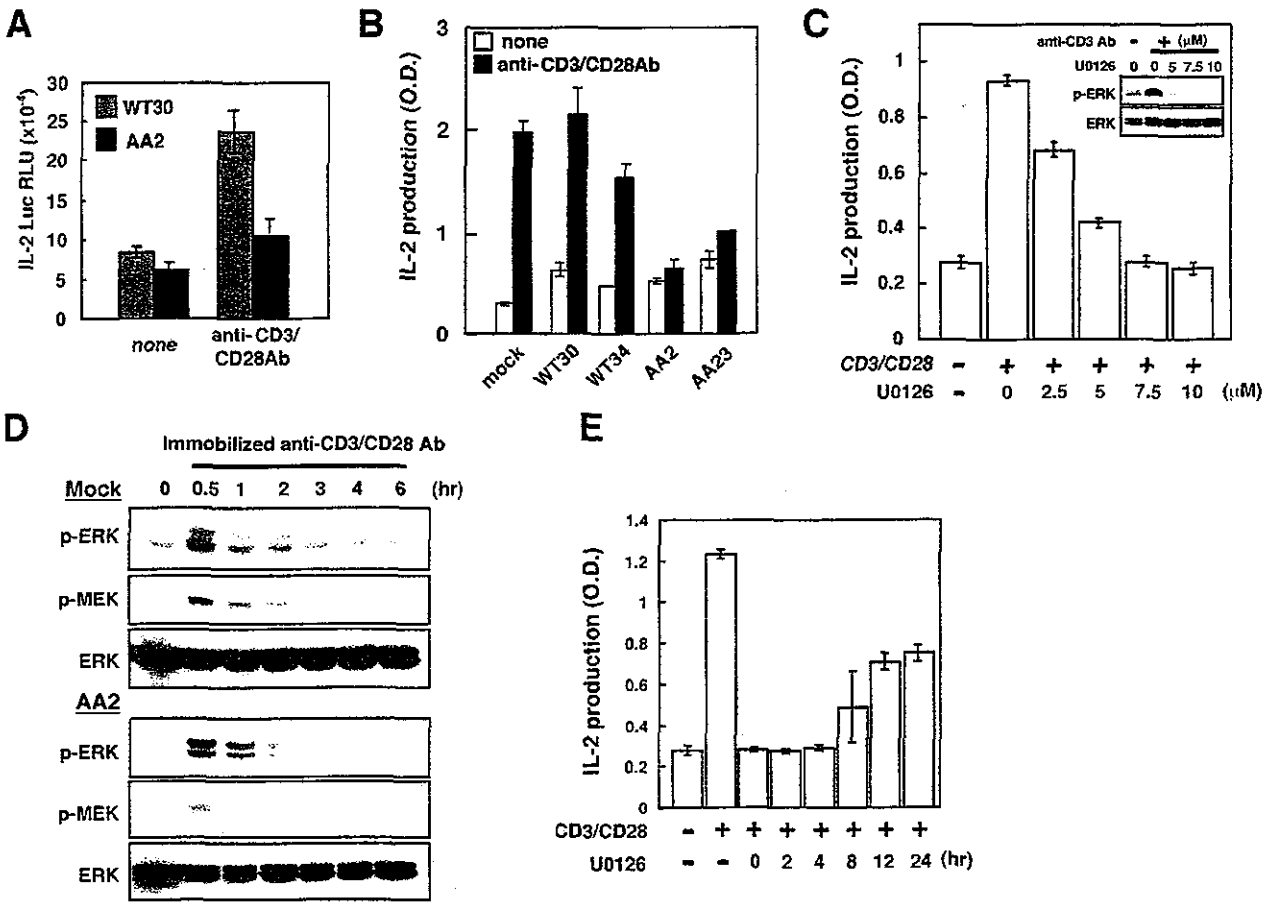

FIG. 5. **Sustained ERK activation controlled by B-Raf is critical in TCR-mediated production of IL-2.** *A*, *luciferase* assay for IL-2 promoter activity. Jurkat clone expressing wild-type B-Raf (WT30) or B-Raf AA (AA2) was transfected with an IL-2-luciferase construct and incubated with or without immobilized anti-CD3 and anti-CD28 antibodies for 12 h. Each luciferase activity was evaluated and normalized by the co-transfected β-galactosidase activity. *RLU*, relative luciferase unit. *B*, Jurkat clones expressing wild-type B-Raf (WT30 and WT34), B-Raf AA (AA2 and AA23), and mock-transfectant were stimulated with immobilized anti-CD3 and anti-CD28 antibodies for 48 h. IL-2 in the culture supernatants was measured by enzyme-linked immunosorbent assay. *C*, Jurkat cells were pretreated with MEK inhibitor U0126 at the indicated concentrations for 30 min before stimulation and then were stimulated with an anti-CD3 antibody for 3 min, and phosphorylation of ERK was analyzed with Western blotting (*insets*). For measurement of IL-2, Jurkat cells pretreated with U0126 at the indicated concentrations were stimulated with immobilized anti-CD3 and anti-CD28 antibodies for 48 h. IL-2 in the culture supernatants were measured by enzyme-linked immunosorbent assay. *D*, mock-transfected (*upper panel*) or B-Raf AA expressing Jurkat clone (AA2; *lower panel*) were stimulated with the immobilized anti-CD3 and anti-CD28 antibodies for the indicated times. The whole cell extract from each sample was analyzed by blotting with anti-phospho-ERK (*top panel*), phospho-MEK (*middle panel*), or ERK (*bottom panel*) antibodies, respectively. *E*, vehicle (Me_2SO) or U0126 (5 μM) was added to the culture at the indicated times after the beginning of stimulation of Jurkat cells with immobilized anti-CD3 and anti-CD28 antibodies. After 48 h from the start of stimulation, each culture supernatant was harvested, and the IL-2 concentration was measured, as described in *C*. Typical data from three independent and reproducible experiments are presented here.

B-Raf for TCR-mediated activation do not entirely overlap with those of other Raf proteins. We elucidated that B-Raf activation couples Ras with TCR-mediated MEK/ERK activation and is indispensable for prolongation of substantial MEK/ERK activation *in vivo*.

This sustained MEK/ERK activation correlates with duration and strength of B-Raf activity. We considered that activation thresholds and the mechanisms regulating each Raf activity lead to distinct activation kinetics of these two Raf kinases. In agreement with this interpretation, the following observations were reported. Although the activities of both Raf-1 (47) and B-Raf (Fig. 3) were dependent on Ras activity, in addition to Ras, Src family kinases regulated Raf-1 activity (48). Upon activation, Raf-1 was shown to be phosphorylated on some tyrosine, serine, and threonine residues, which fulfill the regulatory functions, and the phosphorylation status of these sites in Raf-1 is different from that of B-Raf. First, the major target site of Src is Tyr^{340} in Raf-1; however, B-Raf activity seemed to be less dependent on Src, and Ras activation is sufficient for B-Raf function because B-Raf lacks the Tyr corresponding to Tyr^{340} in Raf-1 (41, 49). Thus, B-Raf activation requires Ras but not Src to activate MEK/ERK, whereas Raf-1 activation needs the synergy of Ras and Src tyrosine kinase(s) (41, 49). Second, conserved B-Raf Ser^{445}, corresponding to Ser^{338} in Raf-1, which is one of the regulatory phosphorylation sites of Raf activity, is constitutively phosphorylated in fibroblasts (49). These results seem to explain the fact that B-Raf exhibits a higher intrinsic kinase activity in a quiescent situation and, once stimulated, a longer activation period than does Raf-1 in our system and other systems.

It should be noted that the dominant negative mutant of B-Raf (B-Raf AA) did not impair the transient MEK/ERK activation but did suppress the sustained MEK/ERK activation although B-Raf was activated and associated with MEK in both the early and the late phase after TCR stimulation. Why was not MEK/ERK activation in the early phase drastically attenuated by B-Raf AA? The most likely explanation is that Raf-1 can compensate for the defects of B-Raf activation due to the

dence has been accumulated that supports the contribution of ERK signaling to NFAT activation. In T cells, the transcriptional activity of NFAT was reported to be regulated by Ras/MEK/ERK acting in synergy with a calcium/calmodulin phosphatase, calcineurin (7, 44). Moreover, the mechanism by which some kinases and phosphatases regulate the NFAT activity implies modulation of nuclear translocation of this factor, its binding to DNA, or transactivation of its target gene expression. Because B-Raf AA abrogated the nuclear localization and transcriptional activity of NFAT, we propose that the model that sustained B-Raf/MEK/ERK activation modulating NFAT-dependent transcription could be achieved by regulation of intrinsic nuclear translocation of NFAT. Supporting this interpretation, it has been reported that ERK1 overexpression augmented the DNA binding activity of NFAT, resulting in NFAT activation in Jurkat cells (50). However, it has been shown that activated ERK binds to and phosphorylates NFAT2, which negatively regulates nuclear translocation and activation of NFAT2 in fibroblasts (51). Conversely, in Jurkat cells, we observed the attenuation of TCR-stimulated nuclear translocation of NFAT by MEK inhibitor.[2] These seemingly discrepant results might be accounted for by use of different systems and cell types. In any case, the formal demonstration of a role for B-Raf and ERK in the regulation of NFAT activation *in vivo* requires more detailed analysis.

It is noteworthy that temporal difference in the Raf-induced ERK activation signal induces qualitatively different cellular responses. In PC12 cells, epidermal growth factor-driven proliferation was coupled with transient ERK activation. On the contrary, neural growth factor-driven differentiation of PC12 cells into sympathetic neurons was induced by sustained ERK activation (10), which was mediated by B-Raf (23). A similar phenomenon was found in T cells. Mariathasan *et al.* (52) demonstrated that in thymocytes, negatively selecting stimuli by agonistic peptides through TCR induced transient and strong ERK activation, resulting in cell death, whereas positively selecting stimuli by the analogue peptides induced sustained and weak ERK activation, resulting in cell survival. In a very recent study, it has been reported that B-Raf but not Raf-1 was activated with TCR stimulation in $CD4^+CD8^+$ double positive thymocytes (53). These observations and our findings that B-Raf and Raf-1 activities regulated the strength and the duration of TCR-mediated ERK activation prompted us to consider that the Ras/B-Raf/MEK/ERK pathway also could play important roles in determining the cell fate such as thymocyte differentiation regulated by temporally distinct ERK activity.

In summary, our data suggest that Ras/B-Raf/MEK/ERK can serve as a novel component of signaling pathways that regulate the duration of ERK activity in response to TCR stimulation. B-Raf and ERK activation with a proper duration determines biological outcomes such as IL-2 production in human T cells.

Acknowledgments—We thank Dr. K. L. Guan for generously providing B-Raf constructs, Dr. V. A. Boussiotis for the IL-2 promoter-luciferase reporter construct, Dr. R. M. Niles for the AP-1-luciferase reporter construct, Dr. T. Saito for the NFAT-GFP reporter construct, Dr. Y. Takai for the GST-MEK expression construct, and Dr. G. R. Crabtree for the TAg-Jurkat cells. We also thank M. Ohara for helpful comments.

REFERENCES

1. Kane, L. P., Lin, J., and Weiss, A. (2000) *Curr. Opin. Immunol.* **12,** 242–249
2. Genot, E., and Cantrell, D. A. (2000) *Curr. Opin. Immunol.* **12,** 289–294
3. D'Ambrosio, D., Cantrell, D. A., Frati, L., Santoni, A., and Testi, R. (1994) *Eur. J. Immunol.* **24,** 616–620
4. Carey, K. D., Dillon, T. J., Schmitt, J. M., Baird, A. M., Holdorf, A. D., Straus, D. B., Shaw, A. S., and Stork, P. J. (2000) *Mol. Cell. Biol.* **20,** 8409–8419
5. Katagiri, K., Hattori, M., Minato, N., and Kinashi, T. (2002) *Mol. Cell. Biol.* **22,** 1001–1015
6. Rincon, M. (2001) *Curr. Opin. Immunol.* **13,** 339–345
7. Whitehurst, C. E., and Geppert, T. D. (1996) *J. Immunol.* **156,** 1020–1029
8. Faris, M., Kokot, N., Lee, L., and Nel, A. E. (1996) *J. Biol. Chem.* **271,** 27366–27373
9. Hogan, P. G., Chen, L., Nardone, J., and Rao, A. (2003) *Genes Dev.* **17,** 2205–2232
10. Marshall, C. J. (1995) *Cell* **80,** 179–185
11. Bommhardt, U., Scheuring, Y., Bickel, C., Zamoyska, R., and Hunig, T. (2000) *J. Immunol.* **164,** 2326–2337
12. van den Brink, M. R., Kapeller, R., Pratt, J. C., Chang, J. H., and Burakoff, S. J. (1999) *J. Biol. Chem.* **274,** 11178–11185
13. Pages, G., Guerin, S., Grall, D., Bonino, F., Smith, A., Anjuere, F., Auberger, P., and Pouyssegur, J. (1999) *Science* **286,** 1374–1377
14. Mariathasan, S., Ho, S. S., Zakarian, A., and Ohashi, P. S. (2000) *Eur. J. Immunol.* **30,** 1060–1068
15. Li, Y. Q., Hii, C. S., Der, C. J., and Ferrante, A. (1999) *Immunology* **96,** 524–528
16. Hagemann, C., and Rapp, U. R. (1999) *Exp. Cell Res.* **253,** 34–46
17. Magnuson, N. S., Beck, T., Vahidi, H., Hahn, H., Smola, U., and Rapp, U. R. (1994) *Semin. Cancer Biol.* **5,** 247–253
18. Eychene, A., Dusanter-Fourt, I., Barnier, J. V., Papin, C., Charon, M., Gisselbrecht, S., and Calothy, G. (1995) *Oncogene* **10,** 1159–1165
19. Barnier, J. V., Papin, C., Eychene, A., Lecoq, O., and Calothy, G. (1995) *J. Biol. Chem.* **270,** 23381–23389
20. Wojnowski, L., Zimmer, A. M., Beck, T. W., Hahn, H., Bernal, R., Rapp, U. R., and Zimmer, A. (1997) *Nat. Genet.* **16,** 293–297
21. Papin, C., Denouel-Galy, A., Laugier, D., Calothy, G., and Eychene, A. (1998) *J. Biol. Chem.* **273,** 24939–24947
22. Vaillancourt, R. R., Gardner, A. M., and Johnson, G. L. (1994) *Mol. Cell. Biol.* **14,** 6522–6530
23. York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W., and Stork, P. J. (1998) *Nature* **392,** 622–626
24. Brummer, T., Shaw, P. E., Reth, M., and Misawa, Y. (2002) *EMBO J.* **21,** 5611–5622
25. Erhardt, P., Schremser, E. J., and Cooper, G. M. (1999) *Mol. Cell. Biol.* **19,** 5308–5315
26. Peraldi, P., Frodin, M., Barnier, J. V., Calleja, V., Scimeca, J. C., Filloux, C., Calothy, G., and Van Obberghen, E. (1995) *FEBS Lett.* **357,** 290–296
27. Qwaki, H., Varma, R., Gillis, B., Bruder, J. T., Rapp, U. R., Davis, L. S., and Geppert, T. D. (1993) *EMBO J.* **12,** 4367–4373
28. Northrop, J. P., Ullman, K. S., and Crabtree, G. R. (1993) *J. Biol. Chem.* **268,** 2917–2923
29. Chen, Y. Z., Matsushita, S., and Nishimura, Y. (1996) *J. Immunol.* **157,** 3788–3790
30. Irie, A., Chen, Y. Z., Tsukamoto, H., Jotsuka, T., Masuda, M., and Nishimura, Y. (2003) *Eur. J. Immunol.* **33,** 1497–1507
31. van den Hoff, M. J., Moorman, A. F., and Lamers, W. H. (1992) *Nucleic Acids Res.* **20,** 2902
32. Ohtsuka, T., Shimizu, K., Yamamori, B., Kuroda, S., and Takai, Y. (1996) *J. Biol. Chem.* **271,** 1258–1261
33. Zhang, B. H., and Guan, K. L. (2000) *EMBO J.* **19,** 5429–5439
34. Boussiotis, V. A., Freeman, G. J., Berezovskaya, A., Barber, D. L., and Nadler, L. M. (1997) *Science* **278,** 124–128
35. Huang, Y., Boskovic, G., and Niles, R. M. (2003) *J. Cell. Physiol.* **194,** 162–170
36. Ohtsuka, M., Arase, H., Takeuchi, A., Yamasaki, S., Shiina, R., Suenaga, T., Sakurai, D., Yokosuka, T., Arase, N., Iwashima, M., Kitamura, T., Moriya, H., and Saito, T. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 8126–8131
37. Uemura, Y., Senju, S., Maenaka, K., Iwai, L. K., Fujii, S., Tabata, H., Tsukamoto, H., Hirata, S., Chen, Y. Z., and Nishimura, Y. (2003) *J. Immunol.* **170,** 947–960
38. Vossler, M. R., Yao, H., York, R. D., Pan, M. G., Rim, C. S., and Stork, P. J. (1997) *Cell* **89,** 73–82
39. Xiang, X., Zang, M., Waelde, C. A., Wen, R., and Luo, Z. (2002) *J. Biol. Chem.* **277,** 44996–45003
40. Chong, H., Lee, J., and Guan, K. L. (2001) *EMBO J.* **20,** 3716–3727
41. Marais, R., Light, Y., Paterson, H. F., Mason, C. S., and Marshall, C. J. (1997) *J. Biol. Chem.* **272,** 4378–4383
42. Borovsky, Z., Mishan-Eisenberg, G., Yaniv, E., and Rachmilewitz, J. (2002) *J. Biol. Chem.* **277,** 21529–21536
43. Deng, T., and Karin, M. (1994) *Nature* **371,** 171–175
44. Genot, E., Cleverley, S., Henning, S., and Cantrell, D. (1996) *EMBO J.* **15,** 3923–3933
45. Wojnowski, L., Stancato, L. F., Zimmer, A. M., Hahn, H., Beck, T. W., Larner, A. C., Rapp, U. R., and Zimmer, A. (1998) *Mech. Dev.* **76,** 141–149
46. Taylor-Fishwick, D. A., and Siegel, J. N. (1995) *Eur. J. Immunol.* **25,** 3215–3221
47. Wotton, D., Ways, D. K., Parker, P. J., and Owen, M. J. (1993) *J. Biol. Chem.* **268,** 17975–17982
48. Pathan, N. I., Ashendel, C. L., Geahlen, R. L., and Harrison, M. L. (1996) *J. Biol. Chem.* **271,** 30315–30317
49. Mason, C. S., Springer, C. J., Cooper, R. G., Superti-Furga, G., Marshall, C. J., and Marais, R. (1999) *EMBO J.* **18,** 2137–2148
50. Park, J. H., and Levitt, L. (1993) *Blood* **82,** 2470–2477
51. Porter, C. M., Havens, M. A., and Clipstone, N. A. (2000) *J. Biol. Chem.* **275,** 3543–3551
52. Mariathasan, S., Zakarian, A., Bouchard, D., Michie, A. M., Zuniga-Pflucker, J. C., and Ohashi, P. S. (2001) *J. Immunol.* **167,** 4966–4973
53. Reynolds, L. F., de Bettignies, C., Norton, T., Beeser, A., Chernoff, J., and Tybulewicz, V. L. (2004) *J. Biol. Chem.* **279,** 18239–18246

[2] H. Tsukamoto, A. Irie, and Y. Nishimura, unpublished data.

Mouse Homologue of a Novel Human Oncofetal Antigen, Glypican-3, Evokes T-Cell–Mediated Tumor Rejection without Autoimmune Reactions in Mice

Tetsuya Nakatsura,[1] Hiroyuki Komori,[1,2] Tatsuko Kubo,[3] Yoshihiro Yoshitake,[1] Satoru Senju,[1] Toyomasa Katagiri,[4] Yoichi Furukawa,[4] Michio Ogawa,[2] Yusuke Nakamura,[4] and Yasuharu Nishimura[1]

Departments of [1]Immunogenetics, [2]Surgery II, and [3]Molecular Pathology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, and [4]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

ABSTRACT

Purpose and Experimental Design: **We recently identified glypican-3 (GPC3) overexpressed specifically in human hepatocellular carcinoma, as based on cDNA microarray analysis of 23,040 genes, and we reported that GPC3 is a novel tumor marker for human hepatocellular carcinoma and melanoma. GPC3, expressed in almost all hepatocellular carcinomas and melanomas, but not in normal tissues except for placenta or fetal liver, is a candidate of ideal tumor antigen for immunotherapy. In this study, we attempted to identify a mouse GPC3 epitope for CTLs in BALB/c mice, and for this, we set up a preclinical study to investigate the usefulness of GPC3 as a target for cancer immunotherapy *in vivo*.**

Results: **We identified a mouse GPC3-derived and K^d-restricted CTL epitope peptide in BALB/c mice. Inoculation of this GPC3 peptide-specific CTL into s.c. Colon26 cancer cells transfected with mouse *GPC3* gene (*C26/GPC3*) led to rejection of the tumor *in vivo*, and i.v. inoculation of these CTLs into sublethally irradiated mice markedly inhibited growth of an established s.c. tumor. Inoculation of bone marrow-derived dendritic cells pulsed with this peptide prevented the growth of s.c. and splenic C26/GPC3 accompanied with massive infiltration of $CD8^+$ T cells into tumors. Evidence of autoimmune reactions was never observed in surviving mice that had rejected tumor cell challenges.**

Conclusions: **We found the novel oncofetal protein GPC3 to be highly immunogenic in mice and elicited effective antitumor immunity with no evidence of autoimmunity. GPC3 is useful not only for diagnosis of hepatocellular carcinoma and melanoma but also for possible immunotherapy or prevention of these tumors.**

INTRODUCTION

Primary hepatocellular carcinoma is one of the common malignancies throughout the world. Because of the global pandemic of hepatitis B and C infections, the incidence of hepatocellular carcinoma is rapidly on the rise in Asian and Western countries (1). This trend is expected to continue for the next 50 years because of the long latency between infection and development of hepatocellular carcinoma. The prognosis of advanced hepatocellular carcinoma remains poor, and effective treatment strategies are urgently needed.

The report of the cloning human melanoma antigen, *MAGE* gene, stated that the human immune system can recognize cancer as a foreign body and can exclude it (2). This genetic approach of T-cell epitope cloning led to identification of a many genes encoding for tumor antigens and antigenic peptides recognized by tumor-reactive CTLs, thereby enhancing the possibility of antigen-specific cancer immunotherapy (3–6). Recently, >1500 types of candidates of tumor antigens have been identified with the SEREX method (7, 8). We also reported cancer antigens identified with this method (9–11). cDNA microarray technology, by which investigators can obtain comprehensive data with respect to gene expression profiles, is rapidly progressing. Studies have shown the usefulness of this technique for identification of novel cancer-associated genes and for classification of human cancers at the molecular level (12–16). We have recently succeeded in identification of a novel cancer rejection antigen specifically expressed in esophageal cancer with cDNA microarray technology (17).

To identify candidates of ideal hepatocellular carcinoma antigen for tumor immunotherapy, which is strongly expressed in almost all hepatocellular carcinomas but not in normal adult tissues, except for immune privilege tissues such as testis and placenta or fetal organs, we used two kinds of data of cDNA microarrays containing 23,040 genes. One is a comparison of expression profiles between 20 hepatocellular carcinomas and their corresponding noncancerous liver tissues (18) and the other is that of various normal human tissues (19). When using these data, we identified *glypican-3* (*GPC3*) overexpressed specifically in hepatocellular carcinoma, and we reported that GPC3 is a novel tumor marker for human hepatocellular carcinoma (20) and melanoma (21). Not only the amino acid sequences but also the expression patterns of human and mouse

Received 6/17/04; revised 9/2/04; accepted 9/15/04.
Grant support: Grants-in-Aid 12213111 (Y. Nishmura) and 14770142 (T. Nakatsura) from the Ministry of Education, Science, Technology, Sports and Culture, Japan.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Yasuharu Nishimura, Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan. Phone: 81-96-373-5310; Fax: 81-96-373-5314; E-mail: mxnishim@gpo.kumamoto-u.ac.jp.

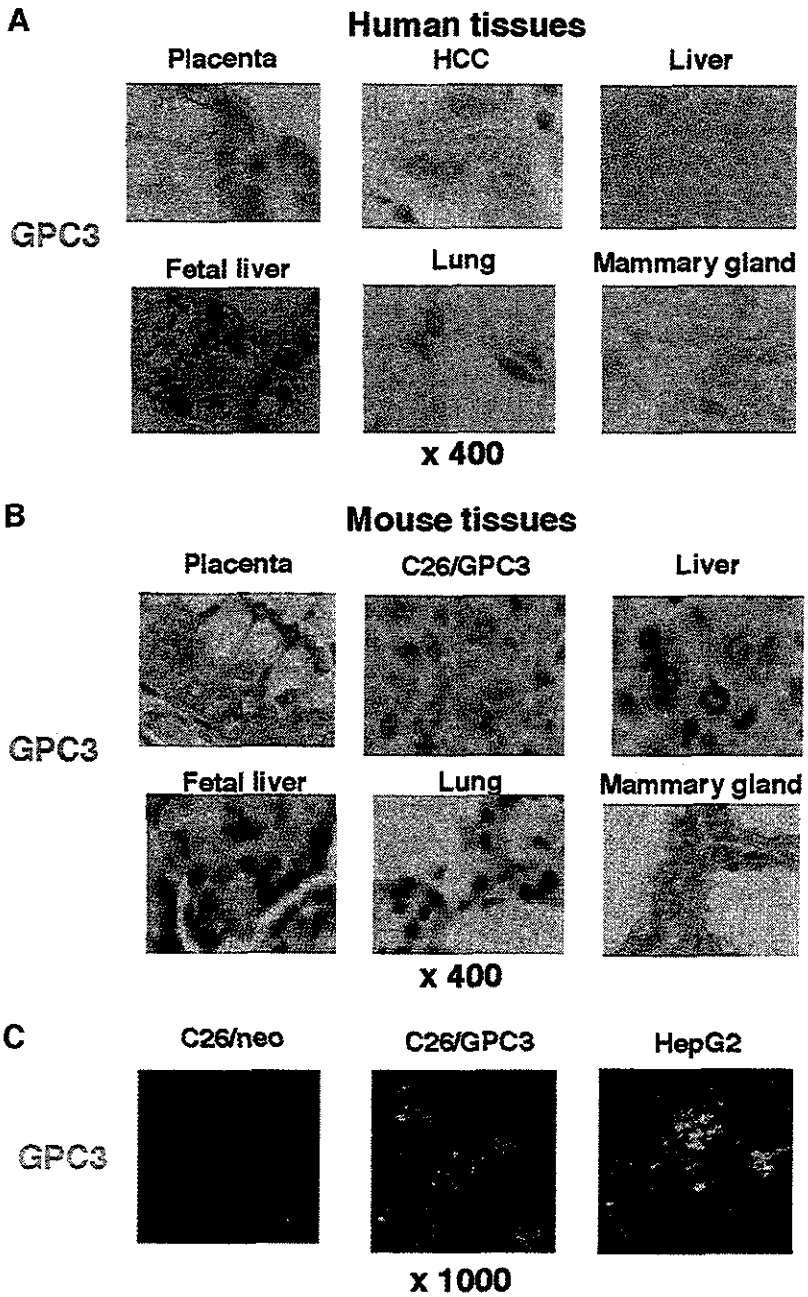

Fig. 1 Expression of GPC3 protein, the candidate of an ideal target for immunotherapy of hepatocellular carcinoma (HCC) and melanoma, in human and mouse tissues and cells. *A* and *B*, expression of GPC3 protein detected by immunohistochemical analysis in various human (*A*) and mouse (*B*) tissues. Objective magnification was 400×. *C*, expression of GPC3 protein detected by FITC-conjugated anti-GPC3 antibodies in HepG2 and GPC3-null mouse colon cancer cell line Colon26 transfected with control vector (C26/neo) or *GPC3* gene (C26/GPC3). Objective magnification was 1000×.

tocellular carcinoma cell line HepG2 (Fig. 1*C*). As a result, the expression levels of GPC3 protein in the human hepatocellular carcinoma, human melanoma, and C26/GPC3 tumor were evidently much higher than those in all adult normal tissues of both human and mouse, including lung and mammary gland, except for placenta and fetal liver (Table 1).

Identification of a GPC3-derived and K^d-restricted CTL Epitope in BALB/c Mice. Structural motifs of peptides bound to human HLA-A24 and mouse K^d are similar. The amino acid sequences of human and mouse GPC3 have a 95% homology. We searched for GPC3-derived peptides of which amino acid sequences were completely shared between human and mouse GPC3. Among these peptides, we selected those carrying binding motifs to both HLA-A24 and K^d molecules, as previously described (10), and prepared 12 different synthetic peptides GPC3-1~12 (Fig. 2*A*). When we tested these peptides for their potential to induce tumor-reactive CTLs *in vitro* from spleen cells derived from mice immunized with GPC3 peptides, only GPC3-8 EYILSLEEL peptide-induced CTLs showed specific cytotoxicity against C26/GPC3 (GPC3+, H-2^d) and T2 cells transfected with the H2-K^d gene (T2K^d) pulsed with GPC3-8 but not against C26 (GPC3−, H-2^d), B16 (GPC3+, H-2^b), and T2K^d cells pulsed with GPC3-7 (Fig. 2, *A* and *B*). These findings indicate

GPC3 protein were very similar. GPC3 is an oncofetal protein overexpressed in almost all human hepatocellular carcinomas and melanomas (20). Both human and mouse GPC3 are expressed in normal tissues, including placenta and fetal liver, but not in other normal adult tissues. In the present study, we set up preclinical studies to investigate the usefulness of GPC3 as a target for cancer immunotherapy *in vivo*, and we found this oncofetal protein to be highly immunogenic in mice in that it elicited effective antitumor immunity with no evidence of autoimmunity.

MATERIALS AND METHODS

Cell Lines. A subline of BALB/c-derived colorectal adenocarcinoma cell line Colon26, C26 (C20) (22) was provided by Dr. Kyoichi Shimomura (Fujisawa Pharmaceutical Co., Osaka, Japan). B16 and HepG2 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer Tohoku University (Sendai, Japan). T2K^d was provided by Dr. Paul M. Allen of Washington University School of Medicine (St. Louis, MO). These cells were maintained *in vitro* in RPMI 1640 or DMEM supplemented with 10% FCS. Expression of H-2K^d was examined with fluorescence-activated cell sorting analysis and an antimouse H-2K^d–specific antibody and a subsequent FITC-labeled antimouse antibody.

Transfection of *GPC3* Gene into Cells. Plasmids, including full-length murine *GPC3* cDNA clones, were purchased (Invitrogen, Osaka, Japan). A cDNA fragment encoding for GPC3 protein was inserted into pCAGGS-IRES-*neo*-R, a mammalian expression vector containing the chicken β-actin promoter and an internal ribosomal entry site (IRES)-*neomycin N-acetyltransferase* gene cassette. We used the empty pCAGGS-IRES-neo-R plasmid as a control. These cDNAs were transfected into C26 (C20) cells by lipofection, as previously described (10), and selected with G418.

Mice. Female 7-week-old BALB/c mice (H-2^d), purchased from Charles River Japan (Yokohama, Japan), were kept in the Center for Animal Resources and Development of Kumamoto University and handled in accordance with the animal care policy of Kumamoto University.

Identification of a CTL Epitope in BALB/c Mice. Peptides were purchased from biologica (Tokyo, Japan), and their purity, as estimated by high-performance liquid chromatography, was >95%. The immunizations were done as follows: we primed the mice with 50 μg of each 12 kinds of GPC3-derived peptides emulsified in 50 μL of complete Freund's adjuvant (Sigma, Tokyo, Japan) diluted with 50 μL of saline s.c. into the left flank and boosted these mice with the same peptides emulsified in incomplete Freund's adjuvant by the same method used for priming 7 days after priming. Splenocytes removed from mice 7 days after the last immunization were harvested, depleted of RBCs by hypotonic lysis, and cultured in 24-well culture plates (2.5×10^6/well) in 45% RPMI/45% AIMV/10% FCS supplemented with recombinant human interleukin 2 (100 units/mL), 2-mercaptoethanol (50 μmol/L), and each peptide (10 μmol/L). Then, 5 days later, cytotoxicity of these cells directed against target cells was assayed in a standard 6-hour ^{51}Cr release assays (10). We purified CD8$^+$ T cells from bulk CTLs with the MACS system with antimouse CD8α (Ly-2) monoclonal antibody, and these CD8$^+$ CTLs were used for adoptive transfer into BALB/c mice.

Bone Marrow-derived Dendritic Cell (BM-DC) Vaccine. BM-DCs were generated as follows: BM cells (2×10^6) were cultured in RPMI 1640 supplemented with 10% FCS, together with granulocyte macrophage colony-stimulating factor (5 ng/mL) for 7 days in 10-cm plates, and these BM-DCs were pulsed with GPC3-8 peptide (10 μmol/L) at 37°C for 2 hours and used as GPC3-8 peptide-pulsed BM-DC vaccine.

***In vivo* Depletion of CD4$^+$ and CD8$^+$ T Lymphocytes.** The mice were given a total of six i.p. transfers (days −18, −15, −11, −8, −4, and −1) of the ascites (0.1 mL/mouse/transfer) from hybridoma-bearing nude mice. The mAbs used were rat antimouse CD4 (clone GK1.5) and rat antimouse CD8 (clone 2.43). Normal rat IgG (Sigma, St. Louis, MO; 200 μg/mouse/transfer) was used as control. Depletion of T-cell subsets by treatment with monoclonal antibodies was confirmed by flow cytometric analysis of spleen cells, which showed a >90% specific depletion.

Histologic and Immunohistochemical Analysis. Immunohistochemical (23) and immunocytochemical (24) detections of GPC3 were done, as described previously. We purchased Human, Normal Organs, and Cancers, Tissue Array, BC4 (SuperBioChips Laboratories, Seoul, Korea) and Human Fetal Normal Multi Tissue Slide (BioChain, Hayward, CA) for immunohistochemical analysis. H&E staining and standard methods were used. Immunohistochemical staining of CD8 was done, as described previously (25). For the terminal deoxynucleotidyl transferase-mediated nick end labeling method, we used ApopTag Fluorescein *In Situ* Apoptosis Detection kits (Serologicals Corporation, Norcross, GA).

Statistical Analysis. We analyzed all data with the StatView statistical program for Macintosh (SAS, Inc., Cary, NC) and evaluated the statistical significance with unpaired *t* test. The percentage of overall survival rate was calculated with the Kaplan-Meier method, and statistical significance was evaluated with the Wilcoxon test.

RESULTS

Limited Expression of GPC3 Protein in Both Human and Mouse Fetal Tissues. We and other investigators found GPC3 to be overexpressed in hepatocellular carcinoma (20, 26–31) and melanoma (21), so we did an immunohistochemical analysis of GPC3 with various human and mouse tissues (Fig. 1 and Table 1). The expression patterns of human and mouse GPC3 protein were very similar. GPC3 protein was expressed in placenta and fetal liver, but no or only an expression was observed in all normal adult human and mouse tissues tested, including brain, lung, heart, liver, kidney, mammary gland, spleen, and thymus (Fig. 1, *A* and *B*). The mouse colorectal cancer cell line Colon26 (C26) did not express GPC3, but after stable transfection of mouse *GPC3* genes, GPC3 protein was expressed in C26/GPC3 (Fig. 1*C*). This C26/GPC3 tumor inoculated s.c. into BALB/c mice expressed GPC3 as evidenced in our immunohistochemical analysis (Fig. 1*B*). The expression level of GPC3 protein in C26/GPC3 is not higher than that of human hepatocellular carcinoma (Fig. 1*A*) or the human hepa

Table 1 The expression levels of GPC3 protein determined by immunohistochemical analysis in various human and mouse tissues

+++*	++	+, +/−	−	−	−
Human hepatocellular carcinoma	Placenta	Lung	Liver	Spleen	Ovary
Human melanoma	Fetal liver	Mammary gland	Brain	Thymus	Uterus
			Heart	Stomach	Prostate
			Kidney	Small intestine	Testis
C26/GPC3 tumor			Pancreas	Colon	C26 tumor

* Expression levels of GPC3 protein determined by immunohistochemical analysis: +++, very strong; ++, strong; +, +/−, weak, −, no or very weak expression.

that this GPC3-8 peptide has the capacity to induce tumor-reactive CTLs and that peptide vaccination primed CTLs reactive to this peptide *in vivo*.

CTL Inoculation Reduced the Growth of C26/GPC3 Tumor in Mice. We determined if these GPC3-8 peptide-induced CTLs were effective against C26/GPC3 tumors inoculated s.c. into BALB/c mice. We separated $CD8^+$ T cells from these GPC3-8 peptide-induced CTLs or from nonspecific cells cultured with interleukin 2, without peptide and injected each of these $CD8^+$ T cells (1×10^7) into each three C26/GPC3 tumors with a diameter of 5 mm (24.2 ± 1.5 mm^2). After 7 days, all three tumors treated with GPC3-8–specific $CD8^+$ CTLs became smaller (15.0 ± 3.2 mm^2), whereas three tumors treated with nonspecific $CD8^+$ T cells became larger (92.3 ± 9.6 mm^2).

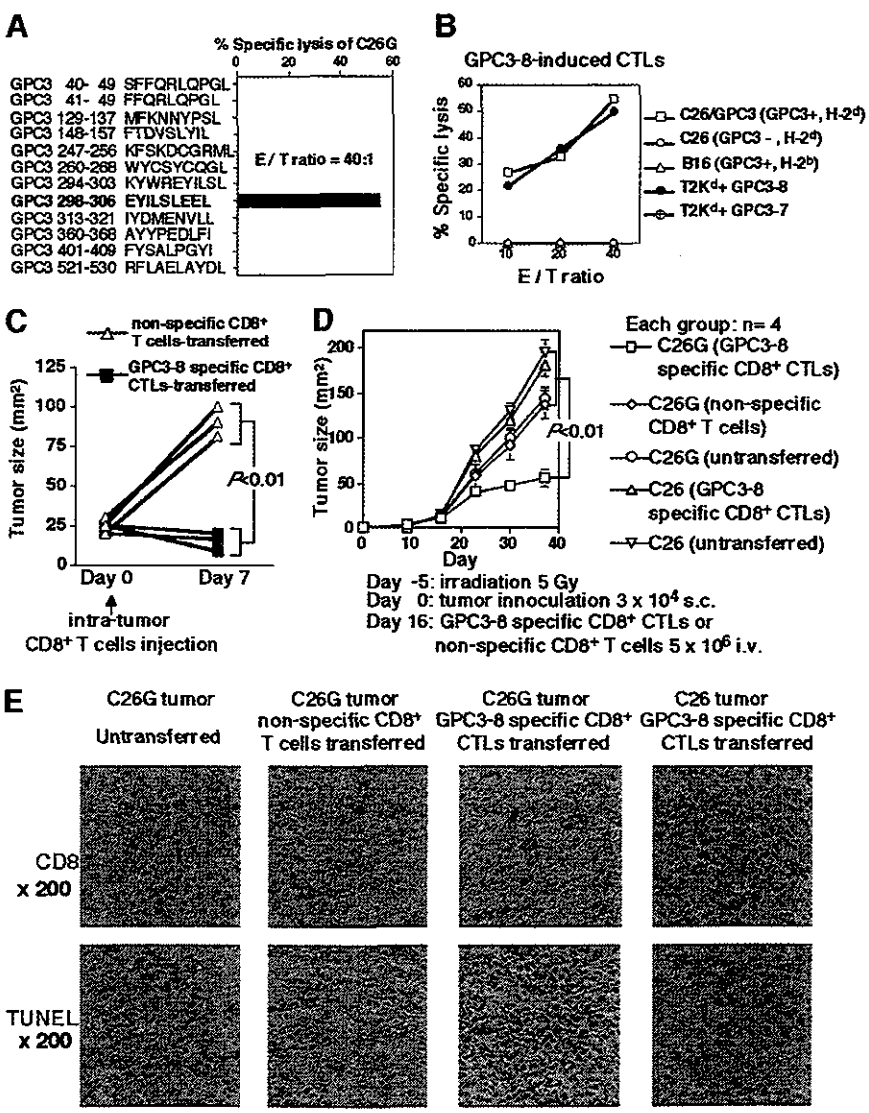

Fig. 2 Identification of a GPC3-derived and K^d-restricted CTL epitope, GPC3-8 EYILSLEEL, and adoptive CTL transfer therapy in BALB/c mice. *A*. BALB/c mice were immunized with 12 GPC3 peptides. Sensitized spleen cells, stimulated *in vitro* with each GPC3 peptide (10 μmol/L) and cultured for 5 days with 100 units/mL interleukin 2, were examined for CTL activity against GPC3-expressing C26/GPC3 cells to identify GPC3-8 EYILSLEEL epitopic peptide. Values represent percent specific lysis calculated based on mean values of triplicate assays. *B*, cytotoxicity of GPC3-8–induced CTLs against various target cells. *C*. $CD8^+$ CTLs (1×10^7) isolated from GPC3-8–induced cells generated as described in *A* or nonspecific cells cultured with interleukin 2, without peptide, were injected into the C26/GPC3 tumor with a diameter of 5 mm in each three mice. The comparison of the tumor size (mm^2) of each three GPC3-8–specific $CD8^+$ CTL-treated tumor and nonspecific $CD8^+$ T-cell–treated tumor was indicated. *D*, suppression of the growth of GPC3-expressing C26/GPC3 tumor inoculated s.c. into sublethally irradiated (5 Gy) mice adoptively transferred with GPC3-8–specific $CD8^+$ CTLs or nonspecific $CD8^+$ T cells. Data are representative of two independent and reproducible experiments. Tumor area was calculated as a product of width and length. Data are presented as mean area of tumor ± SE, and we evaluated the statistical significance with unpaired *t* test. *E*. Immunohistochemical analysis of CD8 or terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells in specimens of C26/GPC3 or C26 tumor on 21 days after adoptive CTL transfer as done in *D*.

There was a statistical significance ($P < 0.01$) in difference of tumor growth between these two groups (Fig. 2*C*). The results indicate that the GPC3-8 peptide-specific $CD8^+$ CTLs reduced the growth of tumors expressing GPC3.

Sublethal Irradiation of Mice Elicited Effective Antitumor-adoptive Immunity. Antitumor responses could be augmented by T-cell homeostatic proliferation in the periphery, involving expansion of T cells recognizing MHC/tumor antigenic peptide ligands (32–34). To investigate tumor growth in a homeostatic CTL proliferation model, we inoculated C26/GPC3 or C26 cells (3×10^4) s.c. into BALB/c mice 5 days after sublethal irradiation (5 Gy). We injected i.v. 5×10^6 of GPC3-8–induced $CD8^+$ CTLs or nonspecific $CD8^+$ T cells derived from spleen cells cultured with interleukin 2, without peptide for 5 days on day 16 after tumor inoculation, when C26/GPC3 or C26 tumors grew to a diameter of 3 to 4 mm (11.9 ± 0.8 mm^2). Mice were placed into five groups: (*a*) C26/GPC3 (GPC3-8–induced $CD8^+$ CTLs); (*b*) C26/GPC3 (nonspecific $CD8^+$ T cells); (*c*) C26/GPC3 (untransferred); (*d*) C26 (GPC3-8–induced $CD8^+$ CTLs); and (*e*) C26 (untransferred). Measurement of tumor size was continued for 37 days after inoculation of the tumor cells when one untreated mouse died (Fig. 2*D*). Each group included four mice, and we obtained reproducible results in two separate experiments. Mean tumor size on day 37 in C26/GPC3 (CTL) group (51.0 ± 6.0 mm^2) was significantly smaller than that in the other four groups (137.2 ± 16.1, 145.3 ± 12.1, 176.2 ± 10.1, and 195.1 ± 10.2 mm^2; $P < 0.01$). Weight of spleen (0.23 ± 0.03 or 0.25 ± 0.05 g) and spleen cell number ($1.20 \pm 0.40 \times 10^8$ or $1.25 \pm 0.25 \times 10^8$) of GPC3-8–induced $CD8^+$ CTLs or nonspecific $CD8^+$ T-cell–transferred groups, C26/GPC3 (GPC3-8–*induced* $CD8^+$ CTLs) and C26/GPC3 (nonspecific $CD8^+$ T cells), were larger than those (0.12 ± 0.03 g, $0.23 \pm 0.03 \times 10^8$) of untransferred mice, C26/GPC3 (untransferred) on day 37. These differences were statistically significant ($P < 0.01$), indicating that homeostatic proliferation of T cells had occurred. GPC3-8–induced $CD8^+$ CTLs, but not nonspecific $CD8^+$ T cells, could infiltrate the C26/GPC3 tumor, but not the C26 tumor, and induced apoptosis of C26/GPC3 tumor cells (Fig. 2*E*). Thus, sublethally irradiated lymphopenic mice transfused with syngeneic GPC3-8–reactive CTLs showed tumor growth inhibition for established C26/GPC3 tumors.

Vaccination of GPC3-8 Peptide-pulsed BM-DCs Induced Complete Rejection of C26/GPC3 Tumor Challenge in Mice. The capacity of GPC3-8 peptide-pulsed BM-DCs to prime GPC3-8–specific T cells *in vivo* was analyzed with a s.c. tumor injection model (Fig. 3*B–G*) and an intrasplenic tumor injection model (Fig. 4, *A* and *B*). The protocol of DC vaccination in this study is shown (Fig. 3*A*). In the s.c. tumor injection model, mice were placed into five groups: (*a*) C26/GPC3 (BM-DC+GPC3-8); (*b*) C26/GPC3 (BM-DC); (*c*) C26/GPC3 (untreated); (*d*) C26 (BM-DC+GPC3-8); and (*e*) C26 (untreated). GPC3-8 peptide-pulsed or unpulsed BM-DCs (5×10^5) were injected i.p. into BALB/c mice twice at 7-day intervals. Death never occurred during the vaccination period. Subcutaneous inoculation of C26/GPC3 or C26 cells (3×10^4) into the right flank was given 7 days after the last vaccination. In groups 2 to 5, s.c. tumor appeared 13 days after the inoculation (Fig. 3*B*). Measurement of tumor size was continued until 38 days after inoculation of the tumor cells when one untreated mouse died. All five mice in group 1 completely rejected 3×10^4 of C26/GPC3 cells but not 3×10^4 of C26 cells. Mean tumor size on day 38 in group 1 mice (0 mm^2) was significantly smaller than that in the other four groups 2 to 5 (234.0 ± 28.4, 251.0 ± 60.0, 170.3 ± 26.1, and 229.0 ± 64.2 mm^2, respectively, $P < 0.01$). All mice in groups 2 to 5 died within 88 days after inoculation of the tumor cells (Fig. 3*C*). In group 1, a tumor was not detected in all five mice 150 days after the inoculation. A statistical significance ($P < 0.01$) of difference was found between group 1 and groups 2 to 5. This experiment was repeated with similar results. However, the transfer of GPC3-8 peptide-pulsed BM-DCs showed no efficacy against the established C26G tumor (data not shown). Therefore, the GPC3-8 peptide-pulsed BM-DC therapy has the potential to prevent growth of tumors expressing GPC3 but could not induce regression of an established tumor.

We also inoculated C26/GPC3 or C26 cells s.c. into three surviving mice that completely rejected the first challenges of C26/GPC3 cells by vaccination with BM-DC+GPC3-8 (Fig. 3, *D* and *E*). These mice also rejected rechallenges of C26/GPC3 cells but not C26 until >150 days after the first challenge. In mean tumor size on day 39 and overall survival, the differences between the C26/GPC3-rechallenged group and the other three groups were statistically significant ($P < 0.01$). These results showed that effects of vaccinations with GPC3-8 peptide-pulsed BM-DCs continued for a long time and that the vaccination can prevent recurrence of GPC3-expressing tumors.

Furthermore, we repeated experiment with another control, BM-DC+GPC3-7 (Fig. 3, *F* and *G*). Binding affinity to K^d of GPC3-8 and that of GPC3-7 is predicted to be similar with Bioinformatics & Molecular Analysis Section.[5] As a result, we obtained similar data with experiments with BM-DCs not pulsed with any peptide.

We next analyzed the effect of the vaccination on an intrasplenic tumor injection model (Fig. 4, *A* and *B*). In this model, mice were placed into two groups: (*a*) C26/GPC3 (BM-DC+GPC3-8) and (*b*) C26/GPC3 (untransferred). Each group included five mice, and the results were reproducible in two separate experiments. Seven days after the last vaccination, inoculation of C26/GPC3 cells (1×10^5) into the spleen was done after laparotomy. Eighteen days after the inoculation, we observed the spleens (Fig. 4*A*) and livers (Fig. 4*B*). Tumor nodules appeared in spleens of all five untreated mice, and multiple metastases appeared in two livers (40%) of such mice. On the contrary, all five vaccinated mice completely rejected 1×10^5 of C26/GPC3 cells inoculated into the spleen, and liver metastasis was nil. Differences in weights of spleen and liver and the rates and the numbers of appearance of tumor nodules in spleen were statistically significant among these two groups. Hence, GPC3-8 peptide-pulsed BM-DCs have the capacity to prevent growth in the spleen and possibly liver metastasis of GPC3-expressing tumors.

[5] Internet address: http://bimas.dcrt.nih.gov/molbio/hla_bind/.

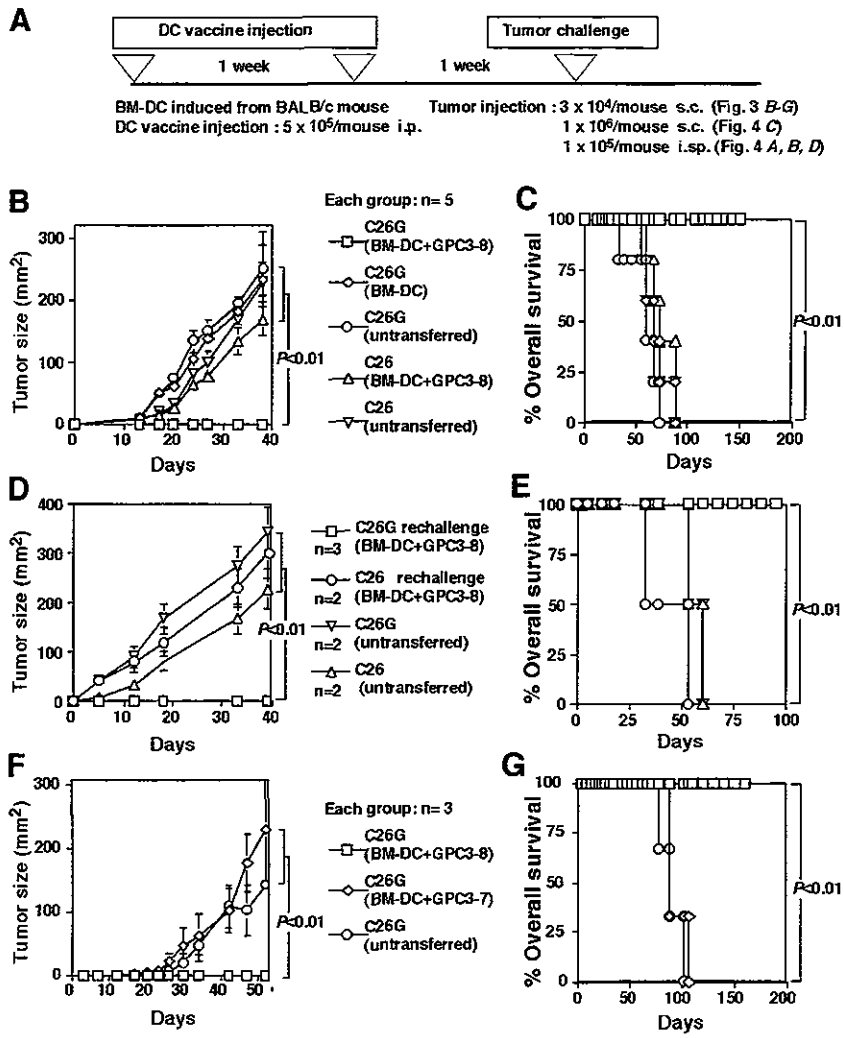

Fig. 3 Mice vaccinated with GPC3-8 peptide-pulsed BM-DCs completely rejected C26/GPC3 tumor challenge. *A*, protocol of peptide-pulsed BM-DC vaccination. *B–G*, s.c. tumor injection model at the first challenge (*B*, *C*, *F*, and *G*) and the rechallenge (*D* and *E*). *B*, *D*, and *F*, suppression of the growth of GPC3-expressing C26/GPC3 tumor inoculated s.c. in mice vaccinated with GPC3-8 peptide-pulsed BM-DCs. Data are representative of two independent and reproducible experiments. Tumor area was calculated as a product of width and length. Data are presented as mean area of tumor ± SE, and we evaluated the statistical significance using unpaired *t* test. *C*, *E*, and *G*. percentage of overall survival was calculated with the Kaplan-Meier method, and the statistical significance of differences between each groups was evaluated with the Wilcoxon test.

Vaccination of GPC3–8 Peptide-pulsed BM-DCs Induced Infiltration of CD8$^+$ T Cells into C26/GPC3 Tumor Cells and Apoptosis of Tumor Cells *In vivo*. In the s.c. tumor injection model, all mice immunized with the BM-DC+GPC3-8 vaccine completely rejected challenges of C26/GPC3 cells (3×10^4). To ascertain that these rejections were induced by CD8$^+$ CTLs, s.c. inoculation of C26/GPC3 or C26 cells (1×10^6) into the right flank was done 7 days after the last vaccination. After tumor formation, we removed the tumor and immunohistochemically stained it with anti-CD8 antibody and the terminal deoxynucleotidyl transferase-mediated nick end labeling method (Fig. 4*C*). Infiltrations of CD8$^+$ T cells into C26/GPC3 tumors and apoptosis of C26/GPC3 tumor cells were observed only in mice vaccinated with GPC3-8 peptide-pulsed BM-DCs but never in mice vaccinated with unpulsed BM-DCs. We also evaluated spleens immunohistochemically with the intrasplenic tumor injection model (Fig. 4*D*). Eighteen days after tumor inoculation, there were fewer CD8$^+$ T cells and terminal deoxynucleotidyl transferase-mediated nick end labeling-positive apoptotic tumor cells in C26/GPC3 tumor nodules in spleens of untreated mice. On the contrary, there were many CD8$^+$ T cells in the considerably enlarged white pulp and some terminal deoxynucleotidyl transferase-mediated nick end labeling-positive apoptotic tumor cells in spleens of mice immunized with the BM-DC+GPC3-8. These results suggest that GPC3-8 peptide-pulsed BM-DCs have the potential to prime a many GPC3-specific CTLs to kill C26/GPC3 tumor cells.

Involvement of CD8$^+$ T Cells in Protection against C26/GPC3 Induced by GPC3-8 Peptide-pulsed BM-DC Vaccination. To determine the role of CD4$^+$ and CD8$^+$ T cells in protection against tumor cells induced by GPC3-8 peptide-pulsed BM-DC vaccination, we depleted mice of CD4$^+$ or CD8$^+$ T lymphocytes by treatment with anti-CD4 or anti-CD8 monoclonal antibody *in vivo*, respectively. With this treatment, >90% of CD4$^+$ and CD8$^+$ T cells were depleted (data not shown). During this procedure, mice were immunized with GPC3-8 peptide-pulsed BM-DCs and challenged with C26/GPC3 cells (each group: $n = 4$). Depletion of CD8$^+$ T cells totally abrogated the protective immunity induced by GPC3-8 peptide-pulsed BM-DCs but that of CD4$^+$ T cells did not do so (data not shown). These results suggest that CD8$^+$ T cells play

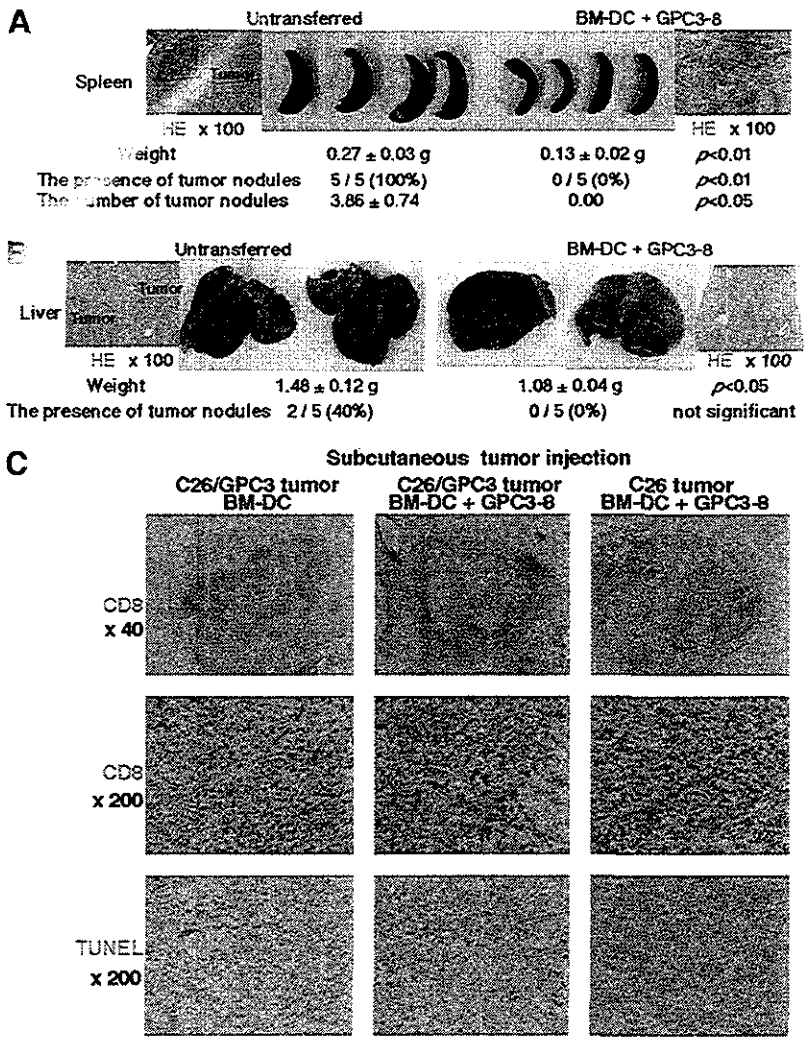

Fig. 4 Vaccination with GPC3-8 peptide-pulsed BM-DCs induced infiltration of $CD8^+$ T cells into C26/GPC3 tumor cells, but not into normal tissues, and induced the apoptosis of C26/GPC3 tumor cells. *A* and *B*, intrasplenic tumor injection model. Eighteen days after inoculation of C26/GPC3 cells (1×10^5) into spleens, the spleens (*A*) and livers (*B*) were observed macroscopically and histologically. *C*. Subcutaneous C26/GPC3 or C26 tumors were analyzed with immunohistochemical staining with anti-CD8 monoclonal antibody and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) methods 4 days after inoculation of 1×10^6 tumor cells. *D*. Spleens were analyzed with immunohistochemical staining with anti-CD8 monoclonal antibody and TUNEL method 18 days after inoculation of 1×10^5 of C26/GPC3 cells. *E*. The normal tissues of BM-DC+GPC3-8–vaccinated or CTL-treated mice were pathologically and immunohistochemically examined. Objective magnification was 200×. *F*. Placenta and mammary gland of BM-DC+GPC3-8–vaccinated female mice and fetal livers were immunohistochemically analyzed. Objective magnification was 200×.

critical roles in antitumor immunity induced by GPC3-8 peptide-pulsed BM-DCs.

No Evidence of Autoimmune Reactions in Surviving Mice that had Rejected Tumor Cell Challenges. GPC3 expression in normal adult mice was not evident in all tissues tested, which suggests a low risk of damage to normal tissue as a result of immune responses to the GPC3 protein. To evaluate the risk of autoaggression by immunization against GPC3-8, the tissues of BM-DC+GPC3-8 immunized or CTL-treated mice were pathologically examined. Mice shown in Fig. 2, *C* and *D*, were sacrificed at 7 and 21 days after $CD8^+$ T cells transfer, respectively. In addition, mice shown in Figs. 3 and 4*A–C* were sacrificed at >150, 25, and 14 days after the last BM-DC+GPC3-8 vaccination, respectively. All mice were apparently healthy without abnormality, suggesting autoimmunity, such as dermatitis, arthritis, or neurologic disorder. The brain, liver, lungs, and heart of these mice were critically scrutinized, and findings were compared with those in normal mice. These tissues had normal structures and cellularity in each of the two mice of groups examined, and pathological changes caused by immune response, such as lymphocyte infiltration or tissue destruction and repair, were nil (Fig. 4*E*). There were no $CD8^+$ T cells in these tissues, which had been immunohistochemically stained (Fig. 4*E*). Although $CD8^+$ T cells infiltrate in the C26/GPC3 tumor 21 days after $CD8^+$ T cells transfer (Fig. 2*E*) and at 14 and 25 days after the last BM-DC vaccination (Fig. 4, *C* and *D*), infiltration of $CD8^+$ T cells was not observed in all adult normal tissues examined at 7 and 21 days after $CD8^+$ T cells transfer (data not shown) and at >150, 25, and 14 days after the last BM-DC vaccination (Fig. 4*E*). These results indicate that lymphocytes stimulated with the GPC3 peptide did not recognize normal self-cells that could express GPC3 at physiologically low levels.

Vaccination of GPC3-8 Peptide-pulsed BM-DCs Induced GPC3-specific CTLs, but did not Induce Damage of Placenta and Fetal Liver Expressing GPC3. In murine tissues, GPC3 protein is expressed in placenta and fetal liver (Fig. 1*B*). To evaluate the risk of autoimmunity against placenta and fetal liver by immunization with BM-DC+GPC3-8, we carried out cross-breeding of BM-DC+GPC3-8–vaccinated female mice with normal male mice and compared events with normal mice pairs. To ascertain induction of GPC3-8–specific CTLs in

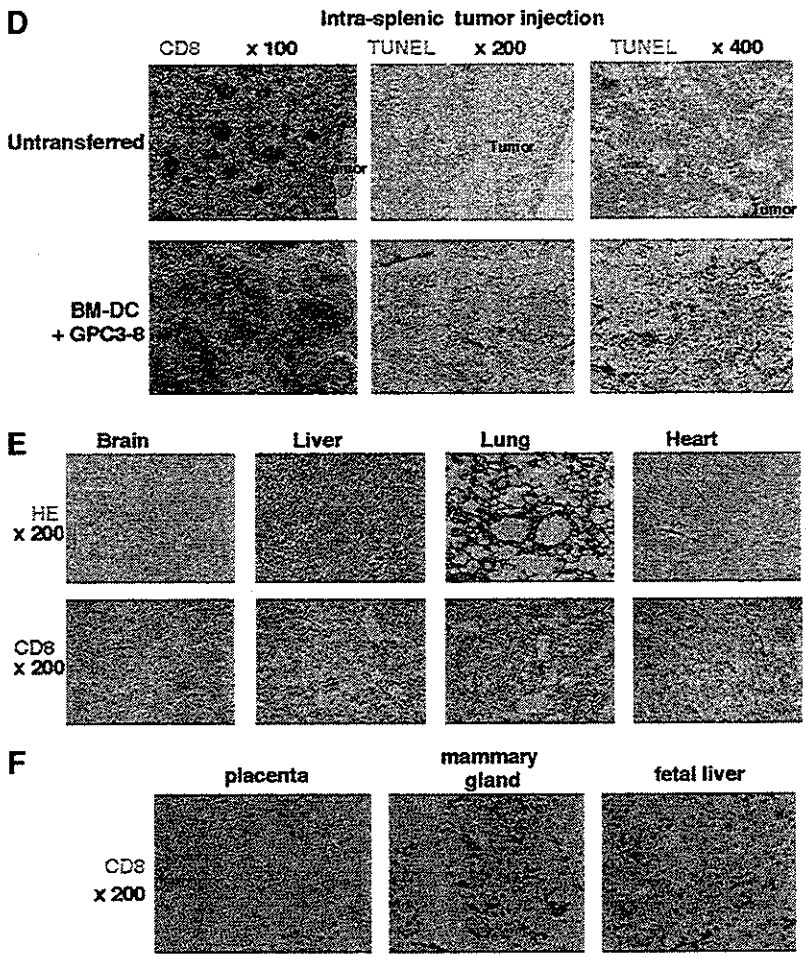

Fig. 4. Continued.

BM-DC+GPC3-8–vaccinated female mice not used for breeding, s.c. inoculation of C26/GPC3 or C26 cells (3×10^4) into the right flank of these female mice was done. All these vaccinated mice completely rejected 3×10^4 of C26/GPC3 cells 100 days after inoculation but not C26 cells (data not shown). Pregnancy and births in vaccinated female mice were normal. Six mice were born from each untreated and vaccinated three female mice, and all neonates were normal. Placenta and mammary gland of BM-DC+GPC3-8–vaccinated female mice and fetal livers had normal structures and cellularity with no pathological changes caused by immune responses, such as lymphocyte infiltration or tissue destruction and repair. There were no $CD8^+$ T cells in these tissues, which had been immunohistochemically stained (Fig. 4*F*). Furthermore, to ascertain induction of GPC3-8–specific CTLs in vaccinated female mice that gave birth, s.c. inoculation of C26/GPC3 or C26 cells (1×10^6) into the right flank of these mice was done. After tumor formation, we removed the tumor and immunohistochemically stained it with anti-CD8 antibody. Infiltration of $CD8^+$ T cells into C26/GPC3 tumors but not into C26 tumors, as observed in Fig. 4*C*, was evidenced (data not shown). These results indicate that in this vaccination model GPC3-8–specific $CD8^+$ CTLs do not attack placenta and fetal livers expressing GPC3.

DISCUSSION

In 1996, Pilia *et al.* (35) reported that *GPC3* is mutated in patients with Simpson-Golabi-Behmel syndrome. This syndrome is an X-linked disorder characterized by pre- and postnatal overgrowth (36). *GPC3*-deficient mice have several abnormalities found in Simpson-Golabi-Behmel syndrome patients, including overgrowth and cystic and dysplastic kidneys (37). Some reports indicated that GPC3 expression is downregulated in tumors of different origin. They showed that, although GPC3 is expressed in the normal ovary, mammary gland, and mesothelial cells, the expressions are undetectable in a significant proportion of ovarian, breast cancer, and mesothelioma cell lines (38). In all cases where GPC3 expression was lost, the *GPC3* promoter was hypermethylated, and mutations were nil in the coding region. Collectively, these data suggest that GPC3 can act as a negative regulator of growth in these cancers.

On the contrary, in the case of hepatocellular carcinoma, tumors originating from tissues that express GPC3 only in the embryo, GPC3 expression tends to reappear with malignant transformation. Whether reexpression of GPC3 plays a role in progression of these tumors is unknown. Why is GPC3 up-

regulated only in hepatocellular carcinoma and melanoma? We are investigating to determine whether GPC3 is involved in the oncogenesis of melanoma and hepatocellular carcinoma.

The genetic approach of T-cell epitope cloning method (2–6) and SEREX method (7–11) led to identification of a many genes encoding for tumor antigens and antigenic peptides recognized by tumor-reactive CTLs, thereby enhancing the possibility of antigen-specific cancer immunotherapy (2–8). MAGE (2) and NY-ESO-1 (8) represent cancer-testis antigen, and MART-1 (39), gp100 (40, 41), and tyrosinase (42) represent melanocyte-differentiation antigen. Cancer-testis antigens, expressed only in tumor cells and not in normal adult tissues, except for immune privilege tissues, including testis, ovary, and placenta, are ideal targets for tumor immunotherapy. One can prevent development of autoimmune diseases by vaccination with cancer-testis antigens, and many cancer-testis antigens are expressed in a variety of cancers (43). However, the rates of expression of cancer-testis antigens in cancers are at most 50% (43), and expression often shows heterogeneity within the same tumor (43, 44).

On the contrary, melanocyte-differentiation antigens are expressed homogeneously in almost all melanoma cells, so one can use these antigens for immunotherapy of melanoma patients. However, autoimmunity, such as vitiligo and uveitis, developed after vaccination with these antigens because these antigens are expressed in normal melanocytes (33). cDNA microarray technology is rapidly progressing (12–16). We have recently succeeded in identifying a novel cancer rejection antigen specifically expressed in esophageal cancer with cDNA microarray technology (17). In the present study, we found that GPC3 is highly immunogenic to stimulate eradication by T cells of tumor expressing GPC3 in mice. GPC3 is an ideal candidate antigen useful for immunotherapy of hepatocellular carcinoma, and vaccination against GPC3 is not expected to induce autoimmune diseases because it has unique tissue specificity regarding protein expression. Our study is the first to show that cDNA microarray technology is useful for identifying ideal cancer antigens.

The *HLA-A24* is the most common *HLA class I* allele in the Japanese population, and 60% of Japanese (95% of whom are genotypically *A*2402*), 20% of Caucasians, and 12% of Africans are positive for *HLA-A24* (45, 46). It is important for especially Japanese to identify HLA-A24–restricted CTL epitope peptides. Structural motifs of peptides bound to human HLA-A24 and BALB/c mouse K^d are similar (47–49), and the amino acid sequences of human and mouse GPC3 have a 95% homology. We searched for GPC3-derived peptides with a common sequence in both and selected peptides carrying binding motifs to both HLA-A24 and K^d molecules, as previously described, and we prepared 12 different synthetic peptides GPC3-1~12. GPC3-derived and K^d- restricted CTL epitope identified in BALB/c mice will be applicable to human HLA-A24–restricted CTLs. Therefore, we selected BALB/c mice in this study. Although we wanted to use hepatocellular carcinoma or melanoma cell lines, these cell lines derived from BALB/c mouse were not available. Furthermore, we found no GPC3-expressing murine tumor cell lines originating from BALB/c mice, and we had already established a system for analysis of mouse tumor immunity *in vivo* with Colon26. Anyway, in this study, with Colon26/GPC3, we could prove that GPC3 could be a cancer rejection antigen in mice. We recently found out that mouse melanoma cell line B16 expressed GPC3, so we are planning to investigate GPC3-mediated antitumor immunity with B16 in C57BL/6 mouse.

We observed the effects of adoptive transfer of highly selected tumor-reactive T cells directed against GPC3-8 peptide, even against established tumors, without causing autoimmune-related destruction. These results show the possibility of therapy of adoptive transfer of GPC3-specific CTLs for both hepatocellular carcinoma and melanoma. We also showed that GPC3-8 peptide-pulsed BM-DCs can prime GPC3-8–specific T cells *in vivo*, and growth of C26 expressing GPC3 was prevented without inducing autoimmune destruction in both s.c. and intrasplenic tumor injection models. The transfer of GPC3-8 peptide-pulsed BM-DCs showed no efficacy against the established C26G tumor (data not shown). On the contrary, vaccination of mice with GPC3-8 peptide emulsified in complete Freund's adjuvant could prime GPC3-8–specific T cells *in vivo*, but could not reject the challenge of C26/GPC3 (data not shown). These results show that GPC3-8 peptide-pulsed BM-DC therapy prevented the appearance or recurrence of tumors, yet this procedure did not induce regression of an established tumor. We suggest that adoptive transfer of GPC3-specific CTLs is a novel treatment strategy for patients with hepatocellular carcinoma or melanoma, and we also suggest that GPC3-derived peptide-pulsed DC vaccination is a novel strategy for prevention of hepatocellular carcinoma or melanoma in patients treated surgically, in patients with liver cirrhosis and chronic hepatitis who are at high risk for development of hepatocellular carcinoma, and in humans at high risk for development of melanoma. Whether GPC3 is an ideal target for immunotherapy in human hepatocellular carcinoma and melanoma, naturally expressing GPC3, will continue to be investigated in our laboratory.

ACKNOWLEDGMENTS

We thank Drs. Mikio Monji, Hidetake Matsuyoshi, Sinya Hirata, Yutaka Motomura, Seiji Hosaka, Yoshiaki Ikuta, and Daiki Fukuma (Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University) for technical assistance, Dr. Paul M. Allen (Washington University School of Medicine), Dr. Kyoichi Shimomura (Fujisawa Pharmaceutical Co.), and the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer Tohoku University for providing cell lines. We also thank Mariko Ohara (Fukuoka) for helpful comments.

REFERENCES

1. Schafer DF, Sorrell MF. Hepatocellular carcinoma. Lancet 1999;353: 1253–7.

2. 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 (Wash. DC) 1991;254:1643–7.

3. Rosenberg SA. Progress in human tumour immunology and immunotherapy. Nature (Lond.) 2001;411:380–4.

4. Morgan RA, Dudley ME, Yu YY, et al. High efficiency TCR gene transfer into primary human lymphocytes affords avid recognition of melanoma tumor antigen glycoprotein 100 and does not alter the recognition of autologous melanoma antigens. J Immunol 2003;171: 3287–95.

5. Maeda Y, Ito M, Harashima N, et al. Cleavage and polyadenylation specificity factor (CPSF)-derived peptides can induce HLA-A2–restricted and tumor-specific CTLs in the majority of gastrointestinal cancer patients. Int J Cancer 2002;99:409–17.

6. Azuma K, Shichijo S, Maeda Y, et al. Mutated p53 gene encodes a nonmutated epitope recognized by HLA-B*4601-restricted and tumor cell-reactive CTLs at tumor site. Cancer Res 2003;63:854–8.

7. Park S, Lim Y, Lee D, et al. Identification and characterization of a novel cancer/testis antigen gene CAGE-1. Biochim Biophys Acta 2003; 1625:173–82.

8. Jager 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 1998;187:265–70.

9. Nakatsura T, Senju S, Yamada K, Jotsuka T, Ogawa M, Nishimura Y. Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. Biochem Biophys Res Commun 2001;281:936–44.

10. Nakatsura T, Senju S, Ito M, Nishimura Y, 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 2002;32:826–36.

11. Monji M, Senju S, Nakatsura T, et al. Head and neck cancer antigens recognized by the humoral immune system. Biochem Biophys Res Commun 2002;294:734–41.

12. Hasegawa S, Furukawa Y, Li M, et al. Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23,040 genes. Cancer Res 2002;62: 7012–7.

13. Zembutsu H, Ohnishi Y, Tsunoda T, et al. Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. Cancer Res 2002;62:518–27.

14. Kitahara O, Furukawa Y, Tanaka T, et al. Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. Cancer Res 2001;61:3544–9.

15. Yu Y, Khan J, Khanna C, Helman L, Meltzer PS, Merlino G. Expression profiling identifies the cytoskeletal organizer ezrin and the developmental homeoprotein Six-1 as key metastatic regulators. Nat Med 2004;10:175–81.

16. Kitahara O, Katagiri T, Tsunoda T, Harima Y, Nakamura Y. Classification of sensitivity or resistance of cervical cancers to ionizing radiation according to expression profiles of 62 genes selected by cDNA microarray analysis. Neoplasia 2002;4:295–303.

17. Yoshitake Y, Nakatsura T, Monji M, et al. Proliferation potential-related protein, an ideal esophageal cancer antigen for immunotherapy, identified using cDNA microarray analysis. Clin Cancer Res 2004;10: 6437–48.

18. Okabe H, Satoh S, Kato T, et al. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 2001;61:2129–37.

19. Saito-Hisaminato A, Katagiri T, Kakiuchi S, Nakamura T, Tsunoda T, Nakamura Y. Genome-wide profiling of gene expression in 29 normal human tissues with a cDNA microarray. DNA Res 2002; 9:35–45.

20. 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;306:16–25.

21. Nakatsura T, Kageshita T, Ito S, et al. Identification of glypican-3 as a novel tumor marker for melanoma. Clin Cancer Res 2004;10: 6612–21.

22. Hattori K, Matsushita R, Kimura K, Abe Y, Nakashima E. Synergistic effect of indomethacin with Adriamycin and cisplatin on tumor growth. Biol Pharm Bull 2001;24:1214–7.

23. Kai M, Nakatsura T, Egami H, Senju S, Nishimura Y, Ogawa M. Heat shock protein 105 is overexpressed in a variety of human tumors. Oncol Rep 2003;10:1777–82.

24. Hirota T, Morisaki T, Nishiyama Y, et al. Zyxin, a regulator of actin filament assembly, targets the mitotic apparatus by interacting with h-warts/LATS1 tumor suppressor. J Cell Biol 2000;149:1073–86.

25. Matsuyoshi H, Senju S, Hirata S, Yoshitake Y, Uemura Y, Nishimura Y. Enhanced priming of antigen-specific CTLs in vivo by embryonic stem cell-derived dendritic cells expressing chemokine along with antigenic protein: application to antitumor vaccination. J Immunol 2004;172:776–86.

26. Hsu HC, Cheng W, Lai PL. Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular carcinoma: biological significance and temporospatial distribution. Cancer Res 1997; 57:5179–84.

27. Zhu ZW, Friess H, Wang L, et al. Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. Gut 2001;48:558–64.

28. Sung YK, Hwang SY, Park MK, et al. Glypican-3 is overexpressed in human hepatocellular carcinoma. Cancer Sci 2003;94:259–62.

29. Midorikawa Y, Ishikawa S, Iwanari H, et al. Glypican-3, overexpressed in hepatocellular carcinoma, modulates FGF2 and BMP-7 signaling. Int J Cancer 2003;103:455–65.

30. Capurro M, Wanless IR, Sherman M, et al. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 2003;125:89–97.

31. 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;64:2418–23.

32. Dummer W, Niethammer AG, Baccala R, et al. T cell homeostatic proliferation elicits effective antitumor autoimmunity. J Clin Investig 2002;110:185–92.

33. Maine GN, Mule JJ. Making room for T cells. J Clin Investig 2002;110:157–9.

34. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science (Wash. DC) 2002;298:850–4.

35. Pilia G, Hughes-Benzie RM, MacKenzie A, et al. Mutations in GPC3, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome. Nat Genet 1996;12:241–7.

36. Neri G, Gurrieri F, Zanni G, Lin A. Clinical and molecular aspects of the Simpson-Golabi-Behmel syndrome. Am J Med Genet 1998;79: 279–83.

37. Cano-Gauci DF, Song HH, Yang H, et al. Glypican-3–deficient mice exhibit developmental overgrowth and some of the abnormalities typical of Simpson-Golabi-Behmel syndrome. J Cell Biol 1999;146: 255–64.

38. Lin H, Huber R, Schlessinger D, Morin PJ. Frequent silencing of the GPC3 gene in ovarian cancer cell lines. Cancer Res 1999;59:807–10.

39. Rivoltini L, Kawakami Y, Sakaguchi K, et al. Induction of tumor-reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by in vitro stimulation with an immunodominant peptide of the human melanoma antigen MART-1. J Immunol 1995; 154:2257–65.

40. Bakker AB, Schreurs MW, de Boer AJ, et al. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. J Exp Med 1994;179:1005–9.

41. Kawakami Y, Eliyahu S, Jennings C, et al. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. J Immunol 1995;154:3961–8.

42. Brichard V, Van Pel A, Wolfel T, et al. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J Exp Med 1993;178:489–95.

43. Jungbluth AA, Chen YT, Stockert E, et al. Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. Int J Cancer 2001;92:856–60.

44. Neumann E, Engelsberg A, Decker J, et al. Heterogeneous expression of the tumor-associated antigens RAGE-1, PRAME, and glycoprotein 75 in human renal cell carcinoma: candidates for T-cell–based immunotherapies? Cancer Res 1998;58:4090–5.

45. Date Y, Kimura A, Kato H, Sasazuki T. DNA typing of the HLA-A gene: population study and identification of four new alleles in Japanese. Tissue Antigens 1996;47:93–101.

46. Ohmori M, Yasunaga S, Maehara Y, Sugimachi K, Sasazuki T. DNA typing of HLA class I (HLA-A) and class II genes (HLA-DR, -DQ and -DP) in Japanese patients with gastric cancer. Tissue Antigens 1997;50:277–82.

47. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature (Lond.) 1991;351:290–6.

48. Maier R, Falk K, Rotzschke O, et al. Peptide motifs of HLA-A3, -A24, and -B7 molecules as determined by pool sequencing. Immunogenetics 1994;40:306–8.

49. Okugawa T, Ikuta Y, Takahashi Y, et al. A novel human HER2-derived peptide homologous to the mouse K(d)-restricted tumor rejection antigen can induce HLA-A24–restricted cytotoxic T lymphocytes in ovarian cancer patients and healthy individuals. Eur J Immunol 2000;30:3338–46.

Identification of Glypican-3 as a Novel Tumor Marker for Melanoma

Tetsuya Nakatsura,[1] Toshiro Kageshita,[2] Shosuke Ito,[3] Kazumasa Wakamatsu,[3] Mikio Monji,[1] Yoshiaki Ikuta,[1] Satoru Senju,[1] Tomomichi Ono,[2] and Yasuharu Nishimura[1]

Departments of [1]Immunogenetics and [2]Dermatology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto; and [3]Department of Chemistry, Fujita Health University School of Health Sciences, Toyoake, Aichi, Japan

ABSTRACT

Purpose: **We reported recently the novel tumor marker glypican-3 (GPC3) for hepatocellular carcinoma. In the present study, we investigated the expression of *GPC3* in human melanoma cell lines and tissues and asked whether GPC3 could be a novel tumor marker for melanoma.**

Experimental Design: **Expression of GPC3 mRNA and protein was investigated in human melanoma cell lines and tissues using reverse transcription-PCR and immunohistochemical analysis. Secreted GPC3 protein was quantified using ELISA in culture supernatants of melanoma cell lines and in sera from 91 patients with melanoma and 28 disease-free patients after surgical removal of primary melanoma. All of the subjects were Japanese nationals.**

Results: **In >80% of melanoma and melanocytic nevus, there was evident expression of *GPC3* mRNA and protein. Furthermore, GPC3 protein was evidenced in sera of 39.6% (36 of 91) of melanoma patients but not in sera from subjects with large congenital melanocytic nevus (0 of 5) and from healthy donors (0 of 60). Twenty-seven of 36 serum GPC3-positive patients were negative for both serum 5-S-cysteinyldopa and melanoma-inhibitory activity, well-known tumor markers for melanoma. The positive rate of serum GPC3 (39.6%) was significantly higher than that of 5-S-cysteinyldopa (26.7%) and of melanoma-inhibitory activity (20.9%). Surprisingly, we detected serum GPC3 even in patients with stage 0 *in situ* melanoma. The positive rate of serum GPC3 at stage 0, I, and II (44.4%, 40.0%, and 47.6%) was significantly higher than that of 5-S-cysteinyldopa (0.0%, 8.0%, and 10.0%). Also observed was the disappearance of GPC3 protein in sera from 11 patients after surgical removal of the melanoma.**

Conclusions: **GPC3 is apparently a novel tumor marker useful for the diagnosis of melanoma, especially in early stages of the disorder.**

INTRODUCTION

The incidence of melanoma is increasing worldwide. In the last decade, several molecules have been evaluated as tumor markers to detect melanoma, including melanin metabolites, adhesion molecules, cytokines, and melanoma-associated antigens [reviewed by Brochez and Naeyaert (1), Hauschild *et al.* (2), and Hartleb and Arndt (3)]. Several investigators reported that 5-S-cysteinyldopa is useful as a marker for melanoma progression or for monitoring metastatic melanoma (4–7). 5-S-cysteinyldopa is usually used as tumor marker for melanoma in Japan, and the usefulness of melanoma-inhibitory activity was reported (8, 9). However, current methods are not sensitive enough to detect organ metastasis at such early stages. There is no available tumor marker that can detect primary melanoma at early stages, with a small size and without metastasis. A simple, inexpensive, and noninvasive method to detect a serum tumor marker would aid the management of high-risk patients.

We recently identified glypican-3 (GPC3) overexpressed specifically in human hepatocellular carcinoma, as based on cDNA microarray data, and we reported that GPC3 is a novel tumor marker for hepatocellular carcinoma (10). Soluble GPC3 protein was detected in sera of hepatocellular carcinoma patients but not in case of other liver diseases or cancers other than melanoma. We also accidentally detected *GPC3*-expression in a B16 mouse melanoma cell line. This observation prompted us to examine the expression of GPC3 in human melanoma, and we asked whether GPC3 might be a novel tumor marker for melanoma. Indeed, GPC3 was detected in the sera of 40% patients with melanoma, irrespective of clinical stages and even in the sera of patients with stage 0 *in situ* melanoma.

MATERIALS AND METHODS

Melanoma and Melanocytic Nevus Tissues, Blood Samples, and Cell Lines. After receiving informed written consent, we obtained tissue and blood samples from melanoma and melanocytic nevus patients treated in the Department of Dermatology, Graduate School of Medical Sciences, Kumamoto University (Kumamoto, Japan). They were stored at −80°C until use. We collected patient profiles from medical records to determine the clinical stages, according to the Unio Internationale Contra Cancrum/American Joint Committee on Cancer Tumor-Node-Metastasis classification (11).

Received 2/24/04; revised 5/15/04; accepted 5/18/04.
Grant support: Grants-in-Aid 14770142, 12213111, and 14570812 from the Ministry of Education, Science, Technology, Sports, and Culture, Japan, and a Grant-in-Aid for Cancer Research (15–11) from the Ministry of Health, Labor, and Welfare, Japan.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Prof. Yasuharu Nishimura, Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan. Phone: 81-96-373-5310; Fax: 81-96-373-5314; E-mail: mxnishim@gpo.kumamoto-u.ac.jp.

Ninety-one consecutive and preoperative patients with melanoma consisted of 43 men and 48 women with an average age of 65.7 years (range, 22 to 89 years); 9 had stage 0 (*in situ*); 25 had stage I; 21 had stage II; 18 had stage III; and 18 had stage IV melanoma. Twenty-eight disease-free patients after removal of primary lesions consisted of 15 men and 13 women; One had stage 0; 8 had stage I; 14 had stage II; and 5 had stage III melanoma. All of the patients were Japanese nationals.

Melanoma cell lines CRL1579, G361, HMV-I, and SK-MEL-28 were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan), and 888mel and 526mel were provided by Dr. Yutaka Kawakami, Keio University (Tokyo, Japan). HMV-I, SK-MEL-28, Ihara, and MeWo were cultured in DMEM supplemented with 10% fetal calf serum, and CRL1579, G361, 888mel, 526mel, 164, SK-MEL-19, and Colo38 were cultured in RPMI 1640 supplemented with 10% fetal calf serum. Human epidermal melanocytes, neonatal, in culture medium 154S supplemented with human melanocyte growth supplement were purchased from KURABO (Osaka, Japan).

Reverse Transcription-PCR. Total RNA was isolated from homogenized tissues and cell lines using the TRIZOL Reagent (Life Technologies, Inc., Rockville, MD). Reverse transcription-PCR (RT-PCR) was done, as described (12). We designed *GPC3* gene-specific PCR primers to amplify fragments of 939 bp, and we used RT-PCR reactions consisting of initial denaturation at 94°C for 5 minutes, and 30 amplification cycles at an annealing temperature of 58°C. *GPC3* PCR primer sequences were: sense, 5′-GTTACTGCAATGTGGTCATGC-3′ and antisense, 5′-CTGGTGCCCAGCACATGT-3′; β-*actin*: sense, 5′-CCTCGCCTTTGCCGATCC-3′ and antisense, 5′-GGATCTTC-ATGAGGTAGTCAGTC-3′. After normalization by β-*actin* mRNA as a control we compared the expression of *GPC3* mRNA in tissues and cell lines.

Immunohistochemical Examination and ELISA. Immunohistochemical examinations were done, as described (13, 14). We stained sections with antihuman GPC3 303–464 antibodies (H-162; Santa Cruz Biotechnology, Santa Cruz, CA). For the negative control, staining replaced the primary antibody with an immunoglobulin fraction from preimmune rabbit serum. The percentage of stained cells in each section was estimated independently by two observers (T. K. and T. O.). ELISA of GPC3 was done as described (10). In ELISA method-1, we used the same anti-GPC3 antibody (H162) and its biotinylated one. To independently confirm the accuracy of this ELISA system for specific detection of GPC3, we used another antihuman GPC3 goat polyclonal antibody (W-18) raised against a NH_2-terminal peptide (Santa Cruz Biotechnology) and used this antibody for ELISA detection of serum GPC3. Sandwich ELISA method-2 was performed by using W-18 fixed on the solid surface and biotinylated H-162.

Statistical Analysis. We analyzed all of the data using the StatView statistical program for Macintosh (SaS, Cary, NC), then evaluated the statistical significance using Student's *t* test, χ^2, and Fisher's exact test. We considered $Ps < 0.05$ to be statistically significant.

RESULTS

Expression of *GPC3* mRNA in Human Melanoma. We examined expression of *GPC3* mRNA using RT-PCR. Expression of *GPC3* mRNA was evidenced in 8 of 11 human melanoma cell lines (Fig. 1*A*). 164, 888mel, Ihara, CRL1579, and MeWo melanoma cell lines showed stronger expression of *GPC3* mRNA than did 526mel, G361, and SK-MEL-28, whereas SK-MEL-19, Colo38, and HMV-I showed no such expression. Primary tumor of melanoma from patients 50, 65, 78, 71 (Fig. 1*B*), and 68 (data not shown), and lymph node metastasis of patient 65 (Fig. 1*B*) showed positive expression, whereas normal skin, including a few melanocytes, showed no such expression (Fig. 1*B*). On the contrary, cultured human neonatal epidermal melanocytes showed moderate expression of *GPC3* mRNA. Tissues of melanocytic nevus also showed positive expression (Fig. 1*B*). Hence, all of the tissues of melanoma and melanocytic nevus we checked showed positive *GPC3* mRNA expression.

Expression of GPC3 Protein in Human Melanoma Tissues. An immunohistochemical analysis of GPC3 was made on 21 primary melanomas and 11 melanocytic nevus tissues. The results of immunostaining are classified by the percentage of stained cells: +++, >75%; ++, 50% to 75%; +, 25% to 50%; ±, <25%; −, negative. The results in melanoma are summarized in Table 1, and representative staining is shown in Fig. 2. Many cases of primary melanoma lesions (17 of 21, 81.0%) showed expression of GPC3 protein in melanoma cells (+++, 6; ++, 6; +, 0; ±, 5; −, 4 cases; Table 1, Fig. 2, *A* and *B*). Ten of 11 melanocytic nevus lesions also showed positive expression (+++, 4; ++, 4; +, 1; ±, 1; −, 1 cases; Fig. 2*C*).

The Presence of Soluble GPC3 Protein in Culture Supernatants of Melanoma Cell Lines and Sera from Melanoma Patients. We next detected soluble GPC3 using ELISA method-1. The evidence that our ELISA system detected soluble GPC3 in culture supernatant of NIH3T3 transfected with mouse *GPC3* gene but not *in* that of wild-type NIH3T3 cells supports the accuracy of ELISA (data not shown). We defined the con-

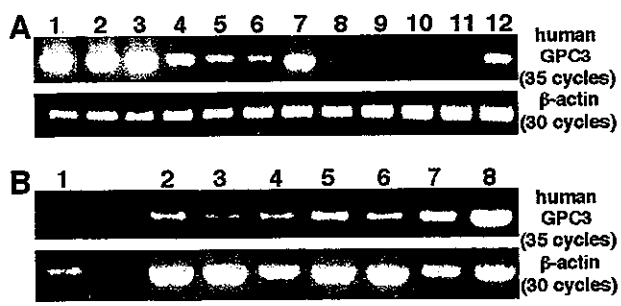

Fig. 1 Expression of *GPC3* mRNA in human melanoma, melanocyte, and melanocytic nevus. *A*, expression of *GPC3* mRNA detected using RT-PCR in human melanoma cell lines (*Lanes 1–11*) and neonatal epidermal melanocytes (HEMn; *Lane 12*). *Lanes 1*: 164, *2*: 888mel, *3*: Ihara, *4*: CRL1579, *5*: 526mel, *6*: G361, *7*: MeWo, *8*: SK-MEL-28, *9*: SK-MEL-19, *10*: Colo38, *11*: HMV-I, *12*: HEMn. *B*, expression of *GPC3* mRNA detected using RT-PCR in human tissues of normal epidermis (*Lane 1*), melanoma (*Lanes 2–6*), and melanocytic nevus (*Lanes 7* and *8*). Primary melanoma tissues originated from patient 50: *Lane 2*, patient 65: *Lane 3*, patient 78: *Lane 5*, patient 71: *Lane 6*, and tissue of metastasis to lymph node of patient 65 is shown in *Lane 4*.

Table 1 Profiles of 91 Japanese patients with melanoma, and quantification of GPC3, 5-S-CD, and MIA in sera of patients

							Serum concentrations of tumor markers			
					GPC3 expression		GPC3 (U/ml)			
Pt. ID	Stage*	Age	Sex	Type	mRNA†	Protein‡	Method 1§	Method 2¶	5-S-CD (nmol/L)‖	MIA (ng/ml)**
1	0	60	M	SSM			0	0	4.6	12.3
2	0	64	F	LM			0	0	2.8	9.8
3	0	78	F	ALM			0	0	2.9	14.1
4	0	74	M	LM			0	0	6.2	19.4
5	0	85	F	ALM		+++	25	24	3.2	9.5
6	0	72	M	LM			0	0	3.8	14.5
7	0	48	F	ALM			38	41	3.3	8.4
8	0	69	F	ALM			22	26	2.6	10.0
9	0	66	F	ALM			8	4	4.7	12.2
10	IA	33	M	MUCOUS		±	103	104	1.9	7.8
11	IA	82	F	LMM			40	41	5.5	17.2
12	IA	75	F	SSM		±	25	19	3.9	16.5
13	IA	41	F	SSM		+++	20	13	2.3	6.8
14	IA	70	F	MUCOUS			0	0	2.4	12.1
15	IA	78	M	ALM			0	0	11.0	17.8
16	IA	60	F	ALM		±	0	0	3.6	16.4
17	IA	61	M	ALM			0	0	1.0	10.1
18	IA	62	F	ALM			0	0	5.9	10.6
19	IA	73	M	ALM			0	0	3.6	10.8
20	IA	70	M	LMM			20	21	8.0	16.2
21	IA	33	F	MUCOUS			10	15	3.6	8.2
22	IA	66	M	ALM			0	0	5.9	13.7
23	IA	76	M	ALM			0	0	5.8	18.5
24	IA	58	F	ALM			0	0	4.4	10.8
25	IA	89	M	LMM			0	0	47.6	17.4
26	IB	58	F	ALM			61	98	2.5	8.9
27	IB	58	F	MUCOUS		−	0	0	8.6	13.4
28	IB	66	F	MUCOUS			23	44	2.9	13.6
29	IB	56	F	SSM			10	11	2.3	13.8
30	IB	64	F	ALM			20	8	7.5	15.5
31	IB	84	F	ALM			0	0	2.0	8.9
32	IB	79	M	ALM		+++	0	0	7.3	18.7
33	IB	76	F	ALM			0	0	5.7	16.3
34	IB	74	F	ALM		++	0	0	2.8	14.7
35	IIA	74	F	SSM			106	108	17.4	10.5
36	IIA	75	M	ALM			54	61	NT††	13.5
37	IIA	74	F	ALM			16	8	3.4	12.5
38	IIA	64	M	ALM		±	0	0	4.6	10.7
39	IIA	47	F	SSM			0	0	4.3	14.0
40	IIA	77	F	LMM			34	39	3.9	9.4
41	IIB	50	F	SSM			75	59	6.4	15.3
42	IIB	72	M	LMM			73	66	7.0	13.1
43	IIB	88	M	ALM		++	0	0	1.2	12.8
44	IIB	63	M	ALM		−	0	0	3.7	11.2
45	IIB	77	M	SSM			0	0	NT	11.8
46	IIB	69	M	ALM			15	16	4.6	9.9
47	IIB	57	M	ALM			0	0	3.4	17.1
48	IIB	69	F	ALM			0	0	3.3	14.7
49	IIB	71	M	ALM		++	0	0	4.7	7.3
50	IIC	79	F	ALM	+		25	30	3.8	6.2
51	IIC	42	M	SSM		−	0	0	6.3	7.9
52	IIC	72	F	ALM			0	0	3.6	12.2
53	IIC	75	F	MUCOUS			0	0	8.7	13.0
54	IIC	77	M	ALM			16	19	7.3	10.9
55	IIC	83	M	SSM			10	9	13.3	11.3
56	IIIA	83	M	ALM		++	0	0	7.7	19.5
57	IIIA	55	M	ALM			0	0	8.2	10.2
58	IIIA	86	F	ALM		++	0	0	9.7	12.5
59	IIIA	79	F	ALM		+++	0	0	6.1	15.9
60	IIIA	70	M	ALM			0	0	4.0	14.8
61	IIIA	63	F	SSM			10	7	11.8	12.1
62	IIIA	79	M	NM			10	10	4.0	14.9
63	IIIA	53	F	MUCOUS			0	0	5.2	13.7
64	IIIB	85	M	ALM			140	126	9.2	24.4
65	IIIB	56	M	LMM	+		0	0	15.5	14.9

Table 1 Continued

Pt. ID	Stage*	Age	Sex	Type	GPC3 expression		Serum concentrations of tumor markers			
							GPC3 (U/ml)			
					mRNA†	Protein‡	Method 1§	Method 2¶	5-S-CD (nmol/L)‖	MIA (ng/ml)**
66	IIIB	59	M	MUCOUS			0	0	1.2	7.0
67	IIIB	77	M	ALM			67	85	7.1	12.1
68	IIIC	35	F	NM	+		132	130	8.4	6.3
69	IIIC	63	F	ALM		±	18	8	4.9	14.0
70	IIIC	50	F	unknown			0	0	5.9	28.6
71	IIIC	47	M	MUCOUS	+		0	0	10.3	5.9
72	IIIC	70	M	ALM		−	22	20	24.2	11.4
73	IIIC	63	M	ALM			0	0	14.4	15.1
74	IV	47	F	SSM		++	35	37	12.7	86.0
75	IV	77	M	ALM			0	0	748	102
76	IV	65	M	unknown			0	0	492	23.1
77	IV	78	M	MUCOUS			0	0	44.6	14.6
78	IV	60	F	SSM	+		0	0	32.4	26.8
79	IV	76	F	MUCOUS			0	0	1.1	9.0
80	IV	72	F	SSM			0	0	981	438
81	IV	73	F	SSM			0	0	56.1	7.7
82	IV	45	F	unknown			10	5	5.5	11.6
83	IV	60	F	MUCOUS			8	13	8.6	11.2
84	IV	72	M	NM			0	0	225	412
85	IV	50	M	SSM			0	0	957	438
86	IV	47	F	NM		+++	0	0	257	419
87	IV	22	M	unknown			57	60	25.8	10.8
88	IV	39	M	NM			0	0	170	15.8
89	IV	74	F	ALM			0	0	395	22.9
90	IV	68	M	unknown			34	73	74.2	11.9
91	IV	66	F	ALM		+++	0	0	246	9.2

Abbreviations: ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma; UICC, Unio Internationale Contra Cancrum; AJCC, American Joint Committee on Cancer; TNM, Tumor-Node-Metastasis; MIA, melanoma-inhibitory activity.

* Clinical stages, according to the UICC/AJCC TNM classification (11).

† The expression of *GPC3* mRNA detected by RT-PCR as shown in Fig. 1*B*.

‡ The expression of GPC3 protein detected by immunohistochemical analysis as shown in Fig. 2. The results of immunostaining are symbolized by the positive rate of all stained melanoma cells: +++, >75%; ++, 50% to 75%; +, 25% to 50%; ±, <25%; −, negative.

§ Soluble GPC3 protein in the sera was quantified by ELISA method 1 using polyclonal anti-GPC3 antibody (H-162). We could obtain reproducible results by using three different batches of antibodies H-162 and representative results were shown. We arbitrarily fixed the cut-off value at 1 unit/mL, and positive values are underlined.

¶ Soluble GPC3 protein in the sera was quantified by sandwich ELISA method 2 using goat polyclonal anti-GPC3 antibody (W-18) raised against a NH_2-terminal peptide of GPC3 and biotinilated H-162. We arbitrarily fixed the cut-off value at 1 unit/mL and positive values are underlined.

‖ 5-S-CD was quantified using high performance liquid chromatography by us. The cut-off value was fixed at 10 nmol/L (6), and positive values are underlined.

** MIA in the sera was detected by ELISA. We arbitary fixed the cut-off value at 17 ng/mL in this study, and positive values are underlined.

†† Not tested.

centration of GPC3 protein in the 1 mL of the culture supernatant of 1×10^5 HepG2 cells after cultivation for 24 hours as 1 units/mL. Soluble GPC3 protein could be detected in culture supernatants of 5 of 11 melanoma cell lines (Fig. 3*A*). The amount of GPC3 protein in the culture supernatants of the 164 was larger than that of the SK-MEL-28, 526mel, G361, and CRL1579. On the other hand, GPC3 was not detected in the 888mel, Ihara, and MeWo (Fig. 3*A*), despite the strong expression of *GPC3* mRNA (Fig. 1*A*). Thus, there was some discrepancy between *GPC3* mRNA expression in melanoma cell lines and the amount of GPC3 protein secreted from these cells into culture supernatants. For example, we did not detect soluble GPC3 protein in the culture supernatant of human epidermal melanocytes, despite the expression of mRNA (Fig. 1*A*).

The quantification by ELISA method-1 of GPC3 protein in sera of 91 preoperative patients with melanoma, 5 patients with large congenital melanocytic nevus, and of 60 healthy donors who have many small melanocytic nevus is indicated in Fig. 3*B* and Table 1. We detected and quantified GPC3 protein in the sera of 36 of 91 melanoma patients (39.6%) but, more importantly, not in sera of patients with large congenital melanocytic nevus and healthy donors, whereas *GPC3* mRNA and protein were expressed in melanocytic nevus tissues. We could obtain reproducible results by using three different batches of polyclonal anti-GPC3 antibody (H-162) indicating that ELISA detection of soluble GPC3 was not dependent on a particular batch of H-162. We arbitrary fixed the cutoff level at 1 units/mL, because all of the healthy donors were completely negative for serum GPC3, and there was no gray area between GPC3-positive and negative patients.

Furthermore, to confirm these results, we performed sandwich ELISA method 2 by using another antihuman GPC3 goat

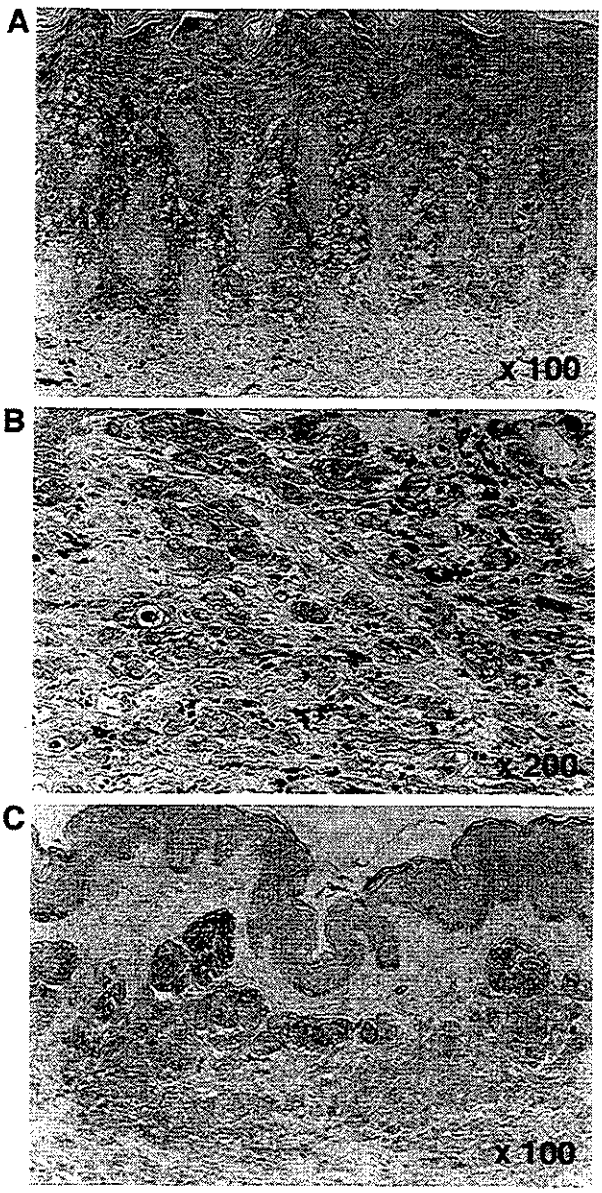

Fig. 2 Expression pattern of GPC3 protein in primary melanoma and melanocytic nevus lesions examined by immunohistochemical staining. *A*, primary melanoma of patient 13; GPC3 immunostaining colored brown was evident in the melanoma cells. *B*, primary melanoma of patient 69; Observation under higher magnification revealed that GPC3 immunoreactivity in melanoma cells was localized mainly in the cytoplasm. *C*, melanocytic nevus (Fig. 1*B*, *Lane 8*) with expression of GPC3. Objective magnifications; *A* and *C*: ×100, *B*: ×200.

polyclonal antibody (W-18) and biotinylated anti-GPC3 303–464 polyclonal antibody (H-162; Fig. 3*C*; Table 1). The results obtained by using these two antibodies were similar ($R^2 = 0.89$) to those obtained by using H-162 alone (method 1), indicating that the detection of serum-soluble GPC3 was not solely dependent on the particular polyclonal antibody H-162. Thus, there was no discrepancy in identification of serum GPC3-positive patients between methods 1 and 2.

The prevalence of GPC3 protein in the sera of melanoma patients was significantly higher than that in other donors ($P < 0.0001$). Although Fig. 1*B* shows that melanoma tumor from patients 50, 65, 78, and 71 expressed *GPC3* mRNA, GPC3 protein was detected only in the serum of patient 50 among these 4 patients. There was no correlation between concentrations of serum GPC3 and its mRNA expressions in the melanoma tissues. There was also no correlation between concentrations of serum secreted GPC3 and levels of GPC3 protein expressed in the tissues.

Among the 21 cases in which immunohistochemical staining of melanoma tissue was done, serum GPC3 was detected in 7 (33.3%) but not in 14 (66.7%; Table 1). In 6 of the 7 (patients 5, 10, 12, 13, 69, and 74), GPC3 protein expression was detected both in the sera and in their melanoma cells, but in the remaining 1 case (patient 72), GPC3 protein expression was detected only in the sera not in melanoma cells. It was thought that almost all of the GPC3 protein produced in melanoma cells of this patient 72 was secreted. On the contrary, in 11 of the 14 (78.6%), serum GPC3 was not detected, despite GPC3 protein expression in their melanoma cells. Only 3 of the 21 cases (14.3%) did not show expression of GPC3 protein in both in melanoma cells and the sera. These results showed that most of melanoma tissues (85.7%) expressed GPC3 protein, and in ~50% of those patients, GPC3 protein was secreted and detected in their sera. On the contrary, although GPC3 was evidenced in most of melanocytic nevus and neonatal epidermal melanocytes, GPC3 protein was not secreted from all of the melanocytic nevus tested and adult epidermal melanocytes.

Comparison of Serum Concentrations of GPC3, 5-S-Cysteinyldopa, and Melanoma-Inhibitory Activity in Patients with Melanoma Classified by Stage. The above results clearly indicated that GPC3 might be a novel tumor marker for melanoma. We next compared the serum concentrations of GPC3, 5-S-cysteinyldopa, and melanoma-inhibitory activity in patients with melanoma classified by stage (Fig. 4; Tables 1 and 2). Fig. 4 shows serum concentrations of GPC3 quantified by ELISA method-1 (Fig. 4*A*), 5-S-cysteinyldopa (Fig. 4*B*), and melanoma-inhibitory activity (Fig. 4*C*) in 91 patients with melanoma (+) and 28 disease-free patients without detectable melanoma (−) classified by stage. We arbitrary fixed the cutoff level at 1 units/ml in GPC3, at 10 nmol/L in 5-S-cysteinyldopa (6), and at 17 ng/mL in melanoma-inhibitory activity, and there were two 5-S-cysteinyldopa false-positive cases in disease-free (−) stage II. Although serum concentrations of 5-S-cysteinyldopa and melanoma-inhibitory activity increased markedly in patients at stage IV, percentages of serum GPC3-positive patients were almost equal among the five clinical stages. To our surprise, we detected GPC3 in the sera of patients with very small melanoma such as stage 0 or I. There was no correlation between the positive state of three tumor markers, GPC3, 5-S-cysteinyldopa, and melanoma-inhibitory activity (Table 1). More importantly, 27 of 36 GPC3-positive patients were negative for both 5-S-cysteinyldopa and melanoma-inhibitory activity (patients 5, 7, 8, 9, and so on), and many were classified as cases of relatively early Unio Internationale Contra Cancrum stages 0, I, and II (Table 1). The positive rate of these three tumor markers in patients with melanoma, classified by stage, is shown in Table 2. Total positive rate of GPC3 (36 of 91, 39.6%) was significantly higher than that of 5-S-cysteinyldopa (26.7%)

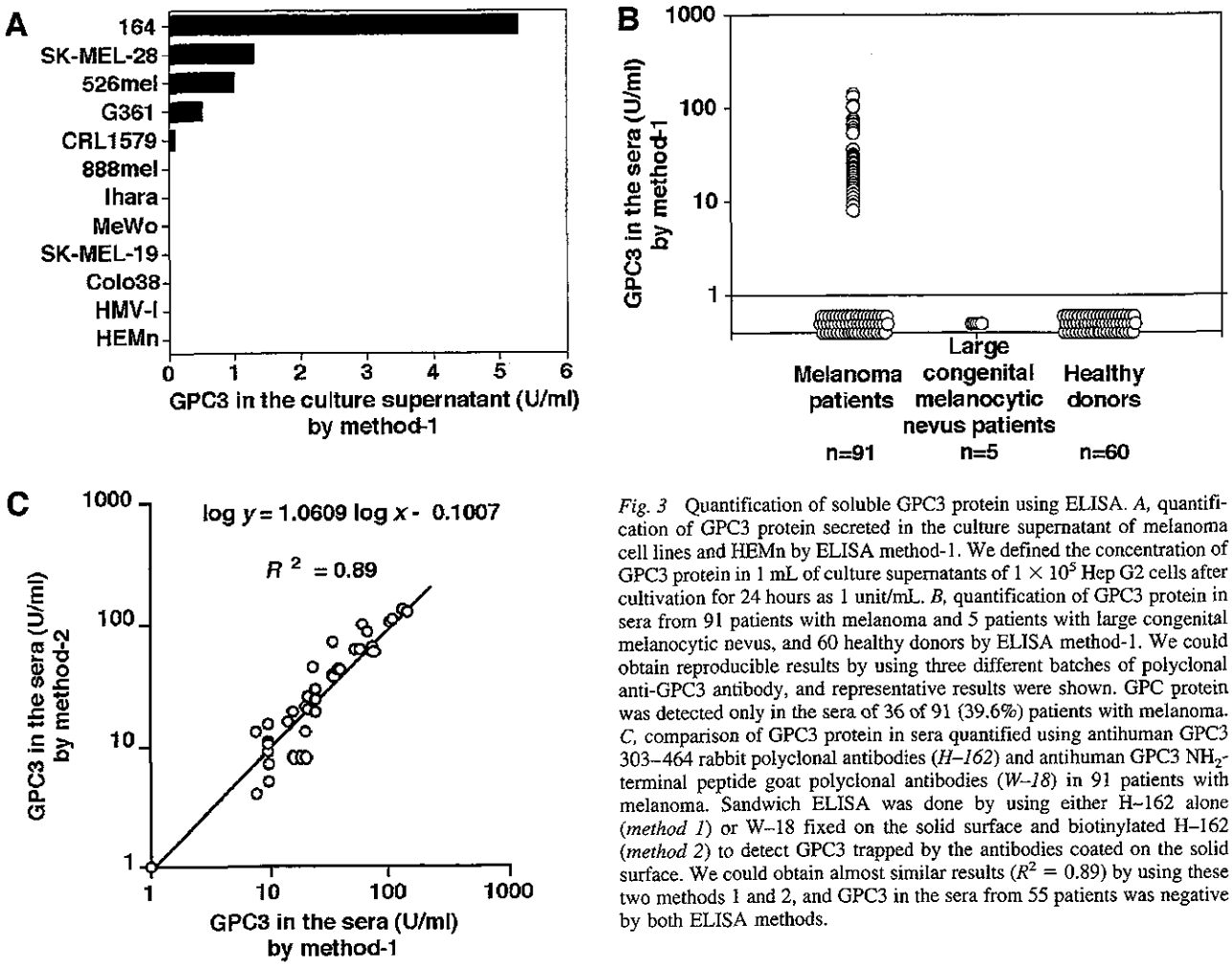

Fig. 3 Quantification of soluble GPC3 protein using ELISA. *A*, quantification of GPC3 protein secreted in the culture supernatant of melanoma cell lines and HEMn by ELISA method-1. We defined the concentration of GPC3 protein in 1 mL of culture supernatants of 1×10^5 Hep G2 cells after cultivation for 24 hours as 1 unit/mL. *B*, quantification of GPC3 protein in sera from 91 patients with melanoma and 5 patients with large congenital melanocytic nevus, and 60 healthy donors by ELISA method-1. We could obtain reproducible results by using three different batches of polyclonal anti-GPC3 antibody, and representative results were shown. GPC protein was detected only in the sera of 36 of 91 (39.6%) patients with melanoma. *C*, comparison of GPC3 protein in sera quantified using antihuman GPC3 303–464 rabbit polyclonal antibodies (*H–162*) and antihuman GPC3 NH_2-terminal peptide goat polyclonal antibodies (*W–18*) in 91 patients with melanoma. Sandwich ELISA was done by using either H–162 alone (*method 1*) or W–18 fixed on the solid surface and biotinylated H–162 (*method 2*) to detect GPC3 trapped by the antibodies coated on the solid surface. We could obtain almost similar results ($R^2 = 0.89$) by using these two methods 1 and 2, and GPC3 in the sera from 55 patients was negative by both ELISA methods.

and melanoma-inhibitory activity (20.9%; $P < 0.01$). Positive rate of GPC3 at stage 0 (4 of 9, 44.4%) was significantly higher than that of 5-S-cysteinyldopa (0.0%; $P < 0.05$), that at stage I (10 of 25, 40.0%) was significantly higher than that of 5-S-cysteinyldopa (8.0%; $P < 0.01$), and that at stage II (10 of 21, 47.6%) was significantly higher than that of 5-S-cysteinyldopa (10.0%) and melanoma-inhibitory activity (4.8%; $P < 0.01$). On the contrary, the positive rate of 5-S-cysteinyldopa at stage IV (15 of 18, 83.3%) was significantly higher than that of GPC3 (27.8%) and melanoma-inhibitory activity (50.0%; $P < 0.01$), and positive rate of melanoma-inhibitory activity at stage IV was significantly higher than that of GPC3 ($P < 0.05$).

Comparison of the Positive Rate of Serum GPC3 in Patients with Melanoma Classified by Clinical Type. We used sera from Japanese patients only in this study. Japanese melanoma has a high frequency of acral lentiginous melanoma, whereas superficial spreading melanoma and lentigo maligna melanoma are frequent types in Caucasians. Some groups have reported that acral lentiginous melanoma differs from other types of melanomas in clinical and histopathological characteristics (15–18). In fact, among 91 melanoma patients investigated in this study, 44 had acral lentiginous melanoma, 16 had superficial spreading melanoma, 9 had lentigo maligna melanoma, 5 had nodular melanoma, 12 had mucous melanoma, and 5 had unknown primary tumors. We next compared the positive rate of serum GPC3 among patients classified by these clinical types (Table 3). There was no significant correlation between the positive rate of serum GPC3 and melanoma type. Therefore, it seems likely that the usefulness of GPC3 as a marker for melanoma is not restricted to Japanese patients.

GPC3 Protein in the Sera of Melanoma Patients Disappeared after Surgical Treatments. Changes in serum levels of three tumor markers, GPC3 quantified by ELISA method-1, 5-S-cysteinyldopa, and melanoma-inhibitory activity, before and after surgical treatments in preoperative GPC3-positive 12 patients (patients 10, 11, 12, 13, 26, 35, 41, 42, 46, 55, 68, and 69) are shown in Table 4. For example, in the case of patient 35, although GPC3 and 5-S-cysteinyldopa were positive in the sera before operation, they disappeared after surgical treatments. GPC3 protein was detected in sera of these 12 patients before surgery but not so after the surgical treatments of patients with melanoma, except for patient 11 who could not be followed after the postoperative day 27. In case of patient 55, although 5-S-cysteinyldopa was weakly positive in serum at postoperative

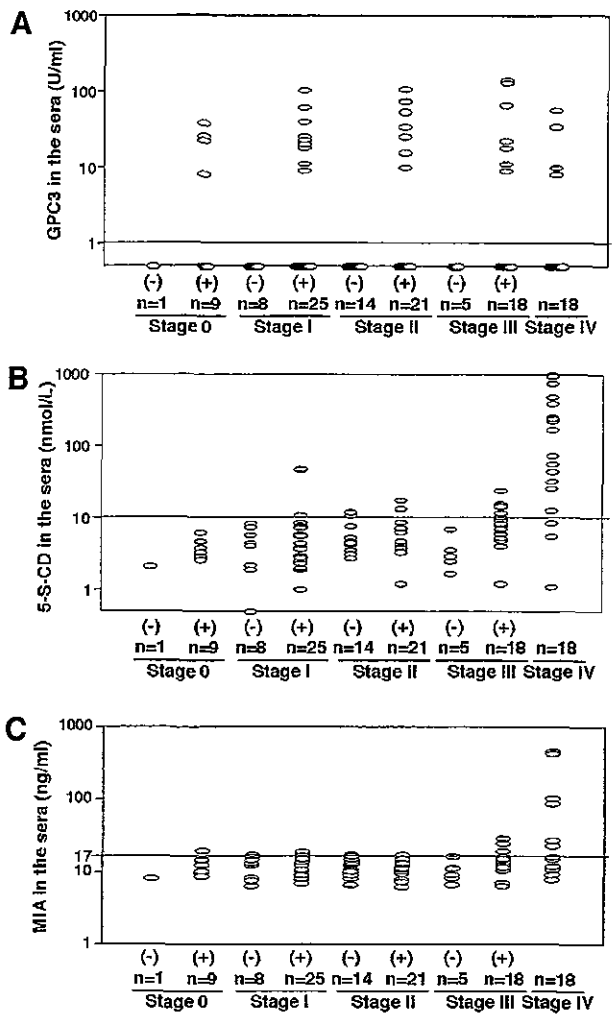

Fig. 4 Comparison of serum concentrations of GPC3, 5-S-CD, and MIA in patients with melanoma classified by stage. *A*, serum concentrations of GPC3 measured by ELISA method-1, (*B*) 5-S-CD, and (*C*) MIA in 91 patients with melanoma (+) and 28 disease-free patients without detectable melanoma (−) classified by stage. We fixed the cutoff level, indicated by a *line* in each panel, at 1 unit/mL in GPC3, at 10 nmol/L in 5-S-CD, and at 17 ng/mL in MIA. MIA, melanoma-inhibitory activity.

day 925, it was not evidenced at the recurrence of the melanoma (clinical data). It must be noted that GPC3 was the only useful tumor marker to follow the efficacy of surgical treatments for patients 12, 13, 26, 41, 42, 46, 68, and 69.

DISCUSSION

There are many tumor markers including carcinoembryonic antigen (19, 20), carbohydrate antigens CA 19–9 (21), and α-fetoprotein (22). Tumor markers have been used in several settings in cancer patients, including screening measures, differentiating malignant from benign lesions, monitoring the response to treatment, and detecting recurrences. In melanoma, several tumor markers have been evaluated for use as prognostic variables, to monitor response to therapy, and to detect recurrence (4–9, 23–25). These markers include 5-S-cysteinyldopa (4–7), melanoma-inhibitory activity (8, 9), ICAM-I (24), and S100 (25, 26) and are useful to detect stage IV metastatic melanoma. However, recurrent disease often cannot be detected at sufficiently early stages, and there is no available tumor marker that can detect primary melanoma at early stages, of a small size, and without metastases.

We found, in this study, that GPC3 could be a novel useful tumor marker for melanoma, especially at early stage, even stage 0 (*in situ*) melanoma. Although we detected no more than 30% of patients with melanoma, using conventional markers, we could diagnose 40% of those, irrespective of clinical stages, by using serum GPC3 as a tumor marker. In our study, the sensitivity of GPC3, 5-S-cysteinyldopa, and melanoma-inhibitory activity was 36 of 91 (39.6%), 24 of 90 (26.7%), and 19 of 91 (20.9%), respectively. We could diagnose only 32 of 91 (35.2%) melanoma patients using 5-S-cysteinyldopa and melanoma-inhibitory activity. But 27 GPC3-positive patients were negative for both 5-S-cysteinyldopa and melanoma-inhibitory activity. Twenty of these 27 patients (74.1%) were classified as being at a relatively early Unio Internationale Contra Cancrum stages 0, I, and II. Therefore, GPC3 is very useful for diagnosis of melanoma at early stages. Finally, we could diagnose 59 of 91 (64.8%) cases of melanoma using 5-S-cysteinyldopa, melanoma-inhibitory activity, and GPC3, a novel tumor marker. Furthermore, GPC3 is superior in specificity to other markers for melanoma. 5-S-cysteinyldopa often gives a false-positivity result. Serum 5-S-cysteinyldopa levels are often increased in patients with a large congenital melanocytic nevus (26). In this study, there were also 2 false-positive cases in disease-free patients. We reported that GPC3 protein in the sera was detect-

Table 2 Positive rates of serum levels of GPC3, 5-S-CD, and MIA in patients with melanoma classified by stage

Stage	GPC3	5-S-CD	MIA
0	4/9 (44.4%)*	0/9 (0.0%)	1/9 (11.1%)
I	10/25 (40.0%)	2/25 (8.0%)	5/25 (20.0%)
II	10/21 (47.6%)	2/20 (10.0%)	1/21 (4.8%)
III	7/18 (38.9%)	5/18 (27.8%)	3/18 (16.7%)
IV	5/18 (27.8%)	15/18 (83.3%)	9/18 (50.0%)
Total	36/91 (39.6%)	24/90 (26.7%)	19/91 (20.9%)

Abbreviations: MIA, melanoma-inhibitory activity

* Values significantly higher than others in the same clinical stage group are underlined.

Table 3 Positive rates of serum levels of GPC3 in patients with melanoma classified by clinical type

Type	Positive rate of GPC3 in sera
ALM	15/44 (34.1%)
SSM	9/16 (56.3%)
LMM/LM	4/9 (44.4%)
NM	2/5 (40.0%)
Mucous	3/12 (25.0%)
Total	33/86 (38.4%)

Abbreviations: ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma.