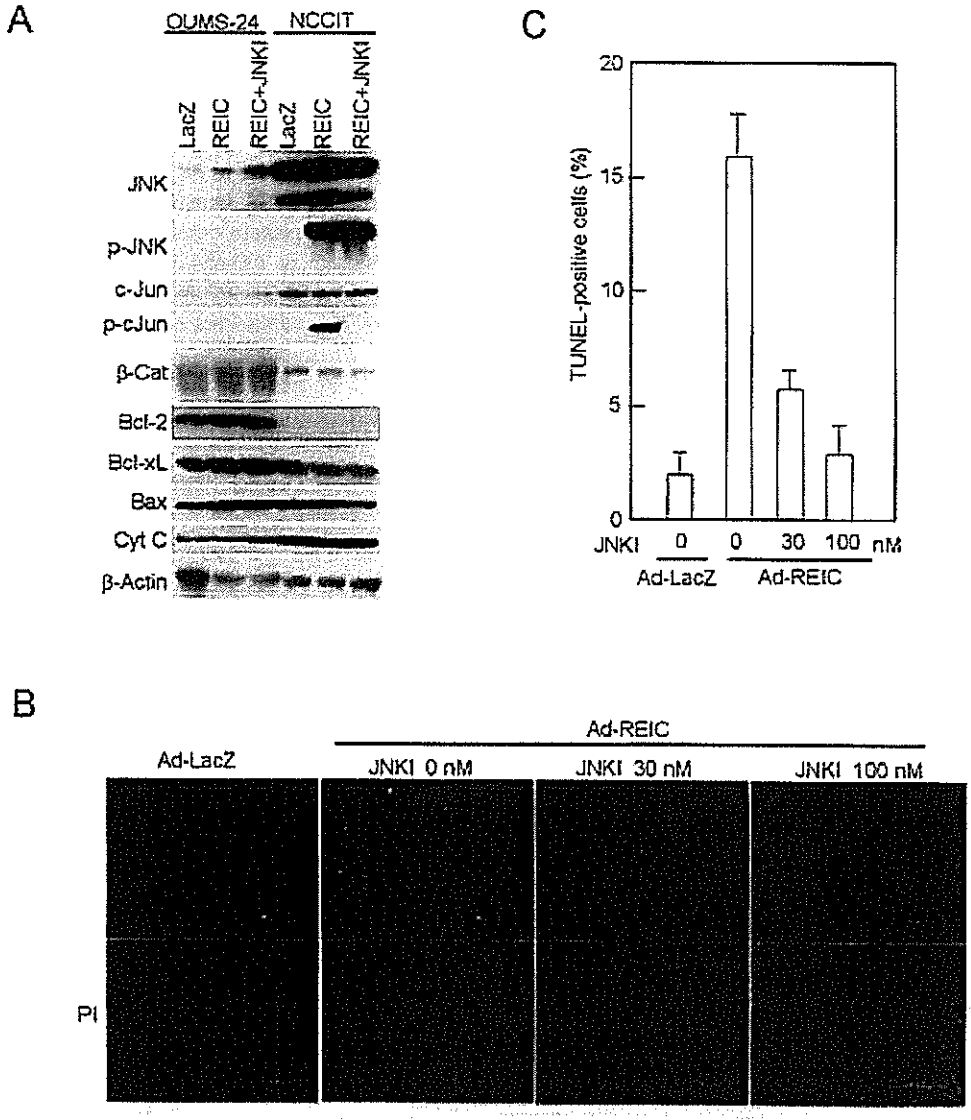

Figure 4. Abrogation of REIC/Dkk-3-induced apoptosis by JNK inhibitor. (A) Western blot analysis for designated proteins of NCCIT cells treated under the same conditions as those in B. The concentration of SP600125 was 100 nM. p-, phosphorylated; β-Cat, β-catenin; Cyt C, cytochrome C. (B) NCCIT cells were infected with Ad-LacZ or Ad-REIC at 20 MOI. JNK inhibitor SP600125 was added 1 h after the infection and the cells were fixed for immunostaining. PI, nuclear staining with propiodium iodide. (C) Quantitation of TUNEL-positive cells shown in A. Vertical bars, standard deviation.

hypermethylation of the REIC/Dkk-3 promoter was indicated to be at least partly involved (19). In our previous study, we noted some discrepancy between the expression level of REIC/Dkk-3 in terms of mRNA and that of protein, i.e., REIC/Dkk-3 protein was null in some cancer cases in which REIC/Dkk-3 mRNA was detected (15). This means that we should determine the protein levels of REIC/Dkk-3 for functionally relevant comparison among different cell types. Significant decrease in REIC/Dkk-3 protein was observed in 6 of the 7 and 13 of the 14 renal clear cell carcinoma cases analyzed by Western blotting and immunohistochemistry, respectively (15). For prostate cancer, twelve cases analyzed in a microarray and 40 cases analyzed in clinically resected tissues showed reduced expression of REIC/Dkk-3 protein depending on Gleason scores (16). Together with the results of the present study, it is now clear that the REIC/Dkk-3 protein level consistently decreased in most of the cases in 3 representative types of urogenital cancer.

Lack of REIC/Dkk-3 expression in NCCIT cells. For further studies on relevance of REIC/Dkk-3 to human testicular cancers, we chose a cell line derived from non-seminomatous germ cell tumor, NCCIT, since the cell line shows hybrid features of seminoma and embryonal carcinoma and can differentiate into various cell types of three embryonic germ layers (20). At first, we examined the expression of REIC/Dkk-3 in NCCIT cells. Quantitative RT-PCR revealed that the REIC/Dkk-3 mRNA level in NCCIT cells was negligible when compared with that in normal human fibroblasts OUMS-24 (Fig. 2A). No REIC/Dkk-3 protein was detected in NCCIT cells by Western blot analysis (Fig. 2B). In accordance with our previous study (16), the protein was detected in normal human fibroblasts as a band of ~62 kDa but not in prostate cancer cell line PC3, which were used as a positive and negative control, respectively. Thus, NCCIT cells share the property lacking REIC/Dkk-3 expression with most testicular tumors and therefore were used for further studies.

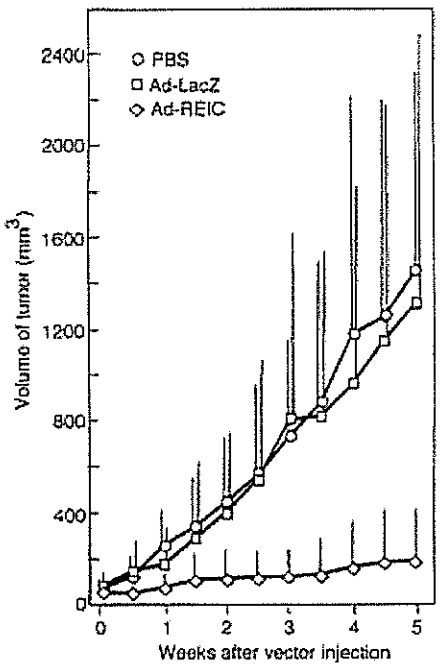

Figure 5. Suppression of the growth of NCCIT cells by Ad-REIC. Three weeks after subcutaneous transplantation of $3x10^7$ NCCIT cells into nude mice, the adenovirus vectors ($2x10^8$ pfu) were injected once into the tumors. Vertical bars, standard deviation of tumor volume in 5 (Ad-REIC) or 6 (PBS or Ad-LacZ) mice.

Induction of apoptosis in NCCIT cells by overexpression of REIC/Dkk-3. Since our previous study showed that overexpression of REIC/Dkk-3 induced apoptosis in prostate cancer cell lines deficient in expression of the gene, we examined its effect on NCCIT cells. Infection of a replication-deficient adenovirus vector carrying either LacZ (Ad-LacZ) or REIC/Dkk-3 (Ad-REIC) to OUMS-24 and NCCIT cells at 20 MOI resulted in overexpression of the proteins at comparable levels (Fig. 3A). Under these conditions, infection efficiency was nearly 100% in both cell types (data not shown). When the apoptotic event was monitored by TUNEL staining 72 h after infection, 31% of NCCIT cells were positive after infection with Ad-REIC. OUMS-24 cells exhibited the positive signal in only 5.3% of the cells infected with Ad-REIC, the rate being comparable to those obtained after Ad-LacZ infection (Fig. 3B and C). These results indicate that overexpression of REIC/Dkk-3 selectively induces apoptotic cell death in NCCIT cells in a similar manner for prostate cancer cell lines as reported previously (16). In the present study, however, direct comparison of NCCIT cells and their normal counterparts was hampered by unavailability of the latter. Although mechanisms underlying the preferential induction of apoptosis in NCCIT cells remain to be clarified, our ongoing study indicates that the functional state of proteins involved in stress-induced apoptosis plays some roles. In this respect, it is noteworthy that the protein level of proapoptotic JNK was higher and that of anti-apoptotic Bcl-2 was lower in NCCIT cells than in the respective counterpart OUMU-24 (Fig. 4A).

Involvement of JNK in apoptotic cell death in NCCIT cells by Ad-REIC. Since our previous study using a prostate cancer cell line indicated the involvement of c-Jun terminal kinase (JNK) in the induction of apoptosis by Ad-REIC, we examined the protein levels and phosphorylation state of JNK and other apoptosis-related proteins by Western blot analysis. As shown in Fig. 4A, infection of Ad-REIC resulted in phosphorylation and activation of JNK in NCCIT cells but not in OUMS-24. The activation of JNK was demonstrated by phosphorylation of the substrate protein c-Jun. Application of an inhibitor of JNK, SP600125, at 100 nM completely abrogated phosphorylation of c-Jun. SP600125 is known to inhibit the activity of JNK1, JNK2, and JNK3 but not their autophosphorylation (21).

To see whether activation of JNK is causally linked to the induction of apoptosis, we examined the effect of SP600126 on Ad-REIC-induced apoptosis. As shown in Fig. 4B and C, addition of SP600126 dose-dependently abrogated apoptotic cell death in NCCIT cells exposed to Ad-REIC, indicating that activation of JNK is essential to the apoptosis-inducing function of Ad-REIC.

Inhibition of tumorigenic growth of NCCIT in nude mice by Ad-REIC. Finally, we investigated the effect of Ad-REIC on the growth of NCCIT cells *in vivo* as a possible therapeutic model. NCCIT cells of $3x10^6$ were subcutaneously transplanted into nude mice and 3 weeks later $2x10^8$ pfu of Ad-REIC or Ad-LacZ in 100 ml was injected intratumorally. In control animals which received PBS or Ad-LacZ, the tumors grew progressively to reach ~1,200 mm^3 in size at the end of the observation period of 5 weeks (Fig. 5). In contrast, the tumor completely disappeared in 2 of the 5 mice injected with Ad-REIC and the mean tumor size among the 5 mice remained largely unchanged.

Concluding remarks. In the present study, we examined the potential utility of REIC/Dkk-3 as a gene-therapeutic agent against testicular cancer. Expression of REIC/Dkk-3 was reduced in all of the human seminoma and NSGCT tissues examined. Adenovirus-mediated overexpression of REIC/Dkk-3 preferentially induced apoptotic cell death in a testicular germ cell carcinoma cell line, NCCIT, defective in expression of REIC/Dkk3. Furthermore, intratumoral injection of the adenovirus vector carrying REIC/Dkk-3 remarkably suppressed tumorigenic growth of NCCIT cells subcutaneously transplanted into nude mice. These results indicate that adenovirus-mediated overexpression of REIC/Dkk-3 may be a promising approach for developing more patient-friendly therapeutic measures against human testicular cancer.

Acknowledgments

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Heat shock proteins play a crucial role in tumor-specific apoptosis by REIC/Dkk-3

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Abstract. We recently showed that overexpression of REIC/Dickkopf-3 (Dkk-3), a tumor suppressor gene, induced apoptosis in a tumor cell-specific manner. The aim of the present study was to determine the mechanisms underlying the selective induction of apoptosis. At first, we found a mouse renal carcinoma cell line, RENCA, to be extremely sensitive to an adenovirus carrying REIC/Dkk-3 (Ad-REIC), and we showed that activation of c-Jun N-terminal kinase (JNK) was a critical step in cell death, i.e. a process similar to that in human prostate and testicular cancer observed in our previous studies. Among the proteins interfering with the activation of JNK, heat shock protein (Hsp)70/72 was reduced in expression in RENCA cells compared with that in NIH3T3 cells. An Hsp70/72 inducer protected RENCA cells from Ad-REIC-induced apoptosis, while an Hsp70/72 inhibitor sensitized NIH3T3 cells for apoptosis induction. These results indicate that functionally active Hsp70/72 is a key factor in tumor cell-specific induction of apoptotic cell death and that analyses of the expression levels of Hsp70/72 may be essential in determining the significance of Ad-REIC-based gene therapy against human cancer.

Introduction

The Dickkopf (Dkk) family is composed of 4 members, Dkk-1-4, that show distinct as well as similar biological functions (1). Dkk-1 and -4 are known to interfere with Wnt signaling through the common receptor LRP5/6 (1-4). Since the Wnt signal pathway has been shown to be aggravated in many types of cancers (5-7), Dkk-1 is thought to behave as a tumor suppressor gene. Expression of endogenous Dkk-1 was shown to be reduced in human melanomas (8), and forced expression of Dkk-1 resulted in increased apoptosis of various tumor cells (9-11). Unlike Dkk-1 and -4, REIC/Dkk-3 does not bind to LPR5/6 or interfere with Wnt signaling (12,13). Nevertheless, expression of the REIC/Dkk-3 gene is suppressed in cells and tissues of many human cancers, including prostate cancer, renal clear cell carcinoma and non-small cell lung cancer (14-16). More notably, expression of REIC/Dkk-3 using an adenovirus vector (Ad-REIC) selectively induced apoptosis in human prostate cancer cell lines (16) and a human testicular cancer cell line (17) with only a marginal effect on normal counterparts. Furthermore, human prostate cancer cells transplanted subcutaneously into nude mice were completely regressed in 4 out of 5 animals by a single injection of Ad-REIC (16). The selective action of REIC/Dkk-3 is of profound significance and prompts further mechanistic study since the selective killing of malignant cells is the most critical issue for successful treatment of human cancer.

Our previous study showed that a membrane-permeable Bax inhibitor V5 completely abrogated the apoptosis induced in the prostate cancer cell line PC3 by infection with Ad-REIC (16). Translocation of Bax protein from the cytoplasm to the mitochondria, a hallmark of the triggering of a Bax-mediated apoptotic pathway, was completely suppressed by V5. The Ad-REIC infection activated c-Jun N-terminal kinase (JNK), one of the upstream activators of Bax in PC3 cells but not in normal human fibroblasts. The JNK-specific inhibitor SP600125 remarkably abrogated the Ad-REIC-induced apoptosis of PC3 cells in a dose-dependent manner. These results indicate that overexpression of REIC/Dkk-3 activates JNK in a malignant cell-specific manner, induces mitochondrial translocation of Bax protein, and leads to apoptotic cell death. In the present study, we analyzed the mechanism of the cell type-specific activation of JNK by Ad-REIC after identification of a cell line highly sensitive to the Ad-REIC-induced apoptosis and thus suitable for the mechanistic study.

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Key words: REIC, Dickkopf-3, testicular tumor, therapy

Materials and methods

Cells and reagents. RENCA, a renal cortical adenocarcinoma cell line of BALB/c mouse origin, was kindly provided by Dr Masatoshi Eto, Kyushu University. NIH3T3 cells were purchased from ATCC. RENCA and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (MEM) and RPMI-1640 (Gibco), respectively, supplemented with 10% FBS (Gibco). A c-Jun N-terminal kinase (JNK) inhibitor, SP600125, was purchased from BioMol (Plymouth Meeting, PA, USA). A heat shock protein (Hsp) 70 inducer, geranylgeranylacetone, and an Hsp70/72 inhibitor (heat shock protein inhibitor I) were purchased from Eisai (Tokyo, Japan) and Calbiochem (La Jolla, CA, USA), respectively.

Immunocytochemistry. Cells were fixed with ethanol or with 4% paraformaldehyde and immunostained with four different anti-human REIC/Dkk-3 antibodies raised in our laboratory against full-length REIC/Dkk-3 and the N-terminal and C-terminal peptides. The specimens were then treated with Alexa fluor R594-conjugated goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA) and mounted in Vectashield R with DAPI (Vector Laboratories, Burlingame, CA).

Overexpression of REIC/Dkk-3 and monitoring of apoptotic cells. REIC/Dkk-3 was overexpressed by infecting cells with an adenovirus vector carrying REIC/Dkk-3 (Ad-REIC) as described previously (16). A vector carrying LacZ (Ad-LacZ) was used as a negative control. Thirty-six hours after infection with the virus vectors at 20 MOI, apoptotic cells were monitored either by the TUNEL method using an In Situ Cell Death Detection kit (Roche) or by using a LIVE/DEAD Viability/Cytotoxicity kit (Invitrogen).

Immunoprecipitation and Western blot analysis. Total cell lysates were prepared at 36 h after adenovirus vector infection with a Triton X-100 lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 200 μg/ml phenylmethanesulfonyl fluoride, 10 mM Tris, pH 7.3). After saving aliquots of the samples for direct Western blot analysis, the cell lysates were cleared by treatment with excess amounts of TrueBlot™ anti-rabbit Ig IP beads (eBioscience, San Diego, CA) and incubated with rabbit anti-JNK/SAPK antibody (Cell Signaling) at 4˚C for 120 min. JNK and its associated proteins were recovered using TrueBlot anti-rabbit Ig IP beads. Western blot analysis was performed under conventional conditions. The antibodies used were as follows: rabbit anti-human REIC/Dkk-3 antibodies raised in our laboratory, a rabbit anti-human c-Jun antibody, a rabbit anti-human phospho-c-Jun (Ser63) antibody, a rabbit anti-human SAPK/JNK antibody, a rabbit anti-human phospho-SAPK/JNK (Thr183/Tyr185) antibody, a rabbit anti-human Hsp70/72 antibody (Cell Signaling Technology, Beverly, MA), a mouse monoclonal anti-human Hsp70/72 antibody (BD Biosciences), a mouse monoclonal anti-human Hsc70 antibody (Santa Cruz), and a mouse monoclonal anti-human ß-actin antibody (Sigma).

In vitro binding assay. Human Hsp70/72 cDNA was cloned and inserted into a pGEX6P3 vector (GE Health Care Biosciences). The Hsp70/72 protein was expressed as a GST-fused protein and purified using glutathione sepharose 4B (GE Health Care Biosciences). Fifty micromolars each of GST-Hsp70/72, JNK1α1 (active, Upstate), and JNK1α1 (inactive, Upstate) were incubated in PBS for 1 h at room temperature, recovered using glutathione sepharose 4B, and analyzed by Western blotting for JNK.

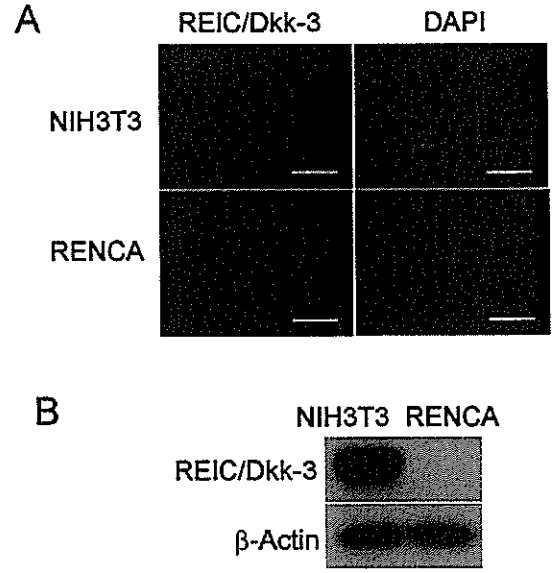

Figure 1. Expression of REIC/Dkk-3 in a mouse renal cancer cell line RENCA, and a normal fibroblastic cell line NIH3T3. (A) Immunohistochemistry for REIC/Dkk-3 using an antibody against the N-terminal region of REIC protein (shown in red) and nuclear staining with DAPI. Scale bars, 20 μm. (B) Western blot analysis for REIC/Dkk-3 in RENCA and NIH3T3 cells. ß-actin was used as a control for the applied amount of protein.

Results and discussion

Lack of REIC/Dkk-3 expression in RENCA cells. We first examined expression of the REIC/Dkk-3 gene in RENCA cells in comparison with that in NIH3T3 cells. Immunocytochemistry for REIC/Dkk-3 using two antibodies against full-length REIC/Dkk-3 protein, an antibody against an N-terminal region, and an antibody against a C-terminal region, consistently revealed a positive signal in the cytoplasm of NIH3T3 cells but never in RENCA cells (Fig. 1A). The lack of expression of REIC/Dkk-3 in RENCA cells was confirmed by Western blot analysis (Fig. 1B). This observation accords well with results of our previous studies showing that REIC/Dkk-3 levels were reduced in tumor cells and tissues derived from various human organs, including the kidney (15) and prostate (16).

Specific induction of apoptosis in RENCA cells by overexpression of REIC/Dkk-3. Since our previous study showed that overexpression of REIC/Dkk-3 induced apoptosis in cancer cells but not in normal cells (16,17), we examined its effect on RENCA and NIH3T3 cells. We infected the cells with either Ad-LacZ or Ad-REIC at 20 MOI and monitored apoptotic cells by TUNEL staining 36 h after the infection.

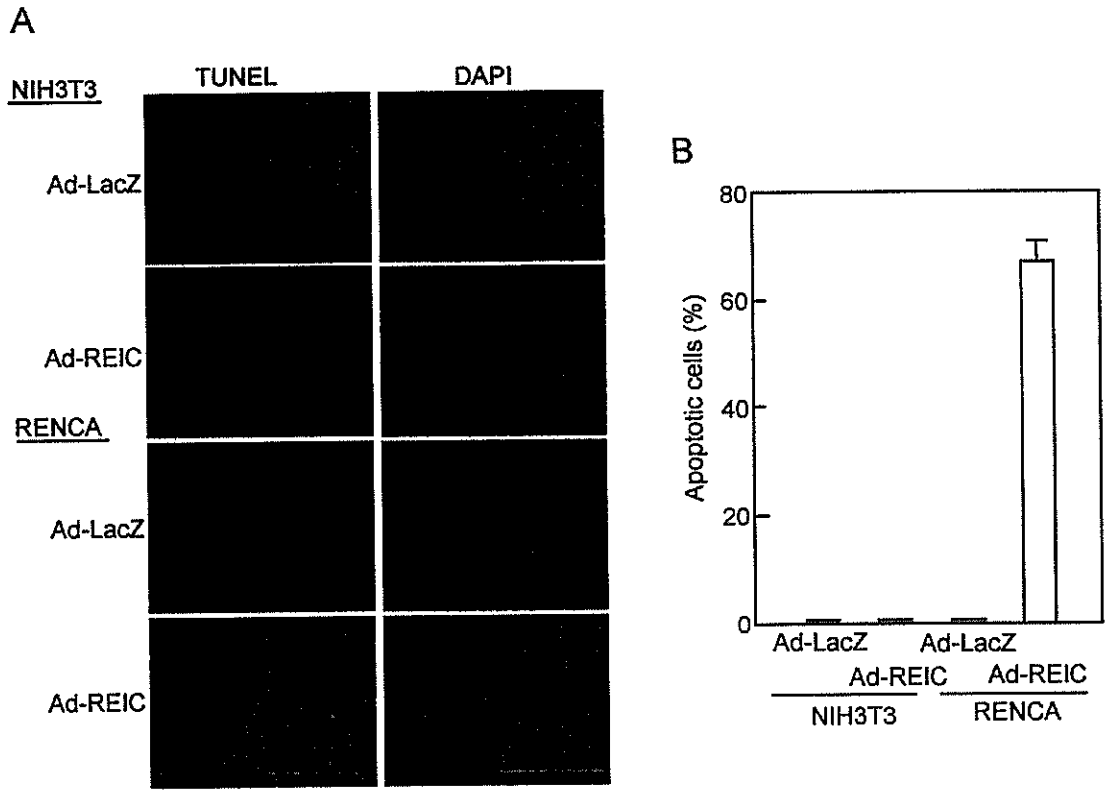

Figure 2. Induction of apoptotic cell death of RENCA cells by overexpression of REIC/Dkk-3. The cells were infected with Ad-LacZ or Ad-REIC at 20 MOI. (A) TUNEL staining for monitoring apoptotic cells and DAPI staining for nuclei 36 h after infection. Scale bars, 200 μm. (B) Quantitation of the TUNEL-positive cells shown in A. Vertical bars, standard deviation.

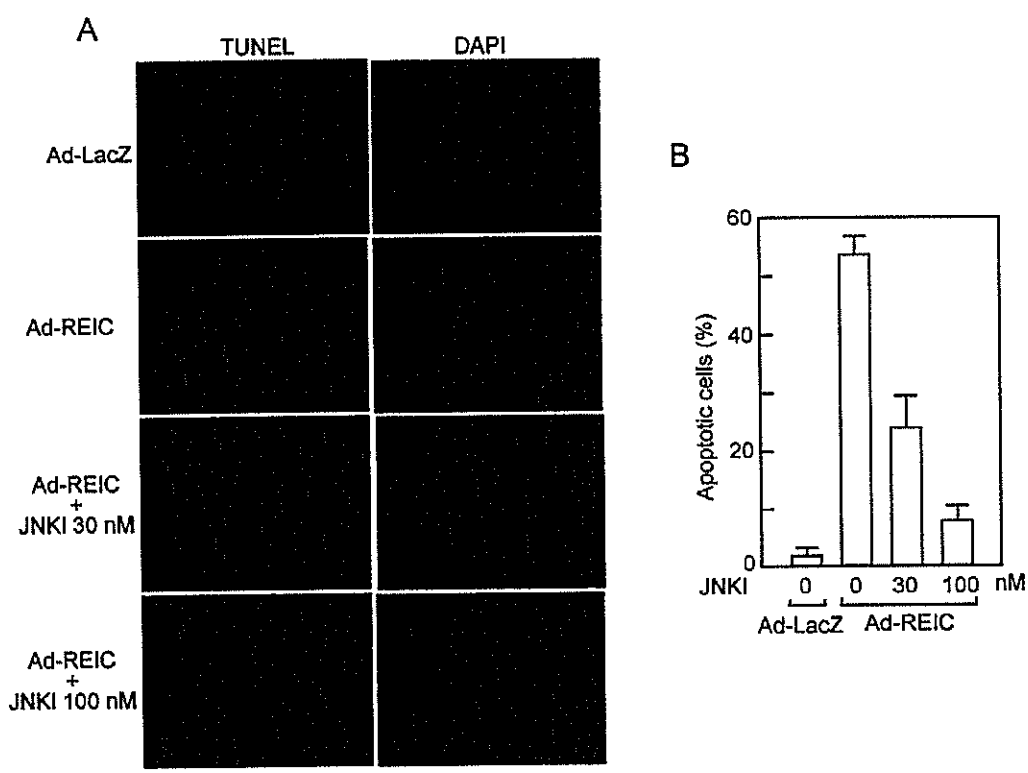

Figure 3. Abrogation of Ad-REIC-induced apoptosis of RENCA cells by a JNK inhibitor SP600125 (JNKI). RENCA cells were infected with Ad-LacZ or Ad-REIC at 20 MOI. The JNK inhibitor was added to the culture immediately after the infection, and the cells were fixed and stained 36 h after infection. (A) TUNEL staining for monitoring apoptotic cells and DAPI staining for nuclei. Scale bars, 200 μm. (B) Quantitation of the TUNEL-positive cells shown in A. Vertical bars, standard deviation.

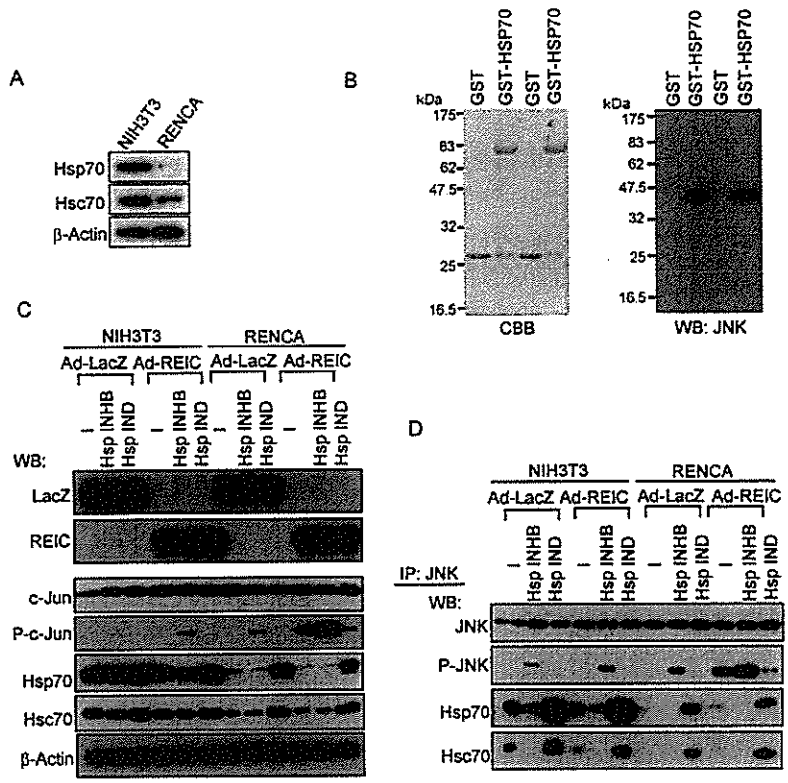

Figure 4. JNK and heat shock proteins (Hsp) in NIH3T3 and RENCA cells. (A) Expression levels of Hsp70/72 and Hsc70 in NIH3T3 and RENCA cells determined by Western blot analysis. ß-actin was used as a control for the applied amounts of protein. (B) Direct binding of JNK to Hsp70/72 *in vitro*. Left panel: recombinant human Hsp70/72 was produced in a GST-fused form and purified using glutathione sepharose. GST was used as a negative control. Proteins were recovered, electrophoresed, and visualized on a PVDF membrane by CBB staining. Right panel: GST-Hsp70/72 was mixed with a mixture of active and inactive JNK proteins (Upstate) and pulled down with glutathione sepharose. The bound fraction of JNK proteins was detected by Western blot analysis. The experiments were performed in duplicate. (C,D) Analysis of JNK and heat shock proteins in REIC-overexpressed cells exposed to Hsp modulators. NIH3T3 and RENCA cells were infected with Ad-LacZ or Ad-REIC at 20 MOI. An Hsp inhibitor (Hsp INHB) (50 μM) was added immediately after the virus infection. An Hsp inducer (Hsp IND) (10 μM) was added 8 h prior to the infection followed by repeated addition of the same dose every 8 h over the cultivation period. The cells were harvested at 36 h after the infection. (C) Western blot analysis for indicated proteins, P-c-Jun and phosphorylated c-Jun protein. ß-actin was used as a control for the applied amount of protein. (D) Proteins were immunoprecipitated with an anti-JNK antibody from the same cell extracts as in A and then analyzed by Western blotting. P-JNK, phosphorylated JNK protein.

Similar expression levels of exogenous REIC/Dkk-3 protein were confirmed in NIH3T3 and RENCA cells (Fig. 4C). As shown in Fig. 2, however, 67% of Ad-REIC-infected RENCA cells were positive for TUNEL staining, while only 0.3% of Ad-REIC-infected NIH3T3 cells were positive. Ad-LacZ exerted a negligible effect on both RENCA and NIH3T3 cells. The highly sensitive and selective feature of REIC/Dkk-3-induced apoptosis in RENCA cells provides a suitable condition for a mechanistic study on tumor-specific induction of apoptosis by overexpression of REIC/Dkk-3 observed in a variety of malignant cells and tissues.

Involvement of JNK in Ad-REIC-induced apoptosis in RENCA cells. Our previous studies showed that JNK plays a major role in the induction of apoptosis by overexpression of REIC/Dkk-3 in human cancer cells (16). To determine whether this is also the case in RENCA cells, we examined the effect of a JNK inhibitor on Ad-REIC-induced cell death in RENCA cells (Fig. 3). The JNK inhibitor SP600125 added to the cultures immediately after the virus infection dose-dependently abrogated the Ad-REIC-induced apoptotic cell death in RENCA cells, i.e. 30 and 100 nM of SP600125 reduced apoptotic cell rate in the untreated cells by 56 and 86%, respectively (Fig. 3B), indicating a critical role of JNK in the apoptotic process. In accordance with this, JNK was shown to be activated in RENCA cells by infection with Ad-REIC but not by infection with Ad-LacZ as demonstrated by phosphorylation of JNK itself and its substrate c-Jun (Fig. 4C). Neither JNK nor its substrate c-Jun was phosphorylated by Ad-REIC in NIH3T3 cells, where no induction of cell death was observed. A critical question to be addressed for understanding the mechanisms of the tumor-specific induction of apoptosis, therefore, is why and how JNK was activated in RENCA cells but not in NIH3T3 cells despite the fact that both types of cells were similarly infected with Ad-REIC.

Activation of JNK was also shown to be a key event in Dkk-1-induced apoptosis by Lee *et al* (11). Although Dkk-1 interferes with Wnt signaling, the activation of JNK by Dkk-1 did not involve the canonical Wnt signal pathway since they used a cell line lacking both alleles of ß-catenin. REIC/Dkk-3 is known not to interfere with Wnt signaling (12,13). We showed that the protein level and intracellular localization of ß-catenin remained unchanged in PC3 cells when the cells

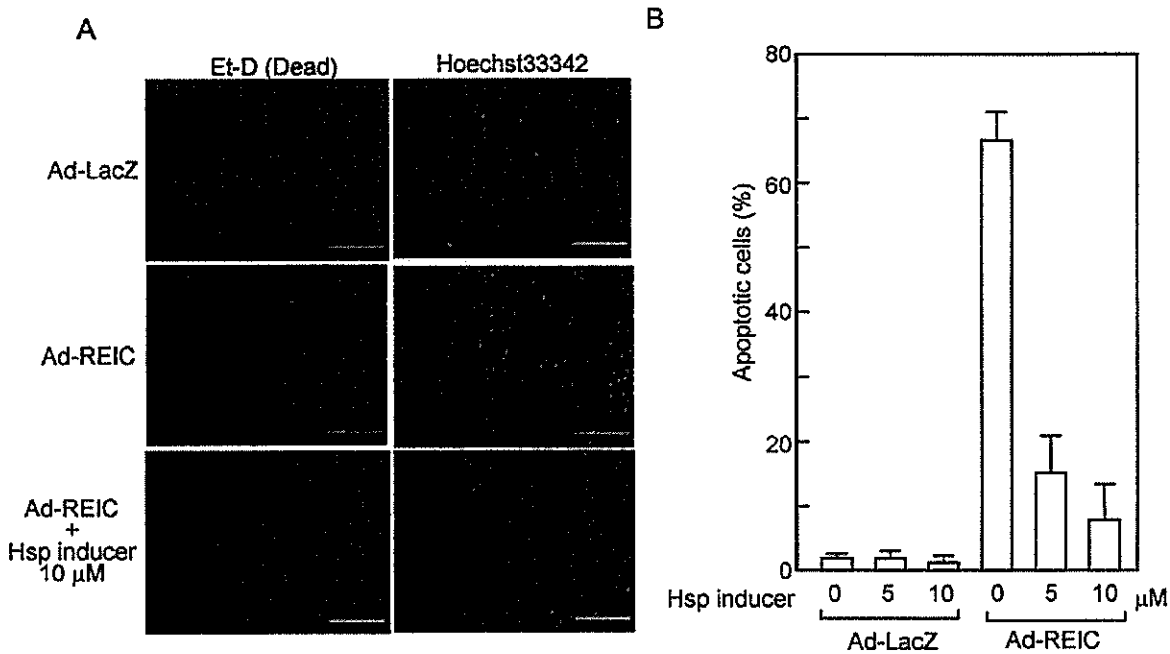

Figure 5. Inhibition of REIC-induced apoptotic cell death of RENCA cells by an Hsp inducer. RENCA cells were treated under the same conditions as those described in the legend of Fig. 4, and viability of the cells was examined using a LIVE/DEAD Viability/Cytotoxicity kit (Cambrex), in which dead cells were stained red with ethidium bromide dimer (Et-D) and all cell nuclei were stained blue with Hoechst 33342. (A) Dead cells and all cell nuclei were stained in red and blue, respectively. Scale bars, 200 μm. (B) Quantitation of the apoptotic cells shown in A. Vertical bars, standard deviation.

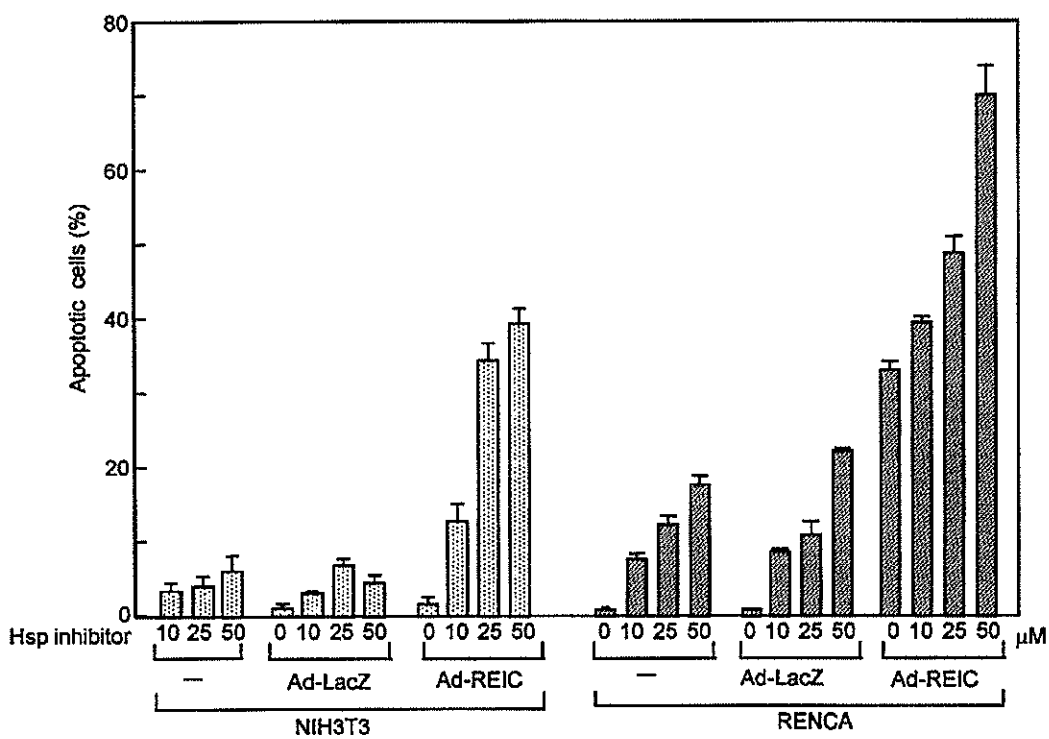

Figure 6. Sensitization of NIH3T3 and RENCA cells to REIC-induced apoptotic cell death by an Hsp inhibitor. The cells were treated under the same conditions as those described in the legend of Fig. 4, and viability of the cells was determined by the same method as that described in the legend of Fig. 5. Vertical bars, standard deviation.

were infected with Ad-REIC and were undergoing apoptotic cell death (16). An alternative and more likely possibility is that Ad-REIC-induced apoptosis may take place through activation of a stress-sensing mechanism for overload of specific proteins known as endoplasmic reticulum (ER) stress. It is well established that JNK is a key molecule leading

cells in ER stress to apoptotic cell death, and inhibition of JNK abrogates the induction of apoptosis (18,19).

Heat shock proteins (Hsps) as a key factor for tumor-specific apoptosis by Ad-REIC. A number of different proteins are known to bind to and inhibit the activity of JNK, including Hsp70/72, p21(WAF1), Rb, and GSTp (20-23). Among these, we focused on Hsp70/72 since the protein level of Hsp70/72 was reduced in RENCA cells compared with that in NIH3T3 cells (Fig. 4A). Expression of a constitutively expressed Hsc70 was also reduced in RENCA cells, though to a lesser extent. Recombinant Hsp70/72 directly bound to JNK *in vitro* (Fig. 4B). Binding of endogenous JNK and Hsp70/72 or Hsc70 was confirmed by immunoprecipitation followed by Western blot analysis (Fig. 4D). Hsp90 and Hsp27 were expressed at lower levels in NIH3T3 cells than in RENCA cells, but neither protein interacted with JNK and hence were not considered to play significant roles in the present context (data not shown).

If the reduced Hsp70/72 level in RENCA cells is causatively linked to the sensitivity to Ad-REIC-induced apoptosis, induction of Hsp70/72 should suppress the apoptotic death of the cells. When RENCA cells were treated with the Hsp70/72 inducer geranylgeranylacetone at 10 μM, Hsp70/72 was induced to a level slightly less than that of untreated NIH3T3 cells (Fig. 4C). The induction of Hsp70/72 resulted in abrogation of activation of JNK (Fig. 4C) and of apoptotic cell death (Fig. 5) by Ad-REIC infection. The apoptotic cell rate determined 36 h after the Ad-REIC infection was reduced from 66.5% in untreated RENCA cells to 15.1 and 7.9% in the cells treated with 5 and 10 μM of geranylgeranylacetate, respectively (Fig. 5B).

On the other hand, exposure of cells to an Hsp70/72 inhibitor sensitized NIH3T3 cells to apoptosis induction by Ad-REIC in a dose-dependent manner from 10-50 μM (Fig. 6). The ratios of apoptotic cells were only 1.6% in NIH3T3 cells simply infected with Ad-REIC and 39.1% in the cells treated with 50 μM of an Hsp70/72 inhibitor in addition to the virus infection (Fig. 6). JNK was activated as assessed by detecting phosphorylated c-Jun in Ad-REIC-infected NIH3T3 cells treated with 50 μM of the inhibitor but not in the untreated cells (Fig. 4C). Treatment with the Hsp70/72 inhibitor dose-dependently induced apoptosis even in non-infected and Ad-LacZ-infected RENCA cells and reinforced apoptotic cell death in Ad-REIC-infected cells (Fig. 6). These results indicate that the tumor cell-specific apoptosis induced by overexpression of REIC/Dkk-3 was mainly due to the functional difference of Hsp70/72.

Hsp70/72 has been shown to inhibit apoptosis induced by a broad variety of agents, including stresses (24-28). Although Park *et al* (29) demonstrated that Hsp72(70) inhibited heat-induced activation of JNK through direct interaction in NIH3T3 cells, many lines of evidence indicate that the mechanism of anti-apoptotic action of Hsp70/72 is pleiotropic depending on cell type and apoptotic stimuli. Hsp70/72 prevented stress-induced apoptosis of a human T-lymphocyte cell line by acting not only on JNK but also at some point downstream of JNK (27). Buzzard *et al* (28) showed that constitutively expressed Hsp70/72 inhibited apoptosis of mouse embryonic fibroblasts by heat, ceramide, or TNFα but did not affect JNK activity. Hsp70/72 exerted its anti-apoptotic action not at the stage of JNK but at a downstream step of caspase 3-like proteases (29).

In the present study, we obtained evidence that the functional state of Hsp70/72 correlates well with resistance to apoptosis induced by overexpression of REIC/Dkk-3. Our preliminary screening revealed that human cancer cells largely differ in sensitivity to Ad-REIC-induced apoptosis and that the sensitivity depends on expression levels of anti-apoptotic proteins, including Hsp70/72. The results of the present study may contribute to the establishment of a convenient and reliable method to distinguish the sensitivity of different human tumors to gene therapy using Ad-REIC.

In conclusion, through the present study we analyzed the mechanism of tumor cell-specific induction of apoptosis by Ad-REIC using a cell line highly sensitive to apoptosis induction. Among the proteins interfering with the activation of JNK, a critical event for Ad-REIC-induced apoptosis was the reduced expression of Hsp 70/72 in RENCA cells compared with that in NIH3T3 cells. An Hsp70/72 inducer protected RENCA cells from Ad-REIC-induced apoptosis, while an Hsp70/72 inhibitor sensitized NIH3T3 cells for apoptosis induction. These results indicate that functionally active Hsp70/72 is a determining factor for tumor cell-specific induction of apoptotic cell death and that analyses of the expression levels of Hsp70/72 may contribute to the identification of human cancers sensitive to gene therapy using Ad-REIC.

Acknowledgements

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Heat shock protein 105 is overexpressed in squamous cell carcinoma and extramammary Paget disease but not in basal cell carcinoma

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Conflicts of interest
None declared.

Summary

Background Heat shock protein (HSP) 105 is a 105-kDa protein, recently discovered by serological analysis of recombinant cDNA expression libraries prepared from tumour cells (SEREX), and is still undergoing intensive research. SEREX can define strongly immunogenic tumour antigens that elicit both cellular and humoral immunity. Previous studies have shown that HSP105 is a cancer testis antigen and is overexpressed in various internal malignancies. The expression of HSP105 has not been studied in skin cancers.

Objectives To assess the expression of HSP105 in skin cancers including extramammary Paget disease (EMPD), cutaneous squamous cell carcinoma (SCC) and basal cell carcinoma (BCC).

Methods Samples of EMPD ($n = 25$), SCC ($n = 23$, of which three were metastatic lesions) and BCC ($n = 23$) were collected from patients treated in our department between January 2002 and December 2004. Western blot and immunohistochemical staining methods were used to investigate the expression of HSP105.

Results Results of Western blot analysis showed overexpression of HSP105 in EMPD and SCC, and minimal expression in BCC. Immunohistochemistry results showed that 56% of EMPD, 60% of primary and 100% of metastatic SCC highly expressed HSP105 while only 13% of BCC lesions showed increased staining.

Conclusions EMPD and SCC overexpress HSP105 while BCC does not. Tumours overexpressing HSP105 present ideal candidates for vaccination by HSP105-derived peptides or DNA.

Heat shock protein (HSP) 105 is a mammalian stress protein that belongs to the HSP110 family.[1] It is released by tissues in response to a wide variety of stresses including infection, ischaemia, heat stress and tumours.[2,3] Studies have shown that the molecule is involved as a biochemical mediator of heat-induced apoptosis by binding to p53 at scrotal temperatures and dissociating from it at suprascrotal temperatures in testicular germ cells.[4] HSP105 consists of α and β components. The α-component is 105 kDa and the β-component is a truncated form, 90 kDa in size, and is specifically induced by heat stress at 42 °C.[5,6] It is important to investigate and isolate tumours which overexpress HSP105 in order to target them for immunotherapy. Studies on colorectal carcinoma and melanoma cell lines in mouse hosts showed that HSP105 DNA vaccination could stimulate HSP105-specific tumour immunity leading to tumour regression.[7]

Kai et al.[8] showed that HSP105 is overexpressed in a variety of intra-abdominal carcinomas such as oesophageal squamous cell carcinoma (SCC), colon and pancreatic adenocarcinoma, and others. No studies have been done to investigate the expression of HSP105 in cutaneous malignancies. We therefore aimed to evaluate the expression of HSP105 in extramammary Paget disease (EMPD), SCC and basal cell carcinoma (BCC). This is the first study to investigate the expression of HSP105 in human skin cancers.

Materials and methods

Tissue specimens

Tissue samples for Western blot analysis were frozen in liquid nitrogen soon after excision and kept at −80 °C. The specimens consisted of one of EMPD, two of SCC, one of BCC and two of normal skin.

Archival formalin-fixed, paraffin-embedded tissues were used for immunohistochemical analysis; the clinical characteristics of

the patients are as follows. Twenty-five specimens of EMPD were obtained from 10 women and 15 men, age range 51–87 years (mean 74). Twenty-three specimens of SCC, of which three were metastatic lymph nodes, were analysed. The SCC population comprised 13 women and 10 men, age range 55–95 years (mean 78·9). Nine women and 14 men with a diagnosis of BCC had age range 51–83 years (mean 70·6).

The specimens were obtained from patients who underwent surgery between January 2002 and December 2004 in the Departments of Dermatology and Plastic and Reconstructive Surgery at Kumamoto University Hospital, from whom informed consent was obtained before surgery. Haematoxylin and eosin-stained sections were used for the initial light microscopy assessment.

Western blot

The frozen tissues were homogenized and 20 μL of the supernatant subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis before transfer to a nitrocellulose membrane. Blocking was achieved by incubating the membrane in 5% skimmed milk/Tris-buffered saline–Tween 20 (TBST) overnight at 4 °C. HSP105 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was used at a concentration of 1 : 500 and incubated with the membrane for 90 min at room temperature. The membrane was then washed and incubated with horseradish peroxidase-conjugated antirabbit antibody (1 : 3000) for 30 min. Membranes were thoroughly washed with 1 mol L^{-1} NaCl/TBST. Signals were detected using the ECL (enhanced chemiluminescence) system (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Immunohistochemistry

Paraffin sections (4 μm) were used for immunohistochemical examination after deparaffinization in xylene and rehydration through graded alcohols. Antigen retrieval was achieved by microwave treatment for 10 min in citric acid (0·01 mmol L^{-1}, pH 6·0), followed by cooling at room temperature for 60 min. Nonspecific staining was blocked with 5% normal horse serum. HSP105 antibody (Santa Cruz Biotechnology) was used at a concentration of 1 : 100 and incubated with the specimens overnight in a humidifying chamber. Endogenous peroxidase was blocked by immersing the sections into 1·5% hydrogen peroxide diluted with methanol for 1 min. Using the Envision method, antirabbit polyclonal antibody (EnVision+; DakoCytomation, Carpinteria, CA, U.S.A.) was used neat and incubated for 60 min at room temperature. Colour signalling was done using the 3, 3′-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan)-based detection method. Haematoxylin was used as a counterstain.

Immunohistochemical evaluation incorporated the percentage of stained cells per specimen and the staining intensity, compared with that of adjacent normal tissues as an internal control. The histochemical score (HSCORE)[9] was used to standardize the results of all three malignancies to allow for comparison. The HSCORE was calculated as follows: HSCORE = P_i (i + 1), where i = 0, 1, 2 and P_i (estimated percentage of stained tumour cells) varies from 0 to 100%. Staining intensity of tumour cells was graded according to the following three grades: 0, weak/negative; 1, distinct staining; 2 strong staining. A mean HSCORE of 100 was considered as overexpression of HSP105.

Statistical analysis

HSP105 expression was analysed using the nonparametric Mann–Whitney test, StatView version 5.0 for Windows (SAS Institute, Cary, NC, U.S.A.). $P < 0·05$ was considered significant.

Results

Western blot

Western blot analysis showed overexpression of HSP105 in EMPD and SCC, and low expression in BCC and normal skin (Fig. 1). HSP105 is constitutively expressed by normal skin in small amounts as evidenced by a visible but light band of HSP105.

Immunohistochemistry

Two investigators (F.C.M., T.K.) viewed all tissue sections. Figure 2 shows the staining pattern of EMPD, SCC and BCC.

EMPD showed positive staining of both the nucleus and cytoplasm, with a heterogeneous staining intensity; however, the sialomucin (mucous) component of the large Paget cells stained negative. Adjacent normal apocrine and eccrine glands were negative. The heterogeneity of staining necessitated the use of the HSCORE, which incorporates both intensity and distribution of staining, for data analysis. The HSCORE for

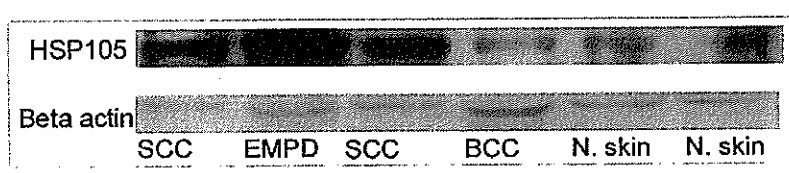

Fig 1. Western blot analysis of heat shock protein (HSP) 105 expression in squamous cell carcinoma (SCC), extramammary Paget disease (EMPD), basal cell carcinoma (BCC) and normal skin. The β-actin band was used as a control.

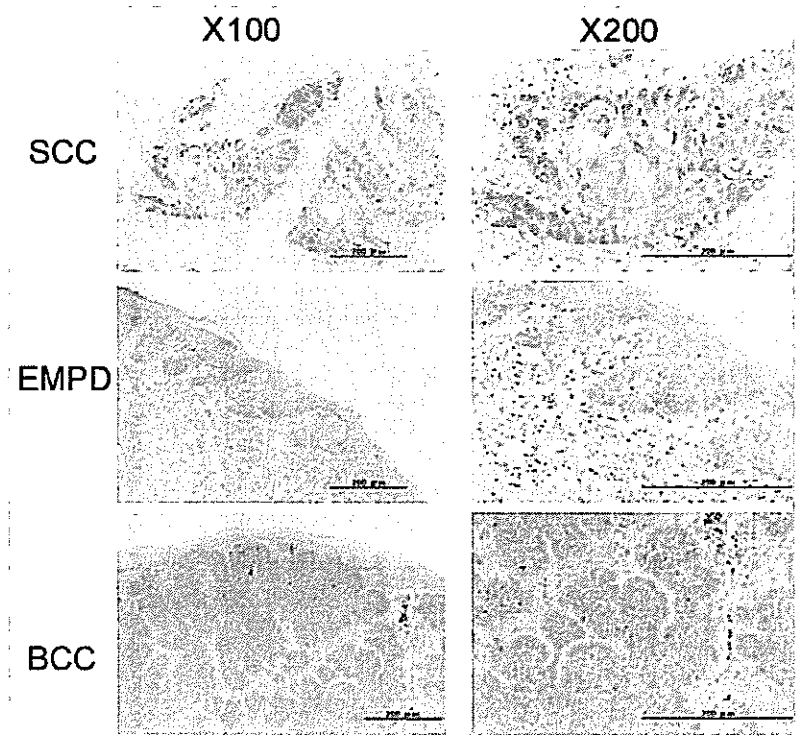

Fig 2. Immunohistochemical staining of heat shock protein (HSP) 105 in squamous cell carcinoma (SCC), extramammary Paget disease (EMPD) and basal cell carcinoma (BCC). The photomicrographs show low and high power views, respectively. SCC and EMPD highly express HSP105 while BCC is negative. Scale bar = 200 μm.

EMPD ranged from 5 to 300 with a mean of 103. Invasive EMPD, with dermal invasion of Paget cells, showed a high-level expression of HSP105 compared with intraepidermal lesions.

SCC highly expressed HSP105 with very strong nuclear and cytoplasmic staining. The HSCORE for primary SCC ranged from 5 to 300 (mean 132). The mean HSCORE for metastatic SCC was 270, indicating a very high level of expression of HSP105.

BCC failed to express HSP105. Even though the overlying epidermis minimally expressed HSP105, the tumour nests exhibited negative expression of the protein (Fig. 2). The HSCORE for BCC ranged from 5 to 60 (mean 17).

Immunohistochemistry results showed that 56% of EMPD, 60% of primary and 100% of metastatic SCC highly expressed HSP105 while only 13% of BCC lesions showed increased staining.

We also investigated by immunohistochemistry the expression of HSP105 in sun-damaged lesions of actinic keratosis; results showed that most specimens exhibited increased staining to HSP105 (data not shown).

Discussion

Although surgical excision is the most common method for treatment of EMPD, SCC and BCC, dermatologists often meet challenging cases. EMPD is an uncommon malignancy arising within the epidermis from native stem cells that differentiate towards glandular cells.[10] The common sites for EMPD are mainly the vulval, penile, scrotal and perianal regions. Often the lesion is so extensive that radical excisions sometimes result in a structural and functional compromise of the adjacent vital structures. Up to 60% of the cases recur[11] and often the tumour extends 2–5 cm beyond that visible under light microscopy; this necessitates repeated excisions, further compromising the integrity of the local structures. We have shown in this study that EMPD and SCC, especially the metastatic lesions, overexpress HSP105, and these tumours present ideal candidates for HSP105-based immunotherapy.

HSPs are present in cells at normal temperatures and act as molecular chaperones, assisting in the folding, unfolding and transport of various proteins. HSP105 is localized in the cytoplasm and nucleus of both stressed and nonstressed cells and it is believed to be translocated from the cytoplasm into the nucleus after heat stress.[12]

In this study we demonstrated by immunohistochemistry and Western blot analysis that HSP105 is overexpressed in EMPD and SCC but not in BCC. Some investigators have implicated HSP105 to be antiapoptotic by binding to p53, the anti-tumour gene product.[4] This study supports the hypothesis that BCC is a stable tumour as it failed to express the HSP105 protein. Some reports have shown that BCC is a relatively benign and slowly progressing tumour, rarely, if ever, metastasizing. It expresses HSP27, a marker of differentiation and proliferation, expressed in keratinocytes, which helps the cell in repair processes.[13] On the other hand, HSP27 is not expressed by SCC, showing the relative aggressiveness of SCC.[14]

Our study has shown that even though surrounding normal tissues in BCC show minimal expression of HSP105, the BCC nests themselves are negative. Kai *et al.* reported negative

staining of the adjacent normal epithelium in the colorectal region;[8] we have found that normal epidermis of skin was slightly reactive to HSP105. However, in grading positivity, a score of 1 was allocated to tumour cells whose staining was more intense than the adjacent normal epidermis.

The skin is the basis of protection from environmental factors and sunlight is a major contributor to skin damage. Sunlight is noted to be an aetiological feature in both SCC and BCC. In our patients, BCC was found mainly on the face. The adjacent epidermis of BCC sections, as noted above, stained slightly more than the tumour nests; we therefore queried whether or not sunburn may have a significant role to play in the expression of HSP105 in photodamaged lesions. Actinic keratosis is a premalignant condition to SCC; if left untreated 2–5% of these cases progress to SCC.[15] Cells that highly express HSP105 have an increased thermotolerance; in malignant cells this phenomenon increases the stability of abnormal cells, leading to progression of the diseases.

The detection of HSP105 by serological analysis of recombinant cDNA expression libraries prepared from tumour cells (SEREX) is of great importance as this method detects tumour antigens that elicit strong humoral and cellular immunity.[16] The task is now for immunologists to engineer an HSP105-based vaccine for testing on tumours that have proved to overexpress it. Development of a specific immunotherapeutic agent that can identify microscopic satellite tumours would be a great leap in the management of EMPD. Tamura *et al.*[17] showed that immunotherapy of mice with pre-existing cancers with HSP preparations derived from autologous cancer cells resulted in retarded progression of the primary cancer, reduced metastatic load and prolongation of life span. The effectiveness of anti-HSP90 drugs in the treatment of paediatric neuroblastoma and osteosarcoma was recently tested. It was found that there was a significant reduction in cell survival after exposure to the drugs, alone or in combination with others.[18] HSP105, by virtue of being discovered by SEREX, is highly immunogenic. Recent studies by Miyazaki *et al.*[7] successfully demonstrated that HSP105 DNA vaccination of mice which were challenged with colorectal and melanoma cell lines could elicit tumour rejection through activation of both cellular and humoral immunity. Therefore, advanced or metastatic EMPD and SCC could be good targets for HSP105 immunotherapy. HSP105 DNA vaccination studies for SCC in mice are under way in our laboratory.

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Identification of HLA-A2- or HLA-A24-Restricted CTL Epitopes Possibly Useful for Glypican-3-Specific Immunotherapy of Hepatocellular Carcinoma

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Abstract **Purpose and Experimental Design:** We previously reported that glypican-3 (GPC3) was overexpressed, specifically in hepatocellular carcinoma (HCC) and melanoma in humans, and it was useful as a novel tumor marker. We also reported that the preimmunization of BALB/c mice with dendritic cells pulsed with the H-2K^d-restricted mouse GPC3$_{298-306}$ (EYILSLEEL) peptide prevented the growth of tumor-expressing mouse GPC3. Because of similarities in the peptide binding motifs between H-2K^d and HLA-A24 (*A*2402*), the GPC3$_{298-306}$ peptide therefore seemed to be useful for the immunotherapy of HLA-A24$^+$ patients with HCC and melanoma. In this report, we investigated whether the GPC3$_{298-306}$ peptide could induce GPC3-reactive CTLs from the peripheral blood mononuclear cells (PBMC) of HLA-A24 (*A*2402*)$^+$ HCC patients. In addition, we used HLA-A2.1 (HHD) transgenic mice to identify the HLA-A2 (*A*0201*)–restricted GPC3 epitopes to expand the applications of GPC3-based immunotherapy to the HLA-A2$^+$ HCC patients.

Results: We found that the GPC3$_{144-152}$ (FVGEFFTDV) peptide could induce peptide-reactive CTLs in HLA-A2.1 (HHD) transgenic mice without inducing autoimmunity. In five out of eight HLA-A2$^+$ GPC3$^+$ HCC patients, the GPC3$_{144-152}$ peptide-reactive CTLs were generated from PBMCs by *in vitro* stimulation with the peptide and the GPC3$_{298-306}$ peptide-reactive CTLs were also generated from PBMCs in four of six HLA-A24$^+$ GPC3$^+$ HCC patients. The inoculation of these CTLs reduced the human HCC tumor mass implanted into nonobese diabetic/severe combined immunodeficiency mice.

Conclusion: Our study raises the possibility that these GPC3 peptides may therefore be applicable to cancer immunotherapy for a large number of HCC patients.

Hepatocellular carcinoma (HCC) is now spreading rapidly, especially in Asian and Western countries. It is clear that patients with hepatitis B or C–based liver cirrhosis are at high risk for developing HCC (1), and patients with hepatitis treated surgically or by other therapies are also at high risk for recurrence (2). Furthermore, the liver function of these patients is often very poor, so further treatment for recurrence is often restricted. As a result, the prognosis of HCC remains poor, and new therapies for the prevention of cancer development and recurrence, i.e., adjuvant therapy, is urgently needed. As for melanoma, the age-adjusted incidence rates have been increasing in most fair-skinned populations in recent decades (3). In 2005, it is estimated that 59,580 Americans will be diagnosed to have melanoma, and 7,770 will die from the disease (4).

We and others previously reported that glypican-3 (GPC3) was overexpressed in most types of HCC (5–9) and melanoma in humans (8), and we also previously reported that an H-2K^d-restricted antigenic peptide, the mouse GPC3$_{298-306}$ (EYILSLEEL) peptide, could be recognized by mouse CD8$^+$ CTLs. In addition, these CTLs rejected tumor expressing mouse GPC3 both *in vitro* and *in vivo* (10). Because the structural motifs of peptides bound to HLA-A24 (*A*2402*) and mouse H-2K^d are similar, we investigated whether the GPC3 peptide was also useful as a cancer immunotherapy modality for HLA-A24$^+$ HCC patients. The gene frequency of HLA-A24 (*A*2402*) is relatively high in Asian populations, especially in the Japanese, whereas it is low in Caucasians. On the other hand, The gene frequency of

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HLA-A2 (*A*0201*) is high among various ethic groups, including both Asians and Caucasians (11). Therefore, it is suggested that the HLA-A2-restricted and GPC3-derived CTL epitopes might be very useful for the immunotherapy of many patients with HCC and melanoma all over the world. In the present study, we identified human GPC3-derived CTL epitopes restricted by HLA-A2 using HLA-A2.1 (HHD) transgenic mice (Tgm) and examined whether these HLA-A2 or HLA-A24-restricted epitope peptides could induce GPC3-reactive CTLs from peripheral blood mononuclear cells (PBMC) of patients with HCC.

Materials and Methods

Mouse. HLA-A2.1 (HHD) Tgm; H-2D$^{b-/-}$β2m$^{-/-}$ double knockout mice introduced with human β2m-HLA-A2.1 (α1 α2)-H-2D^{b} (α3 transmembrane cytoplasmic) (HHD) monochain construct gene were generated in the Department SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, France (12, 13) and kindly provided by Dr. F.A. Lemonnier. Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) female mice at 6 weeks of age were purchased from CLEA Japan (Tokyo, Japan).

Patients, blood samples, and cell lines. Blood samples from patients with HCC were obtained during routine diagnostic procedures after obtaining a formal agreement signed by the patients in Kumamoto University Hospital from April to September 2005. Human liver cancer cell lines, SK-Hep-1 and T2-A0201 (a TAP-deficient and HLA-A*0201-positive cell line; refs. 14, 15), were provided by Kyogo Ito of Kurume University. Human liver cancer cell lines HepG2 and HuH-7 endogenously expressing GPC3, and GPC3^{-} colon cancer cell line SW620, were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). C1R-A*2402 (an *HLA-A*2402* transfectant of C1R cells expressing a trace amount of HLA class I molecule; ref. 15) were generous gifts from Dr. Masafumi Takiguchi. The expression of HLA-A2 and HLA-A24 in these cell lines were examined using flow cytometry with an anti-HLA-A2 monoclonal antibody (mAb), BB7.2 and anti-HLA-A24 mAb (One Lambda, Inc., Canoga Park, CA), respectively, in order to select target cell lines for CTL assays. The origins and HLA genotypes of these cell lines have been described elsewhere (16, 17). These cells were maintained *in vitro* in RPMI 1640 or DMEM supplemented with 10% FCS.

Induction of GPC3-reactive mouse CTLs and IFN-γ enzyme-linked immunospot assay. Human GPC3-derived peptides (purity >90%) sharing the amino acid sequences with mouse GPC3 and carrying binding motifs for *HLA-A*0201*-encoded molecules, were identified using BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD) and we purchased a total of nine peptides carrying HLA-A2 (*A*0201*) binding motifs (Table 1) from Biologica (Tokyo, Japan). The immunizations of mice with peptides were done as previously described (7). In brief, bone marrow (BM) cells (2 × 10^{6}) from HLA-A2.1 (HHD) Tgm were cultured in RPMI 1640 supplemented with 10% FCS, together with granulocyte macrophage colony-stimulating factor (5 ng/mL) and 2ME (0.8 ng/mL) for 7 days in 10-cm plastic dishes, and these BM-dendritic cells (DC) were pulsed with the mixture of GPC3 peptides carrying HLA-A2 binding motifs (1 μmol/L for each peptide) at 37°C for 2 hours. We primed the HLA-A2.1 (HHD) Tgm with this syngeneic BM-DC vaccine (5 × 10^{5}/mouse) into the peritoneal cavity once a week for two weeks. Seven days after the last immunization, the spleens were collected and CD4^{-} spleen cells were isolated by negative selection with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude any nonspecific IFN-γ production by CD4^{+} spleen cells cocultured with the BM-DC. The CD4^{-} spleen cells (2 × 10^{6}/well) were stimulated with syngeneic BM-DC (2 × 10^{5}/well) pulsed with each peptide *in vitro*. Then, 6 days later, the frequency of cells producing IFN-γ/2 × 10^{4} CD4^{-} spleen cells upon stimulation with syngeneic BM-DC (1 × 10^{4}/well), pulsed with or without each peptide, was assayed in an enzyme-linked immunospot (ELISPOT) assay as previously described (18).

Induction of GPC3-reactive human CTLs. We isolated PBMCs from the heparinized blood of HLA-A24^{+} and/or HLA-A2^{+} Japanese patients with HCC or healthy donors by means of Ficoll-Conray density gradient centrifugation, and peripheral monocyte-derived DCs were generated as described previously (19, 20). CD8^{+} T cells were isolated using CD8 microbeads (Miltenyi Biotec) from the PBMC of the same donors, and thereafter, peptide-reactive CD8^{+} CTLs were generated (19, 20). Five days after the last stimulation, the cytotoxic activities of the CTLs were measured by a ^{51}Cr release assay.

CTL responses against cancer cell lines. CTLs were cocultured with each cancer cell line as a target cell (5 × 10^{3}/well) at the indicated effector/target ratio and ^{51}Cr release assay was done as described (21). The blocking of HLA-class I or HLA-DR, was done as follows. Before the coculture of CTLs with a cancer cell line in a ^{51}Cr release assay or ELISPOT assay, target cancer cells were incubated for 1 hour with 10 μg/mL anti-class I mAb W6/32 or 10 μg/mL anti-HLA-DR mAb, H-DR-1, and then the effects of mAbs on either the cytotoxic activity or production of IFN-γ by CTLs were examined as reported previously (22).

Histologic and immunohistochemical analysis. Immunohistochemical staining of CD8 or CD4 in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the GPC3$_{144-152}$ peptides and the staining of

Table 1. GPC3-derived peptides conserved between human and mouse GPC3 and predicted to be bound to HLA-A2 (*A*0201*)

A2-binding peptide	Position	Subsequence residue listing	HLA-A2 binding score*
GPC3A2-1	44-52	RLQPGLKWV	879
GPC3A2-2	102-110	FLIIQNAAV	319
GPC3A2-3	144-152	FVGEFFTDV	828
GPC3A2-4	155-163	YILGSDINV	162
GPC3A2-5	169-177	ELFDSLFPV	1055
GPC3A2-6	254-268	RMLTRMWYC	1259
GPC3A2-7	281-289	VMQGCMAGV	196
GPC3A2-8	326-334	TIHDSIQYV	496
GPC3A2-9	522-560	FLAELAYDL	402

*Binding scores were estimated by using BIMAS software (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker.comboform).

apoptotic cells with terminal deoxynucleotidyl transferase–mediated nick end labeling methods (ApopTag fluorescein *in situ* apoptosis detection kits; Serologicals Corporation, Norcross, GA) in tumor specimens of patients with HCC were done as described previously (23, 24). In addition, immunohistochemical staining of HLA-class I in HCC tumor tissue specimens were done by using anti-HLA-class I mAb, EMR 8-5.[5]

Detection by ELISA of the serum-soluble GPC3 protein. Detection of the serum-soluble GPC3 protein was done by an indirect ELISA using the rabbit anti-GPC3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (7). We used recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) as a standard, and the presence of >106 ng/mL of serum GPC3 protein was considered to be positive.

Transfer of CTLs to the NOD/SCID mice implanted with a human HCC cell line. The transfer of GPC3-reactive CTLs to the immunodeficient mice implanted with a human HCC cell line was done as described previously (7). Briefly, we s.c. inoculated SK-Hep1/GPC3 cells (1×10^7) positive for both HLA-A2 and HLA-A24 at the right flank of NOD/SCID mice. When the diameter of these tumors reached 5×5 mm on day 9 after tumor inoculation into mice, we intravenously injected the mixture of GPC3 peptide-reactive CTL lines or irrelevant HIV peptides; HLA-A2-restricted SLYNTYATL peptide and HLA-A24-restricted RYLRDQQLL peptide, stimulated $CD8^+$ T cells (3×10^6) established from four HLA-A24-positive or two HLA-A2-positive HCC patients, or saline alone. T cells were i.v. injected one more times on day 14. The $CD8^+$ T cells stimulated with HLA-A24-restricted $GPC3_{298\text{-}306}$ peptide or HIV (RYLRDQQLL) peptide and derived from two independent $HLA\text{-}A24^+$ HCC patients were mixed, and injected into three NOD/SCID mice on day 9, and the mixture of peptide-stimulated $CD8^+$ T cells from two other $HLA\text{-}A24^+$ HCC patients distinct from the T cell donors at the first injection, were injected into the mice on day 14. The HLA-A2-restricted peptide-stimulated $CD8^+$ T cells from one $HLA\text{-}A2^+$ HCC patient were also injected into a NOD/SCID mouse on day 9, followed by the injection on day 14 with the peptide-stimulated $CD8^+$ T cells derived from another $HLA\text{-}A2^+$ HCC patient.

Statistical analysis. The two-tailed Student's *t* test was used to evaluate the statistical significance of differences in the data obtained by ELISPOT assay. The statistical significance of the differences in several factors between patients showing a successful CTL induction and other patients was assessed by a χ^2 test. $P < 0.05$ was considered to be significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

Identification of HLA-A2-restricted CTL epitopes by using HLA-A2.1 (HHD) Tgm. To identify HLA-A2-restricted epitopes by using HLA-A2.1 (HHD) Tgm, we selected nine kinds of peptides having amino acid sequences conserved between human and mouse GPC3 and having high predicted binding scores to HLA-A2 (*A*0201*; Table 1). $CD4^-$ spleen cells from HLA-A2.1 (HHD) Tgm immunized i.p. twice with BM-DCs pulsed with the mixture of these nine peptides were again stimulated *in vitro* with BM-DCs pulsed with each peptide, and we found that $CD4^-$ spleen cells stimulated *in vitro* with the $GPC3_{144\text{-}152}$ peptide produced the largest amount of IFN-γ in a peptide-specific manner in ELISPOT assays. These $CD4^-$ spleen cells (2×10^4/well), showed 36 ± 2.85 spot counts/well, in response to the BM-DCs pulsed with the $GPC3_{144\text{-}152}$ peptide, whereas they showed 23 ± 1.84 spot counts/well in the presence of BM-DCs without peptide loading ($P < 0.005$) indicating that about $(36\text{-}23) / 2 \times 10^4 = 0.065\%$ of $CD4^-$ spleen cells were reactive to the GPC3 peptide. When we used syngeneic BM-DCs pulsed with a HLA-A2-binding HIV-derived peptide; SLYNTYATL as a control, no significant response (8.84 ± 1.73) was observed. The summation of the diameter of the IFN-γ ELISPOT observed in $CD4^-$ spleen cells stimulated with the $GPC3_{144\text{-}152}$ peptide pulsed BM-DCs was $1{,}878 \pm 131$ μm, that stimulated with the HIV-derived SLYNTYATL peptide pulsed BM-DCs was 437 ± 77 μm, and that observed in the presence of BM-DC without peptide loading was 762 ± 131 μm ($P < 0.001$). These assays were done thrice with similar results. As shown in Fig. 1B, the differences in the spot counts (left) or spot diameters (right) between stimulations with peptide pulsed BM-DC and BM-DC without peptide loading clearly revealed the $GPC3_{144\text{-}152}$ peptide-specific response of $CD4^-$ spleen cells. As for other peptides, no significant peptide-specific response was observed. These results suggest that the $GPC3_{144\text{-}152}$ peptide could be a CTL epitope peptide in HLA-A2.1 (HHD) Tgm, and we also expected this $GPC3_{144\text{-}152}$ peptide to be an epitope for human CTLs.

The immunization of the HLA-A2-restricted peptide, $GPC3_{144\text{-}152}$, did not induce autoimmunity in HLA-A2.1 (HHD) Tgm. It is well known that melanocyte-differentiation antigens such as MART-1 or gp100 are very useful for immunotherapy of melanoma patients, but they sometimes cause autoimmunity, such as vitiligo or uveitis, following vaccination. We previously reported that the immunization of the $GPC3_{298\text{-}306}$ peptide did not cause autoimmunity in BALB/c mouse (9). To investigate whether the immunization of mice with HLA-A2-restricted GPC3-derived peptides causes autoimmunity, the immunohistochemical staining of several organs with anti-CD4 and anti-CD8 mAb was done in HLA-A2.1 (HHD) Tgm immunized with a mixture of nine GPC3 peptides 7 days before the analysis. As shown in Fig. 2, we could not find any pathologic changes, such as lymphocyte infiltration or tissue destruction and repair in skin, lung, brain, heart, liver, and kidney of HLA-A2.1 (HHD) Tgm. The same result was also observed when mice were vaccinated with the $GPC3_{144\text{-}152}$ peptide alone ($n = 3$; data not shown). These results indicate that the $GPC3_{144\text{-}152}$ peptide-reactive $CD8^+$ CTLs do not attack the normal tissue specimens that we investigated.

Induction of GPC3-reactive CTLs from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. We evaluated the cytotoxic activity of CTLs that were induced with the $GPC3_{298\text{-}306}$ or $GPC3_{144\text{-}152}$ peptide from PBMCs isolated from HCC patients. PBMCs were isolated from HCC patients positive for HLA-A24 and/or HLA-A2, and $CD8^+$ T cells sorted from the PBMCs were cocultured with autologous monocyte-derived DCs pulsed with each peptide as described in Materials and Methods. CTLs from PBMCs of $HLA\text{-}A2^+$ HCC patients stimulated with the $GPC3_{144\text{-}152}$ peptide or CTLs from PBMCs of $HLA\text{-}A24^+$ HCC patients stimulated with the $GPC3_{298\text{-}306}$ peptide exhibited cytotoxicity against peptide-pulsed target cells. The representative data of CTLs restricted by HLA-A2 or HLA-A24 were shown in Fig. 3A. The CTLs induced from PBMCs of patient A2-8 showed cytotoxic activity to T2-A0201 cells (HLA-A2+) pulsed with the $GPC3_{144\text{-}152}$ peptide, but not to T2-A0201 cells without peptide loading by ^{51}Cr release assay. The CTLs induced from PBMCs of patient A24-12 exhibited cytotoxic

[5] T. Torigoe, et al. Immunohistochemical analysis of HLA class I expression in tumor tissues revealed unusually high frequency of down-regulation in breast cancer tissues submitted.

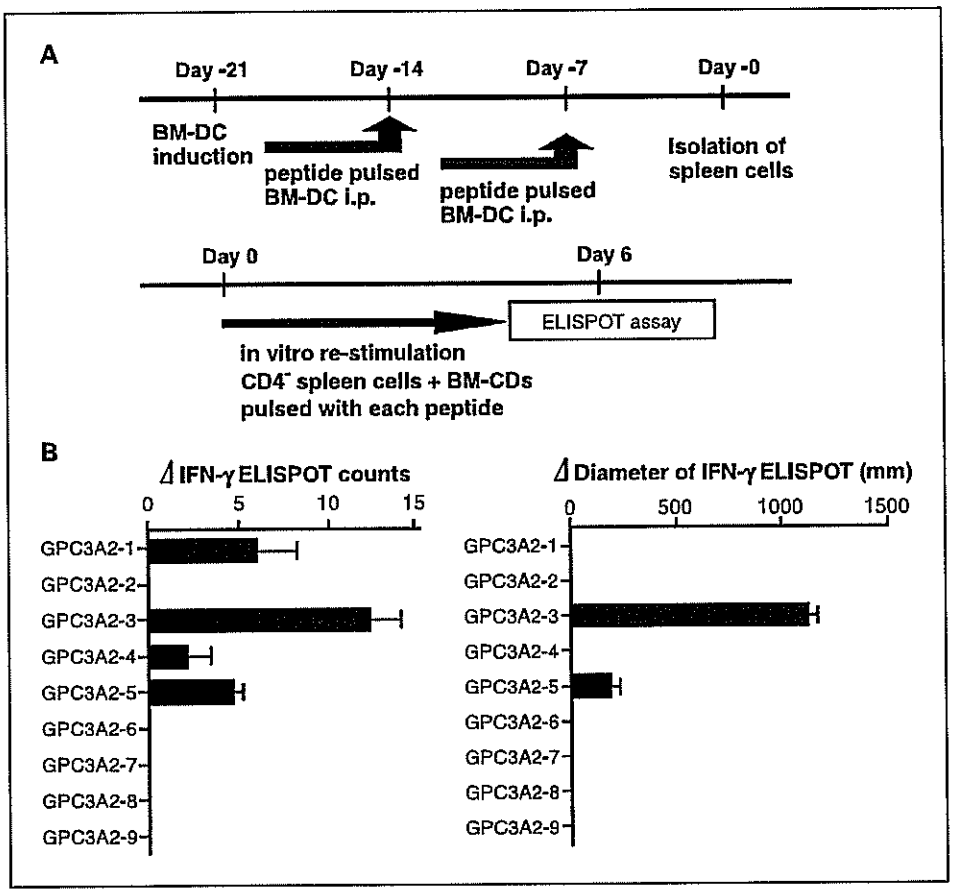

Fig. 1. Identification of HLA-A2-restricted CTL epitopes of GPC3 by using HLA-A2.1 (HHD) Tgm. *A*, protocol for identification of GPC3-derived and HLA-A2-restricted CTL epitopes. We primed the HLA-A2.1 (HHD) Tgm with BM-DCs (5×10^5) pulsed with the mixture of GPC3-derived peptides carrying HLA-A2 (*A*0201*) binding motif into the peritoneal cavity once a week for two weeks. Seven days after the last DC vaccination, spleens were collected and $CD4^-$ spleen cells (2×10^6/well) were stimulated with syngeneic BM-DCs (2×10^5/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured $CD4^-$ spleen cells as responder cells in ELISPOT assay to evaluate GPC3-specific response of CTLs. *B, bar graph*, IFN-γ ELISPOT counts/2×10^4 $CD4^-$ spleen cells cocultured with peptide pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*left*). *Bar graph*, summation of IFN-γ ELISPOT diameters/2×10^4 $CD4^-$ spleen cells cocultured with peptide-pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*right*). *Columns*, mean of triplicate assays; *bars*, SE. All assays were done thrice with similar results.

activity to the C1R-A*2402 cells (HLA-A24+) pulsed with the $GPC3_{298-306}$ peptide, but not to C1R-A*2402 cells without peptide loading. These results indicate that these CTLs had peptide-specific cytotoxicity. Other CTLs induced from the nine patients A2-1, A2-2, A2-3, A2-4, A24-1, A24-3, A24-4, A24-6, and A24-7 similarly exhibited peptide-specific cytotoxicity against peptide-pulsed target cells (data not shown).

Furthermore, we used GPC3 transfectants, SK-Hep1/GPC3 (GPC3+, HLA-A2+, HLA-A24+) or SW620/GPC3 (GPC3+, HLA-A2+, HLA-A24+) as target cells and examined whether we could find GPC3-specific cytotoxic activity of CTLs. As shown in Fig. 3B, the CTLs induced from PBMCs of patient A2-3 by stimulation with the $GPC3_{144-152}$ peptide showed specific cytotoxicity against SK-Hep1/GPC3, but not against GPC3-negative SK-Hep1. Similarly, the $GPC3_{298-306}$ peptide–induced CTLs showed specific cytotoxicity against SW620/GPC3 in patient A24-7 or against SK-Hep1/GPC3 in patient A24-12, but not against SK-Hep1 or SW620, respectively, which did not endogenously express GPC3. These findings indicate that these peptides can be processed naturally in cancer cells, and the peptides in the context of HLA-A2 or HLA-A24 can be expressed on the cell surface of cancer cells to be recognized by the CTLs.

When we think about the application of GPC3 to cancer immunotherapy, the most important point is that these GPC3-reactive CTLs can exhibit specific cytotoxicity to the tumors endogenously expressing GPC3. We thus investigated whether these CTLs could kill human HCC cell lines expressing both endogenous GPC3 and the restriction HLA class I molecules. As shown in Fig. 3C, we could generate GPC3-reactive CTLs by stimulation with the $GPC3_{144-152}$ peptide and these CTLs exhibited cytotoxic activity to HepG2 (GPC3+, HLA-A2+, and

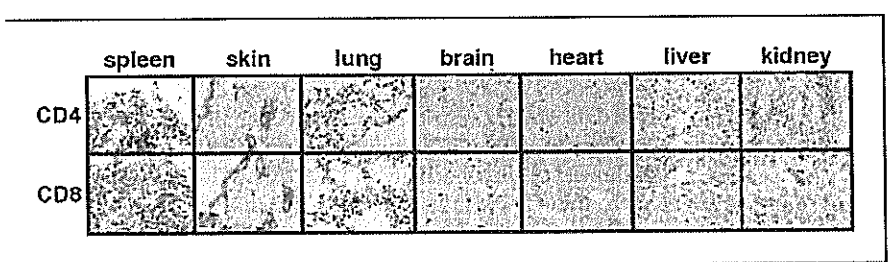

Fig. 2. Immunohistochemical staining with anti-CD4 or anti-CD8 mAb in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the $GPC3_{144-152}$ peptides. These tissue specimens were removed and analyzed 7 days after the second DC vaccination (original magnification, ×200).

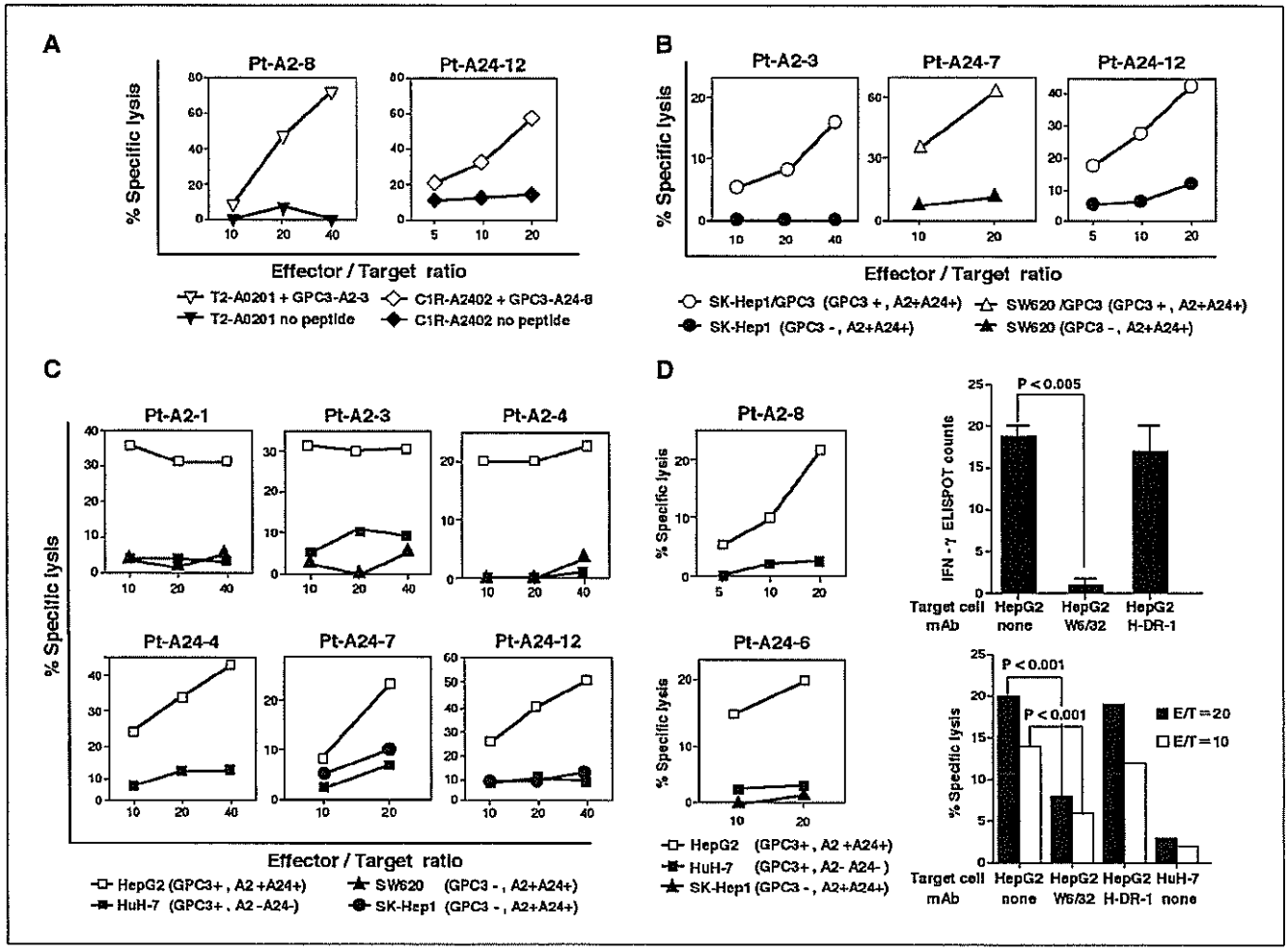

Fig. 3. CTL induction from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. *A* and *B*, GPC3 peptide-reactive CTLs were generated from $CD8^+$ T cells of $HLA\text{-}A2^+$ and/or $HLA\text{-}A24^+$ HCC patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the $GPC3_{144\text{-}152}$ or $GPC3_{298\text{-}306}$ peptide, the CTLs were subjected to a standard ^{51}Cr release assay at the indicated effector/target ratio. Their cytotoxicity against the $GPC3_{298\text{-}306}$ peptide pulsed C1R-A2402 cells or T2-A0201 cells, and each unpulsed cells (*A*), or $GPC3^-$ $HLA\text{-}A2^+$, $HLA\text{-}A24^+$ HCC cell line SK-Hep-1, $GPC3^-$ $HLA\text{-}A2^+$, $HLA\text{-}A24^+$ colon cancer cell line SW620, and those cell lines transfected with the human *GPC3* gene; SK-Hep-1/GPC3 or SW620/GPC3 (*B*) were examined by a ^{51}Cr release assay. *C* and *D*, $GPC3^+$ $HLA\text{-}A2^+$, $HLA\text{-}A24^+$ HCC cell line HepG2, $GPC3^+$ $HLA\text{-}A2^-$, $HLA\text{-}A24^-$ HCC cell line HuH-7, and $GPC3^-$ tumor cell lines SW620 and SK-Hep1 were used as target cells (*left*). *Points*, percentage of specific lysis calculated based on the mean values of a triplicate assay. *D*, inhibition of cytotoxicity by anti-HLA class I mAb (*right*). After the target HepG2 cells were incubated with anti-HLA class I mAb (W6/32, IgG_{2a}) or anti-HLA DR mAb (H-DR-1, IgG_{2a}), respectively, for 1 hour, the CTLs generated from PBMCs of patient A2-8 by stimulation with $GPC3_{144\text{-}152}$ peptide (*top*) or CTLs generated from patient A24-6 using the $GPC3_{298\text{-}306}$ peptide (*bottom*) were added. IFN-γ production (*top*; IFN-γ ELISPOT assay) and cytotoxicity (*bottom*; ^{51}Cr release assay) were markedly inhibited by W6/32, but not by H-DR-1.

HLA-A24+), but not to HuH-7 (GPC3+, HLA-A2−, and HLA-A24−) or SW620 ($GPC3^-$, HLA-A2+, and HLA-A24+) in patients A2-1, A2-3, and A2-4. Similarly, we could generate GPC3-reactive CTLs by stimulation of PBMCs with the $GPC3_{298\text{-}306}$ peptide and these CTLs exhibited cytotoxic activity to HepG2, but not to HuH-7 or SK-Hep-1 ($GPC3^-$, HLA-A2+, HLA-A24+) in patients A24-4, A24-7, and A24-12.

In an HLA-class I blocking experiment, anti-HLA class I mAb W6/32 markedly inhibited the IFN-γ production stimulated with HepG2 cells in ELISPOT assay of CTLs generated from patient A2-8 by stimulation with the $GPC3_{144\text{-}152}$ peptide (Fig. 3D, top), and inhibited cytotoxic activity against HepG2 cells in ^{51}Cr release assay of CTLs generated from patient A24-6 by stimulation with the $GPC3_{298\text{-}306}$ peptide (Fig. 3D, bottom), but anti-HLA-DR mAb, H-DR-1 did not inhibit the response of CTLs. These results clearly indicate that these CTLs recognized HepG2 in a HLA-class I–restricted manner.

As shown in Table 2, we could induce GPC3-reactive CTLs from PBMCs in ~50% of either the HLA-A2− or HLA-A24-positive HCC patients. In patients A2-6, A24-5, A24-9, and A24-11 who did not express GPC3 in tumor tissues, GPC3-reactive CTLs could not be induced from their PBMCs. Among eight HLA-A2-positive HCC patients who expressed GPC3 in HCC tissue or produced soluble GPC3 in sera, patients A2-1, A2-2, A2-3, A2-4, A2-6, A2-7, A2-9, and A2-10, GPC3-reactive CTLs could be generated from the PBMCs of only four patients (50%). In patient A2-6, GPC3 was detected only in the serum but not in HCC tumor tissue. It was thought to be possible that the majority of GPC3 protein was secreted away in this type of HCC cell as described previously (7). Among six HLA-A24-positive patients who expressed GPC3 in tumor tissue, patients A24-1, A24-2, A24-3, A24-6, A24-10, and A24-12, GPC3-reactive CTLs could be generated from the PBMCs of only four patients (67%). We also examined whether it was possible to

induce GPC3-specific CTLs from PBMCs isolated from healthy donors (each HLA type, $n = 3$), but we failed to generate GPC3-specific and HLA-A2- or HLA-A24-restricted CTLs even though PBMCs were stimulated with the peptides thrice *in vitro* (data not shown). These results suggest that GPC3-reactive CTLs could only be induced in patients who expressed GPC3 in tumor tissue, thus, indicating the existence of GPC3-reactive CTL precursors in patients with GPC3$^+$ HCC. We also examined whether GPC3-reactive CTLs could be generated more frequently from PBMCs isolated from HCC patients positive for serum-soluble GPC3. As shown in Table 2, the presence of serum-soluble GPC3 did not correlate statistically with the successful induction of GPC3-reactive CTLs. As a result, we could not observe the enhancement of CTL induction efficiency via possible antigen presentation of soluble serum GPC3 through HLA-class II pathways to CD4$^+$ T cells or cross-presentation through the HLA class I pathway to CD8$^+$ T cells (25, 26) in patients positive for serum GPC3.

Inoculation of the GPC3 peptide-induced CTLs reduced growth of a GPC3$^+$ human HCC tumor cell line implanted into NOD/SCID mouse. To investigate the effects of GPC3 peptide-reactive CTL inoculation into the mice implanted with the GPC3$^+$ human HCC cell line, we s.c. inoculated SK-Hep1/GPC3 cell lines positive for both HLA-A2 and HLA-A24 into NOD/SCID mice, and i.v. injected the mixture of CTLs generated from several HCC patients positive for HLA-A2 or HLA-A24 into mice implanted with SK-Hep1/GPC3 when the diameter of these tumors reached 5 × 5 mm in size as described in Materials and Methods. The CTLs injected into mice were prepared by stimulating peripheral blood CD8$^+$ T cells with HLA-A2- or HLA-A24-restricted GPC3-epitope peptides or control-irrelevant HIV peptides as described in Materials and Methods. The tumor sizes of four individual mice in each group (Fig. 4A) and mean ± SD of tumor sizes in each group (Fig. 4B) were evaluated. After 5 days from the second inoculation of GPC3 peptide-reactive CTLs, the tumor size of SK-Hep1/GPC3 was apparently reduced in comparison to the size of tumor mass implanted into NOD/SCID mice injected with control T cells or saline alone ($P < 0.01$). These results clearly indicate the efficacy of adoptive GPC3 peptide-reactive CTL transfer therapy for GPC3$^+$ tumor in mice.

Discussion

In this article, we identified HLA-A24-restricted or HLA-A2-restricted GPC3 CTL epitope peptides, and found that

Table 2. Expression of GPC3 in HCC tissue, quantification of serum-soluble GPC3, and GPC3-specific CTL induction in HCC patients

	Age	Gender	State of tumor*	GPC3 expression†	Serum GPC3‡	HLA expression§	CTL induction‖
HLA-A2 (*A*0201*) – positive patients							
Pt-A2-1	80	F	IIIa	+	+	+	+
Pt-A2-2	72	M	II	+	+	+	+
Pt-A2-3	67	F	II	ND	+	ND	+
Pt-A2-4	54	M	I	+	–	+	+
Pt-A2-5	57	M	I	ND	–	ND	–
Pt-A2-6	66	M	I	–	+	–	–
Pt-A2-7	54	M	IIIa	+	–	+	–
Pt-A2-8	73	M	II	ND	–	ND	+
Pt-A2-9	68	F	IIIa	+	–	+	–
Pt-A2-10	54	M	II	+	+	+	–
HLA-A24 (*A*2402*)-positive patients							
Pt-A24-1	60	M	IVa	+	+	+	+
Pt-A24-2	57	M	IVa	+	+	+	–
Pt-A24-3	75	F	IIIa	+	+	+	+
Pt-A24-4	59	M	IIIa	ND	–	ND	+
Pt-A24-5	52	M	IVb	–	–	+	–
Pt-A24-6	65	M	I	ND	+	ND	+
Pt-A24-7	61	M	I	ND	–	ND	+
Pt-A24-8	74	M	II	ND	–	ND	–
Pt-A24-9	59	M	IVb	–	–	–	–
Pt-A24-10	69	M	IVa	+	+	+	–
Pt-A24-11	72	M	II	–	–	+	–
Pt-A24-12	61	M	IIIa	+	+	+	+

Abbreviations: F, female; M, male; ND, not determined.
*Tumor-node-metastasis classification.
†Positive (+) or negative (–) staining of tumor cells in contrast with peritumor normal tissue as background staining.
‡Serum levels >106 ng/mL were evaluated as positive.
§Immunohistochemical staining of the membrane of tumor cells was evaluated as positive.
‖Specific lytic activity (≧20%) at E:Tratio = 20 against HepG2 target cells was evaluated as positive by ^{51}Cr release assay.