer-positive CTL among CD8-positive T cells before and after the fourth vaccination. The frequency of tetramer-positive CTL was increased from 0.77% to 2.04% and from 0.05% to 0.20%, in cases 9 and 10 respectively.

Discussion

Many tumor-associated antigens have been identified and clinical trials utilizing them have been conducted.[3–6] However, most such clinical trials were aimed at the treatment of advanced melanoma and there are few reports on the treatment of patients with solid cancers. Although the immunogenicity of these non-melanoma-associated antigens is relatively weak, a specific number of tumor antigens were determined. The HLA-A24-restricted CTL epitope survivin-2B80-88 derived from survivin-2B has high potency for CTL induction in various cancer patients, including those with breast cancer, colorectal cancer, gastric cancer and oral cancer.[10,18–20] Based on the findings of these studies *in vitro*, a phase I clinical study of survivin-2B peptide vaccine therapy began in September 2003 for patients with advanced or recurrent oral cancer, following those for colorectal cancer and breast cancer. In many clinical trials, patients received the peptide in combination with certain adjuvants such as incomplete Freund's adjuvant (IFA) and cytokines for the purpose of enhancing the immune responses against cancer. In the present study, patients received the survivin-2B peptide dissolved in physiological saline without any adjuvant in order to strictly evaluate the clinical effect of the peptide alone.

A dose-escalation trial was chosen to estimate the safe and optimal doses. Dosage groups of 0.1 and 1.0 mg were set up, consisting of six and four patients, respectively. None of the patients had any sign of toxicity. Therefore, the survivin-2B

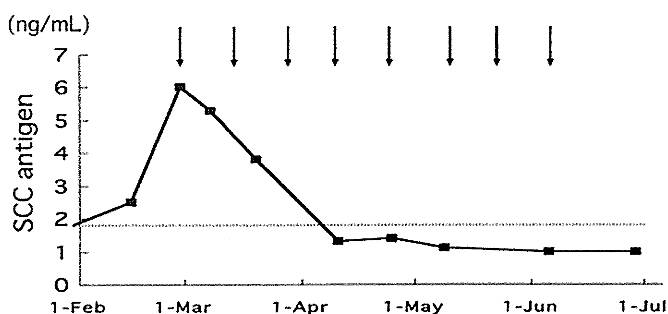

Fig. 2. Changes in the serum squamous cell carcinoma (SCC) antigen level during the vaccination in case 10. The dotted line indicates the cut-off point for the SCC antigen level. The arrows indicate the times of vaccination. The SCC antigen level significantly decreased to 0.7 ng/mL after the sixth vaccination. The cut-off value was 1.5 ng/mL.

peptide vaccine was safe and could be repeatedly injected into patients without serious side-effects. In terms of the clinical responses, the levels of tumor markers were temporarily decreased in comparison with the pretreatment status in two patients in the 1.0 mg dosage group. No patients in the 0.1 mg dosage group experienced a decrease in tumor markers. A PR was observed in one patient who was administered 1.0 mg of peptide. Therefore, the 1.0 mg dosage group appeared to have a better clinical outcome than the 0.1 mg dosage group. Based on these results, the recommended survivin-2B vaccine dose was 1.0 mg. Furthermore, we set up two distinct injection routes, s.c. into the ipsilateral neck or intratumorally. Intratumoral injection was concretely done by

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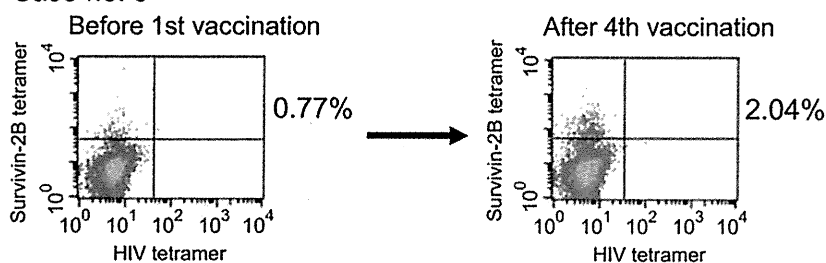

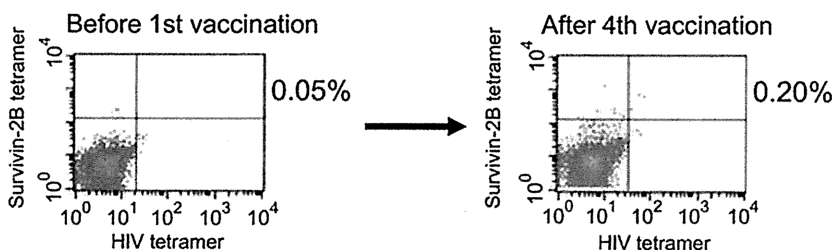

Fig. 3. Tetramer staining before the first vaccination and after the fourth vaccination in cases 9 and 10. Flow cytometric analysis was performed using a FACSCaliber and CellQuest software (Becton Dickinson Biosciences). The frequency of the cytotoxic T lymphocyte (CTL) precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells. The peptide-specific CTL frequency is indicated as the percentage of tetramer-positive CTL among CD8-positive T cells before the first vaccination and after the fourth vaccination. In cases 9 and 10, the frequency of tetramer-positive CTL was increased from 0.77% to 2.04% and from 0.05% to 0.20%, respectively. HIV, human immunodeficiency virus.

submucosal or subcutaneous vaccination into the peripheral parts of tumors, avoiding necrotic areas and vessels, for intraoral tumors and neck tumors, respectively. However, no significantly different findings as a whole were noted for the clinical and immunological responses.

In the present study, one patient (case 10) achieved a clinical PR. This demonstrated that the survivin-2B vaccination could yield an excellent response in oral cancer. The patient had received tegafur/uracil (UFT) as oral adjuvant chemotherapy and limited systemic chemotherapy for a few months prior to the vaccine treatment. She was judged to have PS1 in the ECOG classification. It is possible that peptide-based immunotherapy might be more effective in patients with reduced immune suppression as a result of recent intensive chemotherapy, as suggested by the previous clinical study of survivin-2B vaccination for colon cancer, although the study consisted of only a limited number of patients.[25] The results of the present trial were mostly compatible with the colon cancer studies in terms of the chemotherapeutic background. Furthermore, by immunohistochemistry, we preliminarily examined the infiltration of local immune cells in metastatic progressive tumor samples from her neck obtained before the first vaccination. Infiltration of CD8 T-cells into the peripheral parts of the tumor was markedly observed. On the other hand, a large number of tumor cells with strong survivin and HLA class I expression were observed. It was presumed that these findings indicated good conditions for immune responses in the tumor microenvironment. However, we failed to obtain a specimen during or after vaccination to evaluate the frequency of these cells (data not shown). Further studies to elucidate the immunoregulatory mechanisms of the immune escape by analyzing the infiltrating immune cells in local tumor sites will be necessary.

Although analysis of peripheral blood lymphocytes using HLA-A24/peptide tetramers actually revealed a slight increase in the peptide-specific CTL frequency in six patients, the immune responses had no relevance to the clinical responses in this study. It seems reasonable to conclude that the number of CTL induced by the vaccine was insufficient to induce tumor regression in patients with advanced or recurrent oral cancer, as vaccine-specific CTL might not be recruited into the tumor site, and the cytotoxic function of CTL might be suppressed in the tumor site by certain mechanisms such as regulatory T cells and immunosuppressive cytokines in the tumor microenvironment.

Overall, the survivin-2B peptide vaccination was well tolerated, but it is suggested that this vaccination protocol might provide only marginal immunological and clinical responses in most advanced or recurrent oral cancer patients. It is possible that advanced protocols such as a more intense immunization schedule and delivery in combination with a specific adjuvant and/or an immune-stimulatory cytokine might improve the efficacy of the survivin-2B peptide vaccine against oral cancer. Indeed, vaccination of the survivin-2B peptide mixed with IFA increased the frequency of peptide-specific CTL more than vaccination with the peptide alone in a phase I clinical trial for patients with advanced or recurrent breast cancer.[26] Based on the results of the present study and the other trials, a second clinical study of survivin-2B peptide vaccine has recently been started in combination with IFA and interferon-alpha.

Disclosure Statement

The authors have no conflict of interest.

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Autologous CTL response against cancer stem-like cells/cancer-initiating cells of bone malignant fibrous histiocytoma

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Malignant fibrous histiocytoma (MFH) of the bone is an aggressive tumor with high rates of local recurrence and metastasis. The development of novel therapeutic approaches is critical to improve the prognosis of patients with MFH. We reported previously that the side population (SP) cells of the MFH2003 bone MFH cell line have the characteristics of cancer stem-like cells (CSC)/cancer-initiating cells. In the present study, to establish immunotherapy targeting CSC, we analyzed cell surface immune molecules on SP cells of the MHF2003 cell line, as well as autologous CTL responses against these SP cells in the tumor microenvironment and peripheral circulating lymphocytes, using autologous tumor-infiltrating lymphocytes and autologous CTL clones derived from peripheral blood, respectively. We found that the SP cells expressed human leukocyte antigen (HLA) Class I molecules on the cell surface. The autologous tumor-infiltrating lymphocyte line TIL2003 recognized both the SP and main population cells of the MFH2003 cell line. Next, we induced the CTL clone Tc4C-6 by mixed lymphocyte tumor cell culture using autologous peripheral blood mononuclear cells and freshly isolated SP cells, followed by a limiting dilution procedure. The Tc4C-6 clone showed specific cytotoxicity against the SP cells. Moreover, the cytotoxicity against SP cells was blocked by the anti-HLA Class I antibody W6/32. In conclusion, the findings of the present study support the idea that CSC of bone MFH are recognized by autologous CTL in the tumor microenvironment and peripheral circulating lymphocytes. Thus, CTL-based immunotherapy could target CSC of bone sarcoma to help prevent tumor recurrence. (*Cancer Sci* 2011; 102: 1443–1447)

Malignant fibrous histiocytoma (MFH) of the bone is a rare primary neoplasm, accounting for <5% of primary bone malignancies.[1,2] Histologically, MFH of the bone is composed of fibroblasts and pleomorphic cells with a prominent storiform pattern. It is an aggressive tumor, with high rates of local recurrence and metastasis and a poor prognosis; the 5-year survival has been reported to be <60%.[3,4] Therefore, the development of novel therapeutic approaches is critical to improve the outcomes of patients with MFH.

It was thought that all neoplastic cells within a tumor were capable of tumorigenic growth. However, recent studies have demonstrated that malignant tumors can be generated by a distinct subpopulation of tumor cells, the so-called cancer stem cells (CSC)/cancer-initiating cells (CIC), which have self-renewal ability, differentiation potential, and tumorigenic capacity.[5,6] Thus, CSC could be a therapeutic target for the complete eradication of tumor cells. However, CSC have been reported to be resistant to standard therapeutic modalities, including radiation and drugs.[7,8]

Recently, many clinical trials of CTL-based immunotherapy using peptide vaccination have demonstrated the potency of this new therapeutic modality for various cancers that are resistant to standard chemotherapy.[9] However, it remains unknown whether CTL-based immunotherapy can kill CSC. Previously, we demonstrated that the side population (SP) cells from the bone MFH cell line MFH2003 have CSC characteristics.[10] The SP cells of the MFH2003 cell line exhibited cancer-initiating activity, with *in vitro* sphere formation and *in vivo* tumorigenesis in NOD/SCID mice. In the present study, to characterize the immunogenicity of CSC, we analyzed autologous CTL responses against SP cells of the MFH2003 cell line in the tumor microenvironment, as well as in peripheral circulating blood, using autologous tumor-infiltrating lymphocytes and a CTL clone.

Materials and Methods

The present study was approved under the institutional guidelines for the use of human subjects in research. The patients and their families, as well as healthy donors, provided informed consent for the use of blood samples and tissue specimens in our research.

Cell lines and culture. The cell lines used in the present study were a bone human MFH cell line (MFH2003), an erythroleukemia cell line (K562), and Epstein-Barr virus-transformed B cell lines (LG2-EBV, B2003-EBV). The OS2000, KIKU, MFH2003, and B2003-EBV cell lines were established in our laboratory.[11] The K562 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). ThILG2-EBV cell line was donated by Dr. PG Coulie (Christian de Duve Institute of Cellular Pathology, University of Louvain, Brussels, Belgium). The MFH2003 cells were cultured in Iscove's modified Dulbecco's Eagle's medium (IMDM; Gibco BRL, Grand Island, NY, USA) containing 10% FBS. The LG-2-EBV, B2003-EBV, and K562 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) containing 10% FBS. All other cell lines were maintained in DMEM (Sigma-Aldrich) containing 10% FBS in a 5% CO_2 incubator at 37°C.

Purification of side population cells. The CSC of the MFH2003 cell line were purified by side population analysis, as described previously.[10] Briefly, cell suspensions were labeled with Hoechst 33342 dye (Cambrex Bio Science, Walkersville, MD, USA) at a final concentration of 5.0 μg/mL in the presence or absence of verapamil (75 μM; Sigma-Aldrich) as an inhibitor of the ATP-binding cassette (ABC) transporter. Cells were incubated at 37°C for 90 min with continuous shaking. At the end of the incubation period, cells were washed with ice-cold PBS with 5% FBS, centrifuged at 440*g* for 5 min at 4°C, and resuspended in ice-cold PBS containing 5% FBS. Propidium iodide (final concentration 1 μg/mL; Life Technologies, Carlsbad, CA, USA) was used to gate viable cells. Flow cytometry and cell sorting were performed using a FACSAria II cell sorter (BD

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Biosciences, Bedford, MA, USA). The Hoechst 33342 dye was excited at 357 nm and its fluorescence was analyzed using dual wavelengths (blue, 402–446 nm; red, 650–670 nm).

When the proportion of SP cells was low (≤5%), the SP cells were sorted and subjected to *in vitro* culture in 10 mL IMDM containing 10% FBS for enrichment. Then, after at least 14 days culture, SP analysis and cell sorting were performed again.

Analysis of expression of cell surface molecules. Expression of cell surface molecules was assayed as described previously[11] using an anti-human leukocyte antigen (HLA)-A24 mAb (C7709A2.6), anti-HLA-B&C mAb (B1.23.2), anti-HLA Class I mAb (W6/32), anti-HLA-Class II mAb (L243), and an anti-CD80 mAb (Hybridoma cells for C7709A2.6 were donated by Dr. PG Coulie [Christian de Duve Institute of Cellular Pathology, University of Louvain, Brussels, Belgium] and those for B1.23.2 and L243 were purchased from the American Type Culture Collection [Manassas, VA, USA]. An anti-CD80 mAb was purchased from Immunotech [Marseille, France].). SP, and main population (MP) cells of the MFH2003 cell line, as well as LG2-EBV, B2003-EBV, and K562 cells, were incubated with appropriate mAb for 40 min on ice. Then, the cells were incubated with FITC-labeled secondary antibodies and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

ELISA. Target cells ($1–2 \times 10^4$) were plated in flat-bottomed 96-microwell plates (Corning, Corning, NY, USA) in DMEM containing 10% FBS. Then, TIL2003 cells (5×10^4) in AIM-V medium were added. After 24 h incubation at 37°C, the amount of granulocyte–macrophage colony stimulating factor (GM-CSF) in the supernatant (100 μL) was measured using an ELISA Development kit (TechneCorp, Minneapolis, MN, USA) according to the manufacturer's instructions. All experiments were performed in duplicate.

Establishment of autologous CTL clones against SP cells of the MFH2003 cell line. Autologous CTL clones against SP cells of the MFH2003 cell line were established as described previously. Briefly, peripheral blood mononuclear cells (PBMC) were obtained from an MFH2003 donor patient. The $CD8^+$ T cells were collected from PBMC using magnetic anti-CD8 beads (Miltenyi Biotec, Gladbach, Germany). A total of 5×10^5 irradiated (100 Gy) SP cells of the MFH2003 cell line and 5×10^6 $CD8^-$ T cells were distributed into five wells of a 24-well flat-bottomed culture plate containing 2 mL/well AIM-V and cultured at 37°C. The following day, 20 U/mL recombinant human interleukin-2 (rhIL-2; a kind gift from Takeda Chemical Industries, Osaka, Japan) and 10% AB human serum (HS) were added. The stimulation of T cells was repeated at intervals of 7–10 days using SP cells. After the fourth stimulation, the CTL were plated from all five culture wells at various dilutions in round-bottomed 96-microwell plates (Corning) in AIM-V supplemented with rhIL-2 (200 U/mL) and phytohemagglutinin (PHA; 5 μg/mL; Wako Chemicals, Osaka, Japan). Irradiated LG-2 EBV cells (1×10^4 cells/well) and allogeneic PBMC (1×10^5 cells/well) were added as feeder cells. Cells were incubated at 37°C. After 42 days, three resultant CTL clones were used in the cytotoxicity assays. One CTL clone showing specific cytotoxicity against SP cells of the MFH2003 cell line was selected and designated Tc4C-6. The cytotoxicity assay was performed as described below. Cell surface phenotypes of Tc4C-6 were assayed using an FITC-conjugated anti-CD3 antibody (BD Biosciences), phycoerythrin (PE)-conjugated anti-CD4 antibody (BD Biosciences), PE-Cy5-conjugated anti-CD8 antibody (eBioscience, San Diego, CA, USA), FITC-conjugated anti-CD45RA (BD Biosciences) antibody, and PE-conjugated anti-CCR7 antibody (BD Biosciences). The Tc4C-6 clone and healthy donor PBMC were incubated with these antibodies for 30 min on ice in the dark. After washing with PBS, cells were fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometry.

Cytotoxicity assay. The specific cytotoxicity of CTL clones was measured using the non-radioactive aCella-TOX assay (Cell Technology, Mountain View, CA, USA) according to the manufacturer's instructions. Target cells were plated in triplicate (5000 cells/well) in round-bottomed 96-well microwells in IMDM containing 50 U/well rhIL-2. Effector cells were added at various effector:target (E/T) ratios, as indicated. Spontaneous effector and target cell death was achieved by including control wells of effector and target cells at numbers corresponding to those of their various E/T ratios. To determine maximum release, calculated as total glyceralehyde-3-phosphate dehydrogenase (G3PDH) released, 10 μL lysis reagent (0.5% Nonidet P-40 per 100 μL sample) was added to the target cell positive control 10 min after the end of the assay incubation. After 12 h incubation at 37°C, the culture supernatant from each well was transferred into a corresponding well containing Enzyme Assay Reagent reacting against G3PDH on a white OptiPlate-96 (PerkinElmer, Waltham, MA, USA) and detection reagent was added to each well. The luminescence of each well was analyzed immediately using an ARVO MX/Light 1420 Multilabel Luminescence Counter (PerkinElmer). All experiments were performed in triplicate. Cytotoxicity (%) was calculated as [(experimental G3PDH release − spontaneous G3PDH release from effector cells − spontaneous G3PDH release from target cells)/(maximum G3PDH release from target cells − spontaneous G3PDH release from target cells) × 100].

In blocking experiments, the target cells was incubated with an anti-HLA Class I mAb (W6/32) or anti-HLA Class II mAb (L243) for 30 min at 37°C before the cytotoxicity assay was performed.

Results

Enrichment of SP cells in the MFH2003 cell line. To isolate SP cells as CSC/CIC, SP analysis and cell sorting were performed 95 times. As shown in Figure 1, in independent experiments the proportion of SP cells in the MFH2003 cell line ranged from 0.3% to 7.2% (mean 4.0 ± 1.7%). Figure 1(a) shows typical results from two independent SP analysis experiments. The number of SP cells isolated from bulk MFH2003 cells ranged from 0.1 to 6.7×10^5 (mean $2.4 \pm 1.4 \times 10^5$). Because more than 5×10^5 SP cells were required for each experiment in the present study, the variance in the proportion of SP cells and the low number isolated sometimes made it difficult to complete the experiments. To overcome these problems, we enriched SP cells using *in vitro* SP cell culture. After 7–10 days, the proportion of SP cells increased to between 9.4% and 36.2% (mean 18.6 ± 7.4%). In addition, the resultant number of sorted SP cells increased more than sixfold, ranging from 3.3 to 38.2×10^5 (mean $15.3 \pm 7.7 \times 10^5$; Fig. 1a,b). This improvement in the isolation efficiency of SP cells was useful for further experiments.

Expression profiles of immune molecules on SP and MP cells of the MFH2003 cell line. First, we analyzed the immune molecules on SP and MP cells of the MFH2003 cell line (Fig. 2). The SP cells expressed HLA Class I, HLA-A24, B, and C molecules on their cell surface. The expression of these molecules was greater than that for MP cells. Although MHC Class I and CD80, which provide costimulatory signals necessary for T cell activation and survival, were not expressed on SP cells, the higher expression of MHC Class I on SP cells suggests that CSC can be recognized by the host cellular immunity.

Autologous TIL2003 recognized both SP and MP cells of MFH2003. Next, to evaluate whether CTL can recognize SP cells in the tumor microenvironment, we assessed the response of the autologous tumor-infiltrating lymphocyte line TIL2003 against SP and MP cells. The TIL2003 cell line is a CTL line we established previously from the metastatic lymph nodes of

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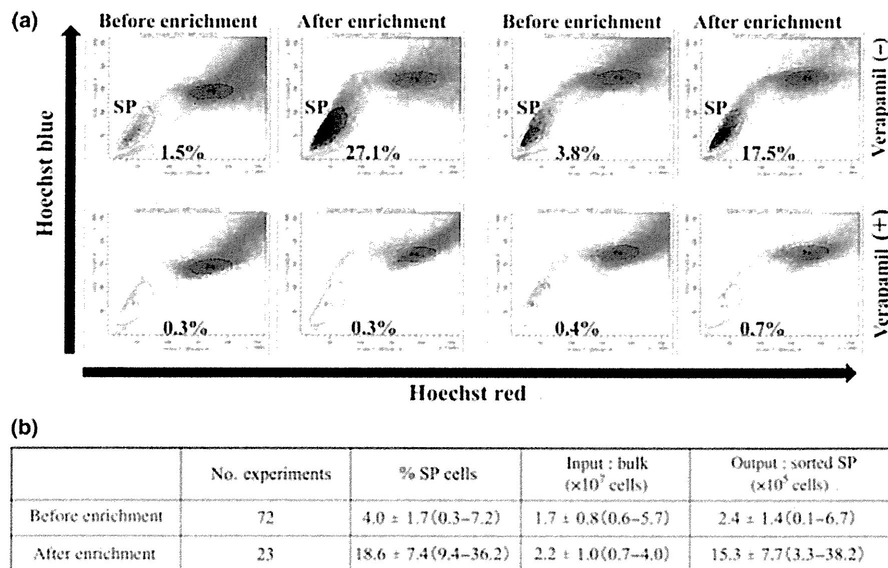

	No. experiments	% SP cells	Input : bulk ($\times 10^7$ cells)	Output : sorted SP ($\times 10^5$ cells)
Before enrichment	72	4.0 ± 1.7(0.3–7.2)	1.7 ± 0.8(0.6–5.7)	2.4 ± 1.4(0.1–6.7)
After enrichment	23	18.6 ± 7.4(9.4–36.2)	2.2 ± 1.0(0.7–4.0)	15.3 ± 7.7(3.3–38.2)

Fig. 1. Enrichment of the side population (SP) cells of the MFH2003 cell line. (a) The SP cells before and after enrichment, in the presence or absence of verapamil, in two independent experiments. The SP cells are encircled by black lines. The proportion of SP cells among total living cells is indicated in each case. (b) Summary of the enrichment of SP cells giving the mean proportion (%) of SP cells in the MFH2003 cell line, the mean number of bulk MFH2003 cells stained with Hoechst 33342 dye, and the mean number of sorted SP cells. Data show the mean ± SD with the range given in parentheses.

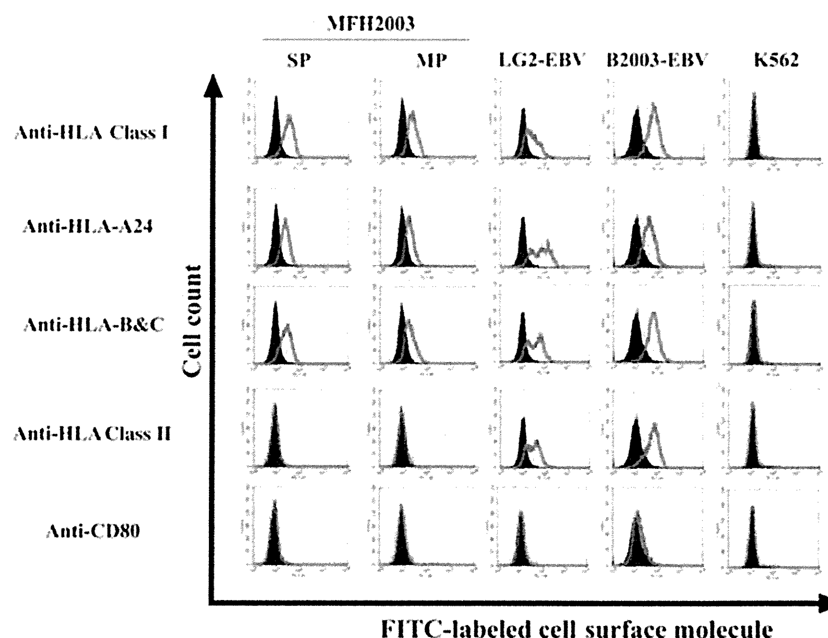

Fig. 2. Expression profile of immune molecules on side population (SP) and main population (MP) cells showing the cell surface expression of human leukocyte antigen (HLA) Class I (HLA-A24, B&C), HLA Class II, and CD80 molecules on MFH2003 (bulk, SP, and MP cells), LG-2, EB-B (B2003-EBV) and K562 cells.

the MFH2003 patient.[11] As shown in Figure 3, TIL2003 cells recognized both SP and MP cells. Although we could not completely rule out the possibility that MP cells triggered the immune response against both SP and MP cells in the context of some antigens expressed in both SP and MP cells, the results do suggest that the CTL response against SP cells was triggered by SP cells in the tumor microenvironment.

Specific response of the CTL clone derived from peripheral blood against SP cells. To detect the peripheral specific CTL response against SP cells of the MFH2003 cell line, we attempted to induce an autologous CTL clone that recognized the SP cells using SP cells as the stimulatory antigen. Cells were stimulated four times by mixed lymphocyte–tumor cell culture using purified SP cells of the MFH2003 cell line and autologous PBMC. Subsequently, conventional limiting dilution was performed. As a result, we obtained one CTL clone, namely Tc4C-6, which showed specific cytotoxicity against SP cells of the MFH2003 line. The Tc4C-6 clone expressed a single Vβ-chain

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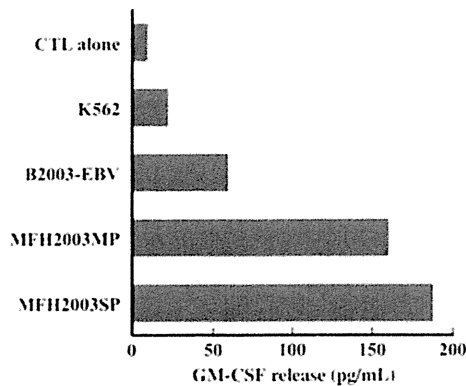

Fig. 3. Autologous tumor-infiltrating lymphocyte TIL2003 cells recognized both side population (SP) and main population (MP) cells. Freshly isolated SP and MP cells from the MFH2003 cell line, autologous EB-B cells (B2003-EBV), and negative control K562 cells were cocultured with TIL2003. After 24 h, the culture supernatant was harvested and the granulocyte–macrophage colony stimulating factor (GM-CSF) released from the TIL2003 was determined using ELISA.

(Vb5.2–3) mRNA, which was also expressed by TIL2003 cells (data not shown). The phenotype of the Tc4C-6 clone was $CD3^{+}CD4^{-}CD8^{+}CCR7^{-}CD45RA^{+}$, a typical effector phenotype (Fig. 4a). Moreover, the Tc4C-6 clone exhibited higher cytotoxicity against SP cells than MP cells of the MFH2003 cell line, as purified by cell sorting (Fig. 4b,c). In addition, the anti-HLA Class I W6/32 antibody apparently blocked the cytotoxicity of the Tc4C-6 clone against MFH2003SP cells (Fig. 4d). These results suggest that SP cells can be killed by autologous CTL in an HLA Class I-restriction manner.

Discussion

In the present study, we showed that: (i) SP cells, as CSC of the MFH200 cell line, expressed more HLA Class I on their cell surface than did MP cells (non-CSC); (ii) SP cells could be recognized by autologous tumor-infiltrating lymphocytes; and (iii) an autologous CTL clone could be induced by mixed lymphocyte–tumor cell culture using SP cells as antigens and that this induced clone killed SP cells rather than MP cells. These results indicate that CTL-recognizing CSC certainly exist in the tumor microenvironment and circulating peripheral blood and that SP cells can be killed by CTL. Thus, CTL-based immunotherapy against the CSC of bone sarcoma is a promising option.

Previous reports have suggested that CSC may be a candidate target for immunotherapy. For example, Pellegatta *et al.*[12] reported that dendritic cell based vaccine therapy resulted in an efficient anti-tumor immune response against glioma stem cells; Todaro *et al.* showed that γδ T cells killed human colon CSC; Pietra *et al.* demonstrated that natural killer (NK) cells killed human melanoma CSC;[13,14] and Weng *et al.*[15] induced CTL against ovarian CSC from HLA-A2^{+} healthy donors using CSC-DC fusion cells and demonstrated that the CTL killed ovarian CSC. However, until now, the autologous CTL response against CSC had not been investigated.

It is well documented that tumors can escape T cell-mediated elimination by downregulating molecules essential for immune recognition.[16] The downregulation of HLA Class I molecules

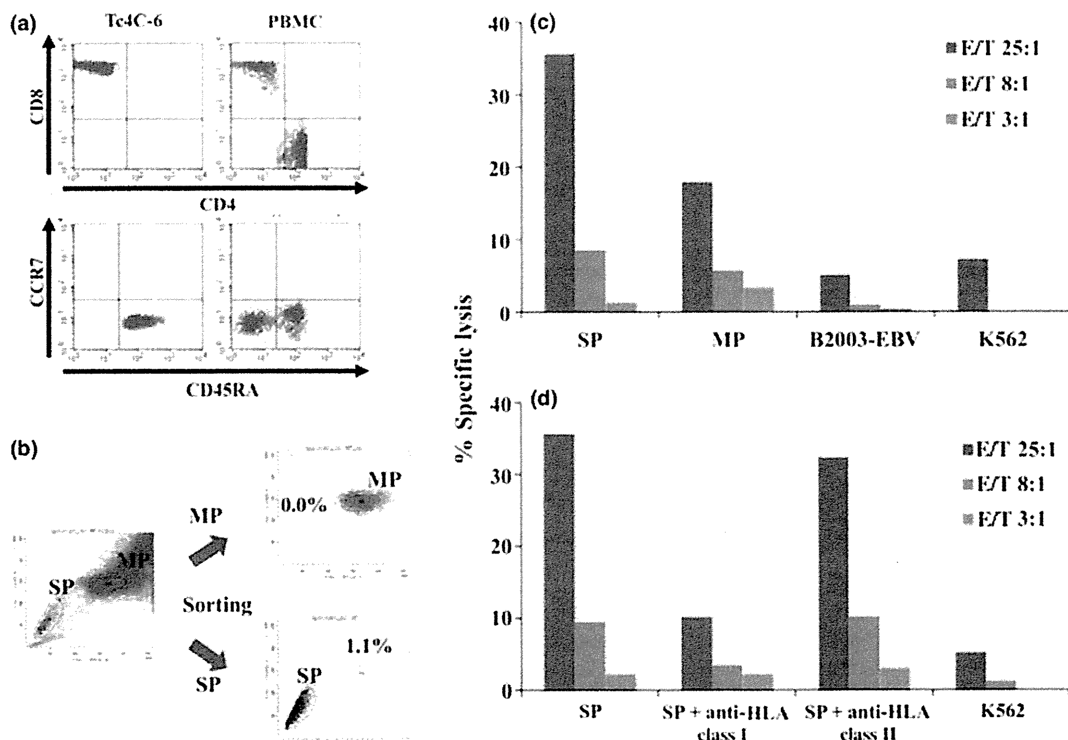

Fig. 4. The autologous CTL clone Tc4C-6 recognized side population (SP) cells. (a) Cell surface expression of CD4, CD8, CCR7, and CD45RA on Tc4C-6 and allogeneic peripheral blood mononuclear cells (PBMC) from a healthy donor. (b) Reanalysis of sorted SP and main population (MP) cells. (c) Cytotoxicity of the CTL clone Tc4C-6. Freshly isolated SP and MP cells, autologous EB-B cells (B2003-EBV), and negative control K562 cells were used as target cells and cocultured with Tc4C-6 at the specified effector:target (E/T) ratios. After 12 h, CTL-mediated cytotoxicity was measured using the aCella-TOX assay (Cell Technology), as described in the Materials and Methods. (d) Blocking assay of Tc4C6-mediated recognition of SP cells from the MFH2003 cell line using anti-human leukocyte antigen (HLA) Class I (W6/32 and anti-HLA Class II (L243) mAbs. Cytotoxicity was also measured with the aCella-TOX assay.

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in tumor tissues is a major prognostic factor and has an important role in tumor immune escape.[17] We have reported previously on the relationship between downregulation of HLA Class I and the poor prognoses of patients with osteosarcoma and Ewing's sarcoma.[18,19] However, as shown in the present study, the expression profile of immune molecules, including HLA Class I molecules on CSC, is preserved. Therefore, CSC may not escape from cellular immune surveillance activated by CTL-based immunotherapy.

The identification of CSC-associated antigens recognized by autologous CTL is very important, especially for the establishment of CTL-based immunotherapy in the adjuvant setting for the prevention of recurrence and metastasis. To this end, establishment of anti-CSC-specific CTL lines is a prerequisite. Although Weng *et al.*[15] assessed the CTL response against allogeneic ovarian CSC, there are no reports regarding CTL lines induced by autologous CSC. Therefore, the CTL clone Tc4C-6 is the first CTL clone against CSC induced by autologous CSC and could serve as a good probe against autologous CTL clone-defined CSC-associated antigen. We are currently trying to isolate the cDNA of the T-cell receptor (TCR) α- and β-chains to develop a permanent probe for cDNA library expression cloning.

In the present study, we evaluated SP cells of the MFH2003 cell line in 72 independent experiments. The proportion of SP cells in the MFH2003 cell line varied among experiments and this was often the main obstacle to completing the experiments using SP cells; thus, we needed to enrich the SP cells. The isolation of SP cells requires high-level technical skills and intensive, hard laboratory work. Although we do not know why the proportion of SP cells in the MFH2003 cell line is so variable, differentiation of SP cells into MP cells in cell culture *in vitro* may contribute to the variance in the proportion of SP cells. Recently, the dynamic regulation theory of cancer stem cells was proposed.[20] A subpopulation of SP cells that were jumonji AT-rich interactive domain 1B (JARID1B) positive was shown to have high proliferative ability. However, not only could individual JARID1B-positive cells become JARID1B negative, but individual JARID1B-negative cells could become JARID1B positive and acquire tumorigenicity. Such a dynamic change in the characteristics of SP and MP cells is another possible reason for the variability of the proportion of SP cells among independent experiments.

In conclusion, we have demonstrated the immunogenicity of CSC of bone MFH using autologous tumor-infiltrating lymphocytes and a peripheral CTL clone. On the basis of our results, we propose that CTL-based immunotherapy could target the CSC of bone sarcoma to prevent tumor recurrence.

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Disclosure Statement

All authors declare that they have no conflict of interest.

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Immunogenic enhancement and clinical effect by type-I interferon of anti-apoptotic protein, survivin-derived peptide vaccine, in advanced colorectal cancer patients

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We previously identified a human leukocyte antigen (HLA)-A24-restricted antigenic peptide, survivin-2B80-88, recognized by CD8+ cytotoxic T lymphocytes (CTL). Subsequently, we attempted clinical trials with this epitope peptide alone for some malignancies, resulting in clinical and immunological responses, although their potential was not strong enough for routine clinical use as a cancer vaccine. In the current study, to assess whether immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we performed clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and a type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect of survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with the vaccination protocol of survivin-2B80-88 plus IFA and IFNα resulted in clinical improvement and enhanced immunological responses of patients. Tetramer analysis of survivin-2B80-88 peptide-specific CTL demonstrated that such CTL were increased at least twofold after vaccination with this protocol in four of eight patients. In these patients, enzyme-linked immunosorbent spot (ELISPOT) results were also enhanced. Subsequent study of single-cell clone separation by cell sorting of peptide-specific CTL showed that each CTL clone was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules. Taken together, these results indicate that vaccination of colon cancer patients with survivin-2B80-88 plus IFA and IFNα can be considered to be a very potent immunotherapeutic regimen, and that this protocol might work for other cancers. (*Cancer Sci* 2011; 102: 1181–1187)

Human tumor immunology research has advanced since the first human melanoma tumor antigen recognized by CD8+ cytotoxic T lymphocytes (CTL) was identified in 1992,[1] and more than 20 melanoma antigens have been reported.[2–7] Some antigens and human leukocyte antigen (HLA) class I-restricted antigenic peptides underwent clinical trials, and their adverse effects and clinical and immunological responses were studied.[8–11] Rosenberg *et al.*[4] reported on a large number of melanoma patients and found that less than 5% of patients who received peptide vaccines such as gp100 and interleukin-2 (IL-2) had a complete response.

Nevertheless, a UK-based pharmaceutical company reported that a 3-year-long observation after melanoma antigen family A, 3 (MAGE-A3) vaccine inoculation indicated a 33% reduction in the post-operative recurrence of non-small-cell lung cancers when compared with a placebo group.[12] This observation gives strong hope for future cancer immunotherapy and has prompted many different investigations for the establishment of human tumor immunotherapy.

Meanwhile, human tumor antigens of non-melanoma tumors such as colon, lung, urinary tract and soft-tissue sarcomas have been analyzed extensively in various laboratories.[13–18] In our laboratory, we have identified tumor antigens using several different experimental systems.[2,3] Using reverse immunological approaches the inhibitor of apoptosis protein (IAP) family members survivin and livin were shown to be highly immunogenic tumor antigens in addition to the fact that these two antigens were selectively expressed in tumor tissues of different tissue origins but not in normal counterparts.[19–21] The HLA-A24-restricted survivin2B80-88 nonamer peptide, which was derived from the survivin splicing variant survivin 2B from cancer patients, appears to have strong immunogenicity as assessed by CTL induction efficiency, tetramer CTL frequency and enzyme-linked immunosorbent spot (ELISPOT).[19,20]

Our group began clinical trials several years ago.[8,9,11] The HLA-A24-restricted survivin2B80-88 peptide was given subcutaneously to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, urinary bladder cancers and lymphomas. There were no severe adverse effects and, clinically, certain patients with colon, lung and urinary bladder cancers showed reductions in tumor markers and growth arrest as assessed on computed tomography (CT). However, these effects were not strong enough for the clinical requirements as decided by the Response Evaluation Criteria in Solid Tumors (RECIST) for cancer chemotherapy. When assessed with the RECIST, which requires more than 30% regression of tumors on CT, only one of 15 patients with colon cancer and three of 15 with urinary bladder cancer had a positive clinical response.

Thus, the therapeutic potential was not strong enough for routine clinical use as a cancer treatment.[2,3] In the current study, to determine if the immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we performed and compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect with survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with survivin-2B80-88 plus IFA

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and IFNα resulted in clinical improvement and enhanced immunological responses of patients. We also analyzed CTL of these patients by single-cell sorting, finding that each CTL clone from the vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.

Materials and Methods

Patient selection. The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University, Japan.[8–11) All patients gave informed consent before being enrolled. Patients enrolled in the present study were required to conform to the following criteria: (i) to have histologically confirmed colon cancer; (ii) to be HLA-A*2402 positive; (iii) to have survivin-positive carcinomatous lesions by immunohistochemistry; (iv) to be between 20 and 85 years old; (v) to have unresectable advanced cancer or recurrent cancer; and (vi) to have Eastern Cooperative Oncology Group (ECOG) performance status between 0 and 2. Exclusion criteria included: (i) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy or other immunotherapy within the previous 4 weeks; (ii) the presence of other cancers that might influence the prognosis; (iii) immunodeficiency or a history of splenectomy; (iv) severe cardiac insufficiency, acute infection or hematopoietic failure; (v) use of anticoagulants; and (vi) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Surgery, Sapporo Medical University Primary Hospital from December 2005 to November 2009.

Peptide preparation. The peptide, survivin-2B80-88 with the sequence AYACNTSTL, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA).[8,9,11] 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.

IFA and IFNα preparation. Montanide ISA 51 (Seppic, Paris, France) was used as IFA. Human IFNα was purchased from Dainippon-Sumitomo Pharmaceutical Co. (Osaka, Japan).

Patient treatment. Two protocols were used in the current clinical study, as illustrated in Figure 1. One was a basic protocol with the survivin-2B80-88 peptide plus IFA, and the other was the survivin-2B80-88 peptide plus IFA and a type-I IFN, IFNα. In this trial, the primary end-point was safety. The second end-point was investigations about anti-tumor effects and clinical and immunological monitoring.

In the first protocol, survivin-2B80-88 at a dose of 1 mg/1 mL plus IFA at a dose of 1 mL were mixed immediately before vaccination. The patients were then vaccinated subcutaneously (s.c.) four times at 14-day intervals. This group included five patients. If patients whose disease was not far advanced hoped for continuation of this peptide vaccination therapy, we vaccinated them in the same manner after the fourth vaccination.

In the second protocol, survivin-2B80-88 plus IFA was vaccinated in a similar manner to the first protocol. In addition, in this protocol, IFNα at a dose of 3 000 000 IU was administered (s.c.) twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and administered at the time of the peptide and IFA biweekly vaccination.

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).[8,9]

Clinical response evaluation. Physical examinations and hematological examinations were conducted before and after each vaccination.[8,9] A tumor marker (carcinoembryonic antigen [CEA]) was examined. Changes in tumor marker levels were evaluated by comparison of the serum level before the first vaccination and that after the fourth vaccination. Immunohistochemical study of the HLA class I expression in patients' primary colon cancer tissues was done with anti-HLA class I heavy chain monoclonal antibody EMR-8-5 (Funakoshi Co., Tokyo, Japan).

Tumor size was evaluated by CT scans or MRI by comparing the size before the first vaccination with that after the fourth vaccination. A complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response (PR) was defined as a ≥30% decrease from baseline in the size of all measurable lesions (sum of maximal diameters). Progressive disease (PD) was defined as an increase in the sum of maximal diameters by at least 20% or the appearance of new lesions. Stable disease (SD) was defined as the absence of criteria matching those for CR, PR or PD.[8,9] Patients who received fewer than four vaccinations were excluded from all evaluations in this study.

***In vitro* stimulation of PBMC.** The PBMC were isolated from blood samples by Ficoll-Conray density gradient centrifugation.

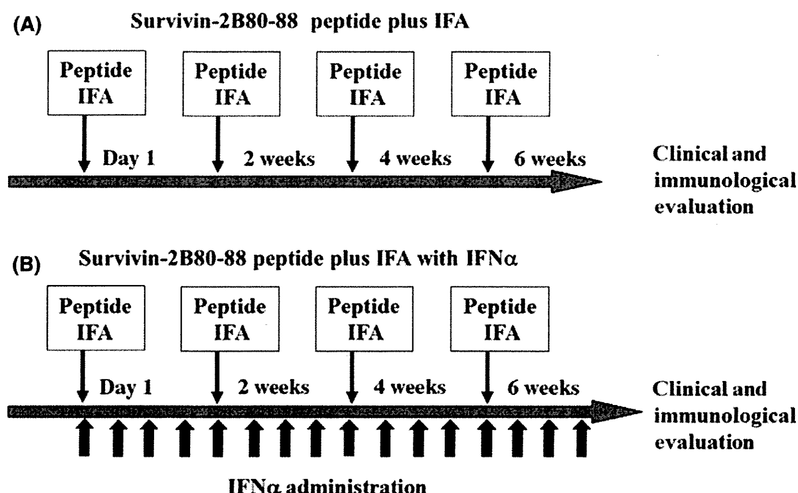

Fig. 1. Protocols of the clinical study. In the current study two protocols were used: (A) survivin-2B80-88 plus IFA and (B) survivin-2B80-88 plus IFA with IFNα. IFA, incomplete Freund's adjuvant; IFN, interferon.

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They were then frozen and stored at −80°C. As needed, frozen PBMC were thawed and incubated in the presence of 30 μg/mL survivin-2B80-88 in AIM-V medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/mL 1 h, 2 days, 4 days and 6 days after the addition of the peptide. On day 7 of culture, the PBMC were analyzed by tetramer staining and ELISPOT assay.

Tetramer staining. FITC-labeled HLA-A*2402-human immunodeficiency virus (HIV) peptide (RYLRDQQLL) and PE-labeled HLA-A*2402-survivin-2B8-88 peptide tetramers were purchased from MBL, Inc. (Nagoya, Japan). For flow cytometric analysis, PBMC, which were stimulated *in vitro* as above, were stained with the PE-labeled tetramer at 37°C for 20 min, followed by staining with a FITC-conjugated anti-CD8 mAb (Beckton Dickinson Biosciences, San Jose, CA, USA) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using FACSCalibur and CellQuest software (Beckton Dickinson Biosciences, San Jose, CA, USA). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells.[8,9,11]

ELISPOT assay. ELISPOT plates were coated sterilely overnight with an IFN-γ capture antibody (Beckton Dickinson Biosciences) at 4°C. The plates were then washed once and blocked with AIM-V medium containing 10% human serum for 2 h at room temperature. CD8-positive T cells separated from patients' PBMC (5×10^3 cells/well), which were stimulated *in vitro* as above, were then added to each well along with HLA-A24-transfected CIR cells (CIR-A24) (5×10^4 cells/well), which had been preincubated with or without survivin-2B80-88 (10 mg/mL) or with a HIV peptide as a negative control. After incubation in a 5% CO_2 humidified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN-γ antibody and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Jena, Germany). In the present study, positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNγ-positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) represents a less than twofold increase.

Single-cell cloning and functional assessment of tetramer-positive CTL. Survivin-2B80-88 peptide tetramer-positive CTL were sorted and subsequently cloned to single cells using FACS (Aria II Special Order, BD, Houston, TX, USA). The peptide-specific cytotoxicity of each of these CTL was determined by pulsing T2A24 cells[8,9,11,20] with survivin-2B80-88 or HLA-A*2402 HIV (RYLRDQQLL) peptides. These CTL were also assessed for live tumor cell cytotoxicity against LK79 (survivin-2B positive and HLA-A24 positive), A549 (survivin-2B positive, HLA-A24 negative) and K562 (survivin-2B negative and HLA-A24 negative) target cells.

Results

Patient profiles and safety. In the first protocol with the survivin-2B80-88 peptide plus IFA, five patients were enrolled in the study (Table 1). None of the treatment interruptions was due to adverse effects of the vaccination. These five patients received the complete regimen including four vaccinations and were evaluated (Fig. 1). They consisted of three men and two women, whose age range was 50–76 years.

In the second protocol with the survivin-2B80-88 peptide plus IFA and IFNα, eight patients were enrolled in the study (Table 2). In this protocol, there were no patients who dropped out because of adverse events due to the vaccination. They consisted of four men and four women, whose age range was 33–76 years.

With respect to safety, the vaccination was well tolerated in all patients in both vaccination protocols. In patients vaccinated with the survivin-2B80-88 peptide plus IFA, no adverse events were observed during or after vaccination except for induration at the injection site. In the second protocol, the survivin-2B80-88 peptide plus IFA and IFNα, approximately half of the patients had a fever reaching almost 39°C after the vaccination, possibly due to the action of IFNα. No other severe adverse events were observed during or after vaccination.

Clinical responses. As shown in Table 1, vaccination with survivin-2B80-88 plus IFA was given to five colorectal cancer patients. The post-vaccination CEA values of four patients were increased compared with the pre-vaccination values. In the other patient (No. 5), the CEA value remained almost the same, although it was beyond the upper limit during the vaccination. As for tumor size, only one patient was considered to have SD, whereas the other four patients were considered to have PD. These outcomes suggested that vaccination with this first protocol was ineffective for clinical responses.

Table 2 summarizes the clinical outcomes for the eight patients in the second protocol with survivin-2B80-88 plus IFA and IFNα. In some patients, particularly No. 6, the post-vaccination CEA value was clearly decreased compared with the pre-vaccination value, and was within the normal limit. Other patients such as Nos 2 and 3 also had decreased post-vaccination levels of CEA, although not so large. As for tumor size evaluated by CT, four patients (Nos 1, 2, 3 and 6) were considered to have SD, but the other four patients (Nos 4, 5, 7 and 8) had PD.

Table 1. Summary of profiles of advanced colorectal cancer patients enrolled in the present study and clinical and immunological responses to vaccination with survivin-2B80-88 peptide and IFA

Patient no.	Age/sex	Adverse effects	Tumor markers pre-/post- (CEA ng/mL)	CT evaluation†	Survivin-2B80-88 peptide		
					Tetramer staining‡		ELISPOT§
					Pre-/post-	% increase	
1	76/M	No	13/20	PD	192/103	53.6	−
2	59/M	No	369/463	SD	13/16	123.1	−
3	60/F	No	685/1010	PD	60/80	133.3	−
4	72/M	No	55/64	PD	11/4	36.4	−
5	50/F	No	8/7	PD	127/97	76.4	+

†Evaluation of CT images was done by the following: PD, progressive disease; SD, stable disease. ‡CTL frequencies of pre- and post-vaccinated patients were assessed with a HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with a HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer used as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTL among 10^4 CD8 T cells is shown. §Positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNg-positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) means a less than twofold increase. CEA, carcinoembryonic antigen; CT, computed tomography; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunosorbent spot; HIV, human immunodeficiency virus; IFA, incomplete Freund's adjuvant; IFN, interferon.

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Table 2. Summary of profiles of advanced colorectal cancer patients enrolled in the present study and clinical and immunological responses to vaccination with survivin-2B80-88 peptide, IFA and IFNα

Patient no.	Age/sex	Adverse effects	Tumor markers pre-/post- (CEA ng/mL)	CT evaluation†	Survivin-2B80-88 peptide		
					Tetramer staining‡		ELISPOT§
					Pre-/post-	% increase	
1	5/M	Leukopenia	Not determined	SD	53/98	184.9	–
2	62/M	No	5/3	SD	17/33	194.1	–
3	65/F	Fever	561/441	SD	11/98	890.9	+
4	72/M	No	6/8	PD	69/81	117.4	+
5	33/F	Fever	4655/8651	PD	8/24	300.0	+
6	44/F	Edema, Fever	12/2	SD	5/76	1520.0	+
7	53/F	Fever	82/117	PD	4/555	13 875.0	+
8	76/M	No	63/140	PD	32/35	109.4	–

†Evaluation of CT images was done by the following: PD, progressive disease; SD, stable disease. ‡CTL frequencies of pre- and post-vaccinated patients were assessed with a HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with a HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer used as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTL among 104 CD8 T cells is shown. §Positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNg-positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (–) means a less than twofold increase. CEA, carcinoembryonic antigen; CT, computed tomography; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunosorbent spot; HIV, human immunodeficiency virus; IFA, incomplete Freund's adjuvant; IFN, interferon.

Consequently, it appeared that there was a close correlation between clinical SD outcomes and a reduced CEA level.

Immune responses, single-cell cloning and subsequent functional assessment of tetramer-positive CTL. We determined if the survivin-2B80-88 peptide vaccination could actually induce specific immune responses in the patients enrolled. The peptide-specific CTL frequency was analyzed using the HLA-A24/peptide tetramer. The CTL frequency before the first vaccination (pre-vaccination) and after the last vaccination (post-vaccination) was assessed with a HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with a HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer for the negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CD8 T cells in 10^4 CD8 T cells was determined. In the current study, ELISPOT was also carried out using these peptides.

The data are summarized in Tables 1 and 2. In the first vaccination protocol, that is, vaccination with survivin-2B80-88 plus IFA, patients did not show obvious survivin-2B80-88-specific CTL responses in the tetramer study. Only patients Nos 2 and 3 exhibited very slight enhancement in that study. Patient No. 5 was positive in the ELISPOT study, but did not show a survivin-2B80-88-specific CTL response in the tetramer study. In these patients, there was no correlation between the clinical and immune responses. However, in the second protocol with survivin-2B80-88 plus IFA and IFNα, many patients demonstrated enhanced tetramer frequency. In particular, four of the eight patients (Nos 3, 5, 6 and 7) had enhanced frequency with a more than 200% increase. It was also interesting that all four of these patients were also positive in the ELISPOT study, and two had SD by CT evaluation, suggesting that immune responses might appropriately reflect, at least in some patients, clinical responses with this second vaccination protocol.

Subsequently, we analyzed tetramer-positive CD8 T cells at the single-cell level, and determined whether these T cells had specificity for the survivin-2B80-88 peptide and cytotoxic potential against live survivin-2B-positive tumor cells in the context of HLA-A*2402. As shown in Figure 2, patient No. 6 (44 years old, female) had positive HLA class I expression in rectal tumor tissue (Fig. 2A), a reduced serum CEA level (Fig. 2B) and obvious immune responses as assessed by the survivin-2B80-88 tetramer and ELISPOT (Fig. 2C) after vaccination with the second protocol. CD8 T cells of the tetramer-positive fraction were sorted by FACS, and then cultured with 1, 3 and 10 cells/well for 7–10 days. As shown in Figure 3, almost all growing T cells were survivin-2B peptide-specific T cells, and we subsequently assessed peptide-specific cytotoxicity by using T cells of well (a) through well (h) as indicated. Consequently, as Figure 4 clearly shows, all T cells except for those in well (b) had very high peptide-specific cytotoxic potential. Furthermore, T cells in well (d) were cloned, and we obtained a T cell clone designated D-13 showing survivin-2B peptide specificity as shown in Figure 5A,B. Finally, the cytotoxic potential against survivin-2B-positive live tumor cells was assessed, confirming that the D-13 T cell clone was clearly cytotoxic against survivin-2B-positive tumor cells in a HLA-A*2402-restricted fashion. Taken together, these data clearly indicate that the second vaccination protocol was capable of inducing a strong CTL response, and in some patients might result in clinical effectiveness.

Discussion

The immunogenicity of HLA class I-restricted tumor peptides is not strong enough for routine clinical use as cancer treatment, and it is important to increase their relative and absolute immunogenicity levels by various means. Previously, our group conducted clinical trials of the survivin-2B80-88 peptide vaccine alone and with IFA in patients with various cancers.[3,8,9,11] In the current study we determined whether the immunogenicity of survivin-2B80-88 could be enhanced with other vaccination protocols. Thus, we compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus IFA; and (ii) survivin-2B80-88 plus IFA and a type-I IFN, IFNα. Our data strongly suggest that although the effect with survivin-2B80-88 plus IFA was not different from that with the survivin-2B80-88 peptide alone, treatment with survivin-2B80-88 plus IFA and IFNα resulted in clear improvement of the clinical and immunological responses of patients.

There has been less evidence concerning the extent to which peptide-specific CTL responses in patients treated with peptide vaccines could occur at the single-cell level. To assess this point, CTL of patients were sorted to the single-cell level, and we confirmed that each CTL obtained from vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.

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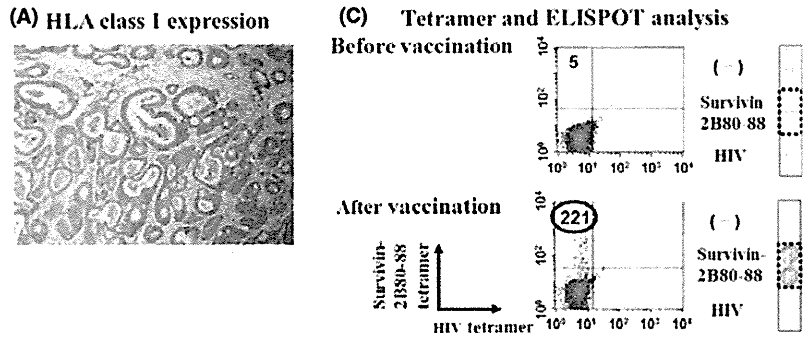

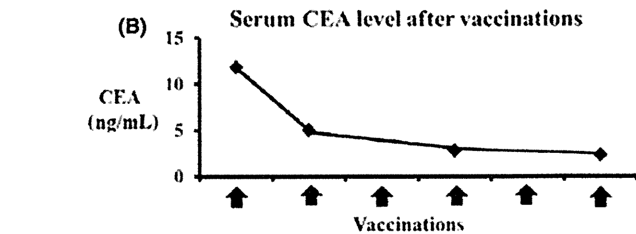

Fig. 2. Representative illustration of clinical effect and immunological analysis in patient No. 6 who was treated with survivin-2B80-88 plus IFA with IFNα. (A) Immunohistochemical analysis of HLA class I expression in primary colon cancer tissue as assessed by anti-HLA class I heavy chain monoclonal antibody EMR8-5, (B) profile of serum CEA level after vaccinations (arrows) and (C) tetramer and ELISPOT analyses before and after vaccinations. The number in the tetramer analysis indicates survivin-2B80-88 peptide-specific CD8+ T cells among 10^4 CD8+ T cells. CEA, carcinoembryonic antigen; ELISPOT, enzyme-linked immunosorbent spot; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; IFA, incomplete Freund's adjuvant; IFN, interferon.

Fig. 3. Single-cell analysis of survivin-2B80-88 peptide tetramer-positive CD8 T cells. Survivin-2B80-88 peptide tetramer-positive CD8 T cells in Figure 2C (circle) were sorted and cultured at 10 (A), 3 (B) and 1 (C) cells/well for 7–10 days. Subsequently, cells were examined for their reactivity to the survivin-2B80-88 peptide tetramer.

It is well known that type-I interferons such as IFNα can work in many immunological manners to activate T cell responses.[22–25] The maturation of dendritic cells (DC) and their effect on the expression of HLA molecules seems to be the main action of this cytokine. Because of difficulties with the availability of patients' samples, we could not actually compare these features of patients' DC and primary tumor tissues before and after treatment with IFNα. However, the evidence of obvious enhancement of CTL responses and improvement of clinical responses in our study favors the two main actions described above. Indeed, a preliminary study of three colon patients who received survivin-2B80-88 plus IFA with GM-CSF did not demonstrate any clinical or immunological response, suggesting that the action of IFNα is remarkable from the aspect of being an immunogenic enhancer for human peptide vaccines. Ideally, patients should be administered IFNα alone before vaccination treatment is undertaken. However, this was practically hard to do since all patients were in the late and advanced disease stages and it was difficult to select such patients. Meanwhile, there is no evidence that administration of IFNα alone results in overt clinical benefits such as those observed in the current study. The second vaccination protocol using IFNα appeared to be highly efficient for enhancing peptide immunogenicity.

Nevertheless, we require more efficient and reasonable ways to enhance peptide immunogenicity *in vivo* with limited side-effects.[26] In our laboratory, it was observed that heat shock protein 90 (HSP90) could highly selectively target substrates such as HLA-class I restricted peptides as well as the innate immunity ligand, CpG, to the Rab5 (+) and EEA1 (+) early static endosomes of DC.[3,27–29] Consequently, HSP90 could strongly induce peptide-specific CTL responses *in vitro* and *in vivo* without obvious side-effects,[20] and it is intriguing that its action may surpass the benefits of IFNα.

Collectively, our present study may reflect one potential vaccination protocol. Since the vaccination protocol with

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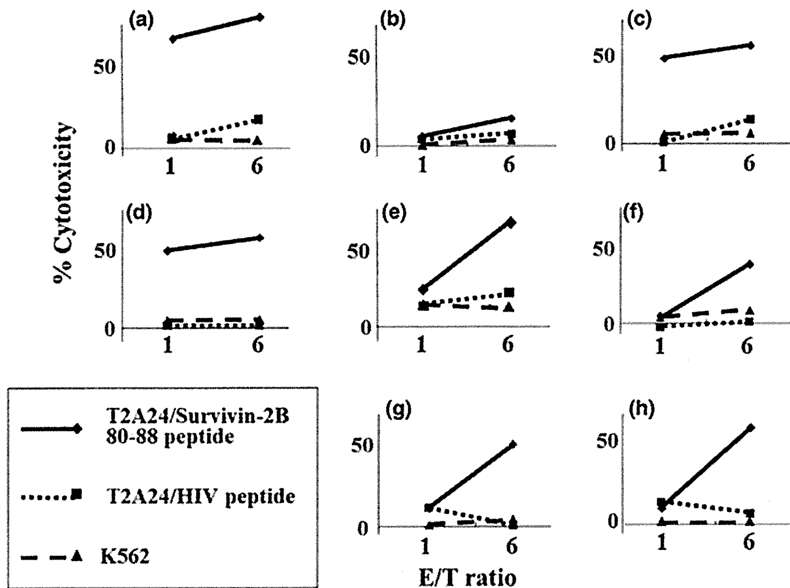

Fig. 4. Analysis of survivin-2B80-88 peptide-specific cytotoxicity. T cells of wells (a) through (h) in Figure 3 were assessed for their peptide-specific cytotoxicity against T2A24 target cells pulsed with the survivin-2B80-88 peptide and HIV peptide, and against control K562 cells. E/T, effector/target; HIV, human immunodeficiency virus.

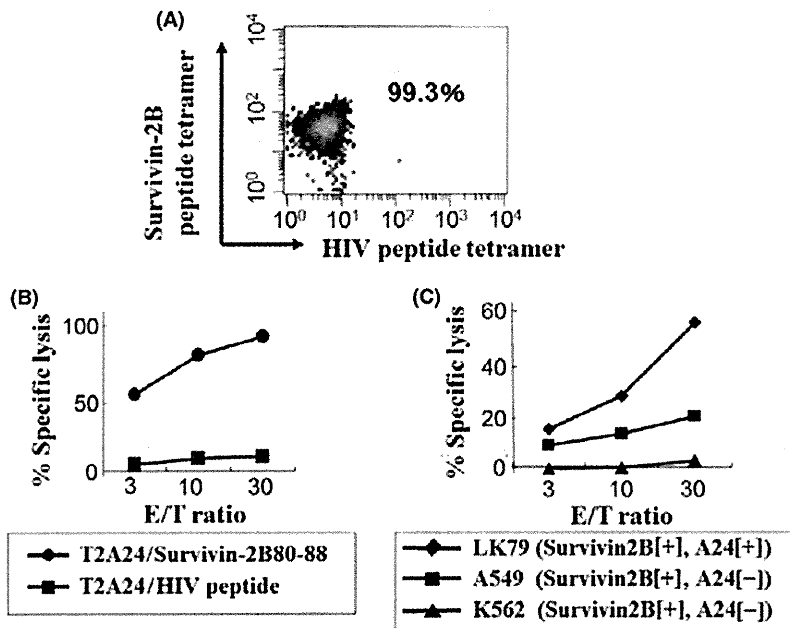

Fig. 5. Clonal analysis of survivin-2B80-88 peptide-specific CD8 T cells. T cells in well (d) in Figure 3 were cultured and CTL clone D-13 was obtained. Subsequently, D-13 was studied for (A) tetramers and (B) cytotoxicity against T2A24 cells pulsed with survivin-2B80-88 and HIV peptides, and (C) cytotoxicity against human tumor cells (LK79, A549 and K562) that express survivin and/or HLA-A24 molecules as indicated. CTL, cytotoxic T lymphocyte; E/T, effector/target; HIV, human immunodeficiency virus; HLA, human leukocyte antigen.

survivin-2B80-88 plus IFA and IFNα appears effective in other cancers such as pancreatic cancer as well, this protocol may be useful as a standard immunotherapy modality.

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Disclosure Statement

The authors have no conflict of interest.

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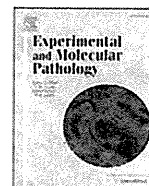

The feasibility of Cep55/c10orf3 derived peptide vaccine therapy for colorectal carcinoma

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ABSTRACT

In our previous study, we demonstrated that a peptide derived from the novel centrosome residing protein Cep55/c10orf3 can be targeted by the cytotoxic T lymphocytes (CTLs) in peripheral blood mononuclear cells (PBMCs) of breast carcinoma patients. In this report, we evaluated the feasibility of cancer immunotherapy using Cep55/c10orf3 peptide for colorectal carcinoma (CRC). To evaluate the expression of Cep55/c10orf3 in CRC tissues, we performed immunohistochemical staining of using anti-Cep55/c10orf3 monoclonal antibody. Sixty-three percent cases showed weak positive for Cep55/c10orf3 in total 70 CRC cases. The Cep55/c10orf3 expression intention was collated with high histological grade of CRC. Thus, we hypothesized that Cep55/c10orf3 can also be the target of CTLs in CRC cases. We generated CTLs from PBMCs of human leukocyte antigen (HLA)-A24-positive colorectal carcinoma patients using HLA-A24-restricted Cep55/c10orf3 peptides. Two of 6 colorectal cancer patients were reactive for the Cep55/c10orf3_193(10) peptide, which was the only immunogenic peptide in breast carcinoma patients. CTL clone specific for Cep55/c10orf3_193(10) recognized and lysed HLA-A24 (+) and Cep55/c10orf3 (+) colorectal carcinoma cell lines. In addition, 1 of 6 colorectal carcinoma patients was reactive for the Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) peptides, but not for Cep55/c10orf3_193(10) with the ELISPOT assay. These observations suggest that the antigenic peptide repertoire presented by HLA-A24 in colorectal carcinoma might be different from that in breast carcinoma. Thus, these peptide vaccination peptide mixture of Cep55/c10orf3_193(10), Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) might be more effective than a single peptide in the treatment of colorectal carcinoma patients.

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Introduction

Colorectal carcinoma is one of the major deadly malignancies in the world. Despite recent progress in the treatment of colorectal carcinoma, recurrence, metastasis or resistance to treatment remain the biggest challenges to control disease and contribute to unfavorable prognosis. In the advanced cases, it is very difficult to treat and control the disease, and new therapeutics are urgently needed. Immunotherapy might represent an effective novel treatment for colorectal carcinoma.

Previously, Cep55/c10orf3 was reported to be overexpressed in colorectal carcinomas (Inoda et al., 2009; Sakai et al., 2006). Cep55/c10orf3 plays essential role in G2/M cell cycle phase (Fabbro et al., 2005; Zhao et al., 2006) and is presumably expressed in all cancer cells. In contrast, Cep55/c10orf3 expression in normal tissues is restricted to testis and thymus (Inoda et al., 2009; Sakai et al., 2006). This makes Cep55/c10orf3 a suitable target for cancer immunotherapy (Hirohashi et al., 2009).

Abbreviations: CTLs, cytotoxic T lymphocytes; TAAs, tumor associated antigens; mAb, monoclonal antibody; PBMCs, peripheral blood mono-nuclear cells; IFN, interferon; B2M, beta-2 microglobulin.

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Table 1
Summary of the Cep55/c10orf3 peptides carrying HLA-A24 binding motief.

Peptides	Position number	Sequence
Cep55/c10or3_169(10)	169-178	EMEIQLKDAL
Cep55/c10orf3_193(10)	193-202	VYVKGLLAKI
Cep55/c10orf3_355(9)	355-363	QMQACTLDF
Cep55/c10orf3_446(10)	446-455	QYPATEHRDL
Cep55/c10orf3_74(11)	74-84	AYQLTEKDKEI
Cep55/c10orf3_94(11)	94-104	RYSTTALLEQL
Cep55/c10orf3_402(11)	402-412	EFAITEPLVTF
Cep55/c10orf3_186(13)	186-198	VYDQQREVYVKGL
Cep55/c10orf3_227(14)	227-240	GYLQEEKQKCYNDL
Cep55/c10orf3_268(12)	268-279	KYEETQKEVHNL
Cep55/c10orf3_283(12)	283-294	LYSQRRADVQHL
HIV-A24		RYLRDQQLLGI

In our previous study, we found that the centrosomal protein Cep55/c10orf3 was overexpressed in breast, colorectal and lung carcinoma tissues. We isolated an HLA-A24-restricited Cep55/c10orf3 peptide [Cep55/c10orf3_193(10)], which has been shown to be the target of CTLs from PBMCs of HLA-A24-positive breast carcinoma patients (Inoda et al., 2009). We established that Cep55/c10orf3 may be used for the cancer immunotherapy of breast carcinoma patients. However, the immunogenicity in colorectal carcinomas is still elusive.

Here we extended our studies to colorectal carcinoma. We evaluated the feasibility of using Cep55/c10orf3 derived peptides for HLA-A24 positive, which is a frequent allele in Japanese and also other populations, colorectal carcinoma immunotherapy. To investigate the immunogenicities of Cep55/c10orf3 derived peptides, PBMCs of colorectal carcinoma patients were stimulated several times using Cep55/c10orf3 peptides, and the CTL reactivity was assessed with the ELISPOT assay and the ^{51}Cr release assay. Two of the 6 colorectal carcinoma patients were positive for the Cep55/c10orf3_193(10) peptide, which is also immunogenic in breast carcinoma patients. The Cep55/c10orf3-specific CTL clone recognized HLA-A24-positive and Cep55/c10orf3-positive colorectal carcinoma cell lines, suggesting that colorectal carcinoma express endogenously processed Cep55/c10orf3 peptide. Furthermore, one of the six colorectal carcinoma patient showed immune reactivity for other peptides, including Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) by the ELISPOT assay. Unfortunately, we could not further analyze the cytotoxic

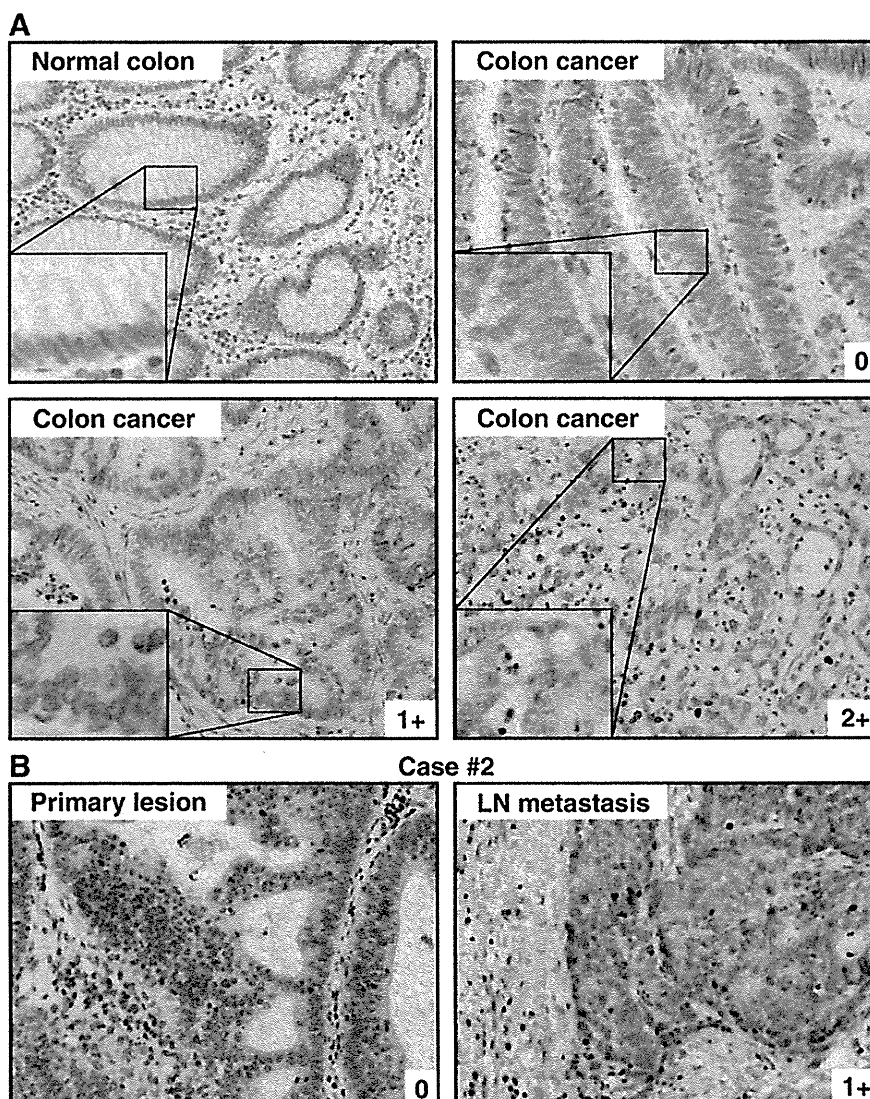

Fig. 1. Immunohistochemical staining of normal colon mucosa and colon cancers. A. Normal colon tissue and colon cancer tissues (summarized in Table 2) were stained with mAb #11-55 (magnification: ×200). Immunoreactivities were classified as follows: 0 as negative staining; 1+, weak positive staining; 2+, strong positive staining. B. Immunohistochemical staining of case #2 primary lesion and lymphnode metastasis lesion (magnification: ×200).

Table 2
Cep55/c10orf3 protein expression in colorectal carcinomas.

Histology*	N	Expression of Cep55/c10orf3		
		0 (Negative) (%)	1+ (Weak positive) (%)	2+ (Strong positive) (%)
Total	70	26 (37%)	36 (51%)	8 (12%)
Well differentiated adenocarcinoma	19	10 (53%)	9 (47%)	0 (0%)
Moderately differentiated adenocarcinoma	44	15 (34%)	25 (57%)	4 (9%)
Poorly differentiated adenocarcinoma	7	1 (14%)	2 (29%)	4 (57%)

*Histology of the colorectal cancer cases were classified according to 'WHO classification Tumors of the Digestive System'. N, case numbers.

activity for these peptides; however, the immune reactivities were specific and vigorous, suggesting that these peptides specific CTL precursors (CTLp) might be activated and expanded clonally *in vivo*. These observations indicate that Cep55/c10orf3 derived peptides, including Cep55/c10orf3_193(10), Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12), may be useful for HLA-A24-positive colorectal carcinoma immunotherapy.

Materials and methods

Immunohistochemical staining of tissue sections

Immunohistochememical staining was done with formalin-fixed, paraffin-embedded sections of 64 CRC, including tissue microarray and 6 biopsy CRC specimens. Sections (4–5-μm thick) were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was done by boiling sections in 120 °C for 5 min in a microwave oven in preheated 0.01-mol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked by 3%hydrogen peroxide in ethanol for 10 min. After blocking with 1%nonfat dry milk in PBS (pH 7.4), the sections were reacted with monoclonal anti-Cep55/c10orf3 antibody #11-55 for 1 hour, followed by incubation with biotinylated anti-mouse IgG (Nichirei) for 30 min. Subsequently, the sections were stained with the streptavidin–biotin complex (Nichirei), followed by incubation, with 3,3'-diaminobenzidine used as the chromogen and counterstaining with hematoxylin. Cytosolic staining was considered positive. We graded the immunoreactivity as follows: 0 as negative staining; 1+, weak positive staining; 2+, strong positive staining. Breast cancer cases were used as positive controls.

Patients

The PBMCs used in this study were obtained from HLA-A24-positive colorectal carcinoma patients who were hospitalized at Higashi Sapporo hospital (Sapporo, Japan) after obtaining their informed consent.

CTL induction, ELISPOT assay and ^{51}Cr release assay

CTLs were generated from PBMCs of HLA-A24 positive colorectal carcinoma as described previously (Hirohashi et al., 2002; Inoda et al., 2009). In brief, dendritic cells (DCs) and PHA-blasts were generated from the PBMCs of HLA-A24-positive colorectal carcinoma patients. CD8-positive T cells were isolated using the MACS separation system (Miltenyi Boitech, Bergish Blabach, Germany) according to the manufacturer's instructions. The CD8-positive T cells were stimulated for three times using DC for the 1st time of stimulation and PHA-blast for the 2nd and 3 rd times of stimulations as antigen presenting cells (APCs) with a mixture of eleven Cep55/c10orf3 peptides carrying HLA-A24 binding motif, as summarized in Table 1, (Inoda et al., 2009) in AIM-V medium (Invitrogen) supplemented with 10% of human serum.

One week later after the last stimulation, the CD8-positive T cells were evaluated the reactivity for peptides with the ELISPOT assay as described previously (Inoda et al., 2009). ELISPOT plates were coated sterilely overnight with an IFN-gamma capture antibody (Beckton Dickinson Biosciences) at 4 °C. The plates were then washed once and blocked with AIM-V medium containing 10% human serum for 2 hours at room temperature. Then, twenty thousand CTLs were incubated with 5×10^4/well T2-A24 (kind gift from Dr. K. Kuzushima, Nagoya, Japan) cells pulsed with Cep55/c10orf3 specific peptide or control peptides (5 μg/mL) or K562 cells. After incubation in a 5% CO2 humidified chamber at 37 °C for 24 hours, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN-gamma antibody and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Germany).

The lytic activity of CTLs was tested by the ^{51}Cr release assay as described previously (Sato et al., 1986). Briefly, ^{51}Cr-labeled target cells (2000 cells/well) were incubated with various numbers of effector cells for 6 hours at 37 °C in 96-well microtiter plates. The radioactivity of the culture supernatant was measured with a gamma counter. The percentage of cytotoxicity was calculated as follows: % cytotoxicity = (experimental release − spontaneous release)/(maximum release − spontaneous release) X100. Colorectal carcinoma cell lines Sw480, HT29 and HCT15 (kind gift from Dr. K. Imai, Sapporo, Japan) were used as target cells. The beta 2 microglobulin (*B2M*) transformant of HCT15 cells (HCT15-B2M) were obtained by the infection of retrovirus coding beta 2 microglobulin (*B2M*) cDNA. The retrovirus was generated as described previously using the retrovirus vector pMXs-puro encoding *B2M* cDNA and PLAT-A packaging cells (kind gift from Dr. T. Kitamura, Tokyo, Japan) (Morita et al., 2000), and

Table 3
Summary of the colorectal carcinoma cases.

Case #	Stage	Histology	Expression of Cep55/c10orf3	Reactive peptides	ELISPOT assay	^{51}Cr release assay
1	IIIb	Adenocarcinoma (mod. diff.)	Primary (1+)	193(10)	+	+
2	IV	Adenocarcinoma (mod. diff.)	Primary (0), LN (1+)	193(10)	-	+
3	IV	Adenocarcinoma (mod. diff.)	Primary (1+)	402(11),283(12)	+	ND
4	IV	Adenocarcinoma (mod. diff.)	Primary (1+)	-	-	-
5	II	Adenocarcinoma (well diff.)	Primary (1+)	-	-	-
6	II	Adenocarcinoma (well diff.)	Primary (0)	-	-	-

Abbreviations: mod. diff., moderatry differentiated; well diff., well differentiated; Primary, Primary lesion; LN, lymph node metastasis lesion; ND, not detected.