GPC3-specific siRNA sequences were used: GPC3-siRNA (#4149), 5'-UUAUCAUUCCAUCACCAGAGCCUCC-3'; GPC3-siRNA (#4150), 5'-GGAGGCUCUGGUGAUGGAAU GAUAA-3': and GPC3-siRNA (#4151), 5'-UAUAGAUGACUG GAAACAGGCUGUC-3'. Synthetic siRNA duplexes were transfected using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocols.

RT-PCR. Using the TRIzol reagent (Invitrogen), we extracted total cellular RNA from untreated or siRNA (GPC3-siRNA or negative-siRNA)-treated JHH-7/HLA-A*02:07. cDNA was synthesized using the PrimeScript II 1st Strand cDNA Synthesis kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. The cDNA was added to a reaction mix that contained 10X Ex Taq Buffer (Takara), 2.5 mM dNTP mixture (Takara), 5 units Ex Taq (Takara), and 10 μM of the *GPC3*- or *β-actin*-specific PCR primers. The following primer sequences (sense and antisense, respectively) were used: for *GPC3*, 5'-AGCCAAAAGGCAGCAAGGAA-3' and 5'-AAGA AGAAGCACACCACCGA-3'; and for *β-actin*, 5'-CCTCGCCT TTGCCGATCC-3' and 5'-GGATCTTCATGAGGTAGTC AGTC-3'. PCR was performed using the 96-well Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA). PCR was performed for 20 cycles of 98°C for 10 sec, 64°C for 30 sec and 72°C for 30 sec, followed by a step of 72°C for 10 sec.

Sequence analysis of TCR-β gene. Using the TRIzol reagent (Invitrogen), total cellular RNA was extracted from established CTL clones. The cDNA of the *TCR-β* gene was synthesized using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions, with the modification that we used 200 nM of the primer specific for the TCR-β chain constant region. The cDNA products were subjected to 2-step PCR, as previously described by Yukie Tanaka-Harada (35,36), and the PCR products were purified and sequenced in the Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems). The *TCR-β* variable (*TRBV*) gene, *TCR-β* joining (*TRBJ*) gene, *TCR-β* diversity (*TRBD*) alleles, and complementarity-determining region 3 (*CDR3*) sequences were identified using the IMGT databases (http://www.imgt.org/).

Results

$GPC3_{144-152}$ peptide-specific CTLs in the peripheral blood of the patient exert a clinical effect. We analyzed the immune responses of the patient who showed a PR following $GPC3_{144-152}$ peptide vaccination. In this patient, the supraclavicular lymph node metastases markedly regressed, two liver tumors disappeared, and the thoracic bone metastasis showed necrosis after the third vaccination (27). The levels of DCP decreased in the patients over the 2-month period. We evaluated the $GPC3_{144-152}$-specific immune responses in the peripheral blood using the *ex vivo* IFN-γ ELISPOT assay. For the HLA-A*02:07-positive patient with advanced HCC, the number and area of the spots increased after two rounds of vaccination, as compared with the pre-vaccination values, and the peak values were noted 10 weeks after the start of the treatment (Fig. 1A).

Establishment of $GPC3_{144-152}$-specific CTL clones from the PBMCs of the patient. To investigate the ability of the $GPC3_{144-152}$-specific CTLs induced by peptide vaccination to recognize antigen, we established CTL clones from the PBMCs of this patient 10 weeks after the start of treatment. The PBMCs were stimulated with the $GPC3_{144-152}$ peptide *in vitro* for 14 days. $CD8^+$ T cells were isolated from the stimulated PBMCs, and then incubated with peptide-pulsed 1-87 cells. $CD8^+$ $CD107a^+$ cells that reacted with the $GPC3_{144-152}$-pulsed 1-87 cells were sorted to the single-cell level. Thus, we established $GPC3_{144-152}$ peptide-specific CTL clones.

Three established CTL clones were analyzed for function using the IFN-γ ELISPOT assay and cytotoxicity assay. All of the CTL clones released IFN-γ in response to the $GPC3_{144-152}$-pulsed 1-87 cells, but not in response to non-pulsed 1-87 cells (Fig. 1B). Moreover, these CTL clones showed cytotoxicity against $GPC3_{144-152}$-pulsed 1-87 cells, but not against non-pulsed or HIV19-27-pulsed 1-87 cells (Fig. 1C). These results indicate that the CTL clones 24-4-2, 24-4-7 and 24-2-10 have specificity for the $GPC3_{144-152}$ peptide.

Functional avidity of the $GPC3_{144-152}$-specific CTL clones. We evaluated the cytotoxicity profiles of the CTL clones for 1-87 cells pulsed with a decreasing concentration series (from 10^{-6} to 10^{-14} M) of the $GPC3_{144-152}$ peptide. The peptide concentration at which the curve reached 50% cytotoxicity was defined as the recognition efficiency of the clone. The recognition efficiencies of CTL clones 24-4-2, 24-4-7 and 24-2-10 were 10^{-11}, 10^{-9} and 10^{-8} M, respectively (Fig. 2). This result suggests that CTL clone 24-4-2 has a higher avidity than the other two clones and, conversely, that CTL clone 24-2-10 has a lower avidity than the other two clones.

A $GPC3_{144-152}$-specific CTL clone recognizes cancer cells that endogenously express GPC3. Next, we tested the reactivities of these CTL clones against cancer cell lines that expressed GPC3 and HLA-A*02:07. We used the JHH-7/mock ($GPC3^+$, HLA-A*02:07-) and JHH-7/HLA-A*02:07 ($GPC3^+$, HLA-A*02:07$^+$) transfectants as the target cells (Fig. 3A). The CTL clone 24-4-2 (with high avidity) produced IFN-γ and was cytotoxic for JHH-7/HLA-A*02:07 cells but not for JHH-7/mock cells (Fig. 3B and C). The other clones did not produce IFN-γ and did not exhibit cytotoxicity for the two target cell lines. These results suggest that only high-avidity CTLs recognize cancer cells that express GPC3 peptide endogenously.

CTL clone 24-4-2 shows specificity for GPC3. To ascertain the GPC3 antigen-specific response of CTL clone 24-4-2, we created a GPC3 knockdown via siRNA treatment of the JHH-7/HLA-A*02:07 cells. GPC3 expression by the JHH-7/HLA-A*02:07 cells was clearly decreased by the GPC3-siRNA, as assessed by RT-PCR (Fig. 4A). We examined the IFN-γ production levels of CTL clone 24-4-2 against JHH-7/HLA-A*02:07 cells treated with GPC3-siRNA. IFN-γ production by CTL clone 24-4-2 was significantly decreased by the GPC3-siRNA (Fig. 4B). These results indicate that the HLA-A2-restricted $GPC3_{144-152}$ peptide is processed naturally by cancer cells, and that both HLA-A*02:07 and HLA-A*02:01 can present the $GPC3_{144-152}$ peptide on the surfaces of cancer cells.

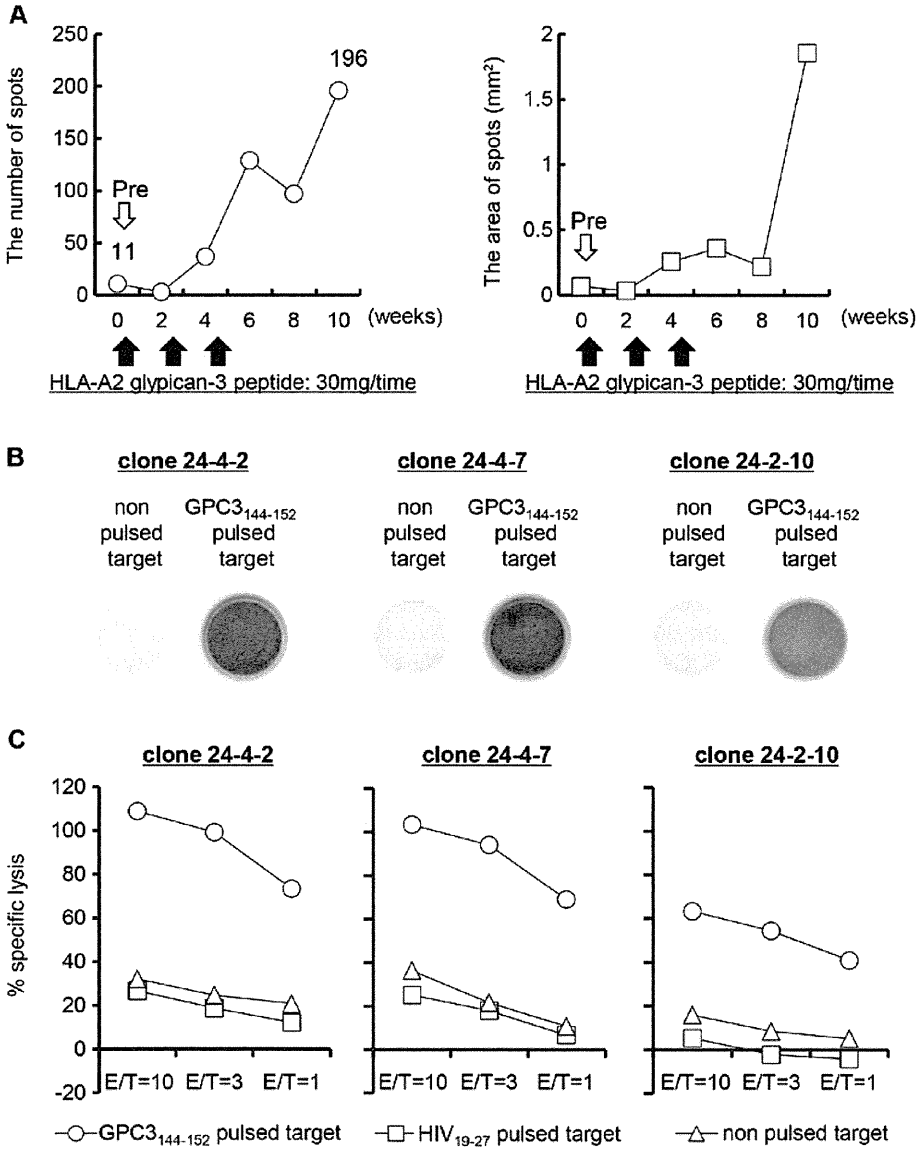

Figure 1. GPC3 peptide-specific CTL clones established from the PBMCs of a patient following GPC3 peptide vaccination. (A) Changes in the frequencies of $GPC3_{144\text{-}152}$ peptide-specific CTLs before and after vaccination in a patient who showed a PR post-vaccination. Changes in the GPC3 peptide-specific CTLs are observed as differences in the number (left) and the area (right) of spots in an *ex vivo* IFN-γ ELISPOT assay. (B) Results of the IFN-γ ELISPOT assay against peptide-pulsed target. HLA-A*02:07$^+$ cancer cell line 1-87 was used as the target. The target was pulsed with the $GPC3_{144\text{-}152}$ peptide. A non-pulsed target was used as the negative control. The ratio of effector cells to target cells (E/T) is 1. (C) Results of the cytotoxicity assay against peptide-pulsed target. The 1-87 cells were used as the target. Non-pulsed and $HIV_{19\text{-}27}$ peptide-pulsed targets were used as negative controls. E/Ts are 10, 3 and 1, respectively. A representative of three experiments is shown.

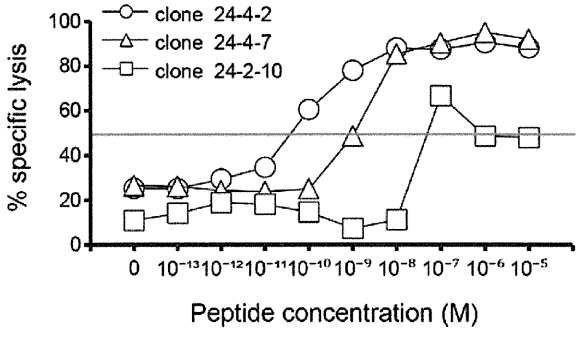

Figure 2. $GPC3_{144\text{-}152}$ peptide-specific avidity of the established CTL clones. The established CTL clones were tested for avidity using 1-87 cells that were pulsed with various concentrations of the $GPC3_{144\text{-}152}$ peptide. The peptide concentration at which the curve crossed the 50% cytotoxicity mark was defined as the recognition efficiency of that clone. E/T is 10. A representative of three experiments is shown.

Established CTL clones have different sets of TCR-β alleles. We analyzed the *TCR-β* gene sequences of the established CTL clones. The *TRBV*, *TRBJ* and *TRBD* alleles were identified using the IMGT databases. Thus, we identified the *TRBV*, *TRBD* and *TRBJ* alleles of the CTL clones (Table I). Each of the established CTL clones had different allele sets.

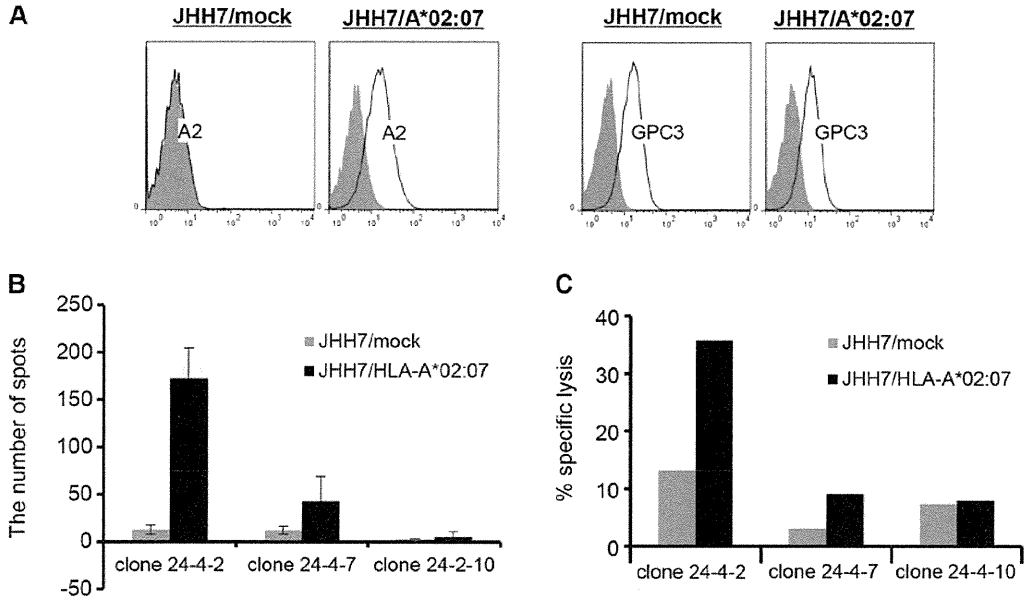

Figure 3. Recognition of GPC3$^+$ cancer cells by the established CTL clones. (A) Expression of HLA-A2 (left panel) and GPC3 (right panel) on established GPC3$^+$ HLA-A*02:07$^+$ cancer cells and control cells. (B) Results of the IFN-γ ELISPOT assay for the GPC3$^+$ cancer cell line. The HLA-A*02:07-overexpressing GPC3$^+$ cancer cell line, JHH7/HLA-A*02:07, was established and used as the target. JHH7/mock cells were used as the negative control. E/T ratio, 1. Data are presented as mean ± SD of three independent batches. (C) Results of the assay for cytotoxicity against the GPC3$^+$ cancer cell line. JHH7/HLA-A*02:07 cells were used as the target. JHH7/mock cells were used as the negative control. E/T is 3. A representative of three experiments is shown.

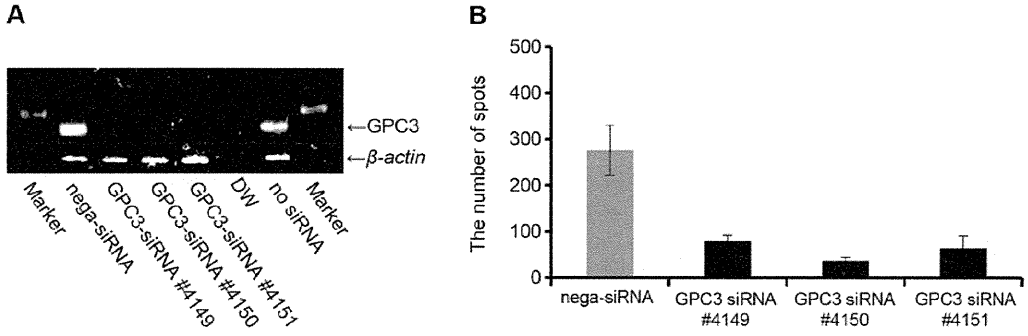

Figure 4. GPC3 specificity of CTL clone 24-4-2. (A) GPC3 expression levels on JHH7/HLA-A*02:07 cells treated with GPC3-siRNA or negative (nega)-siRNA for 48 h, as determined by RT-PCR. (B) Results of the IFN-γ ELISPOT assay for JHH7/HLA-A*02:07 cells treated with GPC3-siRNA or nega-siRNA. E/T is 1. Data are presented as mean ± SD of three independent batches.

*CTL clone 24-4-2 is subject to HLA-A*02:07 restriction.* We investigated whether CTL clone 24-4-2 recognized the $GPC3_{144-152}$ peptide-HLA-A*02:01 complex and the $GPC3_{144-152}$ peptide-HLA-A*02:06 complex, as well as the $GPC3_{144-152}$ peptide-HLA-A*02:07 complex. Healthy donor PBMCs with HLA-A*02:01, HLA-A*02:06, HLA-A*02:07 and HLA-A*24:02 were used as the targets, and an HLA-A*02:01-restricted, GPC3-specific CTL clone, which is a previously established CTL clone (26), was used as the control. The HLA-A*02:01-restricted CTL clone recognized only the $GPC3_{144-152}$ peptide-HLA-A*02:01 complex, and CTL clone 24-4-2 recognized only the $GPC3_{144-152}$ peptide-HLA-A*02:07 complex (Fig. 5). These outcomes indicate that CTL clone 24-4-2 has HLA-A*02:07 restriction.

Table I. TCR-β chain sequencing for established CTL clones.

No.	TRBV	TRBJ	TRBD
Clone 24-4-2	18*01	1-2*01	1*01
Clone 24-4-7	7-3*01	2-7*01	1*01
Clone 24-2-10	7-6*01	2-1*01	2*01

Discussion

Clinical trials of peptide-based vaccines are underway in several parts of the world. However, the monitoring of individual CTL post-vaccination has scarcely been reported in

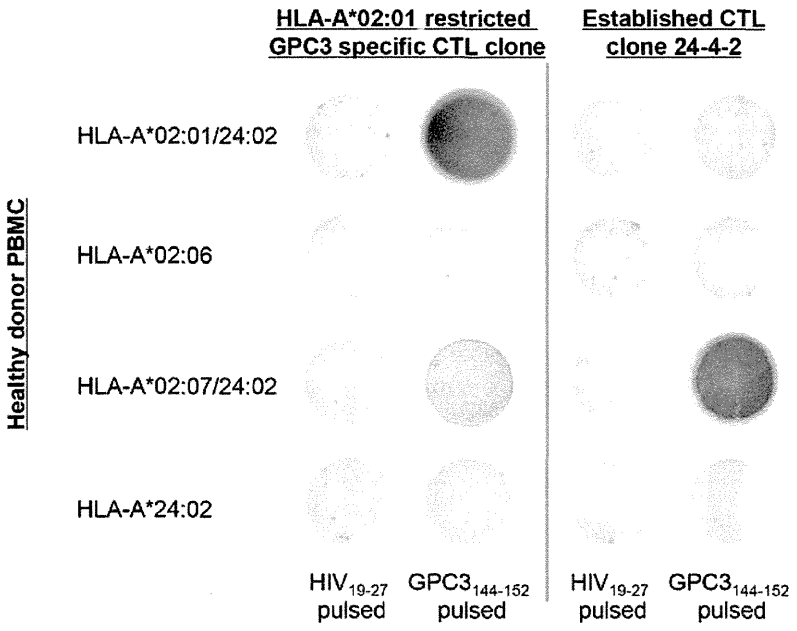

Figure 5. CTL clone 24-4-2 shows HLA-A*02:07 restriction. Results of the IFN-γ ELISPOT assay for healthy donor PBMCs with HLA-A2. The established CTL clone 24-4-2 and the HLA-A*02:01-restricted, GPC3-specific CTL clone were used as effectors. E/T is 0.2. A representative of two experiments is shown.

immunotherapy trials. In the present study, we established HLA-A*02:07$^+$ $GPC3_{144-152}$-specific CTL clones from the PBMCs of a patient who showed a PR following GPC3-derived peptide vaccination and we performed functional analyses against established CTL clones.

This patient showed an increase in the number of CTLs specific for the GPC3-derived peptide in the peripheral blood after vaccination (Fig. 1A) (27,28). Ten weeks after the start of treatment, the $GPC3_{144-152}$-specific CTL counts had increased approximately 18-fold, as compared with the pre-vaccination counts. In this case, analysis of the established CTL clones after vaccination could lend support to the notion that the vaccine-induced CTLs exert an antitumor effect, since few $GPC3_{144-152}$-specific CTLs were detected before vaccination.

In the present study, we confirmed that $GPC3_{144-152}$-specific CTL clones are cytotoxic for both $GPC3_{144-152}$-pulsed 1-87 cells and JHH-7/HLA-A*02:07 cells that express GPC3 peptide endogenously. Confirming that the GPC3 peptide-specific CTL clones kill cancer cells that express endogenously the antigen peptide is important because antigen-derived and CTL-inducible peptides are not necessarily presented by cancer cells that endogenously express the antigen (31-33). Three established CTL clones showed cytotoxic activities related to their avidity for $GPC3_{144-152}$-pulsed 1-87 cells and JHH-7/HLA-A*02:07 cells that expressed the GPC3 peptide endogenously. These results show that although CTLs with different avidity can be isolated, only those CTLs with high avidity can kill cancer cells that express the antigen peptide endogenously. Several investigators have demonstrated a correlation between T-cell avidity and target recognition by T-cell populations that recognize murine tumor models and human cancers (34). Our results strongly support this observation.

The TCR usage of antigen-specific T cells is thought to be influenced by the affinity of the TCR for the antigen peptide-HLA class I complex. Several studies on the TCR usage of tumor-associated antigen (TAA)-specific T cells have used the *TRBV* gene family (35-41). These studies mainly analyzed the frequencies of TAA tetramer positive CD8$^+$ T cells. Although it is important to examine quantitative aspects, such as the frequencies of TAA tetramer positive CD8$^+$ T cells, the cytotoxicity of these T cells against cancer cells that express the TAA peptide endogenously cannot be confirmed. Moreover, GPC3 dextramer positive CD8$^+$ T cells were not detected in the PBMCs of the patients with HCC before GPC3 peptide vaccination (27,28). To analyze biased usage of the *TCR* gene of GPC3 dextramer positive CD8$^+$ T cells in the patients with HCC before and after GPC3 peptide vaccination, a new detection system with greater sensitivity *ex vivo* will be required. In the present study, we analyzed the *TCR-β* genes of the established $GPC3_{144-152}$-specific CTL clones, to confirm that these CTL clones have different TCRs. Our experiments show that the established CTL clones have different TCR-β-chain allele sets, i.e., *TRBV*, *TRBD* and *TRBJ* alleles (Table I), and different CDR3 sequences (data not shown). These results suggest that various GPC3-specific CTLs are induced by $GPC3_{144-152}$ peptide vaccination.

A*HLA-A*02:07 differs from HLA-A*02:01 by a single non-conservative change (Y to C) at residue 99. X-ray crystallographic data have identified position 99 as one of the residues forming the D secondary pocket, which engages the residue at position 3 on peptide ligands (42-44). Although hHLA-A*02:07 was originally not included in the HLA-A2 supertype, cross-reactivity between HLA-A*02:07 and other A2 subtypes was detected at the functional level (44,45). Moreover, this HLA molecule indeed binds a subset of the peptide repertoire bound by other A2 subtypes (44). For these reasons, HLA-A*02:07 should also be included in the A2 supertype (46). Ito *et al* (47) and Nonaka *et al* (48) reported that an HLA-A2-restricted

CTL line established from the tumor-infiltrating lymphocytes (TIL) of an HLA-A*02:07-positive patient showed significant cytotoxicities for HLA-A*02:01-, HLA-A*02:06- and HLA-A*02:07-positive cancer cells. Therefore, we examined whether the $GPC3_{144-152}$-specific CTL clone 24-4-2, which was established from the PBMCs of an HLA-A*02:07- positive patient with HCC, could recognize HLA A-A*02:01 or HLA-A*02:06. However, this CTL clone failed to recognize HLA-A*02:01 or HLA-A*02:06.

We have reported previously on the detection via immunohistochemical staining of massive infiltration of CD8-positive T cells into the remaining liver tumor of this patient (27). It was difficult to confirm that these tumor-infiltrating $CD8^+$ T cells have specificity for GPC3. Currently, we are conducting clinical testing of liver biopsies taken before and after GPC3 peptide vaccination of patients with advanced HCC. Our aim is to reveal the GPC3 peptide-specific immune responses induced by the GPC3-derived peptide vaccine in both the peripheral blood and the tumor. We are analyzing the *TCR* gene sequences of CD8 or GPC3 dextramer positive T cells in both the peripheral blood and tumor. Already in this trial, a remarkable clinical effect has been observed for an HLA-A*02:07-positive patient with HCC who received $GPC3_{144-152}$ peptide vaccination (49).

HLA-A*02:07 is present in the populations of East Asia, South-East Asia (7%), and northern India (11.5%) (26,50-52). In southern China, the frequency of the HLA-A*02:07 allele is reported to be even higher than the frequency of the HLA-A*02:01 allele (53,54). In addition, about 75% of liver cancer cases occur in South-East Asia, including China, Hong Kong, Taiwan, Korea, India and Japan (55). Taking together these previous reports and our results, it appears that HLA-A*02:07-positive patients with HCC are good candidates for $GPC3_{144-152}$ peptide vaccination. Further studies will be necessary to prove the clinical efficacy of GPC3 peptide vaccination for advanced HCC.

In conclusion, we present substantial evidence that $GPC3_{144-152}$-specific CTLs with different TCR allele sets that are induced in patients with HCC who show a PR following $GPC3_{144-152}$ peptide vaccination indicate not only high avidity but also natural antigen-specific killing activity against tumor cells.

Acknowledgements

We thank Dr Ryo Abe and Dr Toshihiro Suzuki for providing the pcDNA3.1 construct that expresses HLA-A*02:07. This study was supported in part by Health and Labor Science Research Grants for Clinical Research and Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor and Welfare, Japan and the National Cancer Center Research and Development Fund.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917, 2010.
3. Zidan A, Scheuerlein H, Schüle S, Settmacher U and Rauchfuss F: Epidemiological pattern of hepatitis B and hepatitis C as etiological agents for hepatocellular carcinoma in iran and worldwide. Hepat Mon 12: e6894, 2012.
4. Llovet JM, Ricci S, Mazzaferro V, *et al*: Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 359: 378-390, 2008.
5. Cheng AL, Kang YK, Chen Z, *et al*: Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 10: 25-34, 2009.
6. Kim HY and Park JW: Molecularly targeted therapies for hepatocellular carcinoma: Sorafenib as a stepping stone. Dig Dis 29: 303-309, 2011.
7. Morimoto M, Numata K, Kondo M, *et al*: Higher discontinuation and lower survival rates are likely in elderly Japanese patients with advanced hepatocellular carcinoma receiving sorafenib. Hepatol Res 41: 296-302, 2011.
8. Greten TF, Manns MP and Korangy F: Immunotherapy of hepatocellular carcinoma. J Hepatol 45: 868-878, 2006.
9. Mizukoshi E, Nakamoto Y, Arai K, *et al*: Comparative analysis of various tumor-associated antigen-specific t-cell responses in patients with hepatocellular carcinoma. Hepatology 53: 1206-1216, 2011.
10. Filmus J, Shi W, Wong ZM and Wong MJ: Identification of a new membrane-bound heparan sulphate proteoglycan. Biochem J 311: 561-565, 1995.
11. Filmus J and Selleck SB: Glypicans: proteoglycans with a surprise. J Clin Invest 108: 497-501, 2001.
12. Nakatsura T, Yoshitake Y, Senju S, *et al*: Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 306: 16-25, 2003.
13. Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E and Filmus J: Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 125: 89-97, 2003.
14. Nakatsura T and Nishimura Y: Usefulness of the novel oncofetal antigen glypican-3 for diagnosis of hepatocellular carcinoma and melanoma. BioDrugs 19: 71-77, 2005.
15. Shirakawa H, Kuronuma T, Nishimura Y, *et al*: Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer. Int J Oncol 34: 649-656, 2009.
16. Shirakawa H, Suzuki H, Shimomura M, *et al*: Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. Cancer Sci 100: 1403-1407, 2009.
17. Motomura Y, Senju S, Nakatsura T, *et al*: Embryonic stem cell-derived dendritic cells expressing glypican-3, a recently identified oncofetal antigen, induce protective immunity against highly metastatic mouse melanoma, B16-F10. Cancer Res 66: 2414-2422, 2006.
18. Motomura Y, Ikuta Y, Kuronuma T, *et al*: HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: preclinical study using mice. Int J Oncol 32: 985-990, 2008.
19. Iwama T, Horie K, Yoshikawa T, *et al*: Identification of an H2-Kb or H2-Db restricted and glypican-3-derived cytotoxic T-lymphocyte epitope peptide. Int J Oncol 42: 831-838, 2013.
20. Komori H, Nakatsura T, Senju S, *et al*: Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 12: 2689-2697, 2006.
21. Nakatsura T, Komori H, Kubo T, *et al*: Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reactions in mice. Clin Cancer Res 10: 8630-8640, 2004.
22. Imanish T, Akaza T, Kimura A, Tokunaga K and Gojobori T: Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: HLA 1991. Vol 1. Tsuji K, Aizawa M and Sasazuki T (eds.) Oxford University Press, Oxford, pp1065-1220, 1992.
23. Sidney J, Grey HM, Kubo RT and Sette A: Practical, biochemical and evolutionary implications of the discovery of HLA class I supermotifs. Immunol Today 17: 261-266, 1996.
24. Yasuda N, Tsuji K, Aizawa M, *et al*: HLA antigens in Japanese populations. Am J Hum Genet 28: 390-399, 1976.
25. Ellis JM, Henson V, Slack R, Ng J, Hartzman RJ and Katovich Hurley C: Frequencies of HLA-A2 alleles in five U.S. population groups. Predominance of A*02011 and identification of HLA-A*0231. Hum Immunol 61: 334-340, 2000.
26. Mehra NK, Jaini R, Rajalingam R, Balamurugan A and Kaur G: Molecular diversity of HLA-A*02 in Asian Indians: predominance of A*0211. Tissue Antigens 57: 502-507, 2001.

27. Sawada Y, Yoshikawa T, Nobuoka D, *et al*: Phase I trial of a glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma: immunologic evidence and potential for improving overall survival. Clin Cancer Res 18: 3686-3696, 2012.
28. Yoshikawa T, Nakatsugawa M, Suzuki S, *et al*: HLA-A2-restricted glypican-3 peptide-specific CTL clones induced by peptide vaccine show high avidity and antigen-specific killing activity against tumor cells. Cancer Sci 102: 918-925, 2011.
29. Nobuoka D, Yoshikawa T, Sawada Y, Fujiwara T and Nakatsura T: Peptide vaccines for hepatocellular carcinoma. Hum Vaccin Immunother 9: 210-212, 2013.
30. Sawada Y, Sakai M, Yoshikawa T, Ofuji K and Nakatsura T: A glypican-3-derived peptide vaccine against hepatocellular carcinoma. Oncoimmunology 1: 1448-1450, 2012.
31. Purbhoo MA, Li Y, Sutton DH, *et al*: The HLA A*0201-restricted hTERT(540-548) peptide is not detected on tumor cells by a CTL clone or a high-affinity T-cell receptor. Mol Cancer Ther 6: 2081-2091, 2007.
32. Nakatsugawa M, Horie K, Yoshikawa T, *et al*: Identification of an HLA-A*0201-restricted cytotoxic T lymphocyte epitope from the lung carcinoma antigen, Lengsin. Int J Oncol 39: 1041-1049, 2011.
33. Guo Y, Zhu Y and Sun S: Identification and functional studies of HLA-A0201 restricted CTL epitopes in the X protein of hepatitis B virus. Acta Virol 55: 107-115, 2011.
34. McKee MD, Roszkowski JJ and Nishimura MI: T cell avidity and tumor recognition: implications and therapeutic strategies. J Transl Med 3: 35, 2005.
35. Harada Y and Kawase I: Single cell-based T cell receptor gene analysis reveals existence of expanded WT1 (Wilms' tumor gene) product-specific T cell clones in leukemia patients but not healthy volunteers. Med J Osaka Univ 50: 1-12, 2007.
36. Tanaka-Harada Y, Kawakami M, Oka Y, *et al*: Biased usage of BV gene families of T-cell receptors of WT1 (Wilms' tumor gene)-specific CD8+ T cells in patients with myeloid malignancies. Cancer Sci 101: 594-600, 2010.
37. Morimoto S, Oka Y, Tsuboi A, *et al*: Biased usage of T cell receptor β-chain variable region genes of Wilms' tumor gene (WT1)-specific CD8+ T cells in patients with solid tumors and healthy donors. Cancer Sci 103: 408-414, 2012.
38. Valmori D, Dutoil V, Lienard D, *et al*: Tetramer-guided analysis of TCR beta-chain usage reveals a large repertoire of melan-A-specific CD8+ T cells in melanoma patients. J Immunol 165: 533-538, 2000.
39. Mandruzzato S, Rossi E, Bernardi F, *et al*: Large and dissimilar repertoire of Melan-A/MART-1-specific CTL in metastatic lesions and blood of a melanoma patient. J Immunol 169: 4017-4024, 2002.
40. Zhou J, Dudley ME, Rosenberg SA and Robbins PF: Selective growth, in vitro and in vivo, of individual T cell clones from tumor-infiltrating lymphocytes obtained from patients with melanoma. J Immunol 173: 7622-7629, 2004.
41. Akiyama Y, Maruyama K, Tai S, *et al*: Characterization of a MAGE-1-derived HLA-A24 epitope-specific CTL line from a Japanese metastatic melanoma patient. Anticancer Res 29: 647-655, 2009.
42. Saper MA, Bjorkman PJ and Wiley DC: Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 A resolution. J Mol Biol 219: 277-319, 1991.
43. Madden DR, Garboczi DN and Wiley DC: The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. Cell 75: 693-708, 1993.
44. Sidney J, del Guercio MF, Southwood S, Hermanson G, Maewal A, Appella E and Sette A: The HLA-A0207 peptide binding repertoire is limited to a subset of the A0201 repertoire. Hum Immunol 58: 12-20, 1997.
45. Rivoltini L, Loftus DJ, Barracchini K, *et al*: Binding and presentation of peptides derived from melanoma antigens MART-1 and glycoprotein-100 by HLA-A2 subtypes: implications for peptide-based immunotherapy. J Immunol 156: 3882-3891, 1996.
46. Sette A and Sidney J: HLA supertypes and supermotifs: a functional perspective on HLA polymorphism. Curr Opin Immunol 10: 478-482, 1998.
47. Ito M, Shichijo S, Tsuda N, Ochi M, Harashima N, Saito N and Itoh K: Molecular basis of T cell-mediated recognition of pancreatic cancer cells. Cancer Res 61: 2038-2046, 2001.
48. Nonaka Y, Tsuda N, Shichijo S, *et al*: Recognition of ADP-ribosylation factor 4-like by HLA-A2-restricted and tumor-reactive cytotoxic T lymphocytes from patients with brain tumors. Tissue Antigens 60: 319-327, 2002.
49. Sawada Y, Yoshikawa T, Fujii S, *et al*: Remarkable tumor lysis in a hepatocellular carcinoma patient immediately following glypican-3-derived peptide vaccination: an autopsy case. Hum Vaccin Immunother 9: Mar 6, 2013 (Epub ahead of print).
50. Krausa P, Brywka M III, Savage D, *et al*: Genetic polymorphism within HLA-A*02: significant allelic variation revealed in different populations. Tissue Antigens 45: 223-231, 1995.
51. Chang CX, Tan AT, Or MY, *et al*: Conditional ligands for Asian HLA variants facilitate the definition of CD8(+) T-cell responses in acute and chronic viral diseases. Eur J Immunol 43: 1109-1120, 2013.
52. Chen KY, Liu J and Ren EC: Structural and functional distinctiveness of HLA-A2 allelic variants. Immunol Res 53: 182-190, 2012.
53. Shieh DC, Lin DT, Yang BS, Kuan HL and Kao KJ: High frequency of HLA-A*0207 subtype in Chinese population. Transfusion 36: 818-821, 1996.
54. Cheng LH, Jin SZ, Gao SQ, Li Z, Zou HY, Wang DM and Wu GG: Difference in HLAA*02 allele distribution between Han populations in south and north China. Di Yi Jun Yi Da Xue Xue Bao 25: 321-324, 2005 (In Chinese).
55. Mohana Devi S, Balachandar V, Arun M, Suresh Kumar S, Balamurali Krishnan B and Sasikala K: Analysis of genetic damage and gene polymorphism in hepatocellular carcinoma (HCC) patients in a South Indian population. Dig Dis Sci 58: 759-767, 2013.

Human Vaccines & Immunotherapeutics 10:2, 1–8; February 2014; © 2014 Landes Bioscience

Significant clinical response of progressive recurrent ovarian clear cell carcinoma to glypican-3-derived peptide vaccine therapy

Two case reports

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Keywords: Glypican-3, peptide vaccine, refractory disease, ovarian clear cell carcinoma, clinical response

Abbreviations: HLA, human leukocyte antigen; UMIN-CTR, University Hospital Medical Information Network Clinical Trials Registry; CT, computed tomography; GMP, Good Manufacturing Practice; RECIST, Response Evaluation Criteria in Solid Tumors; PR, partial response; ^{18}F-FDG PET, Fluorine-18-fluorodeoxyglucose positron emission tomography; IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cell

Carcinoembryonic antigen glypican-3 (GPC3) is expressed by > 40% of ovarian clear cell carcinoma (CCC) and is a promising immunotherapeutic target. We previously reported the safety of and immunological and clinical responses to a GPC3-derived peptide vaccine in a phase I clinical trial of patients with advanced hepatocellular carcinoma (HCC). Although the efficacy of the GPC3-derived peptide vaccine against HCC patients was evaluated, other GPC3-positive cancer patients have not yet been investigated. Therefore, we conducted a phase II trial to evaluate the clinical outcome of ovarian CCC patients treated with a GPC3-derived peptide vaccine. The GPC3 peptide was administered at a dose of 3 mg per body. Patients received an intradermal injection of the GPC3 peptide emulsified with incomplete Freund's adjuvant. Vaccinations were performed biweekly from the first until the 6th injection and were then repeated at 6-week intervals after the 7th injection. Treatment continued until disease progression. We herein present two patients with chemotherapy-refractory ovarian CCC who achieved a significant clinical response in an ongoing trial of a GPC3 peptide vaccine. Case 1, a 42-year-old patient with advanced recurrent ovarian CCC with liver and retroperitoneal lymph node metastases, received the HLA-A24-restricted GPC3 peptide vaccine. Contrast-enhanced CT at week 10 revealed a partial response (PR) using RECIST criteria. Case 2 was a 67-year-old female with multiple lymph node metastases. She was injected with the HLA-A2-restricted GPC3 peptide vaccine. According to RECIST, PR was achieved at week 37. The stabilization of their diseases over one year provided us with the first clinical evidence to demonstrate that GPC3 peptide-based immunotherapy may significantly prolong the overall survival of patients with refractory ovarian CCC.

Introduction

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynecological malignancy. Ovarian clear cell carcinoma (CCC) accounts for 5–25% of all EOC, depending on the geographic location. It accounts for <10% of all EOC diagnosed in the USA.[1] In contrast, the incidence of CCC is reportedly >15% of EOC in Japan.[2] Compared with other EOC subtypes, CCC is associated with a poorer prognosis and increased chemoresistance.[2,3] In particular, the response rate of recurrent CCC to salvage chemotherapy was reported to be less than 10%.[4] Progression-free survival was also less than 6 mo, even in patients who achieved a response when treated with conventional anti-cancer cytotoxic agents.[5] The long-term clinical outcome of patients with recurrent CCC is extremely poor.[6] Therefore, new treatment modalities are urgently required for patients with CCC refractory to chemotherapy.

Immunotherapy is a potentially attractive option for EOC. Glypican-3 (GPC3) is useful not only as a novel tumor marker, but also as an oncofetal antigen for immunotherapy. It is specifically overexpressed in hepatocellular carcinoma (HCC).[7] Previous studies demonstrated that GPC3 was also overexpressed in several malignant tumors, including ovarian CCC.[8-12]

We previously identified the HLA-A24-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptides, both of which can induce GPC3-reactive cytotoxic T cells (CTLs).[13] We recently reported the safety of and immunological and clinical responses to a

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Submitted: 09/11/2013; Revised: 10/26/2013; Accepted: 11/15/2013
http://dx.doi.org/10.4161/hv.27217

GPC3-derived peptide vaccine in a phase I trial for advanced HCC patients.[14] We are currently conducting a phase II trial with a GPC3-derived peptide vaccine in ovarian CCC patients (UMIN-CTR: 000003696).

This study presents, for the first time, two patients with refractory ovarian CCC who achieved a significant clinical response in an ongoing trial of a GPC3 peptide vaccine.

Materials and Methods

Patient eligibility

This study describes two patients from our GPC3 peptide vaccination trial. This clinical trial was approved and monitored by the Institutional Review Board at Nagoya University School of Medicine. Patients with progressive metastatic ovarian CCC were enrolled after providing written, informed consent. The following eligibility criteria were used: diagnosis of ovarian CCC on the basis of histological examinations; no expectation of a response to other therapies; an Eastern Cooperative Oncology Group performance status of 0–2; age between 20 and 80 y; HLA-A24- or HLA-A2-positive status as determined using commercially available genomic DNA typing tests; and adequate organ function (white blood cell count ≥2000/mm^3, platelets ≥50000/mm^3, serum creatinine ≤2.1 mg/dl, total bilirubin ≤3.6 mg/dl, aspartate aminotransferase ≤165 IU/L, alkaline phosphatase ≤1795 IU/L). The following exclusion criteria were applied: other active malignancies; clinically serious infection; active gastrointestinal bleeding; severe cardiac insufficiency; severe interstitial pneumonitis; massive ascites and/or hydrothorax; concurrent treatment with steroids or immunosuppressive agents; and unsuitability for the trial based on a clinical judgment.

Immunohistochemical analysis

Surgical specimens were stained with hematoxylin and eosin or monoclonal antibodies against GPC3 (clone 1G12; dilution 1:300; BioMosaics), CD8 (clone 1A5; dilution 1:80; Novocastra), and HLA class I (clone EMR8/5; dilution 1:1000; Hokudo), according to the manufacturers' directions.

Ex vivo IFN-γ enzyme-linked immunospot assay

An ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assay was conducted to measure the antigen-specific CTL response, as described previously.[14] Non-cultured PBMCs were added to plates in the presence of peptide antigens (10 μg/mL) and incubated for 20 h at 37 °C in 5% CO_2. The numbers of PBMCs plated per well for case 1 and case 2 were 5×10^5 and 2.5×10^5, respectively.

GPC3 double-determinant ELISA

Double-determinant (sandwich) ELISA of GPC3 was performed as described previously.[14] The serum-soluble protein GPC3 was detected by indirect ELISA using an anti-human GPC3 monoclonal antibody (clone 1G12), anti-human GPC3 sheep polyclonal antibody (R&D Systems), and recombinant human GPC3 (R&D Systems).

Case 1

A 42-y-old nulligravid Japanese female was referred to us in November 2011. She had been diagnosed with an ovarian tumor when she presented with bilateral lower extremity deep vein thrombosis. She had undergone laparotomy including total abdominal hysterectomy, bilateral salpingo-oophorectomy, and sampling of the pelvic lymph nodes at a nearby hospital in August 2010. There were residual tumors of peritoneal dissemination and enlarged retroperitoneal lymph nodes. Histopathology revealed ovarian CCC and the presence of pelvic lymph node metastases. Therefore, her initial clinical stage was IIIC. She was treated postoperatively with six cycles of paclitaxel and carboplatin (TC) chemotherapy. After TC chemotherapy, she once again underwent laparotomy including omentectomy and pelvic and para-aortic systemic lymphadenectomy in March 2011, and a complete response was achieved. The histology of the resected tumor revealed retroperitoneal lymph node metastases, and no pathological chemotherapeutic effects were observed. Thus, she received four cycles of postoperative chemotherapy with irinotecan and cisplatin. CT scans revealed the enlargement of the retroperitoneal lymph nodes and calyx of the right kidney in August 2011. The multiple metastases rapidly progressed, and oral opioids were administered to relieve back pain.

After confirming her HLA type as HLA- A*24:02, she was enrolled in a phase II trial of the GPC3 peptide vaccination. She began receiving intradermal injections of 3 mg of HLA-A24-restricted $GPC3_{298-306}$ GMP grade peptide emulsified with incomplete Freund's adjuvant in November 2011. Vaccinations were performed biweekly from the first until the 6th injection and were repeated at 6-wk intervals after the 7th injection according to the trial schedule.

Pretreatment tumor markers were as follows: CA125, 405.4 U/ml and CA72-4, 264.1 U/ml. The serum levels of these tumor markers decreased after the initiation of treatment (**Fig. 1A**). The pretreatment serum GPC3 protein (17.3 ng/ml) was detectable, but changes in the serum levels of GPC3 unlike other tumor markers had been broadly flat while there was an increase or decrease (data not shown). Liver and para-aortic lymph node metastases grew during the first few weeks before tumor regression. According to RECIST, PR was achieved at week 10. Oral opioids were discontinued. Right hydronephrosis, pleural effusion, and ascites disappeared. Liver and para-aortic lymph node metastases were no longer visible on contrast-enhanced CT after 23 wk of exposure to the vaccine (**Fig. 1B**). However, we

Figure 1 (See opposite page). (**A**) Clinical course from the beginning of the GPC3 peptide vaccination. Serum levels of CA125 and CA72–4 decreased after the initiation of therapy. Black arrows indicate vaccinations. The asterisk indicates right inguinal lymph node resection biopsy. The double asterisk indicates bilateral inguinal lymphadenectomy. (**B**) Contrast-enhanced CT scan showing liver (white, red, blue, and orange arrows) and paraaortic lymph node (yellow arrows) metastases. The size of metastases increased immediately following the initiation of the GPC3 peptide vaccination; however, tumor sizes decreased markedly within three months. (**C, D**) Pathological findings of primary ovarian carcinoma (**C**) and right inguinal lymph node biopsy specimens (**D**). A microscopy image of a hematoxylin and eosin (H&E)-stained section shows CCC (**a, i**). Immunohistochemical staining for GPC3 and HLA class I showed positivity in the primary ovarian carcinoma, respectively (**b, c**). Most CCC cells in the resected right inguinal lymph node metastasis appeared to lack GPC3 expression and a reduction in the expression of HLA class I (**ii, iii**). Immunohistochemical analysis showed a few CD8-positive T cells in the primary ovarian CCC tissue (**d**), whereas there was little infiltration of CD8-positive T cells in the resected right inguinal lymph node metastasis (**iv**). Original magnification, x200.

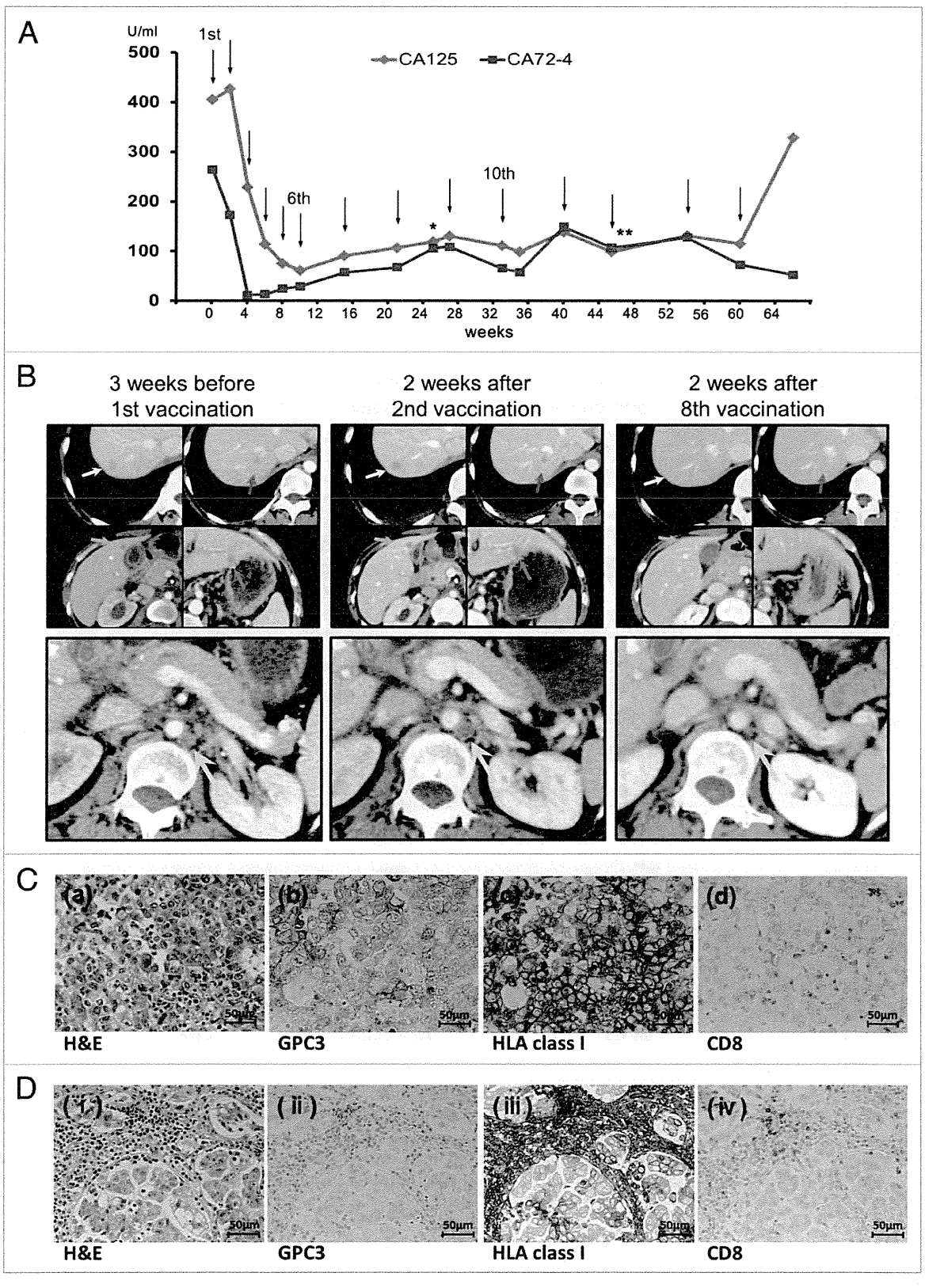

Figure 1. See page 2 for legend.

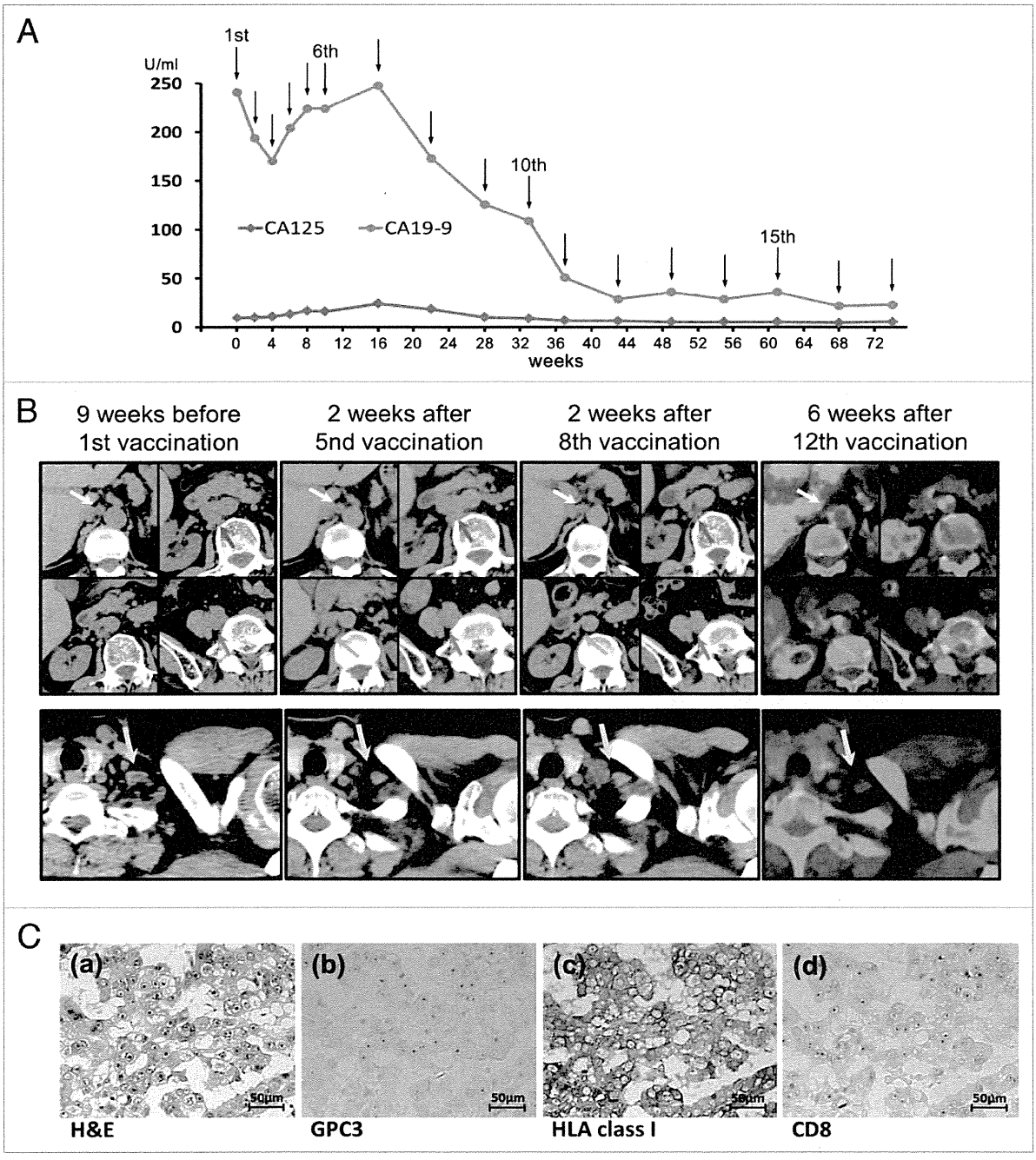

Figure 2. (**A**) Clinical course from the beginning of the GPC3 peptide vaccination. Serum levels of CA19–9 and CA125 decreased after the 7th vaccination. The CA19–9 level decreased to within the normal range. Black arrows indicate vaccinations. (**B**) Plain CT and ^{18}F-FDG PET/CT scans showing retroperitoneal lymph node (white, red, blue and orange arrows) and Virchow's node (yellow arrows) metastases. These metastases were negative on ^{18}F-FDG PET/CT at week 49. (**C**) Pathological findings of primary ovarian carcinoma. A microscopy image of a hematoxylin and eosin (H&E)-stained section shows CCC (**a**). Immunohistochemical staining was performed for GPC3, HLA class I, and CD8. (**b, c, d**). The expression of HLA class I was positive, while that of GPC3 was not, and there was no infiltration of CD8-positive T cells. Original magnification, x200.

observed the slow growth of the bilateral inguinal lymph nodes during the same period. Therefore, right inguinal lymph node resection biopsy was performed at week 25. A histological examination of the biopsied specimen revealed the metastasis of CCC. Immunohistochemical staining was performed with monoclonal antibodies against GPC3, HLA class I, and CD8. Immunohistochemical staining revealed the expression of GPC3 and HLA class I in the cytoplasm and membranes of carcinoma cells and a few CD8-positive T cells in the primary ovarian CCC tissue (**Fig. 1C**), whereas most CCC cells in the resected right inguinal lymph node metastasis appeared to lack GPC3 expression, showed a reduction in the expression of HLA

class I, and there was little infiltration of CD8-positive T cells (**Fig. 1D**).

Because the metastases that had disappeared remained absent, followed by stable disease, we continued to administer the vaccine. The treatment was eventually discontinued due to the development of lower abdominal subcutaneous metastases and progressive disease after the 14th vaccination.

Case 2

A 67-y-old parous Japanese female was referred to our hospital in March 2012. She had undergone laparotomy including total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and sampling of the retroperitoneal lymph nodes in September 2010. Residual enlarged right common iliac and para-aortic lymph nodes were noted. A histological examination showed ovarian CCC. Her initial clinical stage was IIIC due to retroperitoneal lymph node metastases. She was treated postoperatively with nine cycles of weekly TC chemotherapy. Pelvic and para-aortic lymph nodes persisted following weekly TC chemotherapy and increased in size. Two subsequent regimens yielded no response: three cycles of second-line chemotherapy with irinotecan and nedaplatin, and one cycle with gemcitabine and docetaxel as third-line chemotherapy.

She had the HLA-A*02:01 genotype, and began receiving 3 mg of the HLA-A2-restricted $GPC3_{144-152}$ peptide vaccine in April 2012. Pretreatment tumor markers were as follows: CA19-9, 241 U/ml and CA125, 9.8 U/ml. The serum levels of these tumor markers decreased after the 7th vaccination. CA19-9 levels had decreased to within the normal range by week 43 (**Fig. 2A**). The serum GPC3 protein was undetectable.

She achieved stable disease except for Virchow's node by week 24. However, she showed PR at week 37 (**Fig. 2B**). The response resulted in almost the complete resolution of all measurable lesions on plain CT. Unfortunately, she had a past history of allergies to CT contrast media; therefore, we performed ^{18}F-FDG PET/CT to improve the diagnostic accuracy of residual tumors at week 49. ^{18}F-FDG PET/CT showed mild positive FDG uptake in four masses (smaller than 1 cm in size) in the right common iliac lymph node and intraperitoneal dissemination. Although ^{18}F-FDG PET did not always reflect malignancy or detect metastatic lesions, the presence of residual tumors was still suspected.

Because she remained progression free at week 74, she is still receiving the trial treatment. The expression of HLA class I was positive, while that of GPC3 was not positive in the primary ovarian CCC tissue, and there was no infiltration of CD8-positive T cells (**Fig. 2C**).

No adverse effects of the vaccination were observed in either case, except for a local inflammatory response with erythema at the injection site and low-grade fever.

Discussion

Most gynecological oncologists are aware that recurrent or persistent ovarian CCC has a chemoresistant phenotype. Although the different histological types of EOC may represent different diseases with unique clinical and molecular characteristics, ovarian CCC is still currently being treated in the same manner as other EOCs because of its low rate of incidence among EOCs in western countries. Novel treatment approaches should be adopted for ovarian CCC, especially in cases that are recurrent or refractory to previous therapies. Between 5 and 10% of all currently open clinical trials for ovarian cancer patients evaluate approaches using immune-based therapies. Although most immunotherapeutic strategies for ovarian cancer treatment investigated so far are capable of inducing antigen-specific immunity, the unequivocal clinical benefit for these patients has not yet been demonstrated.[15] To date, we have confirmed that a HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone can recognize and kill HLA-A2-positive and GPC3-positive ovarian CCC cell lines.[16]

Based on these conditions, we conducted a trial to assess the clinical outcome of ovarian CCC patients treated with a GPC3-derived peptide vaccine. In the ongoing clinical trial, 20 refractory patients were enrolled until the end of August 2013. Ten of these patients were vaccinated at least six times, and a significant clinical response was achieved in two patients (2/10, 20%) who received the HLA-A24 or A2-restricted GPC3 peptide vaccine. In spite of resistance to multiple chemotherapeutic drugs, the stabilization of their diseases over one year suggests the efficacy of the GPC3 peptide vaccination.

Two patterns of responses, fast and slow, after the initial increase in the total tumor burden were observed in this study. In case 1, a fast response was preceded by an apparent early enlargement in liver and para-aortic lymph node metastases. Metastases were stable for several months in case 2 after beginning the vaccination treatment, except for Virchow's node, which showed radiographical progression; however, all radiographically measurable metastases thereafter almost completely regressed. We hypothesize that the immune response elicited during the first several weeks of vaccination, similarly to ipilimumab,[17] may be mistaken for progressive disease: CTL infiltration and immune-mediated inflammation may not be radiographically distinguishable from a growing tumor. The initial tumor enlargement was suspected to be caused by inflammation. Meanwhile, bilateral inguinal lymph node metastases grew without reductions within the same patient in case 1. Disease progression may occur as part of a "mixed response", i.e., the regression of some lesions and apparent progression of others.

The difference in effectiveness may have been caused by the heterogeneity associated with immune-escape mechanisms, including the downregulation of cancer-specific antigens and/or HLA class I in tumor cells. The intratumor heterogeneity of GPC3 expression was observed at different levels in our preliminary study depending on the locations and timing of biopsies. Although no correlation was observed between the degree of GPC3 expression and $GPC3_{298-306}$ peptide-specific CTL response, GPC3 expression in bilateral inguinal lymph node metastasis in case 1 may be associated with the clinical benefits of GPC3 peptide vaccine therapy. On the other hand, we were unable to perform biopsies of retroperitoneal lymph node metastases in case 2. Therefore, further pathological analysis was limited. Although GPC3 immunohistological expression was

negative in part of the primary ovarian CCC tissue, its expression may be positive in other parts of the tumor. It may be difficult to predict the clinical response against metastatic tumors based on the strength of GPC3 expression in the primary tumor.

In a phase I trial of GPC3-derived peptide vaccination, while GPC3 immunohistological expression was detected in more than 80% of advanced HCC patients, pretreatment serum GPC3 protein was detectable in only approximately half of those patients. Unlike case 1, the serum GPC3 protein in case 2 was undetectable by ELISA using a monoclonal antibody (clone 1G12) and sheep polyclonal antibody. Delayed clinical response may have been due to lower expression of GPC3 than the detection limit of assays using clone 1G12 in metastatic sites.

Ex vivo IFN-γ ELISPOT analysis in these two cases revealed vaccine-induced immune reactivity against the GPC3 peptide (data not shown). Although we were unable to discuss whether there were any differences in the quantity or quality of the responses between the two cases because the number of PBMCs plated per well did not correspond, there were more GPC3 peptide-specific CTL spots and less non-specific background spots in case 2 than in case 1.

It is difficult to confirm whether tumor regression was actually induced by peptide-specific CTLs or other mechanisms. As one of several possibilities, antigen spreading may have occurred following the GPC3 peptide-specific CTL response after the vaccination and contributed decisively to tumor regression.

Although many clinical trials have been conducted with cancer peptide vaccines, none of these have succeeded in Phase 3. The development of a biomarker to select potential responders would contribute significantly to potential success in a Phase 3 trial. In an effort to identify such biomarkers, we consider it possible to predict a response based on HLA class I/peptide complex expression on the cell surface in prevaccine biopsy specimens. Thus, we have attempted to prepare monoclonal antibodies against the HLA-A24/GPC3$_{298\text{-}306}$ peptide complex and HLA-A2/GPC3$_{144\text{-}152}$ peptide complex. Further analyses are needed in the future.

These results provide the first clinical evidence to demonstrate that GPC3 peptide-based immunotherapy is a promising treatment for patients with ovarian CCC. The complexity of the immune response and impact of each individual patient's status on the immune system create challenges for predicting the time course of the response. Ongoing and future trials will yield information on the best clinical use of this vaccine and the most appropriate method for assessing the response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Kennedy AW, Biscotti CV, Hart WR, Webster KD. Ovarian clear cell adenocarcinoma. Gynecol Oncol 1989; 32:342-9; PMID:2920955; http://dx.doi.org/10.1016/0090-8258(89)90637-9
2. Sugiyama T, Kamura T, Kigawa J, Terakawa N, Kikuchi Y, Kita T, Suzuki M, Sato I, Taguchi K. Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. Cancer 2000; 88:2584-9; PMID:10861437; http://dx.doi.org/10.1002/1097-0142(20000601)88:11<2584::AID-CNCR22>3.0.CO;2-5
3. Chan JK, Teoh D, Hu JM, Shin JY, Osann K, Kapp DS. Do clear cell ovarian carcinomas have poorer prognosis compared to other epithelial cell types? A study of 1411 clear cell ovarian cancers. Gynecol Oncol 2008; 109:370-6; PMID:18395777; http://dx.doi.org/10.1016/j.ygyno.2008.02.006
4. Crotzer DR, Sun CC, Coleman RL, Wolf JK, Levenback CF, Gershenson DM. Lack of effective systemic therapy for recurrent clear cell carcinoma of the ovary. Gynecol Oncol 2007; 105:404-8; PMID:17292461; http://dx.doi.org/10.1016/j.ygyno.2006.12.024
5. Takano M, Goto T, Kato M, Sasaki N, Miyamoto M, Furuya K. Short response duration even in responders to chemotherapy using conventional cytotoxic agents in recurrent or refractory clear cell carcinomas of the ovary. Int J Clin Oncol 2013; 18:556-7; PMID:22552358; http://dx.doi.org/10.1007/s10147-012-0404-x
6. Kajiyama H, Shibata K, Mizuno M, Yamamoto E, Fujiwara S, Umezu T, Suzuki S, Nakanishi T, Nagasaka T, Kikkawa F. Postrecurrent oncologic outcome of patients with ovarian clear cell carcinoma. Int J Gynecol Cancer 2012; 22:801-6; PMID:22617480; http://dx.doi.org/10.1097/IGC.0b013e3182540145
7. Nakatsura T, Yoshitake Y, Senju S, Monji M, Komori H, Motomura Y, Hosaka S, Beppu T, Ishiko T, Kamohara H, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003; 306:16-25; PMID:12788060; http://dx.doi.org/10.1016/S0006-291X(03)00908-2
8. Nakatsura T, Kageshita T, Ito S, Wakamatsu K, Monji M, Ikuta Y, Senju S, Ono T, Nishimura Y. Identification of glypican-3 as a novel tumor marker for melanoma. Clin Cancer Res 2004; 10:6612-21; PMID:15475451; http://dx.doi.org/10.1158/1078-0432.CCR-04-0348
9. Saikali Z, Sinnett D. Expression of glypican 3 (GPC3) in embryonal tumors. Int J Cancer 2000; 89:418-22; PMID:11008203; http://dx.doi.org/10.1002/1097-0215(20000920)89:5<418::AID-IJC4>3.0.CO;2-I
10. Toretsky JA, Zitomersky NL, Eskenazi AE, Voigt RW, Strauch ED, Sun CC, Huber R, Meltzer SJ, Schlessinger D. Glypican-3 expression in Wilms tumor and hepatoblastoma. J Pediatr Hematol Oncol 2001; 23:496-9; PMID:11878776; http://dx.doi.org/10.1097/00043426-200111000-00006
11. Aviel-Ronen S, Lau SK, Pintilie M, Lau D, Liu N, Tsao MS, Jothy S. Glypican-3 is overexpressed in lung squamous cell carcinoma, but not in adenocarcinoma. Mod Pathol 2008; 21:817-25; PMID:18469798; http://dx.doi.org/10.1038/modpathol.2008.37
12. Maeda D, Ota S, Takazawa Y, Aburatani H, Nakagawa S, Yano T, Taketani Y, Kodama T, Fukayama M. Glypican-3 expression in clear cell adenocarcinoma of the ovary. Mod Pathol 2009; 22:824-32; PMID:19329941
13. Komori H, Nakatsura T, Senju S, Yoshitake Y, Motomura Y, Ikuta Y, Fukuma D, Yokomine K, Harao M, Beppu T, et al. Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 2006; 12:2689-97; PMID:16675560; http://dx.doi.org/10.1158/1078-0432.CCR-05-2267
14. Sawada Y, Yoshikawa T, Nobuoka D, Shirakawa H, Kuronuma T, Motomura Y, Mizuno S, Ishii H, Nakachi K, Konishi M, et al. Phase I trial of a glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma: immunologic evidence and potential for improving overall survival. Clin Cancer Res 2012; 18:3686-96; PMID:22577059; http://dx.doi.org/10.1158/1078-0432.CCR-11-3044
15. Leffers N, Daemen T, Helfrich W, Boezen HM, Cohlen BJ, Melief K, Nijman HW. Antigen-specific active immunotherapy for ovarian cancer. Cochrane Database Syst Rev 2010; 20:CD007287; PMID:20091627
16. Suzuki S, Yoshikawa T, Hirosawa T, Shibata K, Kikkawa F, Akatsuka Y, Nakatsura T. Glypican-3 could be an effective target for immunotherapy combined with chemotherapy against ovarian clear cell carcinoma. Cancer Sci 2011; 102:622-9; PMID:21205085; http://dx.doi.org/10.1111/j.1349-7006.2011.02003.x
17. Weber JS, Kähler KC, Hauschild A. Management of immune-related adverse events and kinetics of response with ipilimumab. J Clin Oncol 2012; 30:2691-7; PMID:22614989; http://dx.doi.org/10.1200/JCO.2012.41.6750

Identification of HLA-A2 or HLA-A24-restricted CTL epitopes for potential HSP105-targeted immunotherapy in colorectal cancer

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Received October 21, 2013; Accepted November 25, 2013

DOI: 10.3892/or.2013.2941

Abstract. We previously reported that heat shock protein 105 (HSP105) is overexpressed in a variety of human cancers, including colorectal, pancreatic and esophageal cancer and has proven to be a novel biomarker for the immunohistochemical detection of these cancers. In the present study, we used HLA-transgenic mice (Tgm) and the peripheral blood mononuclear cells (PBMCs) of colorectal cancer patients to identify HLA-A2 and HLA-A24-restricted HSP105 epitopes, as a means of expanding the application of HSP105-based immunotherapy to HLA-A2- or HLA-A24-positive cancer patients. In addition, we investigated by *ex vivo* IFN-γ ELISPOT assay whether the HSP105-derived peptide of cytotoxic T cells (CTLs) exists in PBMCs of pre-surgical colorectal cancer patients. We found that four peptides, HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGIYKQDL) and HSP105 A24-7 (EYVYEFRDKL), are potential HLA-A2 or HLA-A24-restricted CTL HSP105-derived epitopes. HSP105-specific IFN-γ-secreting T cells were detected in 14 of 21 pre-surgical patients with colorectal cancer in response to stimulation with these four peptides. Our study raises the possibility that these HSP105 peptides are applicable to cancer immunotherapy in patients with HSP105-expressing cancer, particularly colorectal cancer.

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Key words: cancer immunotherapy, cytotoxic T cell, colorectal cancer, heat shock protein 105, HLA-transgenic mice

Introduction

Colorectal cancer is one of the most prevalent cancers and a major cause of mortality worldwide (1). Although adjuvant systemic chemotherapy or chemoradiation can confer a limited but significant survival advantage, novel and more effective therapies are needed. To improve survival rates, new therapeutic agents have been investigated. Immunotherapy for colorectal cancer is a promising candidate treatment, and there is evidence that host immune responses can influence survival (2). Ideal targets for immunotherapy are gene products overexpressed in cancer cells but silenced in normal tissues, with the exception of immune-privileged tissues, such as that of the testis.

We previously reported that heat shock protein 105 (HSP105), identified by SEREX, is overexpressed in a variety of human cancers, including colorectal, pancreatic and esophageal cancer, but with little to no expression in normal tissues aside from the testis (3,4). HSP105 is a stress protein induced by various stressors and belongs to the HSP105/110 family and plays an important role as a chaperone under physiological conditions (5). Using immunohistochemical analysis, we previously found that HSP105 was specifically overexpressed in 44 of 53 (83.0%) colorectal cancer patients (4). It has also been reported that DNA vaccination with both HSP105 and bone marrow-derived dendritic cells (BM-DCs) pulsed with HSP105 led to tumor rejection of colorectal cancer but did not induce an autoimmune reaction in mice (6-8).

This suggests that HSP105 presents a useful tumor-specific antigen target for immunotherapy. However, HSP105-derived epitope peptides of $CD8^+$ T cells have not been identified. The gene frequency of HLA-A24 (A*24:02) is relatively high in Asian populations, especially the Japanese, but low in Caucasians. On the other hand, the gene frequency of HLA-A2 (A*02:01) is high among several ethnic groups, including Asians and Caucasians (9). Therefore, HLA-A2 or HLA-A24-restricted cytotoxic T cell (CTL) HSP105 epitopes could be extremely

useful for immunotherapy in a large portion of patients worldwide. In the present study, we identified human HSP105-derived CTL epitopes restricted by HLA-A2 or HLA-A24 using HLA-transgenic mice (Tgm) and examined whether these epitope-based peptides could activate HSP105-reactive CTLs in peripheral blood mononuclear cells (PBMCs) of patients with colorectal cancer.

Materials and methods

Mice. HLA-A2.1 (HHD) Tgm, H-2D$^{b-/-}$β2m$^{-/-}$ double-knockout mice introduced with the human β2m-HLA-A2.1(α1 α2)-H-2D^{b} (α3 transmembrane cytoplasmic) (HHD) mono-chain gene construct were generated in the Departmente SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, Paris, France (10,11) and were kindly provided by Dr F.A. Lemonier. HLA-A24.2 (HHD) Tgm were purchased from Japan SLC, Inc. (Shizuoka, Japan). Female 6- to 8-week-old BALB/c mice (H-2K^{d}) and BALB/c nude mice, purchased from Charles River Japan (Yokohama, Japan), were maintained and handled in accordance with animal care policy.

Cell lines. The human colorectal cancer cell line SW620 (endogenously expressing HSP105 and HLA-A*02:01, 24:02) and human liver cancer cell line HepG2 (HSP105-low expressing and HLA-A*02:01, 24:02), were kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). Murine colorectal cancer cells, Colon26 (C26) (endogenously expressing HSP105 and H-2K^{d}) were kindly provided by Dr Kyoichi Shimomura (Fujisawa Pharmaceutical Co., Osaka, Japan). T2 cells (a TAP-deficient and HLA-A*02:01-positive cell line) were provided by Kyogo Ito of Kurume University. Cells were maintained *in vitro* in RPMI-1640 or DMEM supplemented with 10% FCS.

RNA interference. Small interfering RNAs targeting human HSP105 were chemically synthesized by Dharmacon Research (HSP105-siRNA and luciferase; Lafayette, CO, USA) as previously described (12), with the following siRNA sequences: HSP105-siRNA, UUGGCUGCAACUCCGAUU GTT and luciferase, CGUACGCGGAAUACUUCGATT. The transfection of siRNA oligonucleotides was carried out using Oligofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines.

Peptides. Human HSP105-derived peptides, identical in amino acid sequence with mouse HSP105 and expressing the binding motifs for HLA-A*02:01- and HLA-A*24:02-encoded molecules, were designed with BIMAS software (BioInformatics and Molecular Analysis Section; Center for Information Technology, NIH, MD, USA). We purchased a total of 16 versions of peptides carrying the HLA-A2 (A*0201)-binding motifs and 9 versions of peptides carrying the HLA-A24 (A*2402)-binding motifs from Biologica (Tokyo, Japan) (Table I).

Induction of HSP105-reactive CTLs in Tgm. Peptide immunizations in mice were performed as previously described (13). In brief, bone marrow (BM) cells (2x10^{6}) from HLA-A2 or HLA-A24 Tgm were cultured in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (5 ng/ml) and 2-mercaptoethanol (0.8 ng/ml) for 7 days in 10-cm plastic dishes. These BM-DCs were pulsed with the two HSP105 peptide mixtures (1 μmol/l each peptide) for 2 h at 37˚C. We primed the HLA-A2 or HLA-A24 Tgm with the syngeneic BM-DC vaccine (5x10^{5}/mice) into the peritoneal cavity twice, once per week. Seven days following the last immunization, the spleens were collected and CD4^{-} spleen cells were isolated by negative selection with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude any nonspecific IFN-γ production from the CD4^{+} spleen cells co-cultured with the BM-DCs. The CD4^{-} spleen cells (2x10^{6}/well) were stimulated with syngeneic BM-DCs (2x10^{5}/well) that had been pulsed with each peptide *in vitro*. After 6 days, the frequency of cells producing IFN-γ/2x10^{4} CD4^{-} spleen cells upon stimulation with syngeneic BM-DCs (1x10^{4}/well), pulsed with or without each peptide, was assayed using an enzyme-linked immunospot (ELISPOT) assay as previously described (13).

Identification of a CTL epitope in BALB/c mice. The peptide immunizations in mice were performed as previously described (14). Splenocytes removed from mice 7 days following the last immunization were harvested and cultured in 24-well culture plates (2.5x10^{6}/well) in 45% RPMI, 45% AIMV, 10% FCS and supplemented with recombinant human interleukin 2 (100 U/ml), 2-mercaptoethanol (50 μmol/l) and each peptide (10 μmol/l). After 5 days, the cytotoxicity of these cells against target cells was assayed using standard 6-h ^{51}Cr release assays (15).

Blood samples. Blood samples from cancer patients were collected during routine diagnostic procedures after obtaining formal consent from patients at the Kumamoto University Hospital, from April to September 2006 and from patients at the National Cancer Center Hospital East, from December 2006 to March 2007. The study was approved by the local ethics committee, and informed consent was obtained from all patients.

Induction of HSP105-reactive human CTLs. We isolated PBMCs from heparinized blood of HLA-A24^{+} and/or HLA-A2^{+} Japanese patients with colorectal cancer using Ficoll-Conray density gradient centrifugation; peripheral monocyte-derived dendritic cells (DCs) were generated as previously described (16,17). CD8^{+} T cells were isolated with CD8 microbeads (Miltenyl Biotec, Bergisch Gladbach, Germany) from PBMCs of the same donor and peptide-reactive CD8^{+} CTLs were generated. Five days following the last stimulation, the cytotoxic activities of the CTLs against cancer cell lines were measured by ^{51}Cr-release assay as previously described (15). For these assays, CTLs were co-cultured with each cancer cell line, as the target cells (5x10^{3}/well), at the indicated effector/target ratio.

In vivo tumor challenge. Subcutaneous tumors were induced in mice by injecting 1x10^{4} SW620 cells suspended in 100 μl PBS or Hanks' balanced salt solution (Gibco, Grand Island, NY, USA) into the backs of BALB/c nude mice. Tumor incidence and volumes were assessed weekly using calipers and tumor areas were measured. Results are presented as mean tumor areas ± SD.

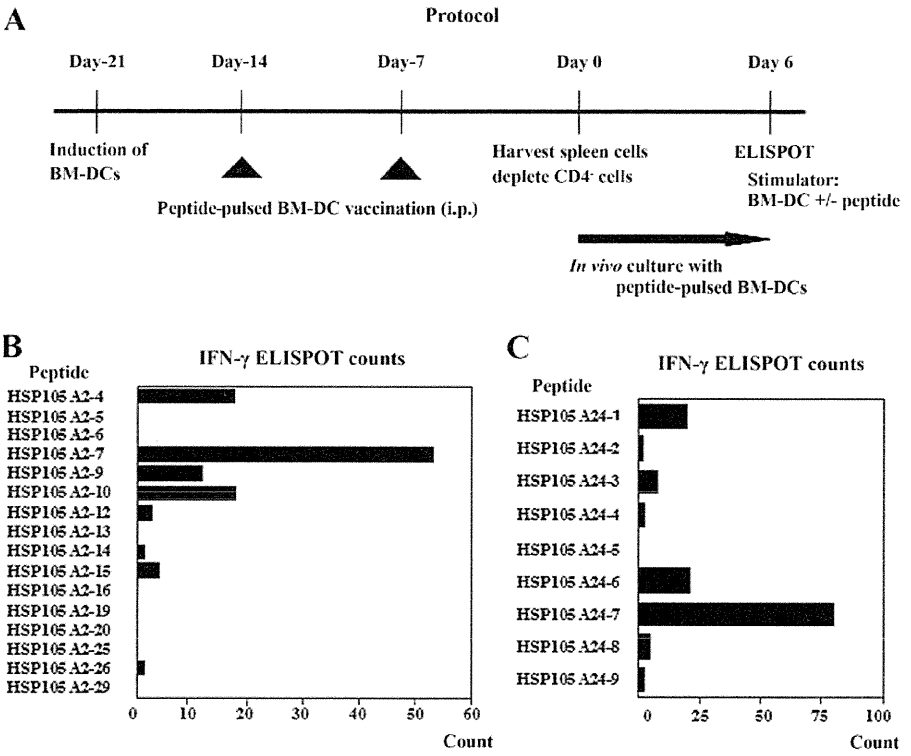

Figure 1. Identification of HLA-A2 or HLA-A24-restricted CTL epitopes of HSP105 using HLA-A2.1 Tgm and HLA-A24 Tgm. (A) The protocol used for identification of HLA-A2 or HLA-A24-restricted CTL epitopes of HSP105 is shown. We primed the HLA Tgm with BM-DCs ($5x10^5$) pulsed with the mixture of HSP105-derived peptides carrying the HLA-A2 or HLA-A24 binding motif into the peritoneal cavity once a week for 2 weeks. Seven days after the last DC vaccination, spleens were collected and $CD4^-$ spleen cells ($2x10^6$/well) were stimulated with syngeneic BM-DCs ($2x10^5$/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured $CD4^-$ spleen cells as responder cells in the IFN-γ ELISPOT assay. (B) The bar graphs show the IFN-γ ELISPOT counts per $2x10^4$ $CD4^-$ spleen cells co-cultured with HLA-A2-restricted peptide-pulsed BM-DCs after normalization to counts from cells co-cultured with BM-DCs without peptide loading. (C) The bar graphs show the IFN-γ ELISPOT counts in the HLA-A24-restricted peptides. The columns represent the means from duplicate assays.

Ex vivo IFN-γ ELISPOT assay in peripheral blood in pre-surgical colorectal cancer patients. Ex vivo IFN-γ ELISPOT assays were performed to determine tumor-specific interferon-γ (IFN-γ)-secreting T cells. The 96-well plates were coated with anti-human IFN-γ (BD Biosciences Co., Ltd., USA). After an overnight incubation at 4˚C, the wells were washed and blocked with complete medium for 2 h at room temperature. A total of $1x10^6$ unfractionated PBMCs were added in duplicate wells and incubated at 37˚C for 18-20 h with or without peptides at 0.2 μl/well (1-10 μM). The plate was washed and then incubated with 5 μg/ml biotinylated anti-human IFN-γ antibody for 2 h at room temperature. After washing away the antibodies, streptavidin-HRP was added for 1 h. Finally, the plate was washed and replaced with fresh substrate solution and the reaction was terminated by washing with distilled water. The HLA-A2-restricted CMV peptide (NLVPMVATV) and HLA-A24 restricted CMV peptide (QYDPVAALF), which includes an epitope derived from the CMV pp65 protein, were used as positive controls.

Histological and immunohistochemical analysis. To investigate whether $CD8^+$ T cells infiltrated normal tissues triggered by the HSP105-derived peptide vaccine, we performed immunohistochemical staining with a monoclonal antibody against CD8 (1:100; LifeSpan BioSciences, Inc., Seattle, WA, USA) in tissue specimens from HLA-A2 Tgm immunized with the HSP105 peptides, as previously described (7). Immunohistochemical staining with rabbit polyclonal antibodies against HSP105 (1:200; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA) was performed according to the manufacturer's instructions.

Results

Identification of HLA-A2-or HLA-A24-restricted CTL epitopes in HLA Tgm. We designed pools of HSP105 peptides possessing amino acid sequences conserved between humans and mice that have a highly predicted binding score to HLA-A2 (pool of 16 different peptides) or HLA-A24 (A*24:02) (pool of 9 different peptides) (Table I). $CD4^-$ spleen cells were obtained from Tgm immunized twice i.p. with BM-DCs that had been pulsed with each peptide mixture; the spleen cells were then stimulated *in vitro*, again with the BM-DCs pulsed with each peptide mixture (Fig. 1A).

The IFN-γ ELISPOT counts, normalized to those of spleen cells co-cultured with BM-DCs without peptide loading, clearly indicated a HSP105 A2-7 peptide-specific response in the $CD4^-$ spleen cells (Fig. 1B). These $CD4^-$ spleen cells ($2x10^4$/well) showed 55±29.7 spot counts/well in response to the BM-DCs pulsed with the HSP105 A2-7 peptide, whereas they showed 23±31.1 spot counts/well in the presence of

Table I. HSP105-derived peptides conserved between human and mouse HSP105 predicted to bind to HLA-A2 or HLA-A24.

Peptides	Position	Subsequent residue listing	HLA-A2 binding score
HSP105 A2-4	120-128	MLLTKLKET	107
HSP105 A2-5	141-149	VISVPSFFT	55
HSP105 A2-6	155-163	SVLDAAQIV	37
HSP105 A2-7	169-177	RLMNDMTAV	591
HSP105 A2-9	202-210	DMGHSAFQV	21
HSP105 A2-10	222-230	VLGTAFDPFL	759
HSP105 A2-12	275-284	KLMSSNSTDL	276
HSP105 A2-13	276-284	LMSSNSTDL	26
HSP105 A2-14	300-309	KMNRSQFEEL	50
HSP105 A2-15	304-313	SQFEELCAEL	32
HSP105 A2-16	313-321	LLQKIEVPL	36
HSP105 A2-19	434-442	FLRRGPFEL	43
HSP105 A2-20	458-467	KIGRFVVQNT	76
HSP105 A2-25	668-676	LLTETEDWL	401
HSP105 A2-26	675-684	WLYEEGEDQA	146
HSP105 A2-29	757-765	EVMEWMNNV	15

Peptides	Position	Subsequent residue listing	HLA-A24 binding score
HSP105 A24-1	180-188	NYGIYKQDL	240
HSP105 A24-2	214-223	AFNKGKLKVL	30
HSP105 A24-3	251-260	KYKLDAKSKI	110
HSP105 A24-4	305-313	QFEELCAEL	47
HSP105 A24-5	433-442	TFLRRGPFEL	33
HSP105 A24-6	613-622	MYIETEGKMI	90
HSP105 A24-7	640-649	EYVYEFRDKL	330
HSP105 A24-8	725-733	HYAKIAADF	140
HSP105 A24-9	739-748	KYNHIDESEM	82

The binding scores were estimated by using BIMAS software: http//bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform.

BM-DCs pulsed with the HSP105 A2-4 peptide. A similarly strong response was observed for the HSP105 A24-7 peptide (Fig. 1C). $CD4^-$ spleen cells ($2x10^4$/well) showed 79.5±27.6 spot counts/well in response to the BM-DCs pulsed with the HSP105 A24-7 peptide, whereas they showed 20.5±14.8 spot counts/well in the presence of BM-DCs with the HSP A24-6 peptide. These assays were performed twice with similar results and they suggest that the HSP105 A2-7 and A24-7 peptides are potential CTL epitope peptides in both HLA Tgm and humans.

Identification of a CTL epitope in BALB/c mice and CTLs that are cytotoxic against C26 tumors in mice. There were similar structural motifs within the peptides that bound to human HLA-A24 and mice K^d. We selected those peptides with binding motifs for both HLA-A24 and K^d molecules and prepared 9 different synthetic peptides (HSP105-1-9). When we tested these peptides for their potential to induce *in vitro* tumor reactive CTLs in spleen cells derived from BALB/c mice immunized with the HSP105 peptides, only the HSP105 24-1 peptide-induced CTLs showed specific cytotoxicity against C26 tumors ($HSP105^+$, $H\text{-}2K^d$) (Fig. 2). The cytotoxicity against C26 was attenuated by HSP105 siRNA. These findings indicate that the HSP105 A24-1 peptide has the capacity to induce tumor reactive CTLs and that peptide vaccination-primed CTLs are reactive to this peptide *in vivo*. We would expect this HSP105 A24-1 (NYGIYKQDL) peptide to also be an epitope for human CTLs.

HSP105-reactive CTLs from PBMCs of HLA-A2-positive colorectal cancer patients and CTLs induce cytotoxicity against HSP105-expressing cancer cells. We generated a CTL line from PBMCs of colorectal patients by stimulation with the HSP105 A2-12 peptide. As shown by ^{51}Cr release assays, the resulting CTLs showed HSP105-specific cytotoxicity against SW620 cells ($HSP105^{+++}$, HLA-A2) and against T2 cells pulsed with the HSP105 A2-12 peptide ($HSP105^-$, HLA-A2), but not against HepG2 cells ($HSP105^{\pm}$, HLA-A2) or T2 cells pulsed with an irrelevant peptide (Fig. 3A). HSP105 siRNA decreased the cytotoxicity against SW620 cells. We investigated the effects of the HSP105 A2-12 peptide-reactive CTL lines on the mice implanted with the SW620 cells. Fourteen days after inoculation of HSP105 A2-12 peptide-reactive CTLs, there was an apparent reduction in tumor size in the SW620 compared to that in untreated mice (Fig. 3B). These results clearly indicate the efficacy of HSP105 A2-12 (KLMSSNSTDL) peptide-reactive CTL injection therapy for $HSP105^+$ tumors in mice.

Detection of HSP105-specific CTLs in peripheral blood of pre-surgical patients with colorectal cancer. Our results suggest that the four peptides, HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGIYK QDL) and HSP105 A24-7 (EYVYEFRDKL), are HSP105-derived, HLA-A2, or HLA-A24-restricted CTL epitopes. To determine the frequencies of the HSP105-derived T cells specific for these peptide in pre-surgical colorectal cancer patients, we analyzed the PBMC responses for each peptide using the ELISPOT assay. HSP105 expression was detected in 20 of 21 (95%) patients, consistent with previous studies (4). HSP105-specific T cells secreting IFN-γ were detected in patients stimulated with the HSP105 A2-7 (4 patients), HSP105 A2-12 (6 patients), HSP105 A24-1 (2 patients) and HSP105 A24-7 (6 patients) peptides (Table II). ELISPOT assay detected positive IFN-γ responses to at least one of the HSP105-derived peptides in PBMCs in 14 of the 21 patients. In contrast to the results for colorectal cancer patients, the 4 peptides were not recognized by PBMCs from healthy donors. Both the ratio of normal donors who showed positive T-cell responses to CMV-derived peptides and the frequencies of the specific T cells were identical to those of the colorectal cancer patients (data not shown).

HSP105-derived peptide immunization does not induce autoimmunity in HLA-A2 Tgm. HSP105 in normal adult mice is expressed in only certain tissues, and expression in these tissues is less than that in C26 tumor cells, suggesting a low

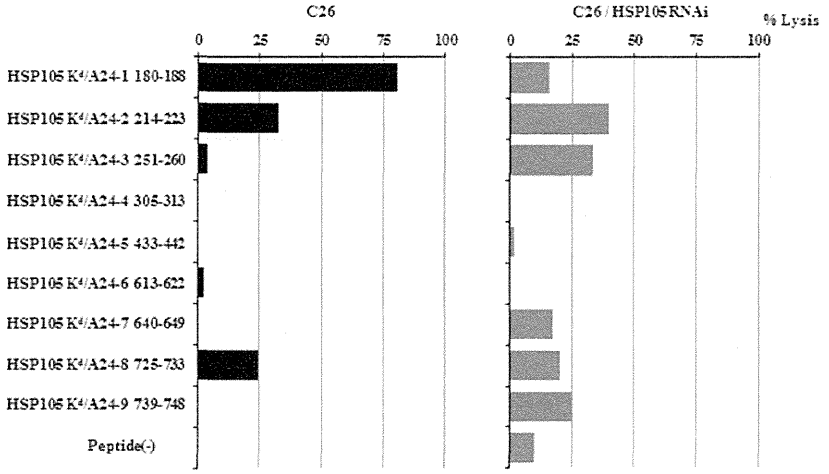

Figure 2. Identification of an HSP105-derived HLA-A24 and K^d-restricted CTL epitope. BALB/c mice were immunized with 9 HSP105 peptides. Using the ^{51}Cr release assay, sensitized spleen cells that had been stimulated *in vitro* with each HSP105 peptide (10 μmol/l) and cultured for 5 days with 100 U/ml interleukin-2 were examined for CTL activity against C26 cells and C26 cells transfected with HSP105 siRNA (C26/HSP105 RNAi). Values represent the percentage of specific cell lysis, based on the mean values from triplicate assays.

Table II. Expression of HSP105 in colorectal cancer tissue and quantification of HSP-specific CTLs in colorectal cancer patients.

						[c]Spot number of peptide-specific CTLs				
HLA-A2-positive patients	Age (yrs.)	Gender	HLA	Stage[a] of tumor	HSP105 expression[b]	HSP105 A2-7		HSP105 A2-12		CMV
1	62	M	0201/2601	IIIB	++	27	+	126	+	160
5	79	M	0207/1101	IIIB	++	0	-	2	-	10
6	51	M	0201/0206	I	+	0	-	49	+	136
8	55	M	0206/2402	I	±	0	-	0	-	66
11	69	M	0206/2402	IIIC	+	143	+	0	-	0
12	61	M	0201/3303	I	±	2	-	45	+	367
13	64	F	0201/2601	IIIC	±	0	-	2	-	254
14	66	M	0206/2402	IIIC	-	13	+	0	-	58
15	78	M	0201/1101	IIA	+	0	-	5	+	57
16	51	F	0206/2601	IV	±	31	+	7	+	15
17	63	F	0206/1101	IIA	++	0	-	25	+	96
HLA-A2402-positive patients						HSP105 A24-1		HSP105 A24-7		CMV
2	64	F	2402	IV	++	2	-	44	+	6
3	60	M	2402/3101	IIIC	++	0	-	0	-	11
4	71	F	2402/3101	IIA	++	25	+	51	+	12
7	47	M	2402/3101	IIIA	++	4	-	6	+	3
9	66	M	2402	IV	++	8	+	6	+	7
10	60	M	2402/3101	I	++	1	-	19	+	26
18	64	M	1101/2402	IV	+	0	-	2	-	40
20	46	F	1101/2402	IIIB	++	4	-	7	+	5
21	66	F	2402	I	++	3	-	0	-	38

F, female; M, male. [a]Stage, staging was performed according to the TNM classification (Union for International Cancer Control; UICC). [b]HSP105 expression, staining intensity of tumor cells was scored on a scale according to the following four grades: -, absent; ±, weak; +, moderate; ++, strong. [c]Spot number indicates the number of peptide-specific CTLs calculated by subtracting the spot number in a well of no peptide. -, Spot number <5; +, Spot number ≥5.

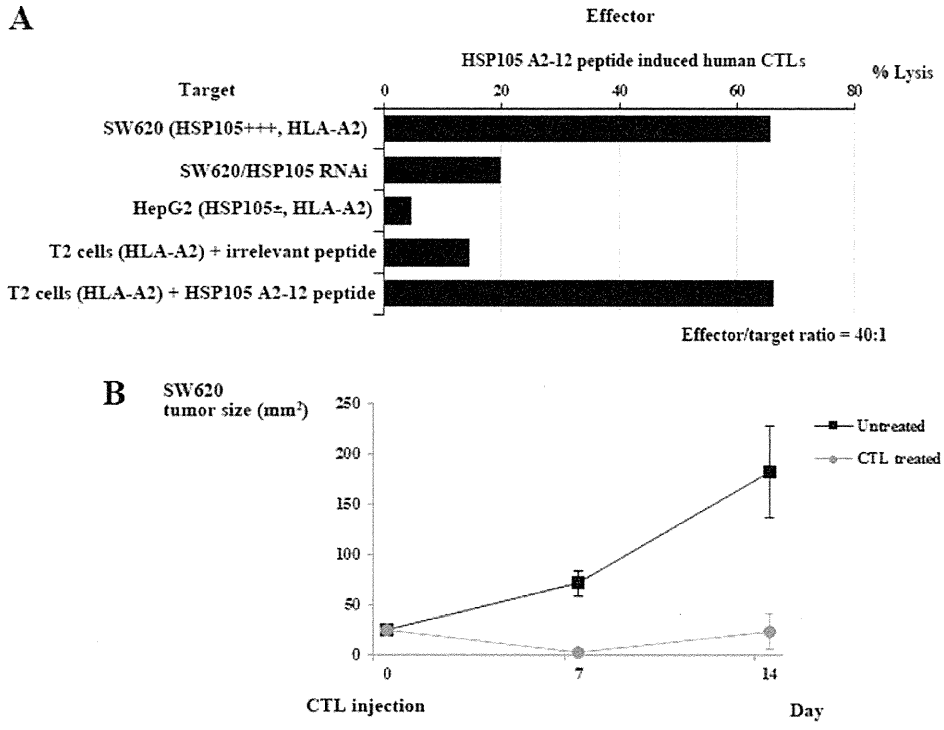

Figure 3. CTL induction from PBMCs of HLA-A2-positive cancer patients. (A) HSP105 peptide-reactive CTLs were generated from $CD8^+$ T cells of HLA-$A2^+$ colorectal cancer patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the HSP105 A2-12 peptides, the CTLs were subjected to a standard ^{51}Cr release assay at the indicated effector/target ratio (40/1). Their cytotoxicity against SW620 cells ($HSP105^{+++}$, HLA-A2), SW620 cells transfected with HSP105 siRNA ($HSP105^-$), HepG2 cells ($HSP105^{\pm}$, HLA-A2), T2 cells pulsed with an irrelevant peptide ($HSP105^-$, HLA-A2) and T2 cells pulsed with the HSP105 A2-12 epitope peptide were all examined by ^{51}Cr release assay. Values represent the percentage of specific cell lysis, based on the mean values from triplicate assays. (B) There was marked growth inhibition of SW620 cells ($HSP105^+$) engrafted into nude mice after intratumoral injection of human CTLs induced by the HSP105 peptides. When tumor size reached 25 mm^2 on day 9 after s.c. tumor implantation, human CTLs ($3x10^6$) reactive to the HLA-A2-restricted HSP105 peptide, generated from an HLA-$A2^+$ donor, were i.t. inoculated. Tumor sizes in nude mice administered the HSP105 epitope peptide-induced CTL lines (n=3), or no treatment (n=3), are shown. The mean tumor size (mm^2) for each group of mice was expressed, and bars represent SD.

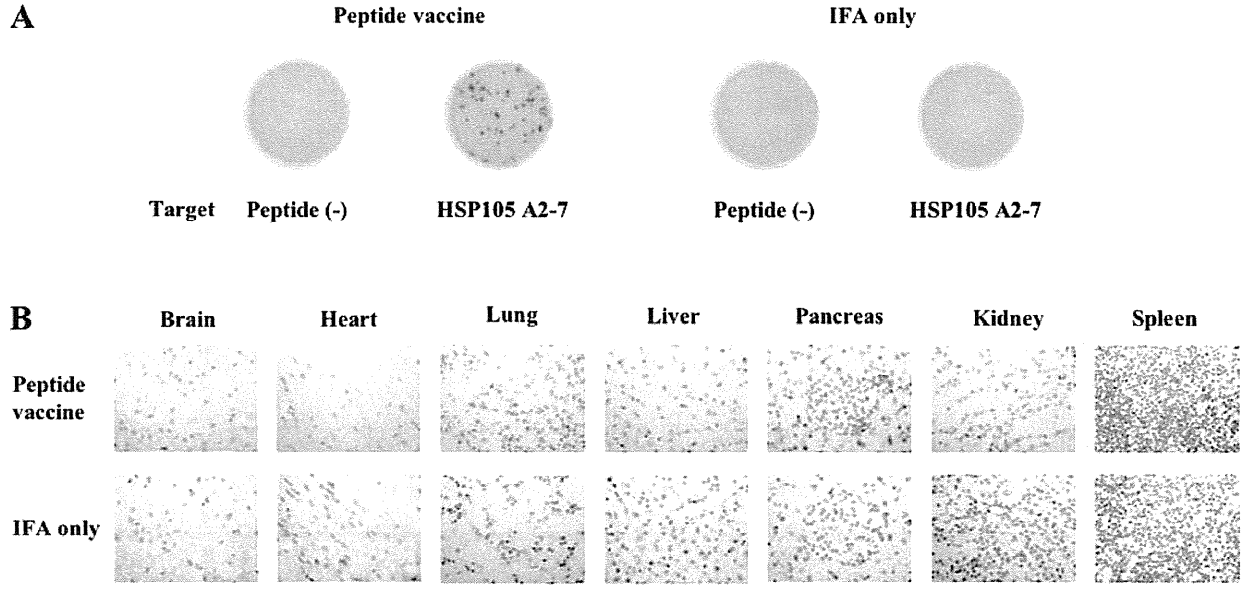

Figure 4. The HSP105 peptide vaccine induces HSP105 peptide-specific CTLs, while CD8 T cells do not infiltrate into normal tissues. (A) HSP105 peptide-specific CTLs were induced in the spleen cells of immunized mice with the HSP105 peptide vaccine. IFN-γ ELISPOT assays were performed using BM-DCs pulsed with HSP105 A2-7 and non-pulsed BM-DCs as target cells. The representative data are shown (n=6). (B) Immunohistochemical staining with anti-CD8 mAb was performed in tissue specimens of HLA-A2 Tgm immunized with the HSP105 A2-7 and A2-12 peptides. The tissue specimens were removed and analyzed 7 days after the second vaccination (original magnification, x400). The representative data are shown (n=3).

risk of damage to normal tissues posed by HSP105 antigen-induced immune responses (6). To investigate whether immunization of the mice with HSP105-derived peptides causes autoimmunity, HLA-A2 Tgm were immunized with the HSP105 A2-7 and A2-12 peptides emulsified in incomplete Freund's adjuvant at 7-day intervals and then sacrificed 7 days after the second vaccination. Using the IFN-γ ELISPOT assay, we confirmed the induction of HSP105 peptide-specific CTLs in the spleen cells of immunized mice (Fig. 4A). We did not detect any pathological changes, such as $CD8^+$ lymphocyte infiltration or tissue destruction/repair, in the brain, heart, lung, liver, pancreas, or kidney of HLA-A2 Tgm (Fig. 4B). These results indicate that the HSP105 peptide-reactive $CD8^+$ CTLs did not attack the healthy tissue specimens that we evaluated.

Discussion

Heat shock proteins (HSPs) have essential functions in the regulation of protein folding, conformation, assembly and sorting. They function as molecular chaperones to maintain the native conformational states of proteins, preventing protein aggregation (18). HSPs are classified into several families based on their molecular weight, including HSP105/110, HSP90, HSP70, HSP60, HSP40 and HSP27 (19). HSP105 is a stress protein within the HSP105/110 family that we previously reported to be overexpressed in a variety of human cancers but with little to no expression in normal tissues, aside from the testis. Thus, HSP105 presents a promising candidate for a target antigen in cancer immunotherapy (3-7). In particular, HSP105 is specifically overexpressed in colorectal cancer (83%) (4). Furthermore, HSP105 is expressed in highly metastatic colon cancer cell lines and its expression is correlated with advanced clinical cancer stages and positive lymph node involvement (20). When considering immunogenic target molecules for cancer immunotherapy, it is important to select a tumor antigen that does not run the risk of becoming lost during immunoediting (21). We reported previously that siRNA-mediated suppression of HSP105 protein expression induced apoptosis in various types of cancer cells, but not in fibroblasts (12). Therefore, it is possible that tumor cells do not lose HSP105 expression, allowing for continued growth.

Advances in molecular biology and tumor immunology have paved the way for identification of a large number of tumor-associated antigens (TAAs) and antigenic peptides recognized by tumor reactive CTLs; hence, peptide-based cancer immunotherapy has become an intensely studied field (22,23). Several HSPs, including HSP70, HSP90 and gp96, bind and deliver (through receptor-mediated endocytosis of HSP) antigenic peptides to the antigen-processing pathway of antigen-presenting cells (APCs) and these peptides are then presented on major histocompatibility complex (MHC) class I molecules. This HSP-mediated pathway has been demonstrated to evoke potent antiviral and antitumor immune responses (24). On the other hand, many researchers have identified MHC class I-presenting peptide epitopes derived from HSP (25). Furthermore, HSP105 itself may induce $CD8^+$ T cells to become reactive towards tumor cells that express HSP105, using HSP105-DNA and HSP105-pulsed DC vaccines in mice (6-8).

We found 4 peptides [HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGI YKQDL) and HSP105 A24-7 (EYVYEFRDKL)] to be potential HSP105-derived, HLA-A2 or A24-restricted CTL epitopes. There was a discrepancy between the expected HSP105 CTL epitopes in Tgm and in PBMCs of colorectal cancer patient. To identify the HSP105-derived CTL epitope peptides, we analyzed the PBMC responses to each of the 4 peptides in colorectal cancer patients using the *ex vivo* IFN-γ ELISPOT assay.

In this study, we used an *ex vivo* assay to detect HSP105-specific IFN-γ-secreting T cells in PBMCs from 14 of 21 pre-surgical patients with colorectal cancer. Generally, CTLs specific for tumor antigens cannot be detected directly *ex vivo*; rather only after expansion by repeated *in vitro* stimulation with the antigenic peptide in the appropriate antigen-presenting cells. This is attributed to assay sensitivity and the low frequency of tumor antigen-specific CTLs (26). HSP105-specific CTLs in PBMCs, which can be detected directly *ex vivo* without *in vitro* stimulation, provide strong immunological evidence of HSP105-derived CTL epitopes, which we were able to identify in this study. However, because the prognosis of the pre-surgical patients was affected by various factors, it was difficult to evaluate the correlation between a positive CTL response before surgery and clinical improvement at the present stage; an increase in the number of patients at each stage and further analyses of this relationship are necessary.

Although the SEREX method facilitated the identification of tumor antigens that could be recognized by antibodies and $CD4^+$ T cells, few of their T-cell epitopes have been determined (27). We previously reported in mice that HSP105-DNA and HSP105-pulsed DC vaccines induced a reaction in $CD4^+$ T cells and $CD8^+$ T cells towards tumor cells expressing HSP105 (6-8). HSP105 was identified by SEREX (3) and thus, HSP105-specific $CD4^+$ T cell reactions may be induced by HSP105 immunization. It was shown that antigen-specific $CD4^+$ T cells are required to activate memory $CD8^+$ T cells into fully functional effector killer cells (28). We are now preparing a clinical trial to investigate HSP105-based immunotherapy for HSP105-expressing tumors, including those from colorectal cancer. We plan to use the HSP105 epitope peptides identified in this study as an initial attempt. We expect that HSP105-based immunotherapy will be a novel treatment strategy for colorectal cancer patients.

Acknowledgements

This study was supported by MEXT KAKENHI grant numbers 12213111, 17015035 and the National Cancer Center Research and Development Fund (25-A-7), as well as Health and Labor Science Research Grants for Research on Hepatitis and Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor and Welfare, Japan. Y.S. would like to thank the Foundation for Promotion of Cancer Research (Japan) for the Third-Term Comprehensive Control Research for Cancer for the award of a research resident fellowship. T.N. is supported by funding from MEDINET Co., Ltd.

References

1. Weitz J, Koch M, Debus J, Höhler T, Galle PR and Büchler MW: Colorectal cancer. Lancet 365: 153-165, 2005.

2. Mlecnik B, Tosolini M, Kirilovsky A, *et al*: Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol 29: 610-618, 2011.
3. Nakatsura T, Senju S, Yamada K, Jotsuka T, Ogawa M and Nishimura Y: Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. Biochem Biophys Res Commun 281: 936-944, 2001.
4. Kai M, Nakatsura T, Egami H, Senju S, Nishimura Y and Ogawa M: Heat shock protein 105 is overexpressed in a variety of human tumors. Oncol Rep 10: 1777-1782, 2003.
5. Hatayama T, Yasuda K and Nishiyama E: Characterization of high-molecular-mass heat shock proteins and 42 degrees C-specific heat shock proteins of murine cells. Biochem Biophys Res Commun 204: 357-365, 1994.
6. Miyazaki M, Nakatsura T, Yokomine K, Senju S, Monji M, Hosaka S, *et al*: DNA vaccination of HSP105 leads to tumor rejection of colorectal cancer and melanoma in mice through activation of both CD4 T-cells and CD8 T-cells. Cancer Sci 96: 695-705, 2005.
7. Yokomine K, Nakatsura T, Minohara M, *et al*: Immunization with heat shock protein 105-pulsed dendritic cells leads to tumor rejection in mice. Biochem Biophys Res Commun 343: 269-278, 2006.
8. Yokomine K, Nakatsura T, Senju S, Nakagata N and Minohara M: Regression of intestinal adenomas by vaccination with heat shock protein 105-pulsed bone marrow-derived dendritic cells in Apc(Min/+) mice. Cancer Sci 98: 1930-1935, 2007.
9. Browning M and Krausa P: Genetic diversity of HLA-A2: evolutionary and functional significance. Immunol Today 17: 165-170, 1996.
10. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA and Pérarnau B: HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. J Exp Med 185: 2043-2051, 1997.
11. Firat H, Garcia-Pons F, Tourdot S, *et al*: H-2 class I knockout, HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumor immunotherapeutic strategies. Eur J Immunol 29: 3112-3121, 1999.
12. Hosaka S, Nakatsura T, Tsukamoto H, Hatayama T, Baba H and Nishimura Y: Synthetic small interfering RNA targeting heat shock protein 105 induces apoptosis of various cancer cells both in vitro and in vivo. Cancer Sci 97: 623-632, 2006.
13. Komori H, Nakatsura T, Senju S, *et al*: Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 12: 2689-2697, 2006.
14. Nakatsura T, Komori H, Kubo T, *et al*: Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reactions in mice. Clin Cancer Res 10: 8630-8640, 2004.
15. Nakatsura T, Senju S, Ito M, Nishimura Y and Itoh K: Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method. Eur J Immunol 32: 826-836, 2002.
16. Yoshitake Y, Nakatsura T, Monji M, *et al*: Proliferation potential-related protein, an ideal esophageal cancer antigen for immunotherapy, identified using complementary DNA microarray analysis. Clin Cancer Res 10: 6437-6448, 2004.
17. Monji M, Nakatsura T, Senju S, *et al*: Identification of a novel human cancer/testis antigen, KM-HN-1, recognized by cellular and humoral immune responses. Clin Cancer Res 10: 6047-6057, 2004.
18. Feder ME and Hofmann GE: Heat-shock proteins, molecular chaperones and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61: 243-282, 1999.
19. Craig EA, Weissman JS and Horwich AL: Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. Cell 78: 365-372, 1994.
20. Hwang TS, Han HS, Choi HK, *et al*: Differential, stage-dependent expression of Hsp70, Hsp110 and Bcl-2 in colorectal cancer. J Gastroenterol Hepatol 18: 690-700, 2003.
21. Kawakami Y and Rosenberg SA: Human tumor antigens recognized by T-cells. Immunol Res 16: 313-339, 1997.
22. 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 254: 1643-1647, 1991.
23. Kawakami Y, Eliyahu S, Delgado CH, *et al*: Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc Natl Acad Sci USA 91: 6458-6462, 1994.
24. Srivastava P: Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. Annu Rev Immunol 20: 395-425, 2002.
25. Hickman-Miller HD and Hildebrand WH: The immune response under stress: the role of HSP-derived peptides. Trends Immunol 25: 427-433, 2004.
26. Romero P, Cerottini JC and Speiser DE: Monitoring tumor antigen specific T-cell responses in cancer patients and phase I clinical trials of peptide-based vaccination. Cancer Immunol Immunother 53: 249-255, 2004.
27. Jäger E, Chen YT, Drijfhout JW, *et al*: Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J Exp Med 187: 265-270, 1998.
28. Gao FG, Khammanivong V, Liu WJ, Leggatt GR, Frazer IH and Fernando GJ: Antigen-specific $CD4^+$ T-cell help is required to activate a memory $CD8^+$ T-cell to a fully functional tumor killer cell. Cancer Res 62: 6438-6441, 2002.