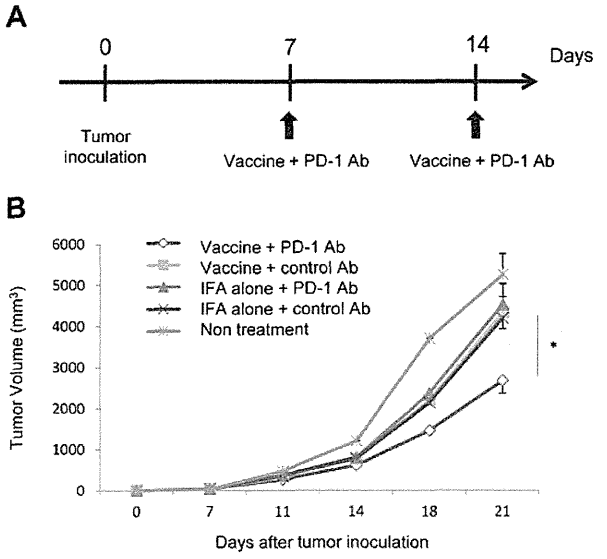

Figure 4. Peptide vaccine and αPD-1 Ab suppress tumor growth synergistically *in vivo*. (A) Mice implanted with RMA were treated with OVA peptide vaccine or IFA alone in combination with αPD-1 Ab or control Ab on days 7 and 14. (B) Tumor growth was expressed as mean tumor volume; bars, SE. Vaccine, OVA peptide emulsified with IFA; IFA alone, vehicle emulsified with IFA. *P<0.05, n=10 using Tukey's test. Two independent experiments were performed, which yielded similar results.

against cancer cells expressing GPC3 endogenously (9,26). Therefore, the CD107a (lysosomal-associated membrane protein-1)-mediated externalization of GPC3 peptide-specific CTL clones was examined upon exposure to liver cancer cell lines. The externalization of CD107a could be a surrogate marker to identify the antigen-specific CTLs that degranulate against tumor cells (27). CTL clones mobilized CD107a in response to SK-Hep1/vec pulsed with $GPC3_{144-152}$ peptide, SK-Hep-1/GPC3, and HepG2 ($GPC3^+$, HLA-A*02:01$^+$), but not in response to pulsed SK-Hep1/vec with HIV_{19-27} (Fig. 3). Furthermore, PD-1 blockade enriched the population of GPC3-specific CTLs that degranulated against only GPC3-positive liver cancer cell lines (SK-Hep1/vec pulsed with $GPC3_{144-152}$ peptide, SK-Hep1/GPC3 and HepG2). These results suggest that blocking the interaction between PD-1 and PD-L1 enhanced the antitumor effect of CTLs in liver tumor cells that evade CTLs via PD-L1 expression.

Combination of a peptide vaccine and αPD-1 Ab suppresses tumor growth in vivo synergistically. Intratumoral injection with $OVA_{257-264}$ peptide (SIINFEKL) effectively inhibited the growth of OVA-negative tumors in a mouse model treated with a peptide vaccine (22). Therefore, we performed *in vivo* therapeutic experiments using intratumoral OVA peptide vaccine and αPD-1 Ab in tumor implanted mice. Mice were implanted with RMA tumor cells on day 0, and established tumors (3-6 mm in diameter) were treated with OVA peptide emulsified with IFA (vaccine) or vehicle emulsified with IFA (IFA alone) in combination with αPD-1 Ab or control Ab on day 7. An additional dose of vaccine and αPD-1 Ab was administered on day 14 after tumor inoculation (Fig. 4A). On day 21, one mouse in the untreated group was dead, and all other mice were alive. The tumor volume of mice treated using the combination therapy of vaccine and αPD-1 Ab was significantly less than those treated with the appropriate control (Fig. 4B, n=10). Treatment with vaccine/control Ab or IFA alone/αPD-1 Ab did not inhibit tumor growth compared with IFA alone/control Ab treatment. These data suggest that the combination of peptide vaccine and αPD-1 Ab had a synergistic antitumor effect.

Vaccine and αPD-1 Ab treatment increases the number of peptide-specific CTLs within mouse tumors. The loading of injected peptide onto major histocompatibility complex (MHC) class I molecules in tumor cells *in vivo* was reported previously using IFN-γ ELISPOT assays (22). In the present study, RMA (OVA-, H-$2K^b$) tumor cells were inoculated onto the backs of C57/BL6 mice. When the tumor diameter reached 3-6 mm, 50 μg H-$2K^b$-restricted $OVA_{257-264}$ peptide was injected into the tumor. After 96 h, the tumors were dissected, cut into small pieces, and digested using collagenase. To investigate whether the injected peptide was loaded onto the MHC class I molecules in the tumor cells in a solid mass, flow cytometry using anti-mouse H-$2K^b$ bound to $OVA_{257-264}$ peptide was performed. The loading of H-$2K^b$-restricted $OVA_{257-264}$ peptide onto MHC class I of tumor cells was detected (Fig. 5A).

To evaluate the immunological response to intratumoral OVA peptide vaccine and αPD-1 Ab, the spleens and tumors of mice treated with the same schedule were analyzed as described previously (Fig. 4A). Peptide-specific immune responses were detected in the spleens of mice treated with intratumoral OVA peptide injection using IFN-γ ELISPOT assays (Fig. 5B). Mice that received the combination of intratumoral OVA peptide injection and αPD-1 Ab exhibited an increased number of OVA peptide-specific CTLs compared with those treated with control Ab on day 14 (n=10).

To obtain direct evidence that the combination of peptide vaccine and αPD-1 Ab led to the local accumulation of antigen-specific CTLs, an OVA tetramer assay was performed in mice. OVA-tetramer-positive CD8 lymphocytes could be detected within a tumor using flow cytometry on day 21. Mice that received the combination of OVA peptide vaccine and αPD-1 Ab had a significantly increased number of OVA peptide-specific CTLs compared with those treated with control Ab (Fig. 5C and D; n=8).

Inhibitory receptors on tumor-infiltrating T lymphocytes and the expression of chemokines. The expression of inhibitory receptors on peptide-specific CTLs at the tumor site was assessed to investigate the mechanism of CTL accumulation in the tumors of mice treated with the combination therapy of peptide vaccine and αPD-1 Ab. RMA-bearing mice were treated with intratumoral OVA peptide injection combined with αPD-1 Ab or control Ab, as described previously (Fig. 4A). The expression of PD-1, CTLA-4, and LAG-3 in OVA tetramer-positive CD8 lymphocytes within the tumor on day 21 was analyzed using flow cytometry. The expression of the inhibitory receptors PD-1 and CTLA-4 was decreased in OVA-tetramer positive CD8 lymphocytes in the αPD-1 Ab group compared with the control Ab group (Fig. 6A). However, αPD-1 Ab treatment did not decrease LAG-3 expression in OVA tetramer-positive CD8 lymphocytes.

The expression of chemokines within the tumor on day 21 was examined using quantitative real-time PCR. The expres-

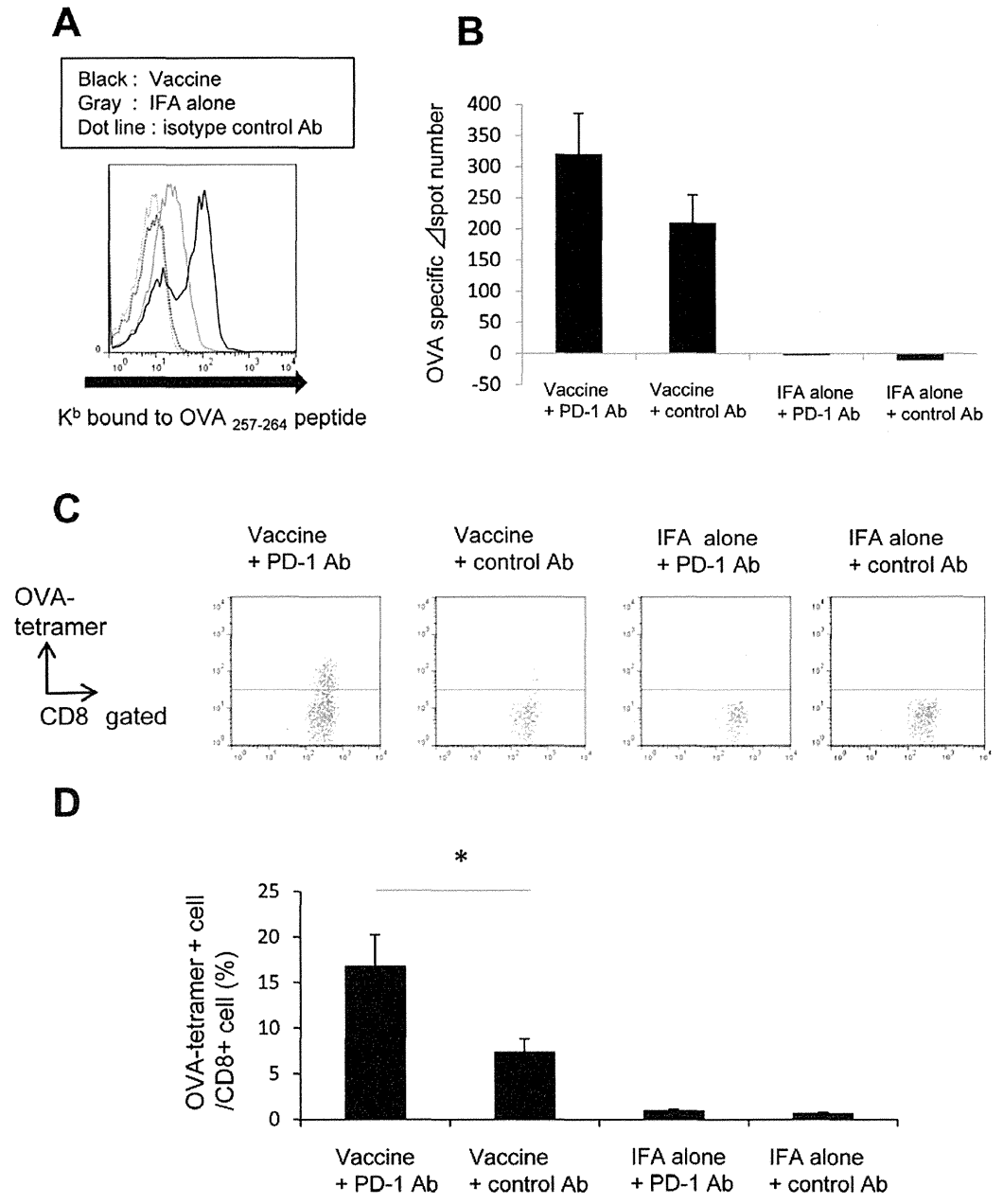

Figure 5. Blocking PD-1 enhanced the infiltration of vaccine-induced CTLs into the tumor. (A) Four days after the intratumoral injection with $OVA_{257-264}$ peptide, isolated RMA tumor cells were stained with anti-mouse H-$2K^b$ bound to $OVA_{257-264}$ peptide or isotype control and analyzed using flow cytometry. Data are presented from a single representative sample (n=3). (B) RMA-bearing mice were treated with OVA peptide vaccine or IFA alone in combination with αPD-1 Ab or control Ab. Spleen cells from treated mice were analyzed using an *ex vivo* IFN-γ ELISPOT assay on day 14. OVA-specific Δspot number, spot number of $OVA_{257-264}$ peptide pulsed BM-DC subtracted by non-pulsed BMDC. Data are presented as means ± SEM (n=10). (C) Tumor-infiltrating T lymphocytes were analyzed using flow cytometry on day 21. Representative plots of OVA tetramer-positive, CD8-positive TILs in the tumors treated with the combination therapy of intratumoral OVA peptide injection and αPD-1 Ab. (D) The percentages of OVA tetramer-positive cells in CD8-positive TILs are shown from three independent experiments using 2-3 mice per group. Data are presented as means ± SEM. *P<0.05, n=8 using Student's t-test.

sion of the chemokine CCL3 was elevated in mice treated with the combination of intratumoral OVA peptide injection and αPD-1 Ab (Fig. 6B). The expression of the chemokines CXCL10 and CXCL12 was unchanged.

Discussion

Many tumor antigens have been identified in HCC, and their potential clinical utility for the development of cancer-specific immunotherapy has been investigated (28-31). GPC3 is a promising target of antigen-specific immunotherapy because it is overexpressed specifically in human HCC (3,4). In addition, it promotes tumor growth by stimulating canonical Wnt signaling (32) or the Hippo pathway (33). A phase I clinical trial of a GPC3-derived peptide vaccine in patients with advanced HCC showed that it had the potential to improve overall survival, which was associated with vaccine-induced CTLs (8). However, the antitumor effects of the peptide-based tumor

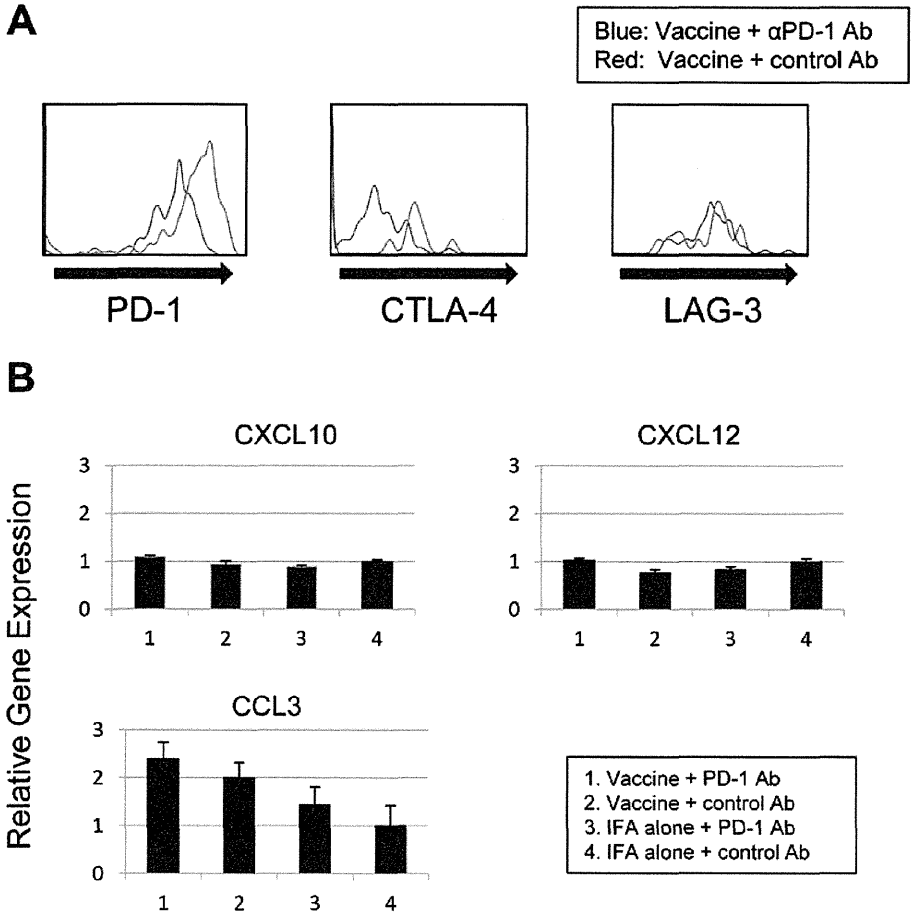

Figure 6. Changes in the expression of inhibitory receptors on tumor-infiltrating T lymphocytes and chemokines at the site of tumors treated using the combination therapy with peptide vaccine and αPD-1 Ab. RMA-bearing mice were treated with intratumoral OVA peptide injection combined with αPD-1 Ab or control Ab. On day 21, mice were sacrificed and the tumors were isolated. (A) Histogram showing the expression of the inhibitory receptors PD-1, CTLA-4, and LAG-3 in OVA tetramer-positive CD8 lymphocytes in tumors from mice treated with intratumoral OVA peptide injection and αPD-1 Ab, as well as from mice treated with intratumoral OVA peptide injection and control Ab. Data are from a single representative sample (n=4-6). (B) The expression levels of chemokines in the tumor were analyzed using quantitative real-time PCR (n=3). Relative expression levels in tumors treated with IFA alone and control Ab were calculated as the control. Data are presented as means ± SEM. Two independent experiments were performed, which yielded similar results.

vaccine alone were not satisfactory in patients with advanced HCC (8,29-31). Several studies identified molecules associated with the tumor escape mechanism, such as PD-1/PD-L1, Fas/FasL, and Decoy receptor 3, which might explain the poor immunogenicity and limitations of the antitumor effects of cancer vaccines alone in patients with advanced HCC (16,17,34,35). Therefore, the present study examined whether blocking PD-1/PD-L enhanced the antitumor effects of peptide vaccines in HCC.

The inhibitory receptor PD-1, was upregulated in GPC3-specific CTLs of HCC patients vaccinated using GPC3 peptide, consistent with previous reports of melanoma vaccine trials (21,27). CTLs for some tumor antigens might not be detected directly *ex vivo*. The *ex vivo* analysis of antigen-specific CTLs from uncultured PBMCs could provide strong and novel immunological evidence in HCC vaccine trials. Fourcade *et al* reported that the upregulation of PD-1 and Tim-3 on CTLs was correlated with the expansion of melanoma-peptide vaccine-induced NY-ESO-1-specific CTLs (21). Further studies are necessary to understand the potential clinical efficacy of vaccine-induced CTLs.

In this experimental model, IFN-γ induced PD-L1 expression in liver cancer cell lines. It was also demonstrated that blocking PD-1 increased the number of GPC3-specific CTL clones that degranulate against these liver cancer cell lines *in vitro*. These results suggest that blocking the interaction between PD-1 and PD-L1 enhanced the antitumor effects of CTL in liver cancer cells that evaded CTLs by expressing PD-L1. In contrast, Xu *et al* reported that αPD-L1 or αCTLA-4 Abs did not enhance cytokine secretion and the proliferation of peripheral GPC3-specific $CD8^+$ T-cell from HCC patients significantly (36). Differences in the effects of blocking PD-1 and PD-L1 might account for the differences between spontaneous GPC3-specific CTLs and vaccine-induced CTLs.

The combination of a peptide vaccine with αPD-1 Ab enhanced tumor suppression and antigen-specific T cell infiltration into the tumors of mouse models. The exact mechanisms by which CTLs accumulate into tumors by blocking PD-1 are unclear. A previous study in a mouse model of adoptive cell transfer demonstrated that blocking PD-1 increased the production of CXCL10 by bone marrow-derived myeloid cells,

which enhanced the recruitment of CTLs in the tumor (25). We hypothesize that the αPD-1 Ab affected chemokine expression, which resulted in recruitment of vaccine-induced CTLs to the tumor. In the present study, the experimental model did not show a change in the expression of CXCL10. However, the expression of CCL3 was elevated by the combination treatment with vaccine and αPD-1 Ab. Furthermore, blocking PD-1 decreased the expression of inhibitory receptors in peptide-specific CTLs at the tumor site. Recently, mouse models revealed that peptide/IFA vaccination increased the antigen-driven expression of the inhibitory receptors PD-1, LAG-3, CTLA-4, and Tim-3 in CTLs, suggesting partial exhaustion (37). PD-1 blockade might be a rational strategy that could be used to rescue CTLs in a state of exhaustion. Interestingly, αPD-1 Ab therapy did not decrease LAG-3 expression in TILs; however, CTLA-4 expression was decreased, suggesting the partial rescue of CTL from exhaustion. A previous study reported that dual treatment with αLAG-3 and αPD-1 Ab was effective in mice with established tumors (38) as well as during the *in vitro* expansion of human NY-ESO-1-specific CTLs (39). Furthermore, Sierro *et al* reported that blocking both PD-1 and PD-L1 might further enhance the antitumor effects of tumor vaccines in mouse models (40).

Based on the results of this clinical trial, the GPC3 peptide vaccine has fewer side effects due to its antigen specificity (8). Enhancing GPC3 peptide vaccine therapy is considered to be promising in terms of sustained tumor control in HCC patients. These data suggest that use of αPD-1 Ab could enhance the antitumor effects of a peptide vaccine, and provide the foundation for the clinical development of a combination therapy.

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References

1. Breous E and Thimme R: Potential of immunotherapy for hepatocellular carcinoma. J Hepatol 54: 830-834, 2011.
2. Greten TF, Manns MP and Korangy F: Immunotherapy of hepatocellular carcinoma. J Hepatol 45: 868-878, 2006.
3. 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.
4. Capurro M, Wanless IR, Sherman M, *et al*: Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 125: 89-97, 2003.
5. 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.
6. 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.
7. 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.
8. 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.
9. 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.
10. Agata Y, Kawasaki A, Nishimura H, *et al*: Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int Immunol 8: 765-772, 1996.
11. Freeman GJ, Long AJ, Iwai Y, *et al*: Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192: 1027-1034, 2000.
12. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T and Minato N: Invovement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci USA 99: 12294-12297, 2002.
13. Iwai Y, Terawaki S and Honjo T: PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. Int Immunol 17: 133-144, 2005.
14. Topalian SL, Hodi FS, Brahmer JR, *et al*: Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366: 2443-2454, 2012.
15. Wolchok JD, Kluger H, Callahan MK, *et al*: Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369: 122-133, 2013.
16. Shi F, Shi M, Zeng Z, *et al*: PD-1 and PD-L1 upregulation promotes CD8(+) T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients. Int J Cancer 128: 887-896, 2011.
17. Gao Q, Wang XY, Qiu SJ, *et al*: Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. Clin Cancer Res 15: 971-979, 2009.
18. McGray AJ, Bernard D, Hallett R, *et al*: Combined vaccination and immunostimulatory antibodies provides durable cure of murine melanoma and induces transcriptional changes associated with positive outcome in human melanoma patients. Oncoimmunology 1: 419-431, 2012.
19. Mkrtichyan M, Najjar YG, Raulfs EC, *et al*: Anti-PD-1 synergizes with cyclophosphamide to induce potent antitumor vaccine effects through novel mechanisms. Eur J Immunol 41: 2977-2986, 2011.
20. Duraiswamy J, Kaluza KM, Freeman GJ and Coukos G: Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine efficacy restorres T cell rejection function in tumors. Cancer Res 73: 3591-3603, 2013.
21. Fourcade J, Sun Z, Pagliano O, *et al*: PD-1 and Tim-3 regulate the expansion of tumor antigen-specific $CD8^+$ T cells induced by melanoma vaccines. Cancer Res 74: 1045-1055, 2014.
22. Nobuoka D, Yoshikawa T, Takahashi M, *et al*: Intratumoral peptide injection enhances tumor cell antigenicity recognized by cytotoxic T lymphocytes: a potential option for improvement in antigen-specific cancer immunotherapy. Cancer Immunol Immunother 62: 639-652, 2013.
23. Li B, VanRoey M, Wang C, Chen TH, Korman A and Jooss K: Anti-programmed death-1 synergizes with granulocyte macrophage colony-stimulating factor - secreting tumor cell immunotherapy providing therapeutic benefit to mice with established tumors. Clin Cancer Res 15: 1623-1634, 2009.
24. Iwama T, Horie K, Yoshikawa T, *et al*: Identification of an H2-K^b or H2-D^b restricted and glypican-3-derived cytotoxic T-lymphocyte epitope peptide. Int J Oncol 42: 831-838, 2013.
25. Peng W, Liu C, Xu C, *et al*: PD-1 blockade enhances T-cell migration to tumors by elevating IFN-γ inducible chemokines. Cancer Res 72: 5209-5218, 2012.

26. Tada Y, Yoshikawa T, Shimomura M, *et al*: Analysis of cytotoxic T lymphocytes from a patient with hepatocellular carcinoma who showed a clinical response to vaccination with a glypican-3-derived peptide. Int J Oncol 43: 1019-1026, 2013.
27. Wong RM, Scotland RR, Lau RL, *et al*: Programmed death-1 blockade enhances expansion and functional capacity of human melanoma antigen-specific CTLs. Int Immunol 19: 1223-1234, 2007.
28. 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.
29. Butterfield LH, Ribas A, Meng WS, *et al*: T-cell responses to HLA-A*0201 immunodominant peptides derived from alpha-fetoprotein in patients with hepatocellular cancer. Clin Cancer Res 9: 5902-5908, 2003.
30. Butterfield LH, Ribas A, Dissette VB, *et al*: A phase I/II trial testing immunization of hepatocellular carcinoma patients with dendritic cells pulsed with four alpha-fetoprotein peptides. Clin Cancer Res 12: 2817-2825, 2006.
31. Greten TF, Forner A, Korangy F, *et al*: A phase II open trial evaluating safety and efficacy of a telomerase peptide vaccination in patients with advanced hepatocellular carcinoma. BMC Cancer 10: 209, 2010.
32. Capurro MI, Xiang YY, Lobe C and Filmus J: Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res 65: 6245-6254, 2005.
33. Feng M, Gao W, Wang R, *et al*: Therapeutically targeting glypican-3 via a conformation-specific single-domain antibody in hepatocellular carcinoma. Proc Natl Acad Sci USA 110: E1083-E1091, 2013.
34. Nagao M, Nakajima Y, Hisanaga M, *et al*: The alteration of Fas receptor and ligand system in hepatocellular carcinomas: how do hepatoma cells escape from the host immune surveillance in vivo? Hepatology 30: 413-421, 1999.
35. Chen C, Zhang C, Zhuang G, *et al*: Decoy receptor 3 overexpression and immunologic tolerance in hepatocellular carcinoma (HCC) development. Cancer Invest 26: 965-974, 2008.
36. Xu Y, Li H, Gao RL, Adeyemo O, Itkin M and Kaplan DE: Expansion of interferon-gamma-producing multifunctional $CD4^+$ T-cells and dysfunctional $CD8^+$ T-cells by glypican-3 peptide library in hepatocellular carcinoma patients. Clin Immunol 139: 302-313, 2011.
37. Hailemichael Y, Dai Z, Jaffarzad N, *et al*: Persistent antigen at vaccination sites induces tumor-specific CD8(+) T cell sequestration, dysfunction and deletion. Nat Med 19: 465-472, 2013.
38. Woo SR, Turnis ME, Goldberg MV, *et al*: Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. Cancer Res 72: 917-927, 2012.
39. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, *et al*: Tumor-infiltrating NY-ESO-1-specific $CD8^+$ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. Proc Natl Acad Sci USA 107: 7875-7880, 2010.
40. Sierro SR, Donda A, Perret R, *et al*: Combination of lentivector immunization and low-dose chemotherapy or PD-1/PD-L1 blocking primes self-reactive T cells and induces antitumor immunity. Eur J Immunol 41: 2217-2228, 2011.

Glypican 3 Expression in Pediatric Malignant Solid Tumors

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Abstract

Purpose Glypican 3 (GPC3) is one of the cell surface heparan sulfate proteoglycans that binds to the cell membrane, and it is known as an oncofetal protein in adult malignant tumors. Clinical trials using a GPC3 peptide vaccine have already been started in Japan as a new immunotherapy for hepatocellular carcinoma in adult patients. To investigate the possibility of GPC3 immunotherapy for pediatric malignant tumors, we assessed the expression of GPC3 in pediatric malignant tumors.

Methods Immunohistochemically, the GPC3 expression was examined in 159 pediatric solid tumors, including 35 cases of neuroblastoma, 30 cases of Wilms tumor, 10 cases of hepatoblastoma, 25 cases of germ cell tumors, 56 cases of rhabdomyosarcoma, and 3 cases of other tumors. In addition, to clarify the physiological expression during the fetal to neoinfantile period, autopsy specimens of subjects without any neoplastic diseases were assessed in 9 fetal cases and 21 neoinfantile cases. The serum levels of GPC3 were also analyzed using specimens obtained from 53 subjects by the sandwich enzyme-linked immunosorbent assay method.

Results Histologically, a high rate of GPC3 expression was noted in 10 (90.9%) of the 11 subjects with yolk sac tumors and 6 (60.0%) of the 10 subjects with hepatoblastoma. In addition, 9 (30.0%) of the 30 subjects with Wilms tumors and 14 (25.0%) of the 56 subjects with rhabdomyosarcoma were positive for the expression of GPC3. Concerning autopsy specimens, most of the 23 subjects younger than 7 months showed positive findings in the liver (94.7%) and kidney (81.8%). Two subjects (100%) with yolk sac tumors and six (75.0%) of the eight subjects with hepatoblastoma serologically demonstrated a high rate of positive expression. Concerning the distribution of the serum GPC3 level according to age, 8 (80.0%) of the 10 subjects younger than 1 year showed a positive finding, while only 16 (37.3%) of the 43 subjects older than 1 year showed a positive finding.

Conclusion Most cases of hepatoblastoma and yolk sac tumor, and some cases of other tumors were found to express GPC3 either histologically or serologically. On the other hand, GPC3 was physiologically expressed during the fetal and neoinfantile period under 1 year of age. Although, more preliminary data and experience are required,

Keywords
- glypican 3
- tumor marker
- immunotherapy

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patients older than 1 year that show a positive finding for GPC3 are considered to be appropriate candidates to receive the new immunotherapy using GPC3 peptide vaccination.

Introduction

Glypican 3 (GPC3) is a cell surface heparan sulfate proteoglycan that is linked to the extracytoplasmic cell-surface membrane by a glycosylphosphatidylinositol anchor.[1] GPC3 is associated with cell growth, development, and the responses to various growth factors.[2] Gonzalez et al described its role as a negative regulator of inhibitory growth factors.[3] GPC3 inactivation has been found to be responsible for X-linked Simpson–Golabi–Behmel (SGB) overgrowth syndrome. In SGB syndrome, 10 to 20% of the patients described have an embryonal malignancy, including hepatoblastoma, neuroblastoma, gonadoblastoma, Wilms tumor, or hepatocellular carcinoma.[4]

Recent studies have shown that there is an overexpression of GPC3 in hepatocellular carcinoma, and has its usefulness as a novel diagnostic marker in many series.[5] Furthermore, the expression of GPC3 has also been reported in other malignant tumors, such as malignant melanoma,[8] clear cell adenocarcinoma of the ovary,[9] and malignant germ cell tumors in adult subjects.[10] Ota et al reported the immunoreactivity of adult testicular tumors, including a yolk sac tumor, teratoma, and choriocarcinoma, as well as a seminoma and embryonal carcinomas. The author demonstrated a high rate of immunoreactivity for the yolk sac tumor.[10]

GPC3 expression has not yet been widely analyzed in pediatric tumors and the roles of GPC3 expression are still unclear. The expression of GPC3 mRNA in several cell lines, including those derived from neuroblastomas, Wilms tumors, and hepatoblastomas, has been reported.[11,12] In addition, Zynger et al examined 65 cases of hepatoblastoma by immunohistochemistry and all subjects exhibited a positive reaction.[13] Zynger et al speculated that GPC3 has a role in the tumorigenesis of hepatoblastoma.

In this study, we analyzed the expression of GPC3 in pediatric malignant solid tumors and assessed the clinical implications of its expression.

Materials and Methods

The immunohistochemical studies examined 159 pediatric solid tumors, including 35 cases of neuroblastoma, 30 cases of Wilms tumor, 10 cases of hepatoblastoma, 25 cases of germ cell tumors (11 yolk sac tumors, 4 immature teratomas, and 10 mature teratomas), and 56 cases of rhabdomyosarcoma and 3 cases of other tumors (2 undifferentiated sarcomas and 1 case of Ewing sarcoma) treated at our institution. The serum levels of GCP3 were also analyzed in samples obtained from 53 subjects, including 13 cases with neuroblastoma, 10 cases of Wilms tumor, 8 cases of hepatoblastoma, 16 cases of germ cell tumors (2 cases with yolk sac tumors, 4 cases with immature teratomas, and 10 cases with mature teratomas), 3 cases of rhabdomyosarcoma, and 3 cases of other tumors by the sandwich enzyme-linked immunosorbent assay (ELISA) method using a GPC3 ELISA kit (Bio Mosaics, Burlington, Vermont, United States).

In addition, to clarify the physiological expression during the fetal to neoinfantile period, autopsy specimens from subjects without any neoplastic disease were assessed by immunohistochemistry. These included samples from 9 fetal cases (age, 19–41 weeks) and 21 neoinfantile cases.

For the immunohistochemical analysis the streptavidin-biotin-peroxidase method (Histofine SAB-PO Kit, Nichirei, Tokyo, Japan) was used. A GPC3 monoclonal antibody (Bio Mosaics) was used at 1:200 dilution.

The serum levels of GPC3 were analyzed by a sandwich ELISA method using an ELISA kit. The samples were diluted at 1:4 and 100 µL of samples or of GPC3 standards were pipetted into the appropriate wells. Covered wells were incubated overnight at 2 to 8°C. After washing the wells five times with wash buffer, 200 µL of a biotin-conjugated anti-GPC3 antibody was pipetted into each well. After overnight incubation, the wells were washed with buffer and 200 µL of streptavidin-horseradish peroxidase conjugated diluents were added to each well. After 30 minutes of incubation, 200 µL of tetramethylbenzidine substrate solution was added to each well for 30 minutes. After these procedures, the absorbance of each well was analyzed by a spectrophotometric plate reader. Based on the data of healthy adult subjects with the standard deviation, the cut-off level for GPC3 was defined as 178 ng/mL in this study.

The patient's parents provided consent for obtaining tumor and tissue preservation and for the subsequent biological analyses. This study was performed according to the Ethical Guidelines for Clinical Research published by the Ministry of Health, Labor, and Welfare of Japan on July 30, 2003.

Results

Histologically, a high rate of GPC3 expression was noted in 10 (90.9%) of the 11 subjects with yolk sac tumors and in 6 (60.0%) of the 10 subjects with hepatoblastoma (►Table 1 and ►Figs. 1a, b). In addition, 9 (30.0%) of the 30 subjects with Wilms tumor (►Fig. 1c), 14 (25.0%) of the 56 subjects with rhabdomyosarcoma, and 1 (2.9%) of the 35 subjects with neuroblastoma were positive for the expression of GPC3.

Similarly, 2 subjects (100%) with yolk sac tumors and 6 (75.0%) of the 8 subjects with hepatoblastoma serologically demonstrated a high rate of positive expression, while 1 (33.3%) of the 3 subjects with rhabdomyosarcoma, 4 (30.7%) of the 13 subjects with neuroblastoma, and 1

Table 1 The results of the immunohistochemical and serological analysis of glypican 3

Histology		GPC3	
		Tissue GPC3 (immunohistochemistry)	Serum GPC3 (ELISA)
HB	Hepatoblastoma	6/10 (60.0%)	6/8 (75.0%)
NBs	Neuroblastoma	1/35 (2.9%)	4/13 (27.3%)
WT	Wilms tumor	9/30 (30.0%)	1/10 (10.0%)
RMS	Rhabdomyosarcoma	14/56 (25.0%)	1/3 (33.3%)
GCT	Yolk sac tumor	10/11 (90.9%)	2/2 (100%)
	Immature teratoma	1/4 (25.0%)	3/4 (75.0%)
	Mature teratoma	0/10 (0.0%)	5/10 (50.0%)
Others	Undifferentiated sarcoma	1/2 (50%)	2/2 (100%)
	Ewing sarcoma	0/1 (0.0%)	0/1 (0.0%)
Total number		159	53

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GCT, germ cell tumor; GPC3, glypican 3; HB, hepatoblastoma; NBs, neuroblastoma and associated tumor; RMS, rhabdomyosarcoma; WT, Wilms tumor.

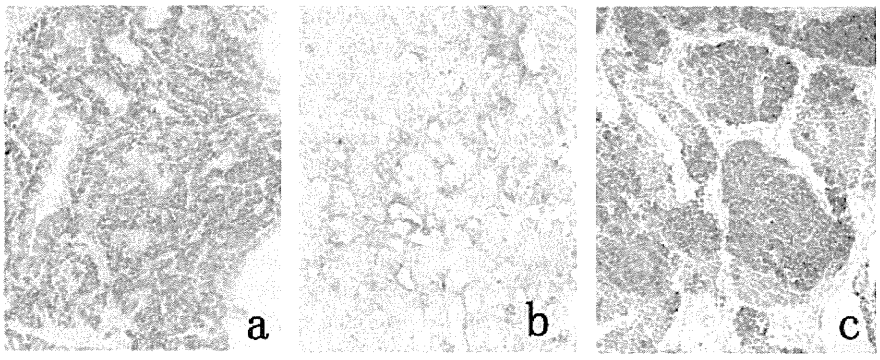

Fig. 1 Immunohistochemical findings: (a) hepatoblastoma, 2-year old; (b) yolk sac tumor, 12-year old; (c) Wilms tumor, 3-year old.

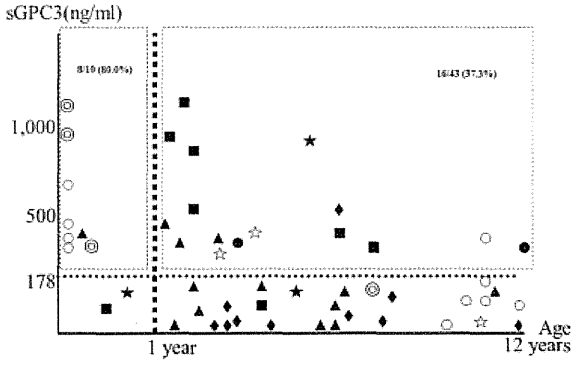

Fig. 2 The distribution of the serum glypican 3 (GPC3) levels according to age. Samples from 8/10 (80.0%) patients younger than 1 year were serologically GPC3-positive, while only samples from 16/43 (37.2%) patients older than 1 year were serologically GPC3-positive. ■, hepatoblastoma; ▲, neuroblastoma and associated tumors; ♦, Wilms tumor; germ cell tumor; ●, yolk sac tumor; ◎, immature teratoma; ○, mature teratoma; ★, rhabdomyosarcoma; ☆, others.

(10.0%) of the 10 subjects with Wilms tumor demonstrated a positive finding (►Fig. 2).

Concerning the distribution of the serum GPC3 level according to age, 8 (80.0%) of the 10 subjects younger than 1 year showed a positive finding. In contrast, only 16 (37.2%) of the 43 subjects older than 1 year showed a positive finding.

Concerning the autopsy specimens, most of the 23 subjects younger than 7 months (including 9 fetal and 14 neoinfantile subjects) showed positive findings in the liver (94.7%) and kidney (81.8%) (►Table 2) (►Figs. 3a–d). The other six subjects older than 1 year did not demonstrate a positive finding in any organ.

The clinical course of one representative case was as follows (►Fig. 4). The subject had an undifferentiated sarcoma that was diagnosed when the patient was 4 years and 6 months old. The serum α-fetoprotein (AFP) level was low, the serum GPC3 level was 334 ng/mL, and the biopsy specimen was immunohistochemically positive for GPC3. The level of serum GPC3 normalized following preoperative intensive

Table 2 The results of the immunohistochemical analysis for autopsy specimens

Age	CNS	Heart	Lung	Liver	Kidney	Pancreas	Spleen	Adrenal gland	Thymus	GI tract
19 wk	–	ND	–	+	+	+	–	ND	ND	–
19 wk	–	ND	–	ND	+	ND	–	ND	–	ND
21 wk	ND	–	–	+	+	–	–	–	ND	ND
21 wk	–	–	–	+	+	–	–	ND	–	–
24 wk	–	–	–	+	+	ND	–	ND	ND	–
24 wk	–	–	–	+	+	ND	–	ND	ND	–
32 wk	–	–	–	+	+	–	–	–	–	–
38 wk	–	–	–	+	+	ND	–	–	+	–
41 wk	–	–	–	+	ND	ND	–	–	–	ND
0 d	–	–	–	+	+	+	–	–	–	–
0 d	ND	–	–	+	+	–	–	–	–	–
0 d	–	–	–	ND	+	ND	–	ND	ND	–
0 d	–	–	–	ND	–	ND	–	ND	ND	–
0 d	ND	–	–	+	+	–	–	–	ND	–
1 d	ND	–	–	+	+	ND	–	–	–	ND
12 d	ND	–	ND	+	–	–	–	–	–	–
14 d	–	–	–	ND	+	+	–	–	–	–
1 mo	ND	–	–	+	–	–	–	–	–	–
3 mo	ND	–	–	+	–	–	–	ND	–	ND
4 mo	ND	–	–	+	+	–	–	ND	–	ND
6 mo	ND	–	–	+	+	–	–	–	ND	–
6 mo	ND	ND	–	–	+	–	–	–	ND	–
7 mo	ND	–	–	+	+	–	–	ND	–	ND
8 mo	ND	–	–	ND	ND	ND	ND	ND	ND	ND
1 y 0 mo	–	–	–	ND	–	–	ND	–	–	ND
1 y 0 mo	–	–	–	–	–	–	–	–	ND	–
1 y 4 mo	–	–	–	ND	–	–	–	–	ND	–
9 y	–	ND	ND	ND	ND	ND	ND	ND	ND	ND
9 y	ND	–	–	ND	ND	ND	ND	ND	–	ND
10 y	–	–	–	–	–	–	–	–	ND	–

Abbreviations: CNS, central nervous system; +, positive; –, negative; ND, not done.

chemotherapy and was maintained within a normal range thereafter. Furthermore, the specimen obtained by radical surgery showed no viable tumor cells and the tissue was immunohistochemically negative for GPC3. After the treatment, the patient has survived for 5 years without any events and the patient's GPC3 level is normal. In this case, the serum GPC3 level was useful as an independent tumor marker.

Discussion

In recent years, several authors have reported the diagnostic value of the serum GPC3 level in hepatocellular carcinomas and other kinds of malignant tumors in adults. In the field of GPC3 research, the expression levels in fetal tissue have been discussed by several authors. Immunohistochemically, three cases of fetal liver tissue were found to be positive, although the benign pediatric liver was negative.[13] In this way, GPC3 has been considered to be a kind of oncofetal protein and to be associated with tumorigenesis in pediatric malignant tumors. However, the clinical implications of the GPC3 expression levels as a diagnostic marker or for the monitoring of tumor progression or curability have not yet been sufficiently analyzed.

From the current data, most hepatoblastomas and yolk sac tumors showed positive findings for both serum and tissue GPC3. In most subjects younger than 1 year, there was a tendency toward a higher level of GPC3 expression compared with subjects older than 1 year. In particular, newborn patients with germ cell tumors, including mature and immature teratomas, exhibited a high level of serum GPC3. Based

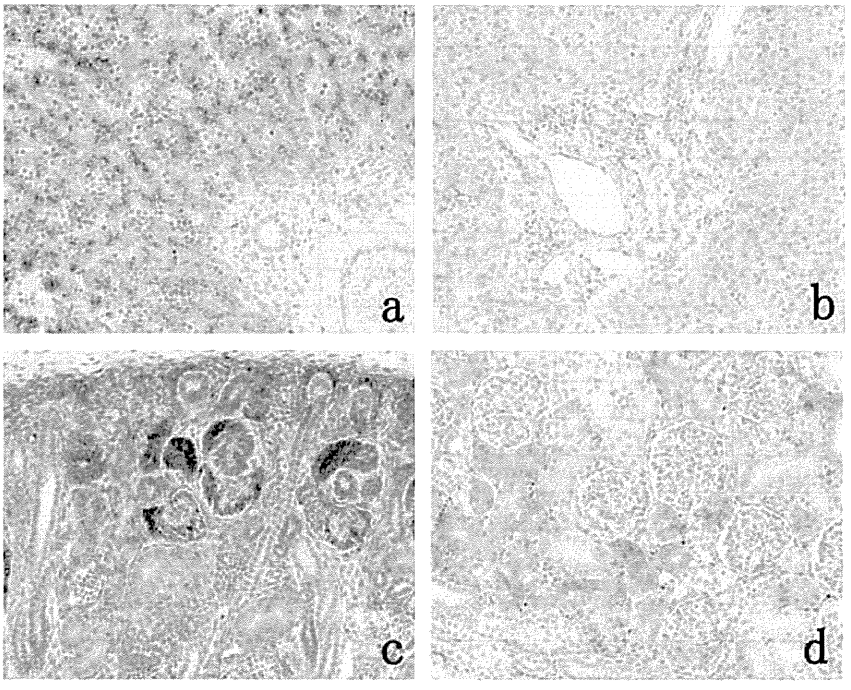

Fig. 3 Immunohistochemical findings for autopsy specimens: (a) fetal liver, 19 weeks, strongly positive; (b) infantile liver, 7 months, moderately positive; (c) fetal kidney, 19 weeks, strongly positive; (d) infantile kidney, 7 months, moderately positive.

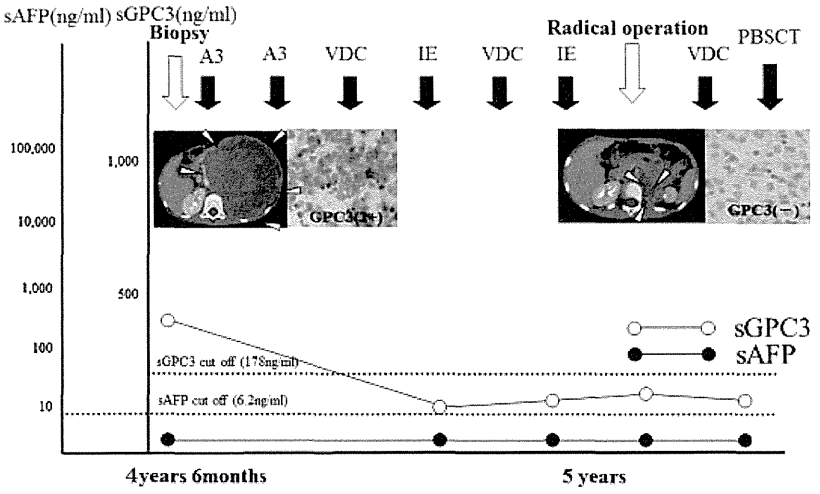

Fig. 4 Clinical course of one case with undifferentiated sarcoma. A3, vincristine + THP-adriamycin + cyclophosphamide + cisplatin; IE, ifosfamide + etoposide; sAFP, serum α-fetoprotein; sGPC3, serum glypican 3; VDC, vincristine + doxorubicin + cyclophosphamide.

on the results of autopsy specimens, we can speculate that GPC3 expression can be observed from the fetal to early neoinfantile period for younger than 1 year regardless of whether malignancy is present. For the subjects older than 1 year, the data from patients who were positive for serum GPC3 but negative for serum AFP imply that GPC3 may be an independent novel tumor marker.

The role of GPC3 as a novel tumor marker for hepatocellular carcinoma in adults has been widely debated in recent studies. A trial using GPC3-targeted immunotherapy for the prevention of cancer development and recurrence has already begun.[14] The same trial protocol would be acceptable to treat and prevent pediatric malignant tumors. However, the number of this series is small, more preliminary data and experience are required to conclude this suitability for immunotherapy.

Conclusion

Most cases of hepatoblastoma, yolk sac tumors and some cases of neuroblastoma, Wilms tumor, and rhabdomyosarcoma were found to express GPC3 either histologically or serologically. On the other hand, GPC3 was also physiologically expressed during the fetal and neoinfantile period in

subjects younger than 1 year. Because the patients older than 1 year who show a positive finding for GPC3 are considered to be appropriate candidates to receive the new immunotherapy using the GPC3 peptide vaccination.

Conflict of Interest
None.

References

1 Filmus J. Glypicans in growth control and cancer. Glycobiology 2001;11(3):19R–23R
2 Song HH, Filmus J. The role of glypicans in mammalian development. Biochim Biophys Acta 2002;1573(3):241–246
3 Gonzalez AD, Kaya M, Shi W, et al. OCI-5/GPC3, a glypican encoded by a gene that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, induces apoptosis in a cell line-specific manner. J Cell Biol 1998;141(6):1407–1414
4 Lapunzina P. Risk of tumorigenesis in overgrowth syndromes: a comprehensive review. Am J Med Genet C Semin Med Genet 2005; 137C(1):53–71
5 Hippo Y, Watanabe K, Watanabe A, et al. Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res 2004;64(7): 2418–2423
6 Capurro M, Wanless IR, Sherman M, et al. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 2003;125(1):89–97
7 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 2003;306(1):16–25
8 Nakatsura T, Kageshita T, Ito S, et al. Identification of glypican-3 as a novel tumor marker for melanoma. Clin Cancer Res 2004;10(19): 6612–6621
9 Maeda D, Ota S, Takazawa Y, et al. Glypican-3 expression in clear cell adenocarcinoma of the ovary. Mod Pathol 2009;22(6): 824–832
10 Ota S, Hishinuma M, Yamauchi N, et al. Oncofetal protein glypican-3 in testicular germ-cell tumor. Virchows Arch 2006;449(3): 308–314
11 Saikali Z, Sinnett D. Expression of glypican 3 (GPC3) in embryonal tumors. Int J Cancer 2000;89(5):418–422
12 Toretsky JA, Zitomersky NL, Eskenazi AE, et al. Glypican-3 expression in Wilms tumor and hepatoblastoma. J Pediatr Hematol Oncol 2001;23(8):496–499
13 Zynger DL, Gupta A, Luan C, Chou PM, Yang GY, Yang XJ. Expression of glypican 3 in hepatoblastoma: an immunohistochemical study of 65 cases. Hum Pathol 2008;39(2):224–230
14 Nishimura Y, Nakatsura T, Senju S. Usefulness of a novel oncofetal antigen, glypican-3, for diagnosis and immunotherapy of hepatocellular carcinoma [in Japanese]. Nihon Rinsho Meneki Gakkai Kaishi 2008;31(5):383–391