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, 338–343; 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

Shiro Suzuki[1,*], Kiyosumi Shibata[1], Fumitaka Kikkawa[1], and Tetsuya Nakatsura[2]

[1]Department of Obstetrics and Gynecology; Nagoya University Graduate School of Medicine; Showa-ku, Nagoya Japan; [2]Division of Cancer Immunotherapy; Exploratory Oncology Research and Clinical Trial Center; National Cancer Center; Kashiwa, Chiba Japan

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

Downloaded by [Kokuritsu Gan Centre Higashi Byoin] at 19:06 27 February 2015

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

*Correspondence to: Shiro Suzuki; Email: shiro-s@med.nagoya-u.ac.jp
Submitted: 09/11/2013; Revised: 10/26/2013; Accepted: 11/15/2013; Published Online: 11/21/2013
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 ≥50 000/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 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**).

Downloaded by [Kokuritsu Gan Centre Higashi Byoin] at 19:08 27 February 2015

Downloaded by [Kokuritsu Gan Center Higashi Byoin] at 19:08 27 February 2015

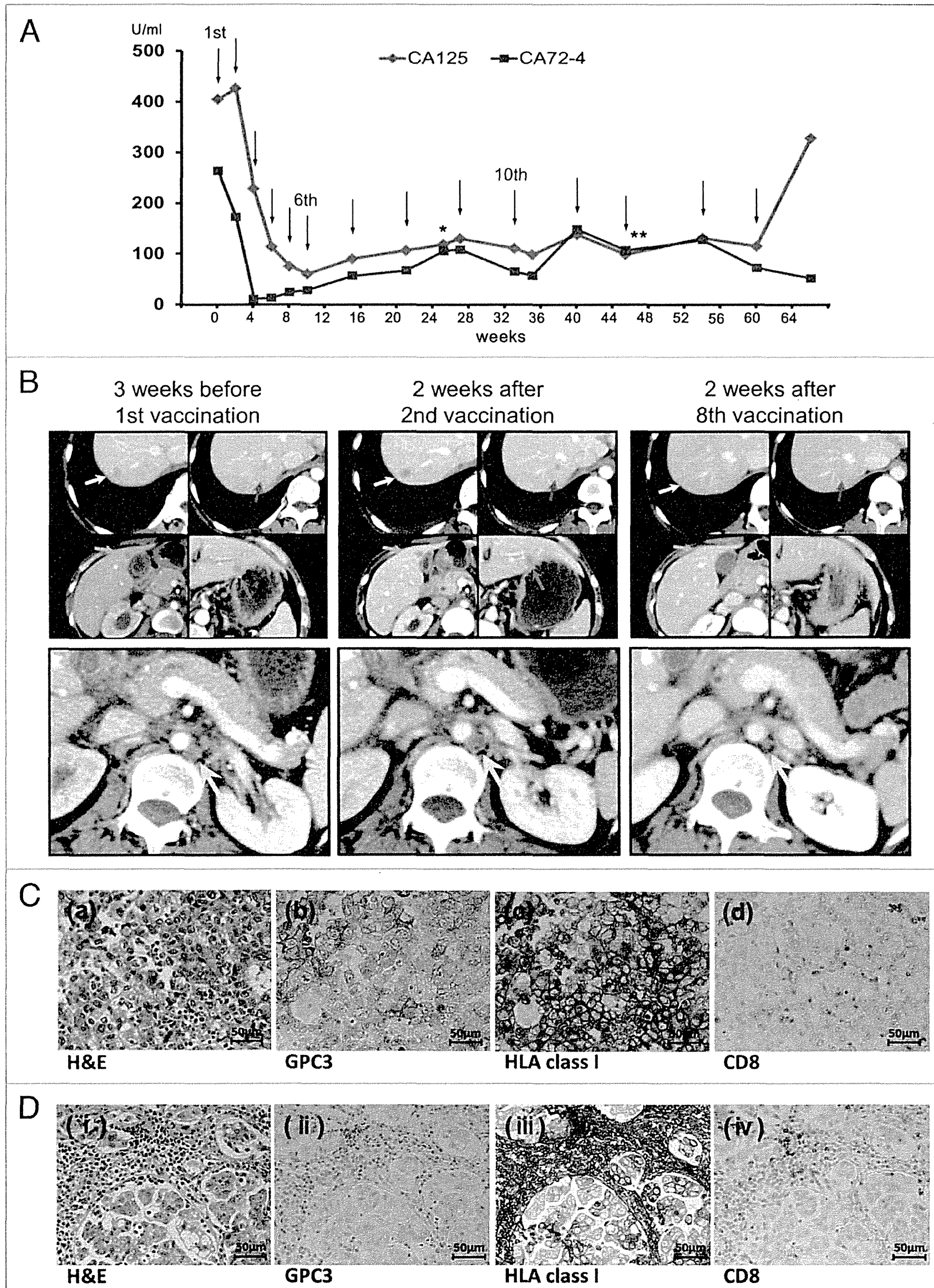


Figure 1. See page 341 for legend.

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.

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

Downloaded by [Kokuritsu Gan Centre Higashi Byoin] at 19:08 27 February 2015

Downloaded by [Kokuritsu Gan Centre Higashi Byoin] at 19:08 27 February 2015

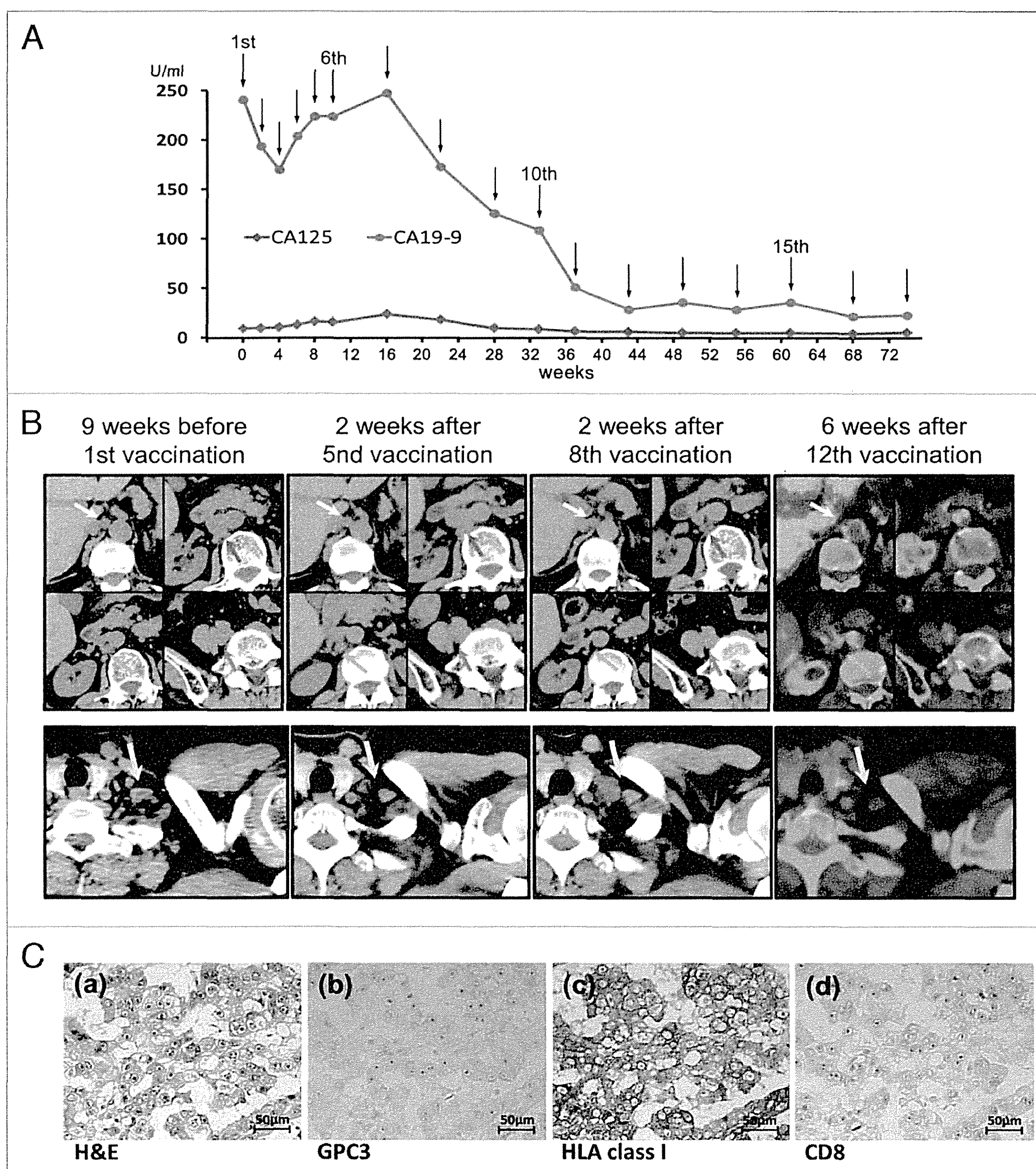


Figure 2. (**A**) Clinical course from the beginning of the GPC3 peptide vaccination. Serum levels of CA19–9 and CA125 decreased after the 7th vaccination. The CA19–9 level decreased to within the normal range. Black arrows indicate vaccinations. (**B**) Plain CT and ^{18}F-FDG PET/CT scans showing retroperitoneal 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.

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$_{298-306}$ peptide complex and HLA-A2/GPC3$_{144-152}$ 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

Downloaded by [Kokuritsu Gan Centre Higashi Byoin] at 19:08 27 February 2015

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

YU SAWADA[1,2*], HIROYUKI KOMORI[3,4*], YOSHIYUKI TSUNODA[1,5,6*], MANAMI SHIMOMURA[1], MARI TAKAHASHI[1], HIDEO BABA[4], MASAAKI ITO[5], NORIO SAITO[5], HIROYUKI KUWANO[6], ITARU ENDO[2], YASUHARU NISHIMURA[3] and TETSUYA NAKATSURA[1]

[1]Division of Cancer Immunotherapy, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Kashiwa 277-8577; [2]Department of Gastroenterological Surgery, Yokohama City University Graduate School of Medicine, Yokohama 236-0004; Departments of [3]Immunogenetics and [4]Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556; [5]Colorectal and Pelvic Surgery Division, National Cancer Center Hospital East, Kashiwa 277-8577; [6]Department of General Surgical Science (Surgery I), Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan

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.

Correspondence to: Dr Tetsuya Nakatsura, Division of Cancer Immunotherapy, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, 6-5-1 Kashiwanoha, Kashiwa 277-8577, Japan
E-mail: tnakatsu@east.ncc.go.jp

*Contributed equally

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.

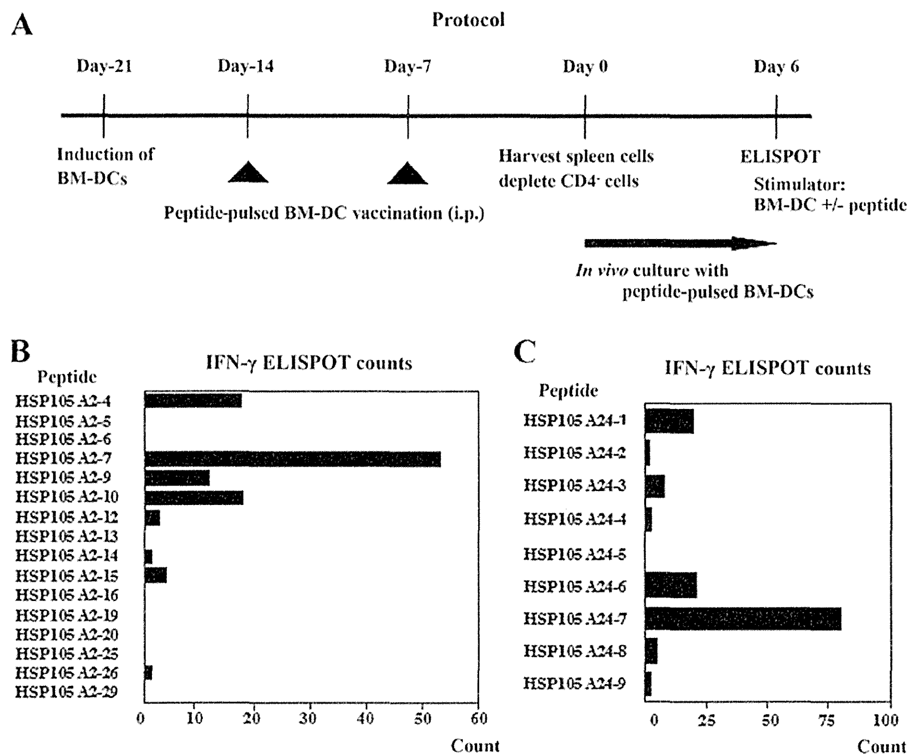


Figure 1. Identification of HLA-A2 or HLA-A24-restricted CTL epitopes of HSP105 using HLA-A2.1 Tgm and HLA-A24 Tgm. (A) The protocol used for identification of HLA-A2 or HLA-A24-restricted CTL epitopes of HSP105 is shown. We primed the HLA Tgm with BM-DCs ($5x10^5$) pulsed with the mixture of HSP105-derived 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 ($HSP105^+$, $H-2K^d$) (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

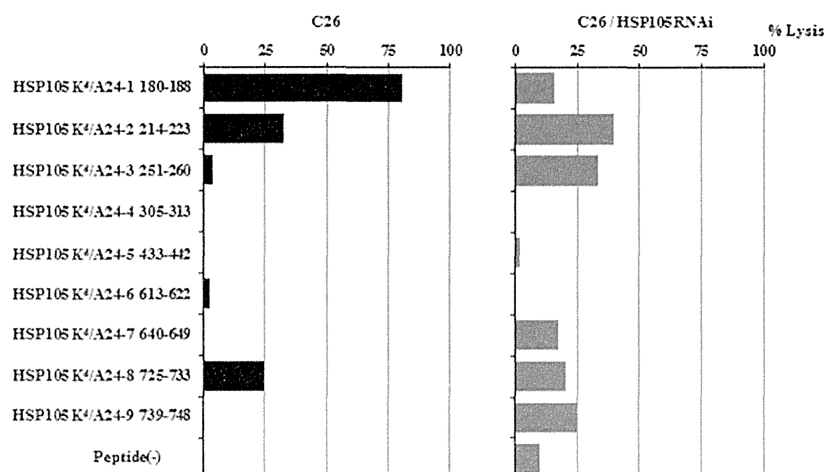


Figure 2. Identification of an HSP105-derived HLA-A24 and K^d-restricted CTL epitope. BALB/c mice were immunized with 9 HSP105 peptides. Using the ^{51}Cr release assay, sensitized spleen cells that had been stimulated *in vitro* with each HSP105 peptide (10 μmol/l) and cultured for 5 days with 100 U/ml 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.

HLA-A2-positive patients	Age (yrs.)	Gender	HLA	Stage[a] of tumor	HSP105 expression[b]	[c]Spot number of peptide-specific CTLs: 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.

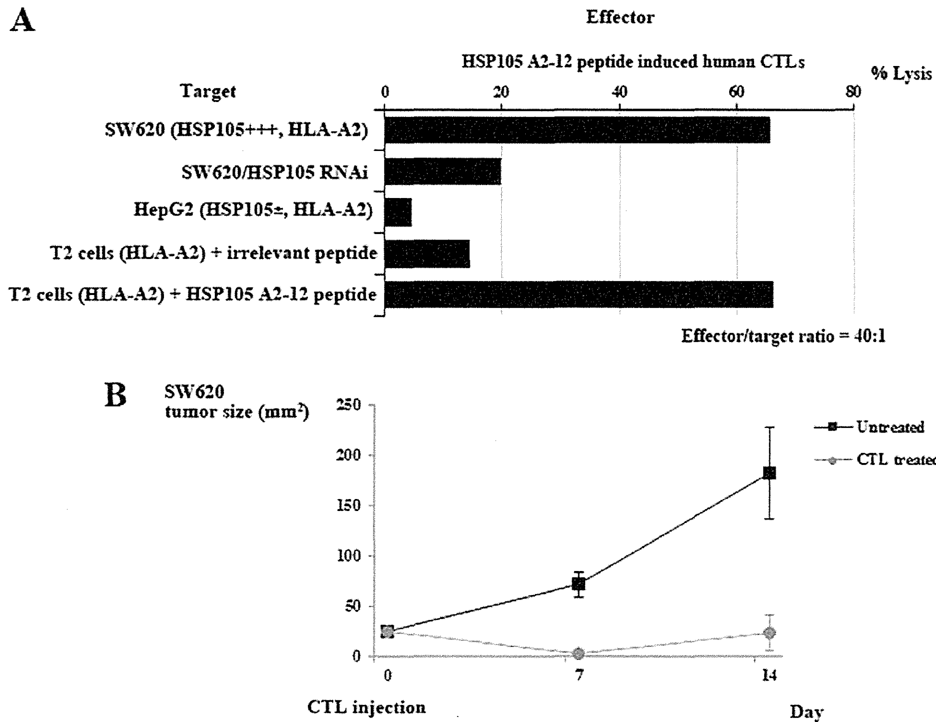


Figure 3. CTL induction from PBMCs of HLA-A2-positive cancer patients. (A) HSP105 peptide-reactive CTLs were generated from $CD8^+$ T cells of HLA-$A2^+$ colorectal cancer patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the HSP105 A2-12 peptides, the CTLs were 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 (3×10^6) 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.

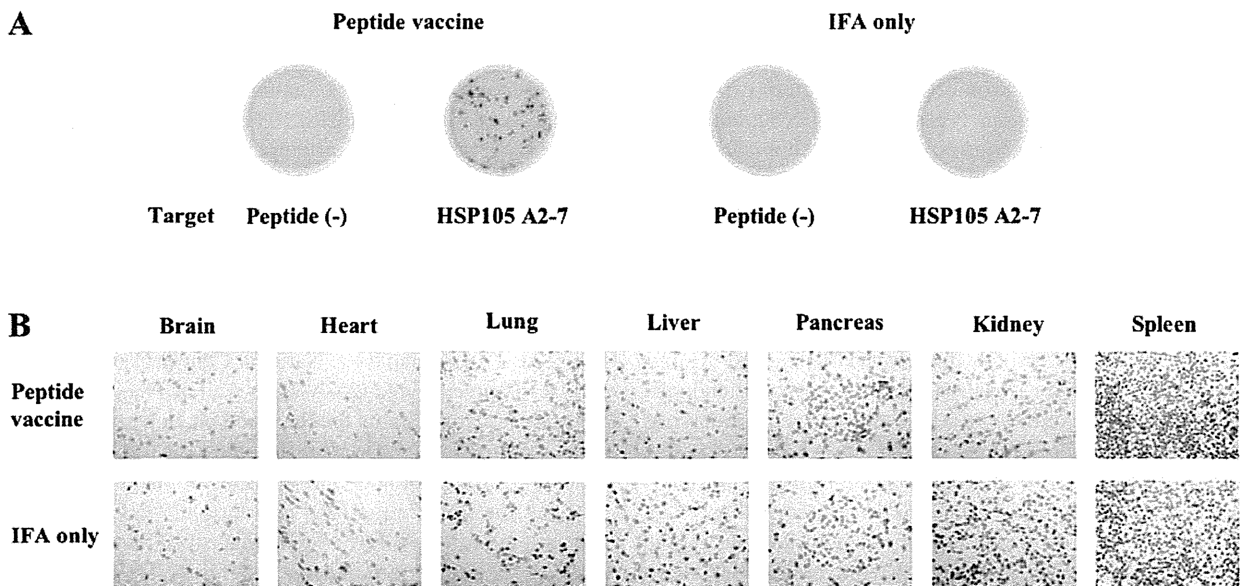


Figure 4. The HSP105 peptide vaccine induces HSP105 peptide-specific CTLs, while CD8 T cells do not infiltrate into normal tissues. (A) HSP105 peptide-specific CTLs were induced in the spleen cells of immunized mice with the HSP105 peptide vaccine. IFN-γ ELISPOT assays were performed using BM-DCs pulsed 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.

Human Pathology (2013) 44, 526–533

Human
PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

Glypican 3 expression in tumors with loss of SMARCB1/INI1 protein expression☆,☆☆

Kenichi Kohashi MD, PhD[a], Tetsuya Nakatsura MD, PhD[b], Yoshiaki Kinoshita MD, PhD[c], Hidetaka Yamamoto MD, PhD[a], Yuichi Yamada MD[a], Tatsuro Tajiri MD, PhD[d], Tomoaki Taguchi MD, PhD[c], Yukihide Iwamoto MD, PhD[e], Yoshinao Oda MD, PhD[a,*]

[a]*Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812–8582, Japan*
[b]*Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwanoha 6-5-1, Kashiwa, Chiba 277–8577, Japan*
[c]*Department of Pediatric Surgery, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812–8582, Japan*
[d]*Department of Pediatric Surgery, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kajii-cho 465, Kamigyo-ku, Kyoto 602–8566, Japan*
[e]*Department of Orthopaedic Surgery, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812–8582, Japan*

Received 10 April 2012; revised 20 June 2012; accepted 27 June 2012

Keywords:
Glypican 3;
SMARCB1/INI1;
Malignant rhabdoid tumor

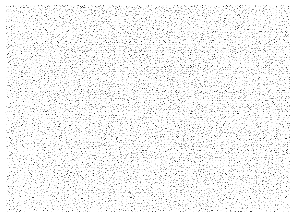
Summary Glypican 3 (GPC3), a membrane-bound heparin sulfate proteoglycan, is mutated in Simpson-Golabi-Behmel syndrome, characterized by tissue overgrowth and an increased risk of embryonal malignancies such as Wilms tumor. Malignant rhabdoid tumor (MRT), originally described as a rhabdomyosarcomatoid variant of Wilms tumor, is a tumor with loss of SMARCB1/INI1 protein expression. We analyzed the frequency of GPC3 protein expression, *GPC3* mRNA, and serum-soluble GPC3 levels in 71 cases of tumors with loss of SMARCB1/INI1 protein expression, including 14 MRTs, 48 epithelioid sarcomas (ES) (proximal-type, 21; distal-type, 27), 4 extraskeletal myxoid chondrosarcomas, and 5 pediatric undifferentiated soft-tissue sarcomas. We found that GPC3 overexpression of more than 10% of the labeling index was recognized in 6 (42.9%) MRTs, 1 (2.1%) proximal-type ES, and 3 (60%) pediatric undifferentiated soft-tissue sarcomas (MRT vs ES, $P =$ .0003). All the remaining cases revealed GPC3-absent expression of less than 1% of the labeling index. The median values of *GPC3* mRNA in the GPC3-absent expression group and overexpression group were 10.2 and 309, respectively, with a statistically significant difference between these 2 groups ($P =$ .004). However, there was no statistically significant difference in the prognoses of these 2 groups of

☆ Disclosure/conflict of interest: The authors declare that there are no conflicts of interest to disclose.

☆☆ This study was supported in part by a Grant-in-Aid for Scientific Research (B) (No. 21390107) and Young Scientists (B) (No.21790356) from the Japan Society for the Promotion of Science, the National Cancer Center Research and Development Fund (23-B-12), and Health and Labor Science Research Grants for Clinical Research from the Ministry of Health, Labor and Welfare, Tokyo, Japan. The English used in this manuscript was revised by KN International (http://www.kninter.com/).

* Corresponding author.
E-mail address: oda@surgpath.med.kyushu-u.ac.jp (Y. Oda).

0046-8177/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.humpath.2012.06.014



MRT (P = .99). In analyzable cases of small-number MRT and pediatric undifferentiated soft-tissue sarcoma, there is no significant correlation between GPC3 immunoreactivity and serum-soluble GPC3 level. Therefore, evaluation of GPC3 immunoexpression may be a useful diagnostic tool to distinguish ES from MRT, especially extrarenal MRT. It was suggested that MRTs with GPC3 overexpression may become a new target of GPC3 immunotherapy.
© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Glypican 3 (GPC3), which is located in Xq26, is a member of the glypican family of heparin-sulfate proteoglycans. This protein is linked to the cell surface through a glycosylphosphatidylinositol anchor, and is thought to regulate cell growth and apoptosis through interactions with morphogenic or growth factors such as Wnt5a, fibroblast growth factor 2, bone morphogenic protein 7, and tissue factor pathway inhibitor [1-3]. Loss-of-function mutations in the human *GPC3* gene result in Simpson-Golabi-Behmel syndrome, an X-linked condition characterized by severe malformations and pre- and postnatal overgrowth. Furthermore, these patients have a high risk of developing embryonal tumors, mostly Wilms kidney tumor and neuroblastoma [4].

Malignant rhabdoid tumor (MRT), which was originally described in 1978 as a rhabdomyosarcomatoid variant of Wilms tumor, is a rare and highly aggressive embryonal tumor in infancy or childhood [5]. SMARCB1/INI1 (INI1) immunohistochemical expression has not been detected in any MRT cases except in rare examples, and this feature has been reported to be useful for the correct diagnosis of MRT [6-8]. However, loss of INI1 protein expression has also been demonstrated in all renal medullary carcinomas, almost all epithelioid sarcomas, half of epithelioid malignant peripheral nerve sheath tumors, about half of pediatric myoepithelial carcinomas, and some extraskeletal myxoid chondrosarcomas [9-13].

In the present study, we analyzed the frequency of GPC3 protein expression in a large series of MRT cases and other tumors with loss of INI1 protein expression. Furthermore, we examined mRNA expressions of *GPC3* in frozen samples by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and serum-soluble GPC3 protein in preoperative blood samples by enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Patients

Tumors with loss of INI1 protein expression in the present study were selected from among more than 15 000 cases of bone and soft-tissue tumors registered in the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, between 1955 and 2010. The primary monoclonal antibodies used in the case selection were BAF47, an antibody to the SMARCB1/INI1 gene product (clone 25; 1:250; 20-minute microwave; BD Transduction Laboratories, San Diego, CA). As a result of this screening, formalin-fixed, paraffin-embedded specimens of 72 sarcoma cases with loss of INI1 protein expression (no staining of tumor nuclei) were available. These tumors with loss of INI1 protein expression were comprised of 14 MRTs, 48 epithelioid sarcomas (proximal-type, 21; distal-type, 27), 4 extraskeletal myxoid chondrosarcomas, and 5 pediatric undifferentiated soft-tissue sarcomas. The clinicopathologic findings of the 14 cases of MRT and the 48 cases of epithelioid sarcoma are summarized in Tables 1 and 2. Immunoreactivities for CAM5.2, AE1/AE3, and CD34 were graded semiquantitatively as 0, no staining; 1+, <5% tumor cells reactive; 2+, 5% to 25% tumor cells reactive; 3+, 26% to 50% tumor cells reactive; and 4+, >50% tumor cells reactive. In addition, we also examined 20 samples of surrounding non-tumorous skeletal muscle that were collected from patients with various types of sarcoma as controls for the quantitative RT-PCR analysis. In all cases, the diagnosis was based on light microscopic examination with hematoxylin and eosin staining according to the most recent World Health Organization classification (MRT, epithelioid sarcoma, extraskeletal myxoid chondrosarcoma) and report of undifferentiated soft-tissue sarcoma [14-17]. Moreover, immunoperoxidase procedures using the streptavidin-biotin-peroxidase method were carried out in all cases. The institutional review board at Kyushu University approved this study (permission code: 22–152).

2.2. Immunohistochemistry for Glypican 3 protein

Immunohistochemistry was performed using the streptavidin-biotin-peroxidase method (Histofine; Nichirei, Tokyo, Japan). A monoclonal antibody against glypican 3 (clone 1G12, 1:200; BioMosaics, Burlington, VT) was employed. We assessed the immunoreactivity with the labeling index (LI). To determine the GPC3 LI, the number of positively stained tumor cells among at least 500 tumor cells were counted by three pathologists (K.K., H.Y., Y.Y.) independently for each tumor. Furthermore, the protein expression in tumors was classified into three categories: −, less than 1% of LI; ±, 1-10% of LI; +, more than 10% of LI.

2.3. RNA extraction

Total RNA was extracted from frozen and paraffin-embedded samples using Trizol reagent (Invitrogen,

Table 1 Clinicopathologic data in malignant rhabdoid tumor patients

Case	Age	Sex	Site	Prognosis	CAM5.2 [a]	AE1/AE3 [b]	CD34 [c]	GPC-3
EMRT-1	0 d	F	Retroperitoneum	2 mo DOD	2+	2+	0	+
EMRT-2	4 mo	M	Liver	12 mo DOD	3+	3+	2+	+
EMRT-3	5 mo	F	Neck	12 mo DOD	1+	1+	2+	+
EMRT-4	6 mo	M	Back	20 mo DOD	4+	4+	0	-
EMRT-5	2 y	F	Back	1 month DOD	4+	1+	0	+
EMRT-6	5 y	F	Buttock	10 mo DOD	1+	1+	0	-
MRTK-1	3 mo	M	Right kidney	8 mo DOD	0	1+	0	-
MRTK-2	5 mo	M	Right kidney	71 mo NED	1+	0	0	+
MRTK-3	1 y	M	Right kidney	Not available	0	1+	1+	+
MRTK-4	1 y	F	Left kidney	4 mo DOD	1+	0	0	-
MRTK-5	1 y	M	Left kidney	10 mo DOD	1+	1+	0	-
MRTK-6	2 y	M	Right kidney	77 mo NED	1+	0	0	-
AT/RT-1	3 y	F	Right cerebello-pontine angle	12 mo DOD	3+	1+	0	-
AT/RT-2	7 y	M	Brain-stem	Not available	4+	4+	0	-

Abbreviations: EMRT, extrarenal malignant rhabdoid tumor; MRTK, malignant rhabdoid tumor of the kidney; AT/RT, atypical teratoid/rhabdoid tumor; DOD, died of disease; NED, no evidence of disease.

[a] Clone CAM5.2; 1:20; 30-minute microwave; Becton-Dickinson, Mountain view, CA.

[b] Clone AE1/AE3; 1:1000; 20-minute microwave; Dako, Glostrup, Denmark.

[c] Clone QBEnd/10; 1:50; 30-minute 0.1% trypsin; Leica Microsystems, Tokyo, Japan.

Carlsbad, CA) according to the manufacturer's instructions. Five micrograms of RNA from each sample were reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) in order to prepare first-strand cDNA.

2.4. TaqMan PCR to detect mRNA quantity of *GPC3*

Quantitative RT-PCR for *GPC3* was performed and analyzed using previously developed TaqMan assay reagents (*GPC3* Hs00170471_m1.; *GAPDH* Hs99999905_m1.; all from Applied Biosystems, Foster City, CA) and an ABI Prism 7700 Sequence Detection system (Applied Biosystems). The PCR reaction was carried out according to the manufacturer's protocol. The obtained data were standardized using data of the international housekeeping gene, *GAPDH*. All the reactions for standard samples and samples of patients were performed in triplicate. The data were averaged from the values obtained in each reaction. The final numerical value (V) in each sample was calculated as follows: V = *GPC3* mRNA value/*GAPDH* mRNA value.

2.5. Detection by ELISA of the serum-soluble glypican 3 protein

Preoperative blood serum samples were available in 4 patients having tumors with loss of INI1 protein expression and 1 congenital mesoblastic nephroma case of GPC3-absent expression. Glypican-3 levels were measured using a commercially available ELISA kit (BioMosaics, Burlington, VT) following the manufacturer's protocol.

3. Results

3.1. Glypican 3 immunoreactivity

The results of the immunohistochemical analysis are summarized in Table 3. In 6 of the 14 MRTs (42.9%), 1 (2.1%) of the 48 epithelioid sarcomas and 3 of the 5 pediatric undifferentiated soft-tissue sarcomas (60%), overexpression of

Table 2 Clinicopathologic data in epithelioid sarcoma patients

Sex		Age (y)		Site		Size		Depth		Rhabdoid cell	
Proximal-type epithelioid sarcoma											
Male	12	≥20	17	Trunk [a]	21	≥5cm	10	Deep	8	(+)	13
Female	9	<20	4	Extremities	0	< 5cm	11	Superficial	13	(−)	8
Distal-type epithelioid sarcoma											
Male	15	≥20	25	Trunk	23	≥5 cm	12	Deep	9	(+)	13
Female	12	<20	2	Extremities	4	<5 cm	15	Superficial	18	(−)	14

[a] Trunk (including genital areas) and head and neck.

Table 3 Glypican 3 expression in tumor with loss of INI1 protein expression

	GPC3(+)	GPC3(−)
Malignant rhabdoid tumor	6 (42.9%)	8
EMRT [a]	4	2
MRTK [b]	2	4
AT/RT [c]	0	2
Epithelioid sarcoma	1 (2.1%)	47
Proximal-type	1	20
Distal-type	0	27
Extraskeletal myxoid CS [d]	0 (0%)	4
US [e]	3 (60.0%)	2

NOTE. Malignant rhabdoid tumor *vs* epithelioid sarcoma: P = .0003. Malignant rhabdoid tumor *vs* proximal-type epithelioid sarcoma: P = .010.
[a] Extrarenal malignant rhabdoid tumor.
[b] Malignant rhabdoid tumor of the kidney.
[c] Atypical teratoid/rhabdoid tumor.
[d] Chondrosarcoma.
[e] Pediatric undifferentiated soft tissue sarcoma.

more than 10% of LI was recognized (Fig. 1A-D). However, all the remaining cases showed absent expression of less than 1% of LI (Fig. 1E-H). GPC3 immunoreactivity was found significantly more frequently in MRT than in epithelioid sarcoma (P = .0003) or proximal-type epithelioid sarcoma (P = .010).

3.2. Glypican 3 mRNA expression by TaqMan PCR

Fig. 2A shows boxplots of *GPC3* mRNA expression (20 non-tumorous skeletal muscle group, median value=10.0; 5 GPC3-overexpression cases of MRT, 270; 5 GPC3-absent expression cases of MRT, 8.73; 2 GPC3-overexpression cases of pediatric undifferentiated soft-tissue sarcoma, 946; 3 GPC3-absent expression cases of proximal-type epithelioid sarcoma, 17.6; 6 GPC3-absent expression cases of distal-type (conventional-type) epithelioid sarcoma, 12.9).

In addition, these analyzed cases were divided into two groups according to the result of immunohistochemistry: a group showing GPC3 overexpression (more than 10% of the LI) and a group showing GPC3-absent expression (less than 1% of the LI). In the GPC3-overexpression and −absent expression groups, median values of *GPC3* mRNA expression were 309 and 10.2, respectively (Fig. 2B). This difference between the two groups was statistically significant (P = .004).

3.3. Serum-soluble glypican 3 protein level

The serum-soluble GPC3 protein levels in tumors with loss of INI1 protein expression are summarized in Table 4 (GPC3-absent expression cases of pediatric undifferentiated soft-tissue sarcoma, 334 ng/mL and 99 ng/mL; GPC3-overexpression cases of MRT, 233 and 151 ng/mL). In the 1 congenital mesoblastic nephroma case of GPC3-absent expression, the serum GPC3 level was 19 ng/mL.

3.4. Prognosis of malignant rhabdoid tumor according to glypican 3 immunoreactivity

Follow-up data were available in 12 of 14 cases (7 cases of less than 1% of LI and 5 cases of more than 10% of LI). However, there was no statistically significant difference in the prognosis of the 2 groups (P = .99, Fig. 3).

4. Discussion

MRT is characterized by rhabdoid cells having a globoid, and hyaline or eosinophilic intracytoplasmic inclusions [8,16]. However, the existence of rhabdoid cells is recognized in a wide variety of tumors, such as epithelioid sarcoma, synovial sarcoma, extraskeletal myxoid chondrosarcoma, leiomyosarcoma, malignant mesothelioma and desmoplastic small round-cell tumors [8]. The histological features of proximal-type epithelioid sarcoma particularly resemble those of MRT, especially extrarenal MRT [12,14]. Some data exist regarding the histologic and immunohistochemical differences between proximal-type epithelioid sarcoma and MRT, but such findings are not yet conclusive. At present, immunoreactivity for CD34 is generally accepted as a differential marker; approximately half of all ES cases are positive for CD34, whereas MRT cases scarcely reveal immunoreactivity for this marker [8].

In the present study, GPC3 immunoreactivity was significantly more frequent in MRT cases (42.9%) than in other cancers with loss of INI1 expression, whereas only one such epithelioid sarcoma case (2.1%) showed a positive reaction (P = .0003). Moreover, as for *GPC3* mRNA expression, it was a predictable result that the expressions in GPC3-positive-immunoreactivity cases of MRT and pediatric undifferentiated soft-tissue sarcoma were higher than that in non-tumorous skeletal muscle of control cases and negative-immunoreactivity MRT and epithelioid sarcoma (including distal-type and proximal-type), and pediatric undifferentiated soft-tissue sarcoma cases. Therefore, GPC3 immunoreactivity has the potential to be a useful ancillary tool in the differential diagnosis of MRT and epithelioid sarcoma, especially proximal-type epithelioid sarcoma.

Meanwhile, GPC3-positive immunoreactivity appeared at almost the same rate in MRT and pediatric undifferentiated soft-tissue sarcoma. Histologically, 4 of the 5 pediatric undifferentiated soft-tissue sarcomas showed a proliferation of small rounded cells having scant cytoplasm without rhabdoid cells, resembling malignant lymphoma or previously reported pediatric undifferentiated soft-tissue sarcoma with loss of INI1 expression [18]. One of these previously reported cases did not have rhabdoid cells with intracytoplasmic inclusions at diagnosis, but did display rhabdoid cells at definitive surgery following chemotherapy [18]. Therefore, it seems that pediatric undifferentiated soft-tissue