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

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

Conclusion

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

Acknowledgments Kumiko Shimozawa and Emiri Nakazawa provided technical assistance. This study was supported in part by a grant-in-aid for Clinical Cancer Research from the Ministry of Health, Labor and Welfare and a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant no. 17390441 to T. Tsukamoto), and research grants from the Stiftelsen Japanese-Swedish Research Foundation, and Gotaro Sugawara Research Fund for Urological Diseases.

References

1. Rosenberg SA (1999) A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 10:281–287
2. Ambrosini G, Adida C, Altieri DC (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 3:917–921
3. Kawasaki H, Altieri DC, Lu CD et al (1998) Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res 58:5071–5074
4. Tamm I, Wang Y, Sausville E et al (1998) IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 58:5315–5320
5. Hirohashi Y, Torigoe T, Maeda A et al (2002) An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. Clin Cancer Res 8:1731–1739
6. Idenoue S, Hirohashi Y, Torigoe T et al (2005) A potent immunogenic general cancer vaccine that targets survivin, an inhibitor of apoptosis proteins. Clin Cancer Res 11:1474–1482
7. Tsuruma T, Hata F, Torigoe T et al (2004) Phase I clinical study of anti-apoptosis protein, survivinderived peptide vaccine therapy for patients with advanced or recurrent colorectal cancer. J Transl Med 2:19–29
8. Tsuruma T, Iwayama Y, Ohmura T et al (2008) Clinical and immunological evaluation of anti-apoptosis protein, survivin-derived peptide vaccine in phase I clinical study for patients with advanced or recurrent breast cancer. J Transl Med 6:24
9. Kitamura H, Torigoe T, Honma I et al (2006) Expression and antigenicity of survivin, an inhibitor of apoptosis family member, in bladder cancer: implications for specific immunotherapy. Urology 67:955–959
10. Koh YT, Higgins SA, Weber JS et al (2006) Immunological consequences of using three different clinical/laboratory techniques of emulsifying peptide-based vaccines in incomplete Freund's adjuvant. J Trans Med 4:42
11. Altman JD, Moss PA, Goulder PJ et al (1996) Phenotypic analysis of antigen-specific T lymphocytes. Science 274:94
12. Sato Y, Sahara H, Tsukahara T et al (2002) Improved generation of HLA class/peptide tetramers. J Immunol Methods 20:177–184
13. Sato Y, Nabeta Y, Tsukahara T et al (2002) Detection and induction of CTLs specific for SYT-SSX-derived peptides in HLA-A24(+) patients with synovial sarcoma. J Immunol 169:1611–1618
14. Date Y, Kimura A, Kato H et al (1998) DNA typing of the HLA-A gene: population study and identification of four new alleles in Japanese. Tissue Antigens 47:93–101
15. Nestle FO, Alijagic S, Gilliet M et al (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 4:328–332
16. Andersen MH, Pedersen LO, Capeller B et al (2001) Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. Cancer Res 61:5964–5968
17. Ojima T, Iwahashi M, Nakamura M et al (2006) The boosting effect of co-transduction with cytokine genes on cancer vaccine therapy using genetically modified dendritic cells expressing tumor-associated antigen. Int J Oncol 28:947–953
18. Svane IM, Nikolajsen K, Walter MR et al (2006) Characterization of monocyte-derived dendritic cells maturated with IFN-alpha. Scand J Immunol 63:217–222
19. Kurotaki T, Tamura Y, Ueda G et al (2007) Efficient cross-presentation by heat shock protein 90-peptide complex-loaded dendritic cells via an endosomal pathway. J Immunol 179:1803–1813
20. Kumagai Y, Takeuchi O, Akira S (2008) TLR9 as a key receptor for the recognition of DNA. Adv Drug Deliv Rev 60:795–804

Cep55/c10orf3, a Tumor Antigen Derived From a Centrosome Residing Protein in Breast Carcinoma

Satoko Inoda,† Yoshihiko Hirohashi,* Toshihiko Torigoe,* Munehide Nakatsugawa,* Kenji Kiriyama,* Emiri Nakazawa,‡ Kenji Harada,§ Hideo Takasu,§ Yasuaki Tamura,* Kenjiro Kamiguchi,* Hiroko Asanuma,‖ Tetsuhiro Tsuruma,† Takeshi Terui,¶ Kunihiko Ishitani,¶ Tosei Ohmura,† Qiang Wang,# Mark I. Greene,# Tadashi Hasegawa,‖ Koichi Hirata,† and Noriyuki Sato**

Summary: Identification of tumor-associated antigens may facilitate vaccination strategies to treat patients with malignant diseases. We have found that the centrosomal protein, Cep55/c10orf3 acts as a novel breast carcinoma-associated tumor-associated antigen. Cep55/c10orf3 mRNA was detectable in a wide variety of tumor cell lines. Expression was barely detectable in normal tissues except for testis and thymus. Moreover, Cep55/c10orf3 protein could be detected by a monoclonal anti-Cep55/c10orf3 antibody (#11-55) in 69.8% of breast carcinoma, 25% of colorectal carcinoma, and 57.8% of lung carcinoma tissues. The expression of Cep55/c10orf3 protein did not show any relationship with the hormone receptors such as estrogen receptor and progesterone receptor or expression patterns of $p185^{HER2/neu}$. We designed 11 peptides which displayed a human leukocyte antigen-A24 binding motif. One Cep55/c10orf3-peptide, Cep55/c10orf3_193(10) (VYVKGLLAKI), induced cytotoxic T lymphocytes (CTLs) in 3 of 3 patients with Cep55/c10orf3 (#11-55)-positive breast carcinoma. A Cep55/c10orf3_193(10)-specific CTL clone could also recognize Cep55/c10orf3 (+) displayed on human leukocyte antigen-A24 (+) cancer cell lines. These data indicate that Cep55/c10orf3 peptides were naturally presented by breast cancer cells and can cause CTL clonal expansion in vivo. Monoclonal antibody #11-55 and the Cep55/c10orf3_193(10) peptides may be useful as part of a therapeutic strategy for hormonal therapy or anti-$p185^{HER2/neu}$ monoclonal antibody therapy-resistant breast carcinoma patients.

Key Words: tumor antigen, breast carcinoma, HLA-A24, CTL, Cep55/c10orf3

(*J Immunother* 2009;32:474–485)

Breast carcinoma is a common malignancy, with an annual incidence of 180,000 and annual death rate of 40,000 in the United States (National Cancer Center Home page). Treatment of this malignancy has progressed, and includes surgical resection, chemotherapy, radiation therapy, hormone therapy, and targeted therapy directed at $p185^{Her2/neu}$ epitopes. Prognosis is improving. However, standard therapy has limited effects against advanced and metastatic cases. A potential systemic treatment for breast carcinoma is immunotherapy, particularly peptide vaccine therapy targeting tumor-associated antigens (TAAs).

Efforts have been made to identify novel TAAs selectively expressed on tumor cells that could represent optimal targets for cytolytic immune responses.[1–3] Initially, TAAs were identified using melanoma patients' peripheral blood mononuclear cells (PBMCs) or tumor-infiltrating lymphocytes. However, this immunotherapeutic approach has had limited success owing to problems related to the specificity, distribution of the antigens among histologically different tumors, and the frequency of expression and immunogenicity of the melanoma TAA.

The centrosome is the principal microtubule organizing center of the mammalian cell, consisting of a pair of barrel-shaped microtubule assemblies that are nonidentical and are referred to as the mother and daughter centrioles.[4,5] Centrioles are surrounded by pericentriolar material that consists of a matrix of predominantly coiled-coil proteins. The centrosome is structurally and functionally regulated in a cell cycle-dependent manner to form the bipolar spindle that guides the proper segregation of replicated chromosomes into 2 daughter cells. Defects in the number, structure, or the function of centrosomes can generate monopolar or multipolar mitotic spindles and cytokinesis defects, resulting in aneuploidy and chromosome instability, which are common characteristics of tumor cells. Thus, abnormal centrosome constituents may be exploited as the therapeutic target for malignantly transformed or

Received for publication November 6, 2008; accepted February 11, 2009.

From the Departments of *Pathology; †Surgery First; ‖Surgical Pathology, Sapporo Medical University, School of Medicine, Chuo-Ku; ‡Department of Cancer Diagnosis, Cancer Diagnosis Laboratory, JST Innovation Plaza Hokkaido, Japan Science and Technology Agency; ¶Department of Surgery, Higashi Sapporo Hospital, Shiroishi-ku, Sapporo; §Pharmaceutical Division, Dainippon Sumitomo Pharma Co, Ltd, Osaka, Japan; and #Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA.

Financial Disclosure: All authors have declared there are no financial conflicts of interest in regards to this work.

This study was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology in Japan.

Satoko Inoda, Yoshihiko Hirohashi, Toshihiko Torigoe, and Noriyuki Sato. designed the research and analyzed the data. Satoko Inoda performed most experiments. Kenji Harada and Hideo Takasu performed microarray analysis. Satoko Inoda, Yoshihiko Hirohashi, Toshihiko Torigoe, Mark I. Greene, and Noriyuki Sato prepared the manuscript.

Reprints: Yoshihiko Hirohashi, Department of Pathology, Sapporo Medical University, School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan (e-mail: hirohash@sapmed.ac.jp).

dysplastic cells. In this context, we hypothesized that TAAs derived from constituents of abnormally expressed centrosomal constituents might be recognized by the immune system and serve as targets of cancer immunotherapy.

We performed screening with a gene chip microarray and isolated about 30 candidate genes expressed in several cancerous tissues which were not detected in a series of normal adult tissues. One of the TAA candidates proved to be a centrosomal protein, Cep55 (centrosome protein 55kd)/c10orf3. As described previously,[6] the localization of Cep55/c10orf3 dramatically changes through the cell cycle. The protein is located in the centrosome during the interphase, dissociates from the centrosome in the M phase, and condenses to the midbody during cytokinesis. Cep55/c10orf3 expression could be detected in several malignant cell lines by the reverse-transcription polymerase chain reaction (RT-PCR) method. To analyze Cep55/c10orf3 protein expression in cancerous tissues, we generated an anti-Cep55/c10orf3 monoclonal antibody (mAb), clone #11-55, and performed immunohistochemical staining of surgically resected specimens. Cep55/c10orf3 could be detected in 69.8% of breast carcinoma, 58.7% of lung carcinoma, and 25% of colorectal carcinoma tissues, but not in normal tissues at the protein level, indicating that it represents an immunogenic protein and may represent a target for cancer immunotherapy.

To assess the immunogenicity of Cep55/c10orf3, we synthesized 11 Cep55/c10orf3-derived candidate peptides carrying the human leukocyte antigen (HLA)-A*2402 binding motif and identified one of these peptides, Cep55/c10orf_193(10), as an immunogenic peptide capable of inducing cytotoxic T lymphocytes (CTL) in the context of HLA-A*2402 (+) cancer patients' PBMCs. We observed cytotoxic reactivity for the Cep55/c10orf3_193(10) peptide in Cep55/c10orf3-positive breast cancer patients in a small number of patients studied. Moreover, the anti-Cep55/c10orf3_193(10) CTL clone could recognize HLA-A*2402 (+) and Cep55/c10orf3 (+) cancer cell lines in an HLA-A*2402-restricted fashion. We propose that the Cep55/c10orf3_193(10) peptide and mAb #11-55 combination may be useful in breast cancer immunotherapy.

MATERIALS AND METHODS

Cell Lines

Breast carcinoma cell lines HMC1 and HMC2, lung adenocarcinoma cell lines LHK-2 and LNY-1, and pancreas adenocarcinoma cell lines PUN and HIP were established in our laboratory. Breast carcinoma cell lines MCF7 and SKBr3 (HLA-A*0201/0301, B*1402/4001, and Cw*0304/0802), lung adenocarcinoma cell lines A549 and Lc817, erythroleukemia cell line K562, and human embryonal kidney cell line 293 T were purchased from ATCC. Colon adenocarcinoma cell lines HCT116 (HLA-A*0101/0201, B*4501, and C*0501/0701), HCT15, Colo205, and Sw480 (HLA-A*0201/2402, B*0702/1508, and Cw*0702/0704) and pancreas adenocarcinoma cell lines BxPC3, CFPAC, Hs766 T, Panc-1, and Su8686 were kind gifts from Prof Kozoh Imai (Sapporo, Japan). Esophageal squamous cell carcinoma cell line KE-4, gallbladder adenocarcinoma cell lines KMG-A, and colon adenocarcinoma cell line KM12LM were kind gifts from Prof Kyogo Itoh (Kurune, Japan). Lung adenocarcinoma cell lines 1-87, LK79, and LC-65 and lung squamous cell carcinoma cell line Sq-1 were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan). Epstein-Barr virus (EBV) transformed B-cell line LG2-EBV was a kind gift from Dr Benoit J. Van den Eynde (Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium). All cell lines except K562 and LG2-EBV were cultured in Dulbecco modified Eagle medium (Sigma Chemical Co, St Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). K562 and LG2-EBV were cultured in Roswell park memorial institute (RPMI)-1640 (Sigma) medium supplemented with 10% FBS. HLA-A*2402 stably transfected transporter associated with antigen processing-deficient cell line T2A-A*2402 cell was a kind gift from Dr K. Kuzushima (Aichi Cancer Research Institute, Nagoya, Japan) and was cultured in RPMI-1640 supplemented with 10% FBS and 0.8 mg/mL of G418 (Invitrogen). HCT116-A*2402, a stable transfectant of HCT116 cells with HLA-A*2402 cDNA, was cultured in Dulbecco modified Eagle medium supplemented with 10% FBS and 1 μg/mL puromycin (Sigma).

RT-PCR Analysis of Cep55/c10orf3

Total RNAs from cultured cell lines were isolated with an RNeasy Mini Kit (QIAGEN). Complement DNA (cDNA) were synthesized from 2 μg of total RNA by reverse transcription using *Superscript II reverse transferase* (Invitrogen) and oligo (dT) primer according to the manufacturer's protocol. A cDNA panel for a set of normal human adult tissues was purchased (Clontech). PCR amplification was performed in 20 μL of PCR mixture containing 1 μL of cDNA mixture, 0.5 μL of *Taq* DNA polymerase (QIAGEN), and 4 pmol of primers. The PCR mixture was initially incubated at 98°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Primer pairs used for RT-PCR analysis were 5′-TGAGTTTGCCATCACAGAGC-3′ and 5′-TTGCTTGCTGGTGCATTAAC-3′ as forward and reverse primers, respectively, with an expected PCR product size of 521 bps. As an internal control, glyceraldehyde-3–phosphate dehydrogenase was detected by using forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and a reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′ with an expected PCR product of 452 bps. The PCR products were visualized with ethidium bromide staining under ultraviolet light after electrophoresis on 1.2% agarose gel. Nucleotide sequences of the PCR products were confirmed by direct sequencing.

Construction of Cep55/c10orf3 Plasmids

Full-length Cep55/c10orf3 cDNA was amplified from cDNA of adenocarcinoma cell line LHK-2 with PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, JAPAN). The primer pair was 5′-CGCGGATCCATGTCTTCCA GAAGTACCAAA-3′ as a forward primer and 5′-CCGCTCGAGCTTTGAAACAGTATTCCACATG-3′ as a reverse primer (underlines indicate BamHI and XhoI recognition sites, respectively). The PCR product was inserted into pcDNA3.1 expression vector (Invitrogen) fused with FLAG-tag with BamHI and XhoI sites. The cDNA sequence was confirmed by direct sequence, and proved to be completely identical as reported previously.[6] For the construct of protein expression, *BamHI* and *XhoI* digested Cep55/c10orf3 cDNA was inserted into pQE30 (QIAGEN) vector, which allows the expression of recombinant

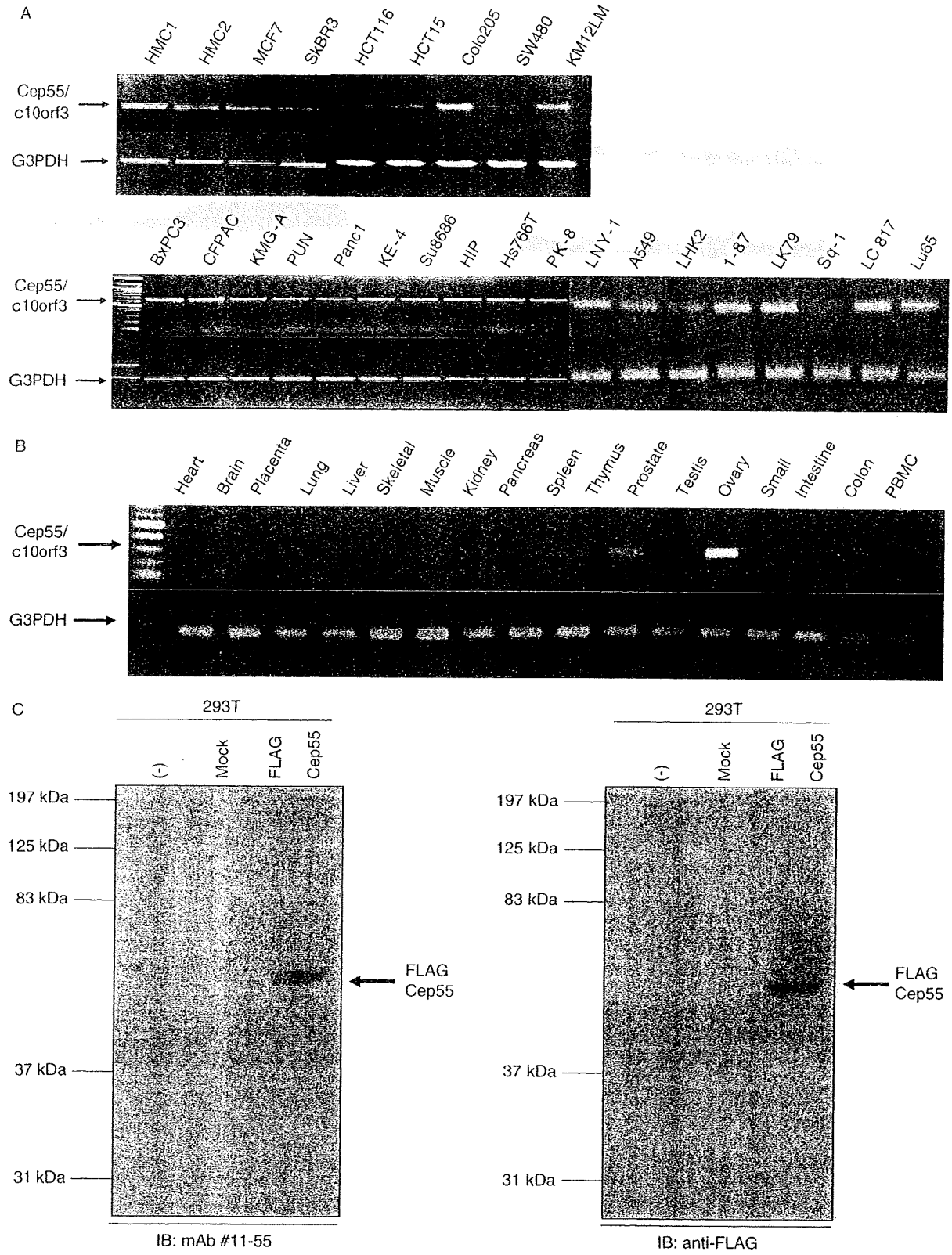


FIGURE 1. Continued.

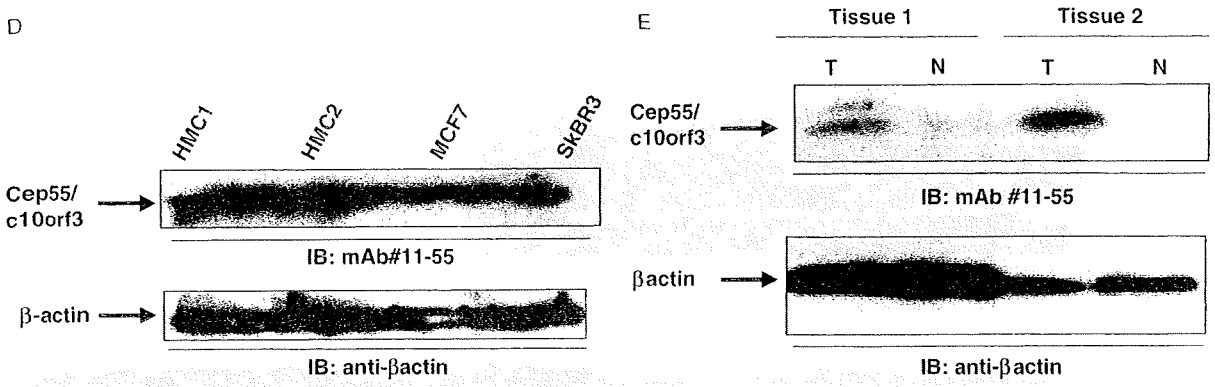


FIGURE 1. Expression profiles of Cep55/c10orf3 mRNA and protein in normal tissues, cancer cell lines, and cancer tissues. A, Cep55/c10orf3 mRNA expression in normal tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, and large intestine, and peripheral blood mononuclear cells were assessed with RT-PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase expression was detected as an internal control. Cep55/c10orf3 mRNA could be detected in the testis and in the thymus at low level. B, Expression of Cep55/c10orf3 breast carcinoma (HMC1, HMC2, MCF7, and SkBR3), colorectal carcinoma (HCT116, HCT15, Colo205, SW480, and KM12LM), pancreatic carcinoma (BxPC3, CFPAC, Panc-1, Su8686, HIP, and Hs766 T), gallbladder carcinoma (KMG-A), esophageal carcinoma (KE4), and lung carcinoma (LNY-1, A549, LHK2, 1-87, LK79, Sq-1, Lc817, and Lu65) were assessed with RT-PCR analysis. Cep55/c10orf3 mRNA was detectable in all the cell lines examined. C, Establishment of anti-Cep55/c10orf3–specific mAb (#11 to 55). The specificity of mAb #11 to 55 was evaluated with Western blotting using FLAG-fused Cep55/c10orf3 overexpressed 293 T cells. Anti-FLAG mAb (M2) was used for positive control. D, Cep55/c10orf3 protein expression in breast carcinoma cell lines were examined with Western blot assay with mAb #11 to 55. Cep55/c10orf3 could be detected in all the breast carcinoma cell lines. E, Cep55/c10orf3 mRNA expression in surgically resected breast carcinoma tissues were assessed with Western blot assay. T exhibits cancerous tissue and N exhibits the normal counterpart tissue from same patient. Cep55/c10orf3 protein were detectable in only cancerous tissues. mAb indicates monoclonal antibody; RT-PCR, reverse-transcription polymerase chain reaction.

proteins with an NH_2-terminal histone (His) 6 tag, at the BamHI and SalI sites.

Cep55/c10orf3 Protein Production and Purification

A pQE30-Cep55/c10orf3 construct was transformed into *Escherichia coli* strain M15 (QIAGEN). His6 tag-fused Cep55/c10orf3 protein was induced with 1 mM isopropyl-â-thiogalactopyranoside for 4 hours at 30°C. Cells were lysed in lysis buffer [6 M guanidine hydrochloride, 20 mM HEPES (pH 8.0), and 50 mM NaCl]. After removing the debris, purification was carried out with nickel-nitrilotriacetic acid resin (Qiagen). The protein was eluted with elution buffer [8 M urea, 20 mM HEPES (pH 8.0), 50 mM NaCl, and 250 mM imidazole). The eluates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Coomassie Blue staining, and Western blotting. Western blotting was carried out according to standard protocols using a horseradish peroxide-conjugated anti-His antibody (Invitrogen).

Establishment of mAb

Cep55/c10orf3 recombinant protein (100 μg) was used for immunization of the BALB/c mice by intraperitoneal injection 4 times at 2-week intervals. One week after the last injection, the spleen cells were collected and fused with the NS-1 mouse myeloma cell line at 4:1 ratio. Screening was performed with enzymes-linked immunosorbent assay using recombinant Cep55/c10orf3 protein and Western blotting.

Immunohistochemical Analysis of Cep55/c10orf3 Expression in Tissue Sections

Immunohistochemical staining was performed with formalin-fixed paraffin-embedded sections of surgically resected tumor specimens of breast cancers. Four micrometer-thick sections were deparaffinized and blocked with 1% nonfat dry milk in phosphate buffered-saline (PBS) (pH 7.4), then the sections were reacted with diluted monoclonal anti-Cep55/c10orf3 antibody for 1 hour, followed by incubation with biotinylated goat anti-rabbit IgG (Nichirei, Tokyo, Japan) for 30 minutes. Subsequently, the sections were stained with streptavidin-biotin complex (Nichirei), followed by incubation with 3,3'-diaminobenzidine, used as chromogen, and counterstaining with hematoxylin.

Western Blotting

Cultured cells were washed in ice cold PBS, lysed by incubation on ice in a lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, and protease inhibitor cocktail; Complete, Roche Diagnostics, Inc, Basel, Switzerland]. The whole-cell lysates were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes (BIO-RAD). After blocking with 5% nonfat dry milk, the membranes were incubated with the anti-Cep55/c10orf3 mAb washed 3 times and incubated with affinity-purified antibody peroxidase labeled goat anti-mouse IgG + IgM (heavy chain + light chain) human serum absorbed peroxidase (2000 ×) (KPL, Gaithersburg, MD). Finally, the membrane was visualized with ECL Western Blotting Detection Reagent (Amersham Biosciences Corp, Piscataway, NJ) according to the manufacturer's protocol.

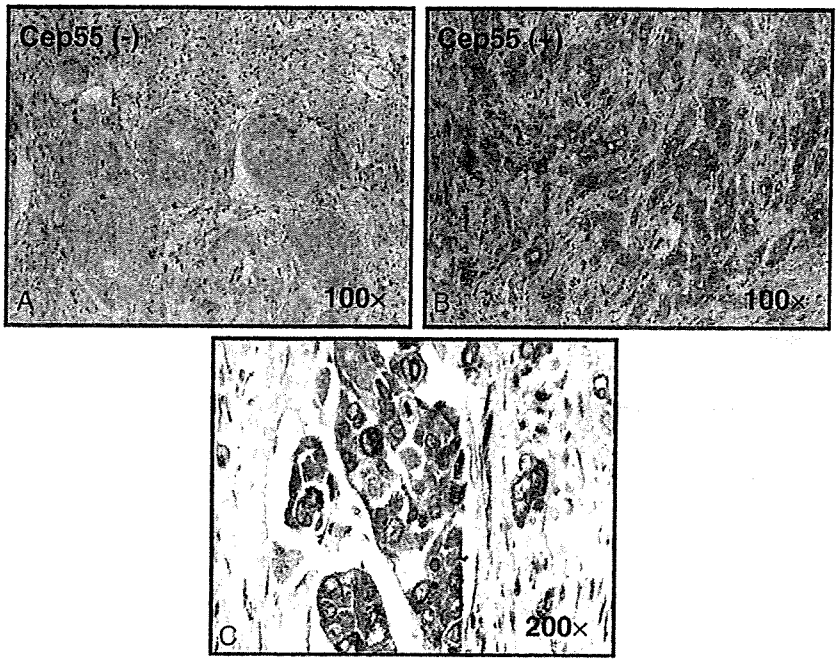


FIGURE 2. Immunohistochemical staining of Cep55/c10orf3 in paraffin-embedded tissue specimens of invasive breast cancer. A, Immunohistochemical staining of Cep55/c10orf3 negative breast carcinoma case. B, Cep55/c10orf3 positive breast carcinoma case. Magnification, 100 ×. C, Representative photographs of invasive breast cancer tissue which can be contrasted to normal lesion with tumor lesion. Magnification, 200 ×.

Synthetic Peptides

Eleven Cep55/c10orf3-derived peptides, EBV LMP2-derived peptide,[7] human immunodeficiency virus envelope-derived peptide,[8] ovalbumin-derived mouse H2-K^d restricted (SL8) peptide (SIINFEKL), and EBV LMP2 HLA-A68 restricted peptide were purchased from Sigma Genosis (Ishikari, Japan). Peptides were dissolved in dimethyl sulfoxide and stored at −80°C before use.

Peptide Binding Assay

Peptide binding affinity to HLA-A24 was assessed by HLA-A24 stabilization assay as described previously.[9] After incubation of T2-A24 transporter associated with antigen processing-deficient cells in culture medium at 26°C for 18 hours, cells (2×10^6) were washed with ice cold PBS and suspended in 1 mL of Opti-MEM (Invitrogen) with or without 100 μg of peptide, followed by incubation at 26°C for 3 hours, and then at 37°C for 3 hours. After washing with ice cold PBS, the cells were stained with an anti-HLA-A24 mAb (c7709A2.6, kindly provided by Dr P. G. Coulie, Ludwig Institute for Cancer Research, Brussels Branch) at 4°C for 30 minutes, followed by incubation with fluorescein isothiocyanate-conjugated rabbit antimouse IgG + IgM (KPL) at 4°C for 30 minutes. The cells were then analyzed

TABLE 1. Summary of Cep55/c10orf3 Candidate Peptides and Binding Affinity for HLA-A24 Molecule

Peptide	Position Number	Sequence	% MFI Increase
Cep55/c10or3_169(10)	169-178	EMEIQLKDAL	4
Cep55/c10orf3_193(10)	193-202	VYVKGLLAKI	83
Cep55/c10orf3_355(9)	355-363	QMQACTLDF	43
Cep55/c10orf3_446(10)	446-455	QYPATEHRDL	10
Cep55/c10orf3_74(11)	74-84	AYQLTEKDKEI	22
Cep55/c10orf3_94(11)	94-104	RYSTTALLEQL	104
Cep55/c10orf3_402(11)	402-412	EFAITEPLVTF	27
Cep55/c10orf3_186(13)	186-198	VYDQQREVYVKGL	10
Cep55/c10orf3_227(14)	227-240	GYLQEEKQKCYNDL	43
Cep55/c10orf3_268(12)	268-279	KYEETQKEVHNL	20
Cep55/c10orf3_283(12)	283-294	LYSQRRADVQHL	52
EBV-A24		TYGPVFMSL	117
HIV-A24		RYLRDQQLLGI	65
EBV-A68		FTASVSTVV	9
SL8		SIINFEKL	13

EBV indicates Epstein-Barr virus; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; MFI, mean fluorescent intensity.

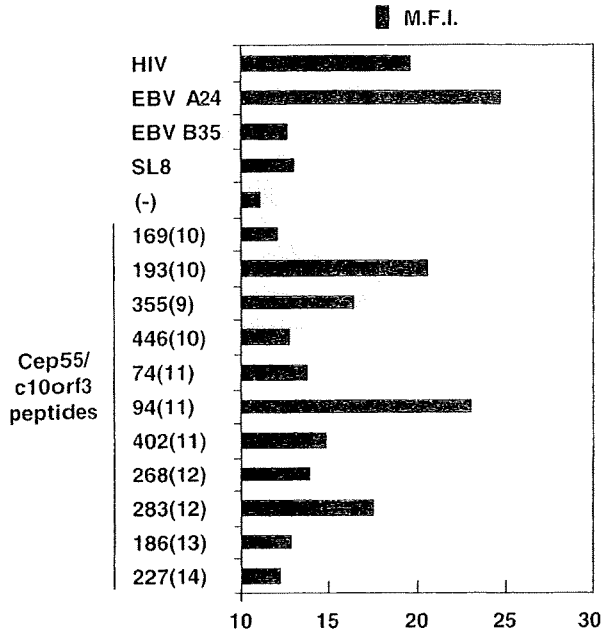


FIGURE 3. Binding assay of synthetic Cep55/c10orf3-derived peptides to HLA-A*2402 peptide binding assay was carried out using T2-A24 transporter associated with antigen processing-deficient cells. After incubation of T2-A24 cells in 26°C for 18 hours, synthetic peptides were added onto the cell culture and cultured at 26°C for 3 hours then at 37°C for 3 hours. The cell surface HLA-A24 expression level was evaluated with FACScan analysis. Cep55/c10orf3_193(10), Cep55/c10orf3_355(9), Cep55/c10orf3_94(11), and Cep55/c10orf3_283(12) peptides showed higher binding affinity to HLA-A24 molecule compared with negative control peptides (EBV B35 and SL8). EBV indicates Epstein-Barr virus; HLA, human leukocyte antigen.

with a FACScan (Becton Dickinson, Mountain View, CA). Binding affinity was evaluated by comparing the mean fluorescence intensity of HLA-A24 expression.

Preparation of Antigen Presenting Cell From PBMCs

PBMCs were isolated by standard density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). For the generation of dendritic cells (DCs), CD14-positive cells were separated by the magnetic cell separation system (Miltenyi Boitech, Bergish Blabach, Germany) according to the manufacturer's instructions. CD14-positive cells were incubated in AIM-V medium (Life Technologies, Inc) supplemented with IL-4 (1000 IU/mL) (R&D systems, Mineapolis) and granulocyte macrophage colony-stimulating factor (1000 IU/mL) (a kind gift from Novartis Pharmaceuticals, Basel, Switzerland) at 37°C for 5 days. On day 5, tumor necrosis factor α (R&D systems) (10 ng/mL) was added to the culture for the maturation of DCs. On day 7, the mature DCs were harvested and used for stimulation of CD8 T cells. Phytohemagglutinin (PHA)-stimulated blasts were obtained as described previously.[10]

CTL Induction and Establishment of CTL Clone

CTL induction was performed according to a procedure described previously with a slight modification.[10,11] Briefly, DCs were incubated at room temperature for 2 hours with peptides (50 μg/mL) then, DCs were irradiated (100 Gy) and washed with AIM-V medium. On day 0, $CD8^+$ T cells were stimulated with 1×10^5 peptide-pulsed DCs in 2 mL of AIM-V supplemented with interleukins (IL)-7 (10 ng/mL) (R&D systems). On day 7, 5×10^5 peptide-pulsed PHA-blasts were irradiated and added to the culture. On day 8, IL-2 [kind gifts from Takeda Pharmaceutical (Osaka, Japan)] was added to each well at a concentration of 20 IU/mL. The peptide stimulations using PHA-blasts were performed every 7 days. During CTL induction, cells were fed with fresh AIM-V medium supplemented with IL-2 (20 IU/mL) every 3 to 4 days. On day 15, $CD8^+$ T cell reactivity was assessed by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. On day 28, the cytotoxic activity of T cells was assessed by conventional 6-hour ^{51}Cr-release assay as described previously.[12] To obtain CTL clones, standard limiting dilution was performed as described previously.[13]

IFN-γ ELISPOT Assay

Multiscreen 96-well plates (Millipore, Bedford, MA) were coated with 100 μL/well of 5 μg/mL anti-IFN-γ capture antibody (PharMingen, San Diego, CA) in PBS at 4°C overnight. Plates were washed once with 200 μL/well complete RPMI 1640 and blocked with 200 μL/well complete RPMI 1640 at room temperature for 2 hours. Then 2×10^4 CTLs were incubated with 5×10^4/well T2-A24 cells pulsed with Cep55/c10orf3 specific peptide or control peptides (5 μg/mL) or K562 cells. After 40-hour incubation at 37°C, IFN-γ spots were developed and counted as per the manufacturer's instructions.

RESULTS

Cep55/c10orf3 Expression in Several Carcinoma Cell Lines and Normal Tissues

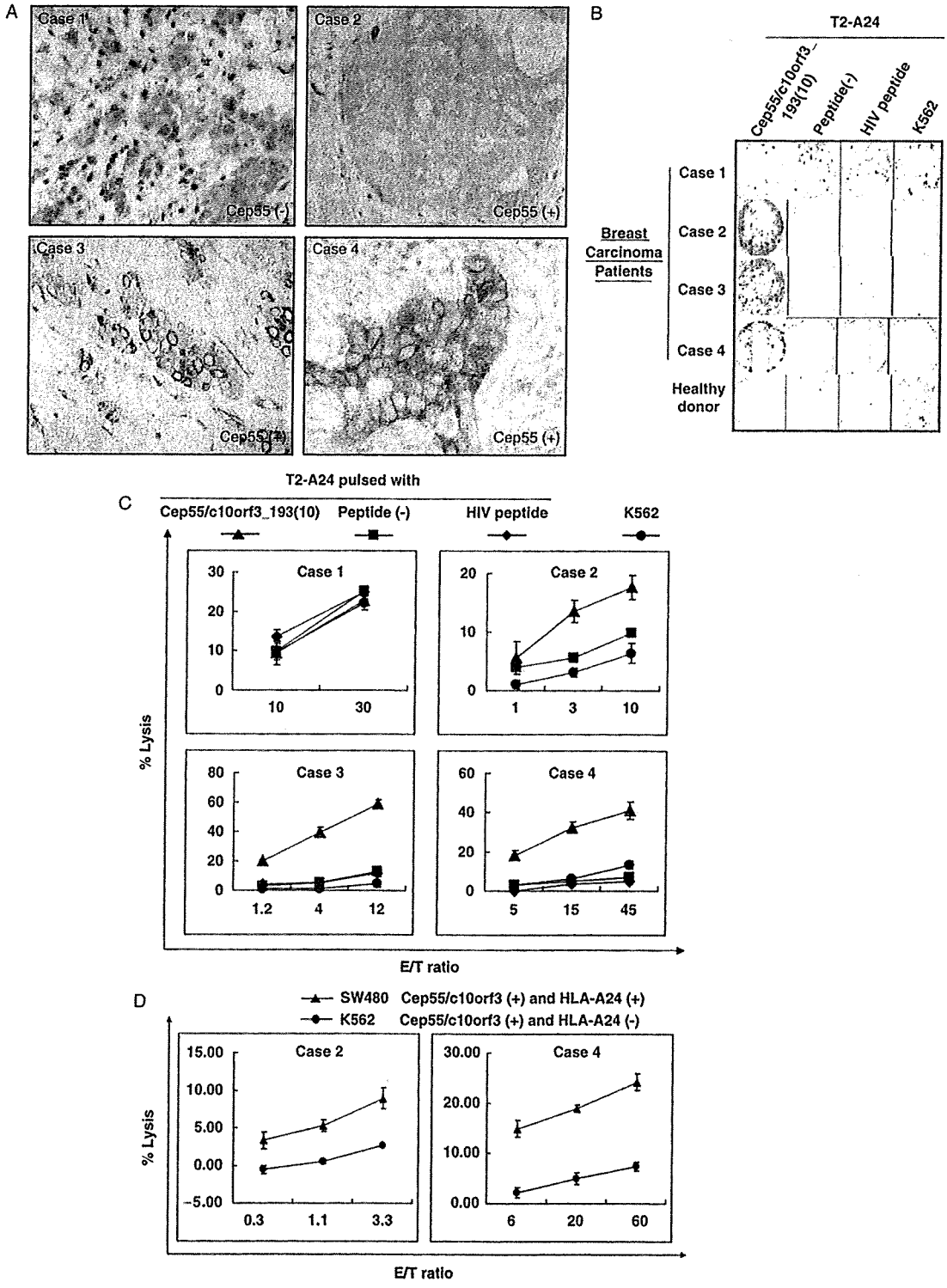
Novel TAAs are essential for the establishment of cancer vaccine therapy. To identify novel TAAs, we initially screened a gene chip microarray expression profile database of more than 700 malignant tissues including breast, colon, pancreas, renal cell, lung, and gastric carcinomas. We isolated 30 over expressed gene products as TAA candi-

TABLE 2. Summary of the HLA-A24 (+) Cancer Patients' Profiles

Case	Stage	Histopathology	Immunohistochemistry	CTL (+) Peptide	ELISPOT Assay	^{51}Cr-release Assay
1	IIIA	Invasive ductal carcinoma	(−)	(−)	(−)	(−)
2	I	Ductal carcinoma in situ	(+)	193(10)	(+)	(+)
3	IIA	Invasive ductal carcinoma	(+)	193(10)	(+)	(+)
4	I	Invasive ductal carcinoma	(+)	193(10)	(+)	(+)

CTL indicates cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunospot; HLA, human leukocyte antigen.

dates (data not shown). The mRNA expression profiles of these genes were confirmed by RT-PCR, and one of the breast cancer-associated antigens was identified as Cep55/c10orf3, previously described as one of the centrosome proteins essential for cytokinesis.[6] As shown in Figure 1A, Cep55/c10orf3 mRNA was overexpressed in various cancer



cell lines, including breast, colorectal, pancreas, esophageal, gallbladder, and lung carcinomas. In contrast, Cep55/c10orf3 mRNA was expressed only in the thymus and testis, and at a very low levels in the spleen and placenta, whereas the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* mRNA can be detected in all tissues (Fig. 1B). Sakai et al[14] also reported that Cep55/c10orf3 was overexpressed in colorectal carcinoma specimens, which support the notion that Cep55/c10orf3 is overexpressed in cancerous tissues. To confirm the expression of Cep55/c10orf3 at the protein level, we established novel anti-Cep55/c10orf3 mAb clone #11-55 (Fig. 1C). With mAb #11-55, we could detect FLAG-fused Cep55/c10orf3 protein with Western blotting. As shown in Figure 1D, breast cancer cell lines showed the expression of Cep55/c10orf3 protein. For the evaluation of Cep55/c10orf3 protein expression in primary cancer tissues, surgically resected breast carcinoma specimens were examined with Western blotting. As shown in Figure 1E, Cep55/c10orf3 expression could be detected in cancerous tissues, but not in noncancerous counterpart tissues. Therefore, the cep55/c10orf3 expression was highly specific to tumor tissues, including breast cancer.

Immunohistochemical Detection of Cep55/c10orf3 in Cancer Tissues

Formalin-fixed, paraffin-embedded breast, colorectal and lung carcinoma specimens were analyzed for the expression of Cep55/c10orf3 by immunohistochemical staining with mAb #11-55. Among the 53 breast cancer specimens, Cep55/c10orf3 protein was positively stained in the cytoplasm of tumor cells in 37 cases (69.8%). Interestingly, Cep55/c10orf3 expression showed no correlations with the levels of the hormone receptors estrogen (ER) and progesterone (PgR) or the protooncogene *HER2*. In the lung and colorectal carcinoma specimens, Cep55/c10orf3 protein could be detected in 17 of 46 cases (58.7%) and 6 of 24 cases (25.0%), respectively. Figure 2 shows the representative staining patterns of the breast cancer specimens, including (a) a Cep55/c10orf3-negative case and (b) a Cep55/c10orf3-positive case. Cep55/c10orf3 protein showed a homogenous staining pattern in the cytoplasm of cancer cells in a higher magnification image (Fig. 2C). These data indicate that Cep55/c10orf3 protein was expressed in breast carcinoma tissues at a high rate regardless of the expression levels of ER, PgR, or *HER2*. Thus, Cpe55/c10orf3 might be a useful target for both ER-, PgR-, and HER2-positive and also HER2-negative breast carcinoma cases.

Binding Assay of Synthetic Cep55/c10orf3-derived Peptides to HLA-A*2402

As Cep55/c10orf3 shows cancer-specific expression profiles in various cancer cell lines, we hypothesized that Cep55/c10orf3 could be a target of CTLs for cancer immunotherapy. To evaluate the immunogenicity of Cep55/c10orf3 as a target of CTLs, we focused on the HLA-A*2402 allele, which is a frequent HLA allele in Japanese and in other ethnics. Initially, we designed Cep55/c10orf3 candidate peptides carrying HLA-A24 binding motifs as follows: (1) 9-mer to 14-mer length, (2) tyrosine, phenylalanine, methionine, or tryptophan at the second position, and (3) leucine, isoleucine, phenylalanine, or methionine at the COOH-terminal residue as described previously with minor variations.[15] Ninemer and 10-mer peptides are commonly presented with the HLA-A24 molecule, but peptides longer than 11-mer also have potential binding ability to HLA class I molecules,[16,17] so we included peptides longer than 11-mer as candidate CTL targets. As shown in Table 1, 11 candidate peptides with length of 9-mer and 14-mer were synthesized. The HLA-A24 binding ability was assessed by HLA-A24 binding assay. The Cep55/c10orf3_193(10), Cep55/c10orf3_355(9), Cep55/c10orf3_94(11), and Cep55/c10orf3_283(12) peptides showed significantly higher binding affinity to HLA-A24 molecules than the negative control peptides (Fig. 3).

CTL Induction From PBMCs of HLA-A*2402+ Breast Carcinoma Patients

To evaluate the immunogenicity of Cep55/c10orf3-derived peptides, we performed ELISPOT assay and ^{51}Cr-release assay as follows. To identify the highly immunogenic peptides among the 11 candidates, we started with a peptide cocktail for the induction of CTLs. PBMCs were collected from the blood of HLA-A24 (+) breast cancer patients, and the CD8 T cells were stimulated several times with antigen-presenting cells pulsed with the 11-peptides cocktail as described in Materials and Methods. After several rounds of stimulation, the CD8 T cells were assessed with ELISPOT assay to evaluate the peptide-specific reactivity (Table 2).

The Cep55/c10orf3 protein expression in surgically resected specimens is shown in Figure 4A. Case 1 was Cep55/c10orf3 negative and cases 2, 3, and 4 were Cep55/c10orf3 positive. As shown in Figure4B, we could detect specific IFN-γ secretion with the Cep55/c10orf3_193(10) peptide in Cep55/c10orf3 strongly positive breast cancer patient cases 2, 3, and 4, but not in Cep55/c10orf3 negative breast carcinoma case 1 or healthy volunteer. To confirm the peptide-specific cytotoxic activity, we also performed

FIGURE 4. Induction of HLA-A24-restricted CTLs from PBMCs of HLA-A24-positive breast carcinoma patients' PBMCs. A, Immunohistochemical staining of breast carcinoma patient cases 1 to 4 with monoclonal antibody #11 to 55. Case 2, 3, and 4 showed strong positive staining, but, case 1 showed negative staining. B, CTLs were induced by stimulating HLA-A24 (+) breast carcinoma patients' PBMCs with Cep55/c10orf3 HLA-A24 binding candidate 11 peptides and allyl phenyl carbinols. Peptide-specific reactivity were assessed with interferon-γ enzyme-linked immunospot assay with peptide-pulsed T2-A24 cells. Human immunodeficiency virus (HIV)-derived peptide was used as a negative control. K562 is a natural killer target cell. Case 2, 3, and 4 showed Cep55/c10orf3_193(10) specific reactivity. C, Cytotoxicity of the CTLs from breast carcinoma patients' PBMCs against the peptide-pulsed T2-A24 cells and K562 cells were analyzed ^{51}Cr-release assay at various effector-to-targe (E/T) ratios. Case 2, 3, and 4 showed Cep55/c10orf3_193(10) specific lysis compared with peptide nonpulsed T2-A24, irrelevant peptide pulsed T2-A24, and K562 cells. In contrast, case1 showed Cep55/c10orf3 peptide-specific lysis. D, Cep55/c10orf3_193(10) specific CTLs were assessed the cytotoxic activity against Cep55/c10orf3 (+) and HLA-A24 (+) target cell (SW480). Case 2 and 4 CTLs showed specific lytic activity of SW480 compared with K562 cell as a negative control. CTL indicates cytotoxic T lymphocytes; HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cells.

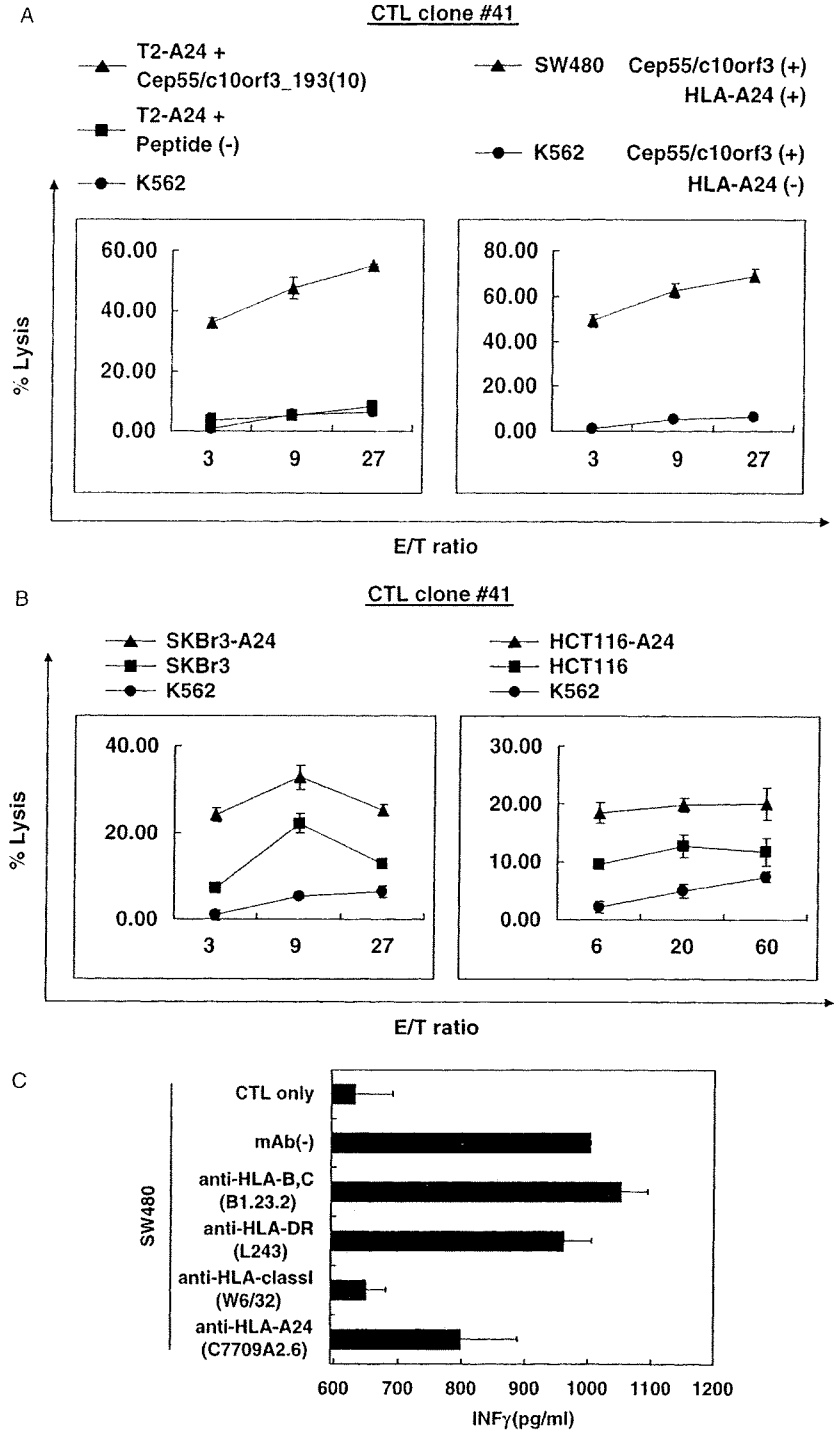


FIGURE 5. Establishment and characterization of Cep55/c10orf3_193(10) specific CTL clone. CTL clone #41 was established from breast carcinoma case 4 CTL. A, Cep55/c10orf3_193(10) peptide-specific lysis was confirmed with ^{51}Cr-release assay. CTL clone #41 lysed Cep55/c10orf3 (+) and HLA-A24 (+) target cell SW480. B, HLA-A24 transfected Cep55/c10orf3 (+) and HLA-A24 (−) cells were used to confirm the HLA-A24 restriction of CTL clone #41. CTL clone #41 lysed HLA-A24 transfected HCT116-A24 and SkBR3-A24 cells. C, Blocking assay with mAbs. CTL clone #41 was assessed with IFN-γ ELISA assay using several mAbs. IFN-γ secretion was inhibited with anti-HLA-class I mAb (W6/32) and anti-HLA-A24 mAb (c7709A2.6), but not by anti HLA-B, C mAb (B1.23.2) and anti-HLA-class II DR mAb (L243). All antibodies were used at a final concentration of 20 μg/mL each. CTL indicates cytotoxic T lymphocytes; HLA, human leukocyte antigen; IFN, interferon; mAb, monoclonal antibody.

TABLE 3. Summary of the Breast Carcinoma Associated Antigens and CTL Induction Rates From Breast Carcinoma Patients

Gene	Patients	Peptide	HLA	% CTL Positive	References
Cep55/c10orf3	Cep55/c10orf3 (+) breast carcinoma	Cep55/c10orf3_193 (10)	A24	100	
Survivin	Breast carcinoma	Sur1	A2	33	18
	Breast carcinoma	Sur9	A2	50	18
	Breast carcinoma	Sur20-28	A24	50	19
	Breast carcinoma	Sur46-54	B35	nd	20
	Breast carcinoma	Sur51-59	B35	nd	20
Bcl-XL	Breast carcinoma	Bcl-XL173–182	A2	33	21
TRAG-3	Breast carcinoma	T_37.9	A2	56	22
	Breast carcinoma	T_4	A2	33	22
	Breast carcinoma	T_57.10	A2	44	22
Mammaglobin-A	Breast carcinoma	Mam-A2.1	A2	63	23
	Breast carcinoma	Mam-A2.2	A2	75	23
	Breast carcinoma	Mam-A2.3	A2	63	23
	Breast carcinoma	Mam-A2.4	A2	50	23
	Breast carcinoma	Mam-A2.7	A2	63	23
HER2	Breast carcinoma	HER2 342-350	A24	50	24
	Breast carcinoma	HER2 485-493	A24	63	24
	Breast carcinoma	HER2 553-561	A24	25	24

CTL indicates cytotoxic T lymphocyte; HLA, human leukocyte antigen; nd, not determined.

^{51}Cr-release assay. As shown in Figure 4C, CTLs from HLA-A24 (+) breast cancer patients 2, 3, and 4 showed cytotoxic activity against Cep55/c10orf3_193(10) peptide-pulsed T2-A24 cells in a dose-dependent manner, but not against natural killer target K562 cells or T2-A24 cells that were either not pulsed or pulsed with the irrelevant human immunodeficiency virus peptide.

To confirm the findings of reverse-immunogenetics analysis, it is essential to determine that the peptide-specific CTLs exhibit specific cytotoxic activity against HLA-A24 (+) and Cep55/c10orf3 (+) cancer cells, because some antigenic peptides cannot be naturally presented by HLA molecules. Therefore we performed ^{51}Cr-release assay against HLA-A24 (+) and Cep55/c10orf3 (+) targets with Cep55/c10orf3_193(10)-specific CTLs. As shown in Figure 4D, Cep55/c10orf3_193(10)-specific CTLs from breast cancer patients 2 and 4 recognized HLA-A24 (+) and Cep55/c10orf3 (+) target SW480 cells but did not recognize K562 HLA-A24 (−) cells. These data supported the idea that the endogenously processed Cep55/c10orf3_193(10) peptide could be presented by HLA-A24 molecules. We could not generate such CTLs from the PBMCs of 4 healthy HLA-A24 (+) volunteers (data not shown) and concluded that this peptide was highly immunogenic for Cep55/c10orf3 strongly positive HLA-A24 (+) cancer patients. These data indicate that Cep55/c10orf3_193(10) peptide might be presented and evoke CTL clonal expansion in vivo, and thus be suitable for cancer peptide vaccine therapy.

We established the Cep55/c10orf3_193(10) peptide-specific CTL clone #41 for further analysis. As shown in Figure 5A, clone #41 mediated Cep55/c10orf3_193(10) peptide-specific lysis, and also recognized SW480 HLA-A24 (+) and Cep55/c10orf3 (+) target cells. Furthermore, clone #41 did not recognize HLA-A24 (−) and Cep55/c10orf3 (+) targets SKBr3 or HCT-116 but recognized HLA-A24 transfected SKBr3 and HCT-116 cells (Fig. 5B). These data also indicated that Cep55/c10orf3_193(10) peptide was naturally processed and presented by HLA-A24 molecules.

To confirm the HLA restriction of CTL clone #41, we performed the blocking assay using HLA molecule-specific mAbs followed by IFN-γ enzymes-linked immunosorbent assay. As shown in Figure 5C, the reactivity of CTL clone #41 against SW480 HLA-A24 (+) and Cep55/c10orf3 (+) target cells was inhibited by anti-HLA class I mAb W6/32 and anti-HLA-A24 mAb C7709A2.6. but not by control mAbs B1.23.2 (anti-HLA-B, Cw) and L243 (anti-HLA DR), suggesting that T-cell recognition is HLA-A24 restricted.

Relative Immunogenic Potential of Breast Carcinoma-related Antigenic Peptides

There have been several reports of antigen peptide-specific CTLs from breast carcinoma patients (summarized in Table 3). The CTL induction rates from breast carcinoma patients' PBMCs were from 25% to 75%, and Cep55/c10orf3_193(10) peptide-specific CTLs were detectable in 3 of 4 breast carcinoma patients. The Cep55/c10orf3 CTL induction efficiency is relatively high when compared with other breast carcinoma-related antigens. In addition, it is should be noted that Cep55/c10orf3_193(10)-specific CTLs could be generated only from Cep55/c10orf3-positive cases, with a 100% success rate. These data indicate that Cep55/c10orf3 is a highly immunogenic TAA compared with other breast carcinoma-related antigens. Thus, Cep55/c10orf3 might be an ideal target for breast carcinoma immunotherapy.

DISCUSSION

A rational approach for treating human cancers includes vaccination with tumor specific antigens. CD8-positive T lymphocytes recognize peptides derived from the degradation of endogenous cellular proteins which are presented by major histocompatibility complex class I molecules. Antigenic peptides have been identified by extracting major histocompatibility complex class I-bound peptides from the cancer cell surface, followed by analysis of autologous CTL responses against the peptide-pulsed

target cells. TAAs have been characterized for several types of malignant tumors.[2,3]

However, CTL-based immunotherapy has been disappointing and hampered by the lack of knowledge of the peptide antigens that are involved in inducing immune response to tumors. Reverse immunologic techniques have been used to identify a number of cancer antigen proteins, such as the Bcr-Abl protein in chronic myeloid leukemia,[25] SYT-SSX protein in synovial sarcoma,[26] and the hTERT protein.[27] Although success has been limited with these antigens identification of potent CTL epitopes derived from cancer antigen proteins may ultimately improve therapy of cancers.

In this study we used a relatively standard approach and identified cancer antigens by gene chip microarray screens. We identified 30 TAA candidates that are frequently overexpressed in cancer cells, but not in normal organs. Among TAA candidate genes, we focused on a widely expressed antigen, Cep55/c10orf3, which is localized to the centrosome. The proteins mediate an essential role in cytokinesis.

Cep55/c10orf3 cDNA is transcribed from chromosome 10q23.33 and consists of 9 exons, of which exon 1 is noncoding. The Cep55/c10orf3 protein is composed of 464 amino acid residues and has 3 centrally located coiled-coil domains, a structure commonly found in centrosomal proteins. Fabbro et al[6] reported that Cep55/c10orf3 localizes to the centrosome of the interphase cells. Cep55/c10orf3 relocates to the midbody to mediate an incompletely understood function in mitotic exit and cytokinesis, cells with diminished Cep55/c10orf3 levels undergo cytokinesis failure, leading to aneuploidy and multiple spindle poles. Furthermore, over expression of Cep55/c10orf3 induces aneuploidy, albeit to a lesser extent than Cep55/c10orf3 depletion. Centrosome amplification may be involved in the origin of chromosomal instability during tumor development.

Cep55/c10orf3 is overexpressed in several cancer cell lines, whereas the expression is barely detected in normal tissues by expression-profile analyses using microarrays.[14] In this study, we confirmed Cep55/c10orf3 expression in a variety of cancerous tissues and cell lines at the protein level. To establish the cancer immunotherapy for Cep55/c10orf3, we focused on CTL epitopes restricted to HLA-A*2402, because HLA-A*2402 is frequently expressed in human populations (60% of the Japanese and 17% of the White).

We demonstrate for the first time an HLA-A24–restricted CTL epitopes derived from Cep55/c10orf3, which may serve as a widely applicable antigen for cancer immunotherapy of HLA-A24 positive patients. Anti-Cep55/c10orf3 CTLs can be activated and clonally expanded in vivo with the Cep55/c10orf3 peptide. Thus the Cep55/c10orf3_193(10) peptide might be suitable for Cep55/c10orf3 positive breast cancer patients' immunotherapy. In addition, the anti-Cep55/c10orf3 mAb #11-55 may be used to identify the individuals that are responsive to the anti-Cep55/c10orf3 peptide immunotherapy.

Previous studies have indicated that Cep55/c10orf3 plays an essential role in G2/M phase and is localized to the centrosome at interphase of the cell cycle, and the midbody at cytokinesis. We also detected a small pool of Cep55/c10orf3 protein in the cytoplasm of cancer cells independent of the cell cycle stage (Fig. 2). Moreover, we did not detect Cep55/c10orf3 protein expression in normal epithelial cells at M phase (data not shown). These findings suggest that the expression of Cep55/c10orf3 protein is deregulated in cancer cells, leading to ectopic accumulation of the protein in the cytoplasm. It is conceivable that the aberrant subcellular localization of Cep55/c10orf3 may contribute to the high immunogenicity of the Cep55/c10orf3_193(10) peptide. We noted that a number of other mitotic regulatory proteins, such as the Aurora-A kinase and survivin, also accumulate in the cytosol of cancer cells. It is not clear whether these phenomena are caused via a common mechanism.

Previously, we identified HLA-A24 restricted cancer-related antigenic peptide derived from surviving.[10] Survivin has been reported to localize to the mitotic apparatus, including the kinetochore, mitotic spindle, centrosome, and midbody. Cep55/c10orf3 can also be detected in the centrosome and midbody. The mitotic apparatus undergoes dynamic changes throughout the cell division cycle.

The proteins of the apparatus can become closely associated with the proteasome, which is involved in the breakdown and turnover of the structural and the regulatory proteins. The proteasome also plays a key role in processing the antigenic peptides. Hence the proteasome-enriched mitotic apparatus may provide a source of antigenic peptides and TAAs localized to this domain might be more immunogenic than other TAAs. For example, Hung et al[28] showed that DNA vaccination with centrosome targeting HPV 16 E7 construct caused more powerful CD8 and CD4 T cell reactions. In this respect, our studies provide additional evidence that localization of TAAs to the mitotic apparatus may give rise to higher immunogenicity.

We have generated a specific anti-Cep55/c10orf3 mAb #11-55 and found up-regulation of Cep55/c10orf3 in a large portion of human cancers. In addition, we have identified a novel HLA-A24 restricted antigenic peptide, namely Cep55/c10orf3_193(10), which is derived from Cep55/c10orf3 and modulates CTL-mediated immune response to Cep55/c10orf3 positive cancer cells. Cep55/c10orf3 represents a promising target for cancer immunotherapy and mAb #11-55 may represent a tool to identify cancer patients who are responsive to such treatment.

ACKNOWLEDGMENTS

The authors thank Drs K. Imai, K. Itoh, B.J. van den Eynde, P.G. Coulie, and K. Kuzushima for kindly providing cell lines.

REFERENCES

1. van der Bruggen P, Traversari C, Chomez P et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science.* 1991;254:1643–1647.
2. Boon T, Coulie PG, Van den Eynde BJ, et al. Human T cell responses against melanoma. *Annu Rev Immunol.* 2006;24:175–208.
3. Rosenberg SA. Progress in human tumour immunology and immunotherapy. *Nature.* 2001;411:380–384.
4. Wang Q, Hirohashi Y, Furuuchi K, et al. The centrosome in normal and transformed cells. *DNA Cell Biol.* 2004;23:475–489.
5. Fukasawa K. Oncogenes and tumour suppressors take on centrosomes. *Nat Rev Cancer.* 2007;7:911–924.
6. Fabbro M, Zhou BB, Takahashi M, et al. Cdk1/Erk2- and Plk1-dependent phosphorylation of a centrosome protein,

Cep55, is required for its recruitment to midbody and cytokinesis. *Dev Cell.* 2005;9:477–488.
7. Lee SP, Tierney RJ, Thomas WA, et al. Conserved CTL epitopes within EBV latent membrane protein 2: a potential target for CTL-based tumor therapy. *J Immunol.* 1997;158:3325–3334.
8. Ikeda-Moore Y, Tomiyama H, Miwa K, et al. Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1. *J Immunol.* 1997;159:6242–6252.
9. Nakao M, Shichijo S, Imaizumi T, et al. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. *J Immunol.* 2000;164:2565–2574.
10. Hirohashi Y, Torigoe T, Maeda A, et al. An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. *Clin Cancer Res.* 2002;8:1731–1739.
11. Maeda A, Ohguro H, Nabeta Y, et al. Identification of human antitumor cytotoxic T lymphocytes epitopes of recoverin, a cancer-associated retinopathy antigen, possibly related with a better prognosis in a paraneoplastic syndrome. *Eur J Immunol.* 2001;31:563–572.
12. Sato T, Sato N, Takahashi S, et al. Specific cytotoxicity of a long-term cultured T-cell clone on human autologous mammary cancer cells. *Cancer Res.* 1986;46:4384–4389.
13. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med.* 1995;333:1038–1044.
14. Sakai M, Shimokawa T, Kobayashi T, et al. Elevated expression of C10orf3 (chromosome 10 open reading frame 3) is involved in the growth of human colon tumor. *Oncogene.* 2006;25:480–486.
15. Kondo A, Sidney J, Southwood S, et al. Prominent roles of secondary anchor residues in peptide binding to HLA-A24 human class I molecules. *J Immunol.* 1995;155:4307–4312.
16. Probst-Kepper M, Stroobant V, Kridel R, et al. An alternative open reading frame of the human macrophage colony-stimulating factor gene is independently translated and codes for an antigenic peptide of 14 amino acids recognized by tumor-infiltrating CD8 T lymphocytes. *J Exp Med.* 2001; 193:1189–1198.
17. Tsukahara T, Nabeta Y, Kawaguchi S, et al. Identification of human autologous cytotoxic T-lymphocyte-defined osteosarcoma gene that encodes a transcriptional regulator, papillomavirus binding factor. *Cancer Res.* 2004;64:5442–5448.
18. Andersen MH, Pedersen LO, Capeller B, et al. Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. *Cancer Res.* 2001;61:5964–5968.
19. Andersen MH, Soerensen RB, Becker JC, et al. HLA-A24 and survivin: possibilities in therapeutic vaccination against cancer. *J Transl Med.* 2006;4:38.
20. Reker S, Becker JC, Svane IM, et al. HLA-B35-restricted immune responses against survivin in cancer patients. *Int J Cancer.* 2004;108:937–941.
21. Andersen MH, Reker S, Kvistborg P, et al. Spontaneous immunity against Bcl-xL in cancer patients. *J Immunol.* 2005;175:2709–2714.
22. Meier A, Reker S, Svane IM, et al. Spontaneous T-cell responses against peptides derived from the Taxol resistance-associated gene-3 (TRAG-3) protein in cancer patients. *Cancer Immunol Immunother.* 2005;54:219–228.
23. Jaramillo A, Narayanan K, Campbell LG, et al. Recognition of HLA-A2-restricted mammaglobin-A-derived epitopes by CD8+ cytotoxic T lymphocytes from breast cancer patients. *Breast Cancer Res Treat.* 2004;88:29–41.
24. Azuma K, Shichijo S, Shomura H, et al. Identification of HER2/neu-derived peptides capable of inducing both cellular and humoral immune responses in HLA-A24 positive breast cancer patients. *Breast Cancer Res Treat.* 2004;86:19–29.
25. Bosch GJ, Joosten AM, Kessler JH, et al. Recognition of BCR-ABL positive leukemic blasts by human $CD4^+$ T cells elicited by primary in vitro immunization with a BCR-ABL breakpoint peptide. *Blood.* 1996;88:3522–3527.
26. Sato Y, Nabeta Y, Tsukahara T, et al. Detection and induction of CTLs specific for SYT-SSX-derived peptides in HLA-A24(+) patients with synovial sarcoma. *J Immunol.* 2002; 169:1611–1618.
27. Vonderheide RH, Hahn WC, Schultze JL, et al. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. *Immunity.* 1999;10:673–679.
28. Hung CF, Cheng WF, He L, et al. Enhancing major histocompatibility complex class I antigen presentation by targeting antigen to centrosomes. *Cancer Res.* 2003;63:2393–2398.

Review Article

The functioning antigens: beyond just as the immunological targets

Yoshihiko Hirohashi,[1] Toshihiko Torigoe,[1] Satoko Inoda,[1] Jun-ichi Kobayasi,[1] Munehide Nakatsugawa,[1] Takashi Mori,[2] Isao Hara[2] and Noriyuki Sato[1,3]

[1]Department of Pathology, Sapporo Medical University School of Medicine, South-1 West 17, Chuo-ko, Sapporo, 060-8556 Japan; [2]Department of Urology, Wakayama Medical University, Kimiidera 811-1, Wakayama, 641-0012 Japan

(Received December 16, 2008/Accepted January 30, 2009/Online publication March 25, 2009)

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

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

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

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

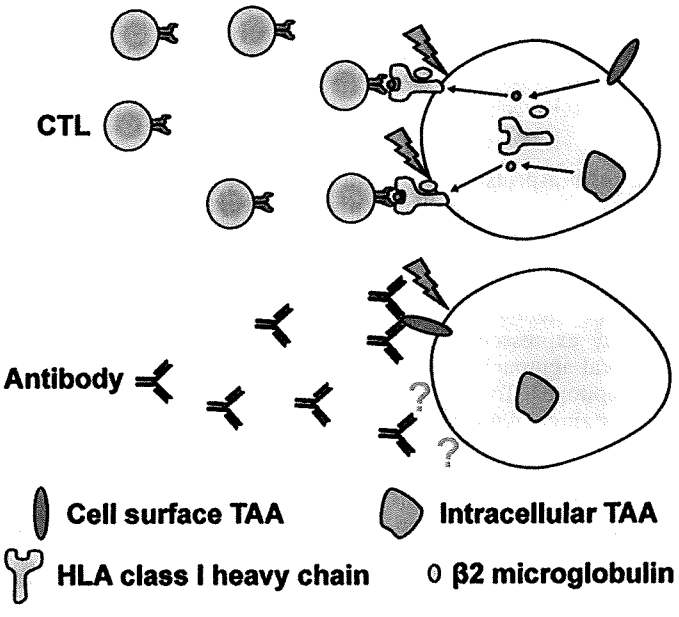


Fig. 1. Cancer-specific immunotherapy based on cytotoxic T lymphocytes (CTLs) and antibodies. In a variety of immuno systems, CTLs and B cell (antibody) show antigen-specific reactions through their antigen-specific receptors. CTL recognizes endogenously processed antigenic peptides presented with human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

[3]To whom correspondence should be addressed. E-mail: nsatou@sapmed.ac.jp
The abbreviations used are:
TAA, tumor associated antigen; CTL, cytotoxic T lymphocyte; HLA, human leukocyte antigen.

doi: 10.1111/j.1349-7006.2009.01137.x

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

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

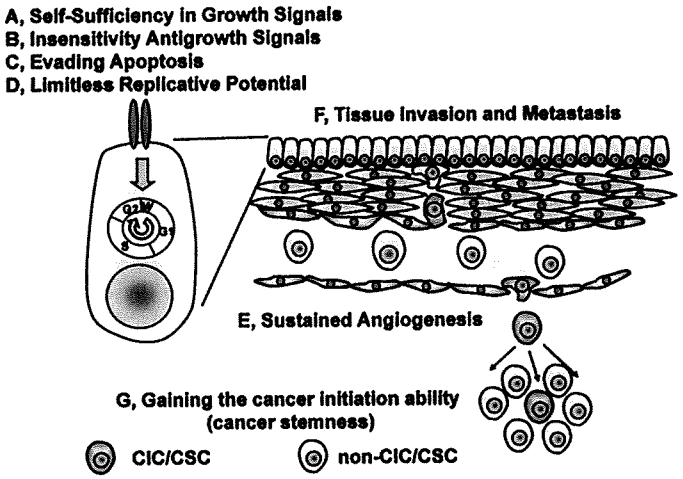


Fig. 2. The 'Functioning antigens'. The 'Functioning antigens' play several roles in carcinogenesis, tumor initiation, invasion and metastasis.

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

Self-Sufficiency in Growth Signals

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

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

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

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

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

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

doi: 10.1111/j.1349-7006.2009.01137.x

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

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

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

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

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

Insensitivity Antigrowth Signals

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

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

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

Evading Apoptosis

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

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

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

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

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

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

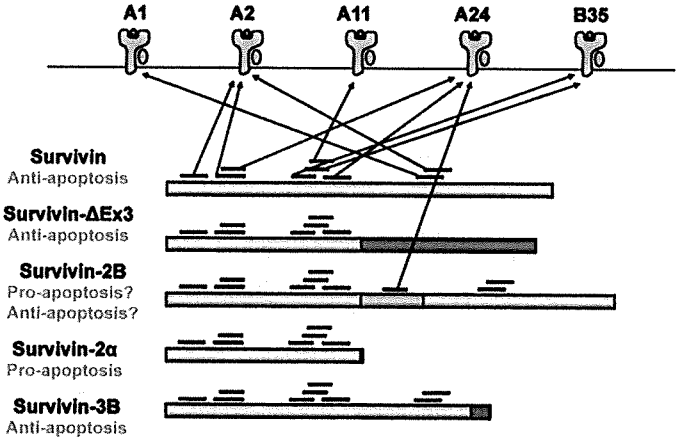


Fig. 3. Several antigenic peptides from survivin and variants. There are five survivin transcripts already reported. Wild-type survivin, survivin-ΔEx3 and survivin-3B have anti-apoptotic function. Survivin-2α has pro-apoptotic function. Survivin consists of four exons. Survivin-ΔEx3 skips exon 3 causing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

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

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

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

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

doi: 10.1111/j.1349-7006.2009.01137.x

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

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

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

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

Limitless Replicative Potential

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

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

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

Sustained Angiogenesis

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

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

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

Tissue Invasion and Metastasis

In the process of malignant tumor cell growth, the cells need to invade the normal connective tissue barrier. Tissue invasion and metastasis are landmarks of malignant diseases that distinguish

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

MMP-2. Matrix metalloproteinase-2 plays an essential role in tumor progression; however it is expressed ubiquitously, and the peptides derived from MMP-2 might be tolerated. Godefroy *et al.* showed how an MMP-2-derived peptide could be presented by tumor cells specifically and recognized by CTLs.[79] They reported that an HLA-A*0201-restricted peptide from the MMP-2 (GLPPDVQRV) was cross-presented by αvβ3-expressing tumor cells following clathrin-coated pit-mediated uptake of secreted extracellular MMP-2 and proteasome activity. The classic endogenous cytosolic pathway did not present this peptide. Hence, an MMP-2-derived peptide vaccine might be useful for MMP-2 secreting and αvβ3-expressing melanoma cells.

Gaining Cancer Initiation Ability (Cancer Stemness)

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

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

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

Conclusion

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

Acknowledgments

We are grateful for the kind cooperation in the current work by Drs Y. Tamura, S. Ichimiya, T. Tsukahara, T. Kikuchi of our laboratory, N. Takahashi of the Marine Biomedical Institute, T, Tsuruma, Y. Iwayama, and K. Hirata of Department of Surgery, S. Kawaguchi, T. Wada, and T. Yamashita of the Department of Orthopedic Surgery, T. Tsukamoto of the Department of Urology, A. Miyazaki and H. Hiratsuka of the Department of Oral Surgery and, T. Himi of the Department of Otolaryngology, Sapporo Medical University School of Medicine as well as H. Sahara of Laboratory of Biology, Azabu University School of Veterinary Medicine, N. Yamanaka of the Department of Otolaryngology, Wakayama Medical University School of Medicine, N. Shijubo of JR Sapporo Hospital and M. Ogasawara of Sapporo Hokuyu Hospital. We also thank Dr K. Kikuchi, Professor Emeritus, Sapporo Medical University School of Medicine, for his helpful assistance over many years.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant nos. 16209013, 17016061 and 15659097), for Practical Application Research from the Japan Science and Technology Agency and for Cancer Research (15-17 and 19-14) from the Ministry of Health, Labor and Welfare.

References

1 Tanaka K, Tanahashi N, Tsurumi C, Yokota KY, Shimbara N. Proteasomes and antigen processing. *Adv Immunol* 1997; **64**: 1–38.
2 Hammer GE, Kanaseki T, Shastri N. The final touches make perfect the peptide-MHC class I repertoire. *Immunity* 2007; **26**: 397–406.
3 van der Bruggen P, Traversari C, Chomez P *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991; **254**: 1643–7.
4 Boon T, van der Bruggen P. Human tumor antigens recognized by T lymphocytes. *J Exp Med* 1996; **183**: 725–9.
5 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
6 Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008; **8**: 755–68.
7 Eccles SA. The role of c-erbB-2/HER2/neu in breast cancer progression and metastasis. *J Mammary Gland Biol Neoplasia* 2001; **6**: 393–406.
8 Fisk B, Blevins TL, Wharton JT, Ioannides CG. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by

doi: 10.1111/j.1349-7006.2009.01137.x