PFU, plaque-forming unit; IRES, internal ribosome entry site; FITC, fluorescent isothiocyanate; MFI, mean fluorescence intensity.

CONFLICT OF INTEREST

Y Urata is an employee of Oncolys BioPharma, Inc., the manufacturer of OBP-401 (Telomescan). The remaining authors declare no conflict of interest.

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Radiofrequency ablation for hepatocellular carcinoma induces glypican-3 peptide-specific cytotoxic T lymphocytes

DAISUKE NOBUOKA[1,4*], YUTAKA MOTOMURA[1,5*], HIROFUMI SHIRAKAWA[1], TOSHIAKI YOSHIKAWA[1], TOSHIMITSU KURONUMA[1], MARI TAKAHASHI[1], KOHEI NAKACHI[2], HIROSHI ISHII[2], JUNJI FURUSE[2], NAOTO GOTOHDA[3], SHINICHIRO TAKAHASHI[3], TOSHIO NAKAGOHRI[3], MASARU KONISHI[3], TAIRA KINOSHITA[3], HIROYUKI KOMORI[5], HIDEO BABA[5], TOSHIYOSHI FUJIWARA[4] and TETSUYA NAKATSURA[1]

[1]Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, [2]Division of Hepatobiliary and Pancreatic Oncology, [3]Division of Digestive Surgery, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577; [4]Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558; [5]Department of Gastroenterology and Hepatology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

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Abstract. Glypican-3 (GPC3), a carcinoembryonic antigen, is an ideal target for anticancer immunotherapy against hepatocellular carcinoma (HCC). In this study, we attempted to compare the induction of the GPC3-specific T-cell-mediated immune response after locoregional therapies in HCC patients and tumor-bearing mice. Twenty-seven HCC patients treated with locoregional therapies, including radiofrequency ablation (RFA), surgical resection and transcatheter arterial chemoembolization (TACE), were prospectively enrolled in this study. Additionally, we performed RFA experiments using a mouse model. GPC3-specific T-cell response was investigated pre-treatment and post-treatment by an interferon-γ enzyme-linked immunospot assay using peripheral blood mononuclear cells from HCC patients and lymph node cells from tumor-bearing mice. Circulating GPC3-specific cytotoxic T lymphocytes (CTLs) were increased in 5 of 9 patients after RFA and in 4 of 9 patients after TACE, but in only 1 of 9 patients after surgical resection. All 7 patients with GPC3-expressing HCCs exhibited an increase in GPC3-specific CTLs after RFA or TACE, whereas none of the 7 patients did after surgical resection. The number of increased GPC3-specific CTLs after RFA was significantly larger than that after surgical resection (P=0.023). Similarly, the frequency of GPC3-specific CTLs after RFA was significantly greater than that after surgical resection in the mouse model (P=0.049). We validated for the first time the stronger effect on the immune system brought by RFA compared with surgical resection for HCC patients and tumor-bearing mice. Combined treatment of RFA and immunotherapy is a reasonable strategy against HCC.

Correspondence to: Dr Tetsuya Nakatsura, Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan
E-mail: tnakatsu@east.ncc.go.jp

*Contributed equally

Abbreviations: GPC3, glypican-3; HCC, hepatocellular carcinoma; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization; CTL, cytotoxic T lymphocyte; CT, computed tomography; TNM, tumor-node-metastasis; UICC, the Union for International Cancer Control; PBMC, peripheral blood mononuclear cell; IFN, interferon; ELISPOT, enzyme-linked immunospot; HSP105, heat shock protein 105; CMV, cytomegalovirus; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist II; hTERT, human telomerase reverse transcriptase; MRP3, multidrug resistance-associated protein 3

Key words: hepatocellular carcinoma, radiofrequency ablation, glypican-3, cytotoxic T lymphocyte, immunotherapy

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and most serious cancers worldwide (1). Locoregional therapies, including radiofrequency ablation (RFA), surgical resection, and transcatheter arterial chemoembolization (TACE), are recognized as the gold-standard therapies for HCC patients whose cancer lesions are limited to the liver (2). However, the recurrence rate remains quite high despite potentially curative treatment (3,4). The reasons for this are as follows: first, a multicentric new tumor frequently occurs from underlying active hepatitis or cirrhosis and, second, a small tumor undetectable by imaging modalities frequently exists before treatment and would be left untreated (5). Therefore, the establishment of effective adjuvant therapy to prevent recurrence is urgently required, and

clinical trials are ongoing throughout the world (6). However, at the present time, there is no universal consensus (2,7,8).

Previous studies have reported that local tumor ablation treatments, such as RFA and cryoablation, not only destroy tumor tissue but also induce a marked inflammatory response both locally and systemically (9,10). Unlike surgical resection, tumor ablation treatment generates tumor cell necrosis (11), followed by the release of tumor-associated antigens (12). These antigens can be uptaken, processed, and presented by dendritic cells (10,13), and then an antigen-specific T-cell-mediated immune response can be induced (9). If this induction is sufficiently steady and reliable, it may provide the basis for adjuvant immunotherapy, which is an attractive strategy.

Glypican-3 (GPC3) belongs to the glypican family of heparan sulfate proteoglycans that are linked to the outer surface of the cell membrane through a glycosylphosphatidylinositol anchor (14). GPC3 is one of the carcinoembryonic antigens overexpressed in HCC (15-17). We have shown that GPC3 is an ideal target for anticancer immunotherapy because its expression is specifically detected in ~80% of HCCs even in the early stages and is correlated with a poor prognosis (18-21). Moreover, GPC3-specific cytotoxic T lymphocytes (CTLs) have a high level of killing activity against HCC tumor cells (22). We have finished the phase I clinical trial of a GPC3-derived peptide vaccine for patients with advanced HCC (unpublished data), and just started the phase II clinical trial for adjuvant therapy after curative resection or RFA.

In this study, our aim was to determine if the GPC3-specific T-cell-mediated immune response is strengthened after locoregional therapies in HCC patients and tumor-bearing mice. Moreover, we evaluated the hypothesis that the post-treatment immune response may provide the basis for adjuvant immunotherapy.

Materials and methods

Patient population and treatment of HCC. Twenty-seven patients with primary HCC were prospectively enrolled in this study from January to November 2007 at the National Cancer Center Hospital East, in Japan. The eligibility criteria included primary HCC, which would undergo locoregional therapies with curative intent. Three treatment groups of nine patients each would undergo RFA, surgical resection, or TACE, respectively. Treatment selection in each patient was in accordance with the Japanese HCC treatment guidelines (2). Other inclusion criteria included HLA-A24 or HLA-A2 gene-positive status, as determined by commercially-available genomic DNA typing tests (Mitsubishi Chemical Medience, Tokyo, Japan), and no other active malignancy. HCC was diagnosed using dynamic computed tomography (CT). Tumor stage was assigned according to the tumor-node-metastasis (TNM) classification of the Union for International Cancer Control (UICC) (23). All RFA procedures were performed percutaneously under ultrasound guidance. Curative treatment was defined as complete necrosis of the tumor lesion confirmed by dynamic CT after RFA, a negative surgical margin confirmed histopathologically after resection, and complete lipiodol deposition after TACE.

All patients gave written informed consent before entering the study and this study was approved by the Ethics Committee of the National Cancer Center, conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

Collection of blood samples and preparation of peripheral blood mononuclear cells. Venous blood (20-30 ml) from each patient was collected both before treatment and one month after treatment. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using LeucoSep® tubes (Greiner Bio-One, Frickenhausen, Germany) by means of density gradient centrifugation.

Identification of GPC3-specific CTLs in HCC patients. In order to identify GPC3-specific CTLs, the proportion of cells producing interferon (IFN)-γ upon stimulation with GPC3 peptide was assessed by an *ex vivo* IFN-γ enzyme-linked immunospot (ELISPOT) assay using pooled PBMCs from HCC patients. Defrosted PBMCs ($1x10^6$ cells/well) were cultured in duplicate using 96-well flat-bottomed plates (BD Biosciences, San Jose, CA) with HLA-A24-restricted $GPC3_{298-306}$ peptide (EYILSLEEL) or HLA-A2-restricted $GPC3_{144-152}$ peptide (FVGEFFTDV) (10 μmol/l) with 100 U/ml recombinant human interleukin-2 (IL-2) for 20 h. The negative control consisted of medium alone or HLA-A24- or HLA-A2-restricted heat shock protein 105 (HSP105) peptide, and the positive control included the HLA-A24- or HLA-A2-restricted cytomegalovirus (CMV) peptide. The number of spots, which indicated the presence of IFN-γ secreting cells, was automatically counted using the Eliphoto system (Minerva Tech, Tokyo, Japan). For an exact comparison of the frequency of GPC3-specific CTLs existing at pre- and post-treatment, the obtained mean values of the number of spots with non-peptide-pulsed samples ($1x10^6$ PBMCs) at pre- and post-treatment were equalized and set to zero, and then the actual number of GPC3-, CMV-, or HSP105-specific spots was calculated. The Δspot was defined as the difference in the number of spots with each antigen between pre- and post-treatment.

Mice. Female BALB/c mice ($H\text{-}2^d$), 6-8 weeks of age, were obtained from Charles River Laboratories Japan (Yokohama, Japan). The mice were maintained under specific-pathogen-free conditions. All animal procedures were performed in compliance with the guidelines by the Animal Research Committee of the National Cancer Center, Japan.

Tumor cell lines. A subline of the BALB/c-derived GPC3-negative colorectal adenocarcinoma cell line, Colon 26 (24), was provided by Dr Kyoichi Shimomura (Astellas Pharma, Tokyo, Japan). Colon 26/GPC3 is an established stable GPC3-expressing cell line (18). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in humidified 5% CO_2 at 37°C.

RFA experiment using a mouse model. The mice were shaved at the tumor area and the contralateral flank. After attachment of the electricity-conducting pad (ground pad) onto the contralateral side, an RFA needle with 5-mm active tip (Cool-tip™, Valleylab, Boulder, CO) was inserted into the middle of the tumor. Impedance could be evaluated on the RFA lesion generator system (RFG-3B model, Radionics, Burlington, MA).

Treatment was started by delivering RFA energy. During two treatment cycles of 10 sec, the temperature could be monitored using the thermistor and thermocouple in the tip of the probe. Treatment was considered successful if a tip temperature of 60-70°C was reached.

Identification of GPC3-specific CTLs in mice. BALB/c mice were immunized beforehand by peptide vaccination with K^d-restricted $GPC3_{298-306}$ peptide (50 μg/mouse) emulsified with incomplete Freund's adjuvant twice at a 7-day interval as described previously (20). The day after the second vaccination, the mice were challenged subcutaneously with Colon 26/GPC3 tumor cells ($1x10^5$ cells/100 μl) on their shaved back and, 5 days later, the mice underwent therapeutic RFA or surgical resection for the established tumor. After the next 5 days, the mice were sacrificed and bilateral inguinal lymph nodes were obtained. $CD8^+$ T cells were isolated from lymph node cells using anti-mouse CD8α (Ly-2) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and an IFN-γ ELISPOT assay was performed without prior *in vitro* stimulation. For the IFN-γ ELISPOT assay, $CD8^+$ lymph node cells ($3x10^5$ cells/well) were used as effector cells, and Colon 26 and Colon 26/GPC3 cells ($3x10^4$ cells/well) as target cells. These cells were cultured in duplicate using 96-well flat-bottomed plates (BD Biosciences) with 100 U/ml recombinant murine IL-2 for 20 h. The number of spots after RFA or surgical resection was compared with that without treatment.

Immunohistochemical analysis. To investigate GPC3 expression in HCC tissues, we performed immunohistochemical staining of GPC3 in biopsy specimens or resected specimens from HCC patients. The paraffin-embedded blocks were analyzed using monoclonal anti-GPC3 antibody (dilution 1:300, BioMosaics, Burlington, VT) as described previously (17,21). The results were classified into two groups according to the area of GPC3-positive staining cells as follows: -, negative (<10%) and +, positive (≥10%).

To investigate tumor-infiltrating lymphocytes, we performed immunohistochemical staining of CD4 and CD8 in resected specimens from an HCC patient using monoclonal anti-CD4 or CD8 antibody (dilution 1:20, Novocastra, Newcastle upon Tyne, UK).

Statistical analysis. Statistical analyses were performed using χ^2 test, Mann-Whitney U test, or Kruskal-Wallis rank test. Differences were considered significant at $P<0.05$. Data were analyzed with the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

Demographics and clinical characteristics. The characteristics of all 27 patients are represented in Table I. The three groups of 9 patients received RFA (RFA1-9), surgical resection (RES1-9), or TACE (TAE1-9), respectively. Among them, 21 patients had the HLA-A24 gene and 7 had the HLA-A2 gene. One patient had both HLA-A24 and -A2, and the HLA-A2-restricted $GPC3_{144-152}$ peptide was used for the IFN-γ ELISPOT assay in this patient. Among the three treatment groups, tumor size in the RFA group (mean: 16.4 mm) was significantly smaller than that in the resection group (mean: 43.2 mm) (P=0.001) and the TACE group (mean: 44.1 mm) (P=0.001). Similarly, tumor stage in the RFA group was less advanced than that in the resection group (P=0.018) and TACE group (P=0.005). There was no statistically significant difference in Child-Pugh classification grade among the three groups (P=0.128). In this study, all treatments were considered to be curative according to the definitions described in Materials and methods. Moreover, all groups reduced the levels of α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II) in most of HCC patients after treatment (data not shown). The diagnosis of HCC was histopathologically confirmed by biopsy specimens or resected specimens from 21 patients. GPC3 expression was detected by immunohistochemical staining in 14 of 21 patients.

Analysis of GPC3-specific CTLs in HCC patients. As shown in Table I, GPC3-specific CTLs were detected in 11 and 15 of 27 patients at pre- and post-treatment, respectively. In total, 19 patients had GPC3-specific CTLs at either pre- or post-treatment. There was no statistically significant correlation between the presence of GPC3-specific CTLs and clinical features, including HLA-A type (P=0.126), age (P=0.750), gender (P=0.764), HCV infection (P=0.674), HBV infection (P=0.764), Child-Pugh classification grade (P=0.404), tumor multiplicity (P=0.674), tumor size (P=0.650), HCC staging (P=0.155), serum AFP level (P=0.288), and serum PIVKA-II level (P=0.094). Among the 21 patients who had the information about GPC3 expression in their HCC tissue, patients with GPC3-expressing HCCs had GPC3-specific CTLs more frequently than those with GPC3-negative HCCs, but the difference was not statistically significant (P=0.053).

Changes in GPC3-specific CTLs between before and after treatment. In order to analyze the effect of anticancer treatment on GPC3-specific T-cell response, we compared the frequency of GPC3-specific CTLs in PBMCs before treatment with that after treatment. As shown in Table I and Fig. 1, an increase in GPC3-specific CTLs was found in 5 of 9 patients after RFA and in 4 of 9 after TACE, but in only 1 of 9 patients after resection. Of note, all of the 7 patients with GPC3-expressing HCCs exhibited an increase in GPC3-specific CTLs after RFA or TACE, whereas none of the 7 patients with GPC3-expressing HCCs did after surgical resection. The Δspot of GPC3 in the RFA group (mean: 24.4 spots) was larger than that in the resection group (mean: -7.2 spots) (P=0.023). The Δspot of GPC3 in the TACE group (mean, 36.9 spots) was also larger than that in the resection group, but the difference was not statistically significant (P=0.096). In contrast, the Δspot of CMV showed no difference among the three groups (P=0.498). Neither the existence of GPC3-specific CTLs before or after treatment, nor the changes between before and after treatment had statistically significant correlation with patient survival according to the log-rank test in each treatment group (neither disease-free nor overall), with the 27-month mean follow-up period (data not shown).

The representative data on changes in CT images and serum levels of tumor markers between before and after treatment is shown in Fig. 2. All three patients (RFA3, RES6, and TAE5) had GPC3-expressing HCCs. Both the CT images and

Table I. Patient characteristics and glypican-3-specific cytotoxic T lymphocytes.

Patient	HLA	Age (yrs.)	Gender	Etiology	Child-Pugh	No. of tumor	Tumor size (mm)	T[1]	N[1]	M[1]	AFP (<9.5 ng/ml)	PIVKA-II (<40 mAU/ml)	GPC3 expression[2]	GPC3-specific CTLs[3]			
														Pre	Post	Change	Δspot[4]
RFA1	A24	73	F	HBV	A	2	26	2	0	0	4.0	228	-	4	0	-	-4
RFA2	A24	68	M	HCV	B	1	20	1	0	0	5.0	300	+	10	24	+	+14
RFA3	A2	50	M	HCV	A	1	15	1	0	0	63.3	25	+	0	88	+	+88
RFA4	A24	79	F	HCV	A	1	10	1	0	0	484.2	30	+	0	10	+	+10
RFA5	A24	69	M	HCV	A	1	15	1	0	0	2.3	57	-	0	0	+/-	0
RFA6	A24	60	M	HCV	A	1	17	1	0	0	15.1	23	-	0	0	+/-	0
RFA7	A2	73	M	HCV	A	1	20	1	0	0	97.3	51	+	3	88	+	+85
RFA8	A2/A24	64	M	HBV/HCV	B	1	15	1	0	0	39.9	17	+	0	31	+	+31
RFA9	A2	60	M	HCV	B	1	10	1	0	0	92.0	19	-	19	15	-	-4
RES1	A24	48	M	HBV	A	1	20	1	0	0	19.7	38	+	32	15	-	-17
RES2	A24	66	F	HCV	A	1	26	2	0	0	63.4	77	+	20	3	-	-17
RES3	A24	64	M	HCV	A	2	30	2	0	0	10.1	276	+	12	0	-	-12
RES4	A2	72	M	-	A	1	60	2	0	0	9.2	1500	+	3	1	-	-2
RES5	A24	70	M	HCV	A	1	20	1	0	0	4.2	25	+	0	0	+/-	0
RES6	A24	42	M	HBV/HCV	A	2	98	3	0	0	1515.0	22477	+	50	30	-	-20
RES7	A2	75	M	-	A	1	75	2	0	0	22.8	10341	-	0	3	+	+3
RES8	A24	52	M	HCV	A	1	30	1	0	0	16.0	234	+	0	0	+/-	0
RES9	A24	60	M	HBV	A	1	30	1	0	0	15.6	23	-	0	0	+/-	0
TAE1	A2	64	M	-	A	3	30	2	0	0	10.7	98	+	0	330	+	+330
TAE2	A24	78	F	HCV	B	1	60	1	0	0	2483.0	3932	ND	34	0	-	-34
TAE3	A24	77	F	-	A	>5	35	3	0	0	180.2	11538	ND	0	3	+	+3
TAE4	A24	77	M	HCV	A	2	80	4	0	0	20014.0	241	ND	0	0	+/-	0
TAE5	A24	55	M	HBV	A	2	30	2	0	0	3.7	24	+	0	23	+	+23
TAE6	A24	77	M	-	A	>5	42	2	0	0	1407.0	1661	ND	0	20	+	+20
TAE7	A24	63	F	HCV	A	>5	32	2	0	0	640.3	270	ND	0	0	+/-	0
TAE8	A24	74	M	-	A	1	18	1	0	0	3.8	12	-	0	0	+/-	0
TAE9	A24	62	M	HCV	A	3	70	3	0	0	46.8	1907	ND	10	0	-	-10

[1]Tumor stage was assigned according to the tumor-node-metastasis (TNM) classification of the Union for International Cancer Control (UICC). [2]GPC3 expression was evaluated by immunohistochemical staining; +, positive; -, negative. [3]Peripheral blood was taken from each patient before and after treatment, and GPC3-specific CTLs were measured by *ex vivo* interferon-γ enzyme-linked immunospot assay; +, increase; -, decrease; +/-, no change. [4]The Δspot was defined as the difference in the number of spots with each antigen between pre- and post-treatment. F, female; M, male; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist II; GPC3, glypican-3; ND, not determined.

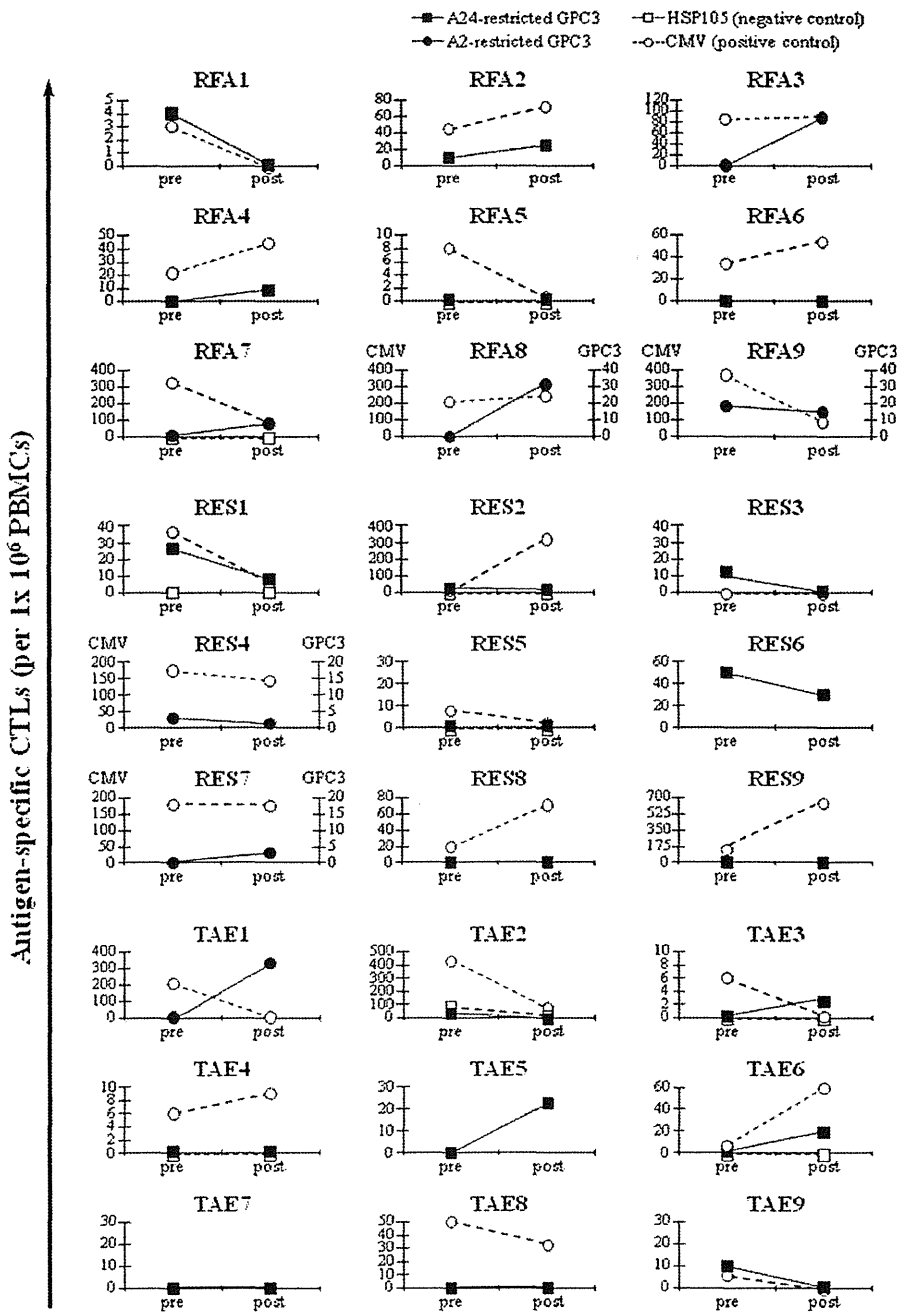

Figure 1. Kinetics of glypican-3 (GPC3)-specific CTLs between before and after treatment in each patient. A direct *ex vivo* interferon-γ enzyme-linked immunospot assay of PBMCs was performed before treatment and one month after treatment. The data are expressed as the number of interferon-γ producing cells, which indicate the CTLs specific with HLA-A24-restricted $GPC3_{298-306}$ peptide (EYILSLEEL) (■) or HLA-A2-restricted $GPC3_{144-152}$ peptide (FVGEFFTDV) (●). Heat shock protein 105 (HSP105) peptide (□) and cytomegalovirus (CMV) peptide (○) were used as the negative and positive control, respectively.

kinetics of tumor markers indicated that their treatment was effective. The frequency of GPC3-specific CTLs increased after RFA (RFA3) and TACE (TAE5), whereas it decreased after surgical resection (RES6).

RFA has the potential to strongly induce T-cell-mediated immune response: A case report. A 70-year-old woman was admitted because of recurrent HCCs. Thirteen months earlier, the patient had undergone RFA for primary HCC located in the S5/8 region of the liver. CT detected two recurrent HCCs: one was contiguous to the previously ablated S5/8 region and the other was a distant tumor located in the S6 region. We performed surgical resection for these recurrent HCCs. Immunohistochemical examination of CD8 in the resected tumors revealed that a marked number of $CD8^+$ T cells had infiltrated not only into the surrounding recurrent tumor but also into the distant recurrent tumor after RFA (Fig. 3). On the other hand, few $CD4^+$ T cells were observed in these tumors (data not shown). Immunohistochemical analyses showed the expression of GPC3 and HLA class I in these tumors (data not

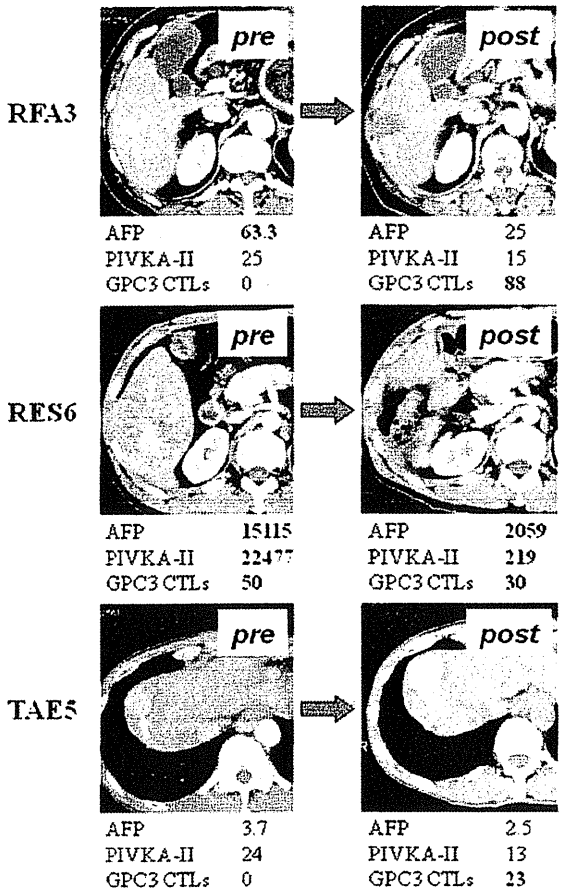

Figure 2. Changes in computed tomography images, serum levels of tumor markers, including α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II), and glypican-3 (GPC3)-specific CTLs in PBMCs between before and after treatment in patients RFA3, RES6, and TAE5. White arrows indicate nodules of hepatocellular carcinoma at pre- and post-treatment. The bold letters show the abnormal levels of tumor markers or the positive response of GPC3 specific CTLs.

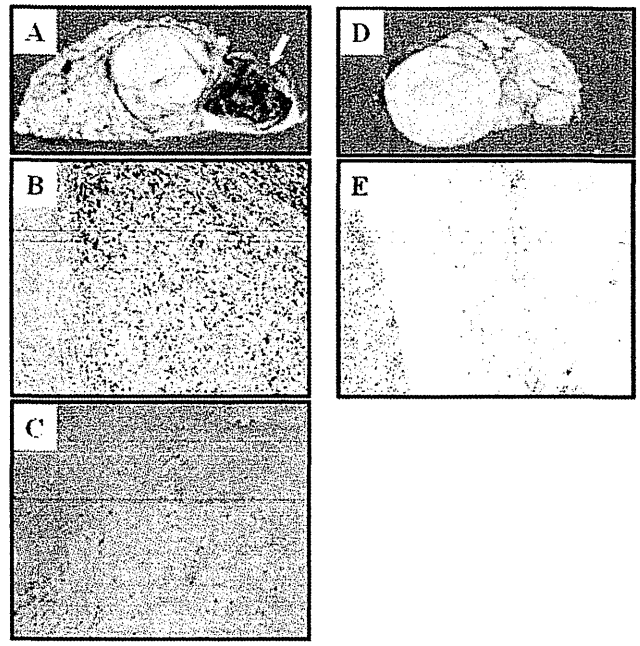

Figure 3. Macroscopic features and immunohistochemical examination of $CD8^+$ T cells in the resected tumors that had recurred after radiofrequency ablation. (A and D) show the cut surface of the resected specimens. (A) The white arrow indicates the post-ablated lesion to which a recurrent tumor was contiguous. The other recurrent tumor was distant from the post-ablated lesion (D). A marked number of $CD8^+$ T cells had infiltrated into the contiguous recurrent tumor (B) and the distant recurrent tumor (E), whereas few $CD8^+$ T cells had infiltrated into the post-ablated necrotic lesion (C). Magnification x100 (B and C) and x40 (E).

shown). These findings suggest that RFA not only activates the immune response systemically but also induces local infiltration of CTLs into the tumors.

Analysis of immune response induced by RFA in a mouse model. The experimental schedule is shown in Fig. 4A. The IFN-γ ELISPOT assay with $CD8^+$ T cells from the lymph nodes of mice demonstrated that the number of spots against both Colon 26 (P=0.049) and Colon 26/GPC3 (P=0.049) was larger after RFA compared to without treatment. On the other hand, the number of spots did not increase after surgical resection. These results suggest that RFA induced a significantly larger number of both Colon 26- and Colon 26/GPC3-reactive CTLs compared to no treatment or surgical resection (Fig. 4B).

The difference in number of spots between Colon 26 and Colon 26/GPC3 in each mouse, which represents GPC3-specific CTLs, is shown in Fig. 4C. As an effect of prior peptide vaccination, GPC3-specific CTLs were detected in the no treatment group. The frequency of GPC3-specific CTLs increased after RFA and decreased after surgical resection. As a result, the frequency of GPC3-specific CTLs after RFA was significantly greater than that after surgical resection (P=0.049). These results suggest that RFA induced a significantly larger number of GPC3-specific CTLs compared to surgical resection (Fig. 4C).

Discussion

We previously reported that 39% of HCC patients had detectable GPC3-specific CTLs by a direct *ex vivo* IFN-γ ELISPOT assay (25). In this study, GPC3-specific CTLs were detectable before treatment in 11 of 27 patients (41%). Additionally, when we analyzed the patients with a prior treatment for HCCs using the same methods, 11 of 21 (52%) patients had detectable GPC3-specific CTLs (data not shown). These results are favorable for anticancer immunotherapy because the antigen-specific T-cell-mediated immune response could be detected without *in vitro* stimulation. As for frequency, GPC3-specific CTLs were detectable in ~40% of HCC patients, whereas AFP-, human telomerase reverse transcriptase (hTERT)-, and multidrug resistance-associated protein 3 (MRP3)-specific CTLs have been detected in 5-20, 6-12, and 14-21% of HCC patients with a single epitope peptide, respectively (26-28). As for tumor stages, a GPC3-specific immune response is frequently detected even in the early stages (24), whereas AFP-specific CTLs are more frequently detected in patients with advanced HCC (26). These results suggest that GPC3 has strong immunogenicity and GPC3-specific T-cell-mediated immunotherapy is suitable for adjuvant therapy against HCC because the induction of tumor-specific immune response in

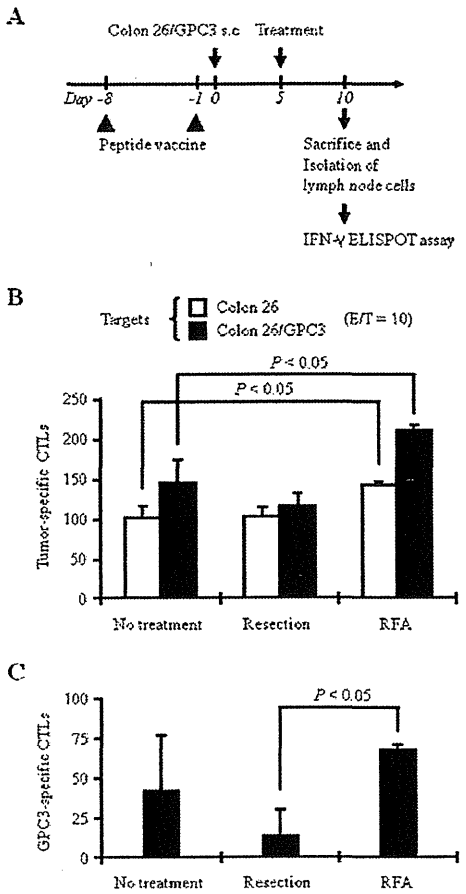

Figure 4. Investigation of the glypican-3 (GPC3)-specific immune response in a mouse model. (A) Experiment schedule. (B) An *ex vivo* interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay of $CD8^+$ lymph node cells (effector, $3x10^5$ cells/well) against Colon 26 and Colon 26/GPC3 (target, $3x10^4$ cells/well). No treatment column indicates the group of mice that received only the peptide vaccination and no therapy for the established tumor. The data are expressed as the mean + SD. Three mice were used for each group. Effector/target ratio=10. (C) The frequency of GPC3-specific CTLs, which is calculated from the difference in the number of spots between Colon 26 and Colon 26/GPC3 in each mouse.

the early stages would be more effective for suppression of tumor growth.

The association between the induction of an antigen-specific immune response and the antigen expression in tumor tissue remains unclear. In this study, we obtained the result that the presence of GPC3-specific CTLs in PBMCs potentially had a positive correlation with GPC3 expression in tumor tissue, but the correlation was not statistically significant. On the other hand, Mizukoshi *et al* showed a negative correlation between the frequency of MRP3-specific CTLs and MRP3 expression level (28). Moreover, Benavides *et al* showed that even antigen-naïve patients had pre-existing immunity (29). First, this may be because of tumor heterogeneity of cancer tissue. In most cases, the whole tumor cannot be evaluated and, in the case of truly antigen-naïve patients, antigen-specific CTLs cannot exist in theory. Second, antigen expression may be negative if antigen-specific CTLs have killed all of the antigen-expressing tumor cells as described by Jäger *et al* (30). As for the changes in an antigen-specific immune response between before and after treatment, in this study, we showed impressive data that all patients with GPC3-expressing HCCs exhibited an increase in GPC3-specific CTLs after RFA or TACE, whereas no patient with GPC3-expressing HCCs did after surgical resection.

This is the first study to compare locoregional therapies, including RFA, surgical resection, and TACE, in terms of antigen-specific T-cell response in HCC patients and tumor-bearing mice. Half the patients after RFA or TACE showed an increase in GPC3-specific CTLs, which might have been induced by the treatment, whereas only 1 of 9 patients after resection showed an increase and more than half the patients after resection showed a decrease. Similarly, the frequency of GPC3-specific CTLs increased after RFA and decreased after resection in a mouse model. These results suggest that RFA induced a stronger GPC3-specific immune response compared to surgical resection. RFA destroys tumor tissue and causes local necrosis followed by the release of tumor-associated antigens (12), whereas all of the tumor-associated antigens must be completely removed after resection. With regard to TACE, whereas the results of an IFN-γ ELISPOT assay after TACE were as encouraging as that after RFA, we have no other favorable data on the immune response after TACE. Although further investigation is required, TACE, which is also a necrosis-inducing treatment, might induce an antigen-specific immune response.

A limitation of this study is the patient selection in the three kinds of locoregional therapy. Current treatment guidelines for HCC including the Japanese ones, which we followed in this study, recommend RFA to earlier HCCs and TACE to more advanced HCCs than those which receive surgical resection (2,31-33). Therefore, selection bias is unavoidable under the circumstances. To overcome this problem, we added a murine study. The advantage of RFA over surgical resection in the induction of GPC3-specific CTLs was demonstrated also in a mouse model.

The correlation between antitumor immune response and clinical response is controversial. In this study, a significant contribution of GPC3-specific CTLs toward an optimal prognosis was not demonstrated. Mizukoshi *et al* reported that enhancement of T-cell response did not last for long and did not contribute to the prevention of HCC recurrence (34). In view of the highly complex nature of the human immune system, patient prognoses might not be determined only by the CTL response. Previous studies have demonstrated that the release of tumor-derived antigens by necrosis-inducing treatment causes sufficient signaling to activate not only antigen-specific CTL response but also antigen-specific helper T-cell response (35,36), antigen-specific antibody response (36), and non-antigen-specific natural killer cell response (37). However, the mechanisms for cancer escape from immunosurveillance would suppress the efficiency of these immune responses (38). In the literature, tumor-infiltrating lymphocytes in HCC are associated with better prognosis (39), but, in our case, tumor-infiltrating CTLs were actually insufficient for suppression of cancer recurrence despite the massive infiltration. For successful anticancer immunotherapy, the development of an innovative strategy to link antitumor immune response with clinical response and to provide a survival benefit for cancer patients is necessary, and so we have just started the clinical trial of a GPC3-derived peptide vaccine for adjuvant therapy after RFA.

In conclusion, our results demonstrate that RFA has a stronger effect on the immune system compared with surgical resection. Although further investigation is necessary, the data on immune response support the rationale for combined immunotherapy for HCC patients.

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A novel apoptotic mechanism of genetically engineered adenovirus-mediated tumour-specific p53 overexpression through E1A-dependent p21 and MDM2 suppression

Yasumoto Yamasaki [a], Hiroshi Tazawa [a,b], Yuuri Hashimoto [a], Toru Kojima [a], Shinji Kuroda [a], Shuya Yano [a], Ryosuke Yoshida [a], Futoshi Uno [a], Hiroyuki Mizuguchi [c], Akira Ohtsuru [d], Yasuo Urata [e], Shunsuke Kagawa [a], Toshiyoshi Fujiwara [a,*]

[a] *Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan*
[b] *Center for Gene and Cell Therapy, Okayama University Hospital, Okayama 700-8558, Japan*
[c] *Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan*
[d] *Takashi Nagai Memorial International Hibakusha Medical Center, Nagasaki University Hospital, Nagasaki 852-8501, Japan*
[e] *Oncolys BioPharma Inc., Tokyo 105-0001, Japan*

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KEYWORDS
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p21

Abstract Oncolytic viruses engineered to replicate in tumour cells but not in normal cells could be used as tumour-specific vectors carrying the therapeutic genes. We previously developed a telomerase-specific oncolytic adenovirus, OBP-301, that causes cell death in human cancer cells with telomerase activities. Here, we further modified OBP-301 to express the wild-type *p53* tumour suppressor gene (OBP-702), and investigated whether OBP-702 induces stronger antitumour activity than OBP-301. The antitumour effect of OBP-702 was compared to that of OBP-301 on OBP-301-sensitive (H358 and H460) and OBP-301-resistant (T.Tn and HSC4) human cancer cells. OBP-702 suppressed the viability of both OBP-301-sensitive and OBP-301-resistant cancer cells more efficiently than OBP-301. OBP-702 caused increased apoptosis compared to OBP-301 or a replication-deficient adenovirus expressing the *p53* gene (Ad-p53) in H358 and T.Tn cells. Adenovirus E1A-mediated p21 and MDM2 downregulation was involved in the apoptosis caused by OBP-702. Moreover, OBP-702 significantly suppressed tumour growth in subcutaneous tumour xenograft models compared to monotherapy with OBP-301 or Ad-p53. Our data demonstrated that OBP-702 infection expressed adenovirus E1A and then inhibited p21 and MDM2 expression, which in turn efficiently induced apoptotic cell death. This novel apoptotic mechanism suggests that the p53-expressing OBP-702 is a promising antitumour reagent for human cancer and could improve the clinical outcome.

* *Corresponding author:* Address: Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-chó, Kita-ku, Okayama 700-8558, Japan. Tel.: +81 86 235 7257; fax: +81 86 221 8775.
E-mail address: toshi_f@md.okayama-u.ac.jp (T. Fujiwara).

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1. Introduction

Replication-selective oncolytic viruses have emerged as promising antitumour reagents for induction of tumour-specific cell death.[1–4] Recent evidence from several clinical studies of oncolytic virotherapy has suggested that oncolytic viruses are well tolerated by cancer patients.[5–8] We previously developed a telomerase-specific replication-competent oncolytic adenovirus OBP-301 (Telomelysin), in which the human telomerase reverse transcriptase (*hTERT*) promoter drives the expression of the *E1A* and *E1B* genes that are linked to an internal ribosome entry site (IRES).[9–11] A phase I clinical trial of OBP-301 in patients with advanced solid tumours has been recently completed and OBP-301 was well tolerated by these patients.[12] However, the antitumour effect of OBP-301 was limited in some of the OBP-301-injected tumours. Therefore, to efficiently eliminate tumour cells using OBP-301, and to improve the clinical outcome of cancer patients, enhancement of the OBP-301-mediated antitumour effect is required.

Genetically engineered armed oncolytic viruses that express several types of therapeutic transgenes have recently been reported that were aimed at enhancing the antitumour effect of an oncolytic virus.[6,13] Among candidate therapeutic transgenes, the tumour-suppressor *p53* gene is a potent therapeutic transgene for induction of cell cycle arrest, senescence and apoptosis.[14] Indeed, a p53-expressing replication-deficient adenovirus (Ad-p53, Advexin) has been reported to induce an antitumour effect in both *in vitro* and *in vivo* settings[15,16] as well as in various clinical studies.[17–20] Recently, p53-expressing armed replication-selective oncolytic adenoviruses have been shown to induce a stronger antitumour effect than a non-armed oncolytic adenovirus or Ad-p53.[21–23] However, the molecular mechanism of the enhanced antitumour effect of a p53-armed oncolytic adenovirus remains unclear. We recently showed that, in combination therapy, OBP-301 enhanced Ad-p53-mediated apoptosis through p53 upregulation and by suppression of the p53-downstream target p21,[24] which is not only transcriptionally activated and mainly induces cell cycle arrest, but also suppresses apoptosis.[25] These results suggest that this p53-expressing oncolytic adenovirus has a strong antitumour effect through apoptosis induction.

In the present study, we first investigated whether the p53-expressing telomerase-specific replication-competent oncolytic adenovirus OBP-702 has efficient *in vitro* antitumour activity compared with OBP-301. We next compared the induction of apoptotic cell death of human cancer cells infected with OBP-301, OBP-702 and Ad-p53. The molecular mechanism of OBP-702-mediated apoptosis induction was further addressed. Finally, the *in vivo* antitumour effect of OBP-702 was evaluated using two subcutaneous human tumour xenograft models.

2. Materials and methods

2.1. Cell lines

The human non-small cell lung cancer cell lines H1299 (p53 null), H358 (p53 null) and H460 (wild-type p53) were obtained from the American Type Culture Collection (Manassas, VA, USA). The human oesophageal cancer cell line T.Tn (mutant-type p53) was purchased from the Japanese Collection Research Bioresources (JCRB, Osaka, Japan). The human oral squamous cell carcinoma cell line HSC4 (wild-type p53) was obtained from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). The human colon cancer cell lines (SW620 (mutant-type p53) and LoVo (wild-type p53)) and the human liver cancer cell line HepG2 (wild-type p53) were obtained from the American Type Culture Collection (Manassas, VA, USA). The human liver cancer cell line Huh-7 (mutant-type p53) was obtained from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). H1299, H358, H460, T.Tn, SW620 and LoVo cells were maintained in RPMI 1640 medium. HSC4, HepG2 and Huh-7 cells were maintained in Dulbecco's modified Eagle's medium. All media were supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were routinely maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Recombinant adenoviruses

The recombinant telomerase-specific, replication-competent adenovirus OBP-301 (Telomelysin), in which the promoter element of the *hTERT* gene drives the expression of *E1A* and *E1B* genes that are linked with an IRES, was previously constructed and characterised.[9–11] For OBP-301 induction of exogenous *p53* gene expression, a human wild-type *p53* gene expression cassette derived by the Egr-1 promoter was inserted into the E3 region of OBP-301 (Fig. 1A). The E1A-deleted adenoviral vector dl312 and the wild-type adenovirus type 5 (Ad5) were used as control vectors. Recombinant viruses were purified by ultracentrifugation using caesium chloride step gradients, their titres were determined by a plaque-forming assay using 293 cells, and viruses were stored at −80 °C.

2.3. Western blot analysis

Cells were seeded in a 100-mm dish at a density of 1×10^5 cells/dish 12 h before infection and were infected with OBP-301, OBP-702 or Ad-p53 at the indicated multiplicity of infection (MOI). Whole cell lysates were prepared in a lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Complete Mini; Roche, Indianapolis,

IN, USA) at the indicated time points. Proteins were electrophoresed on 6–15% SDS polyacrylamide gels and were transferred to polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK). Blots were blocked with 5% non-fat dry milk in TBS-T (Tris-buffered saline and 0.1% Tween-20, pH 7.4) at room temperature for 30 min. The primary antibodies used were: mouse anti-p53 monoclonal antibody (mAb) (Calbiochem, Darmstadt, Germany), mouse anti-$p21^{WAF1}$ mAb (Calbiochem), mouse anti-MDM2 mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-BAX polyclonal antibody (pAb) (Santa Cruz Biotechnology), rabbit anti-poly (ADP-ribose) polymerase (PARP) pAb (Cell Signaling Technology, Beverly, MA, USA), mouse anti-Ad5 E1A mAb (BD PharMingen, Franklin Lakes, NJ, USA) and mouse anti-β-actin mAb (Sigma–Aldrich, St. Louis, MO, USA). The secondary antibodies used were: horseradish peroxidase-conjugated antibodies against rabbit IgG (GE Healthcare) or mouse IgG (GE Healthcare). Immunoreactive bands on the blots were visualised using enhanced chemiluminescence substrates (ECL Plus; GE Healthcare).

2.4. Cell viability assay

Cells were seeded on 96-well plates at a density of 1×10^3 cells/well 12 h before infection and were infected with OBP-301 or OBP-702 at MOIs of 0, 0.1, 1, 10 or 100 plaque-forming units (PFU)/cell. Cell viability was determined on days 2, 3 and 5 after virus infection using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA), which is based on an XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulphonic acid hydrate assay, according to the manufacturer's protocol. The 50% inhibiting dose (ID_{50}) value of OBP-301 and OBP-702 for each cell line was calculated using cell viability data obtained on day 5 after virus infection.

2.5. Flow cytometric analysis of active caspase-3 expression

Cells were incubated for 20 min on ice in Cytofix/Cytoperm solution (BD Biosciences, Franklin Lakes, NJ, USA), were labelled with phycoerythrin-conjugated rabbit anti-active caspase-3 mAb (BD Biosciences) for

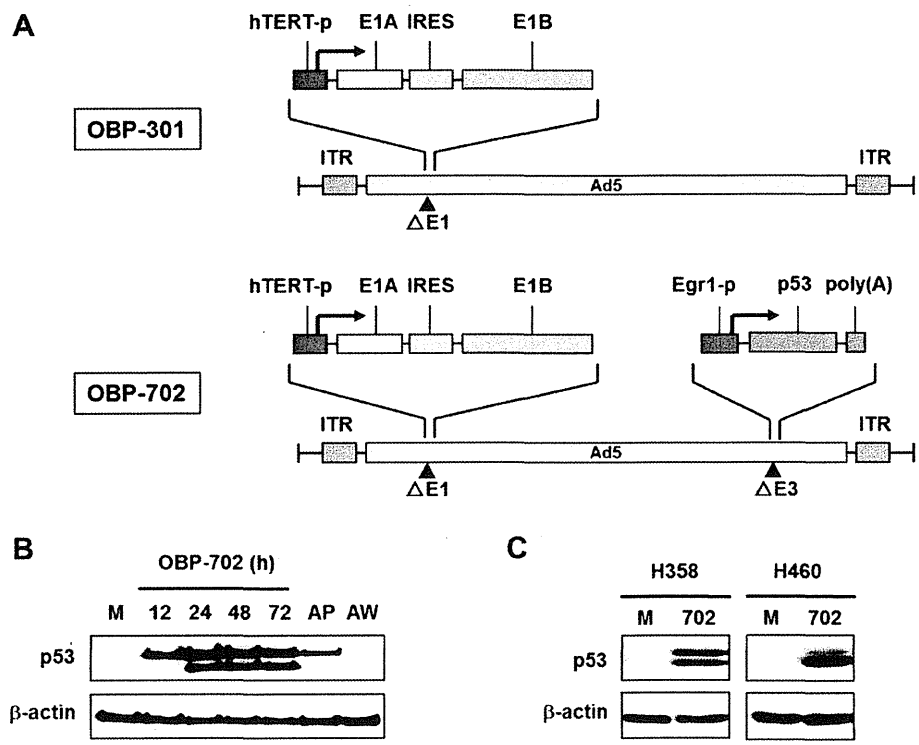

Fig. 1. p53 upregulation in human cancer cells infected with OBP-702. (A) Schematic diagrams of OBP-301 and OBP-702 structures. OBP-301 is a telomerase-specific replication-competent adenovirus, in which the *hTERT* promoter drives the expression of *E1A* and *E1B* genes that are linked with an IRES. OBP-702 is a p53-armed OBP-301, in which the *Egr-1* promoter drives expression of the *p53* gene that is inserted into the E3 region. (B) Expression of the p53 protein in p53-null H1299 cells infected with OBP-702 (10 MOI) at the indicated time points. A replication-deficient p53-expressing adenovirus Ad-p53 (AP) and a wild-type adenovirus Ad5 (AW) were also infected at an MOI of 10 for 24 h as a positive and negative control, respectively. Cell lysates were subjected to Western blot analysis with an anti-p53 antibody. β-Actin was assayed as a loading control. (C) Expression of the p53 protein in H358 and H460 cells infected with OBP-702 (702) at an MOI of 10 for 24 h. Mock-infected cells (M) were used as controls.

30 min, and were then analysed using FACS array (BD Biosciences).

2.6. In vivo subcutaneous H358 and T.Tn xenograft tumour models

Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine. The H358 and T.Tn cells (5×10^6 cells per site) were inoculated into the flanks of 5-week-old female athymic nude mice (Charles River Laboratories, Wilmington, MA, USA). When tumours reached approximately 5–6 mm in diameter, a 50 μl volume of solution containing OBP-301, OBP-702 or Ad-p53 at a dose of 1×10^8 PFU or phosphate buffered saline (PBS) was injected into the tumours for three cycles every 2 days. Tumour size was monitored by measuring tumour length and width using calipers. Each tumour volume was calculated using the following formula: tumour volume (mm^3) = $L \times W^2 \times 0.5$, where L is the length and W is the width. The survival rate of mice with H358 tumours or T.Tn tumours was assessed until 90 or 180 days, respectively, after first treatment.

2.7. Statistical analysis

Data are expressed as means ± standard deviation (SD). Student's t test was used to compare differences between groups. Log-rank test was also used to compare differences between groups in the survival rate of mice. Statistical significance was defined as a P value less than 0.05.

3. Results

3.1. p53 induction in human cancer cells infected with OBP-702

To examine the level of p53 expression induced by OBP-702 in human cancer cells, we first evaluated p53 expression of p53-null human lung cancer H1299 cells after OBP-702 infection using Western blot analysis. The p53 expression level was increased within 24 h after OBP-702 infection, and a high expression level was maintained for up to 72 h (Fig. 1B). OBP-702-induced p53 expression was higher than Ad-p53-induced p53 expression 24 h after infection. Detectable 40 kDa protein expression in OBP-702-infected H1299 cells may be due to higher p53 expression. In contrast, no p53 expression was induced by OBP-301 infection (data not shown). OBP-702 further induced p53 expression in other human lung cancer cells (H358 (p53-null) and H460 (wild-type p53)) and in human colon cancer cells (SW620 (mutant p53), LoVo cells (wild-type p53)) and human liver cancer cells (HepG2 (wild-type p53) and Huh7 (mutant p53)) (Fig. 1C and Supplementary Fig. 1A). These results indicate that OBP-702 efficiently induces exogenous p53 expression in human cancer cells independent of the status of endogenous p53.

3.2. OBP-702 has enhanced antitumour activity against human cancer cells compared to OBP-301

To compare the *in vitro* antitumour activity of OBP-702 and OBP-301, we used the two OBP-301-sensitive human cancer cells (H358 and H460) and the two OBP-301-resistant human cancer cells (T.Tn and HSC4) that were previously reported.[11] OBP-301-resistant cells showed lower the coxsackie and adenovirus receptor (CAR) expression compared to OBP-301-sensitive cells (data not shown). The cell viability of each cell line was assessed over 5 days after infection using the XTT assay. OBP-702 suppressed the viability of OBP-301-sensitive and OBP-301-resistant cells more efficiently than OBP-301, although at least 48 h are required for the sufficient viral replication (Fig. 2A). Furthermore, OBP-702 also showed increased antitumour activity against human colon and liver cancer cells compared to OBP-301 (Supplementary Fig. 1B). Calculation of the ID_{50} values indicated that all cell lines were more sensitive to OBP-702 than to OBP-301 (Supplementary Table S1). These results suggest that OBP-702 is more cytopathic for human cancer cells than OBP-301.

3.3. Increased induction of apoptosis by OBP-702 compared to OBP-301 or Ad-p53

We next investigated whether OBP-702 has a greater apoptotic effect than OBP-301 or Ad-p53. OBP-301-sensitive H358 cells and OBP-301-resistant T.Tn cells were each infected with OBP-702, OBP-301 or Ad-p53 at MOIs of 10 and 100 for 48 h, and apoptosis was analysed. Western blot analysis showed that OBP-702, but not OBP-301 or Ad-p53, induced the cleavage of PARP at 48 and 72 h after infection (Fig. 3A). Furthermore, flow cytometric analysis demonstrated that OBP-702 infection significantly increased the percentage of apoptotic H358 and T.Tn cells that expressed active caspase-3 compared to Ad-p53 infection (Fig. 3B and C). However, no apoptosis was induced after OBP-301 infection. These results suggest that OBP-702 has a stronger effect on apoptosis than Ad-p53 or OBP-301.

3.4. Induction of apoptosis by OBP-702 through p53-dependent BAX upregulation and E1A-dependent p21 and MDM2 downregulation

Overexpression of p53 is well known to induce apoptosis through induction of p53-downstream target genes.[14] To investigate the molecular mechanism of OBP-702-induced apoptotic cell death, the expression

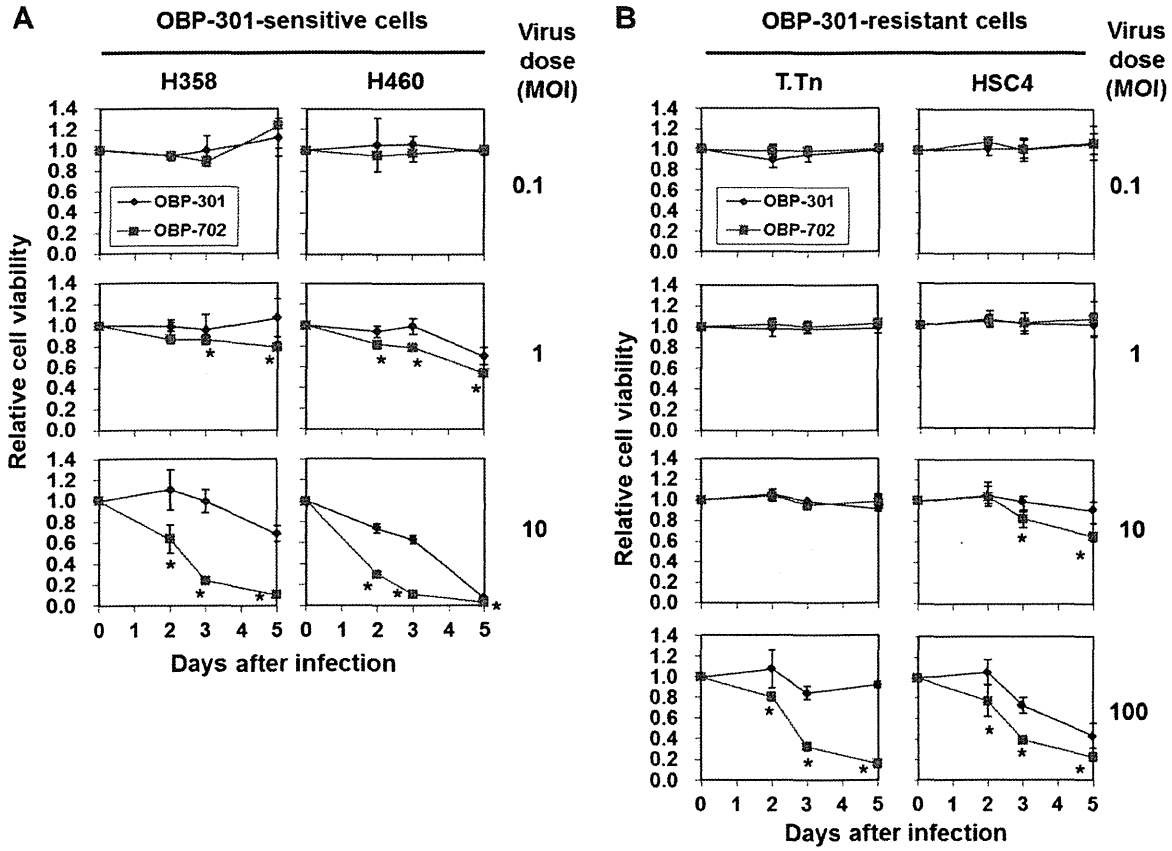

Fig. 2. OBP-702 has enhanced antitumour activity against human cancer cells compared to OBP-301. OBP-301-sensitive cells (H358 and H460) (A) and OBP-301-resistant cells (T.Tn and HSC4) (B) were infected with OBP-301 or OBP-702 at the indicated doses and cell viability was measured using the XTT assay on days 2, 3 and 5 after infection. Cell viability was calculated relative to that of the mock-treated group on each day, which was set at 1.0. Cell viability data are expressed as mean values ± SD ($n = 5$). Statistical significance was determined using Student's t test. $^{*}P < 0.05$. The data are representative of three separate experiments.

level of p53, and p53-downstream target proteins such as p21, BAX and MDM2, was evaluated by Western blot analysis. OBP-702 infection induced higher p53 expression than that induced by Ad-p53 between 24 and 72 h after infection (Fig. 4A). Ad-p53 infection upregulated the expression of p21, MDM2 and BAX proteins. In contrast, OBP-702 infection upregulated the BAX protein as well as Ad-p53, but expression of p21 and MDM2 was low despite strong p53 activation. PARP cleavage was observed 48 and 72 h after OBP-702 infection, consistent with suppression of p21 and MDM2 expression. Overexpression of the adenoviral E1A protein was observed in OBP-702-infected cells. These results suggest that OBP-702 upregulates p53 expression and subsequent BAX expression, but downregulates p21 and MDM2 expression, resulting in the induction of apoptosis.

We recently reported that OBP-301 enhances Ad-p53-induced apoptosis through p53 overexpression and p21 suppression.[24] Furthermore, adenovirus-mediated E2F1 overexpression also enhanced Ad-p53-induced apoptosis through MDM2 downregulation.[26] Since adenoviral E1A is known to activate E2F1 expression,[27] we hypothesised that OBP-702-mediated E1A expression may enhance Ad-p53-induced apoptosis through suppression of p21 and MDM2 expression. To address this hypothesis, H358 cells were coinfected with E1A-deficient dl312 or E1A-expressing wild-type Ad5 after Ad-p53 infection. Ad-p53-induced p53 overexpression was enhanced in the Ad5-coinfected H358 cells, but not in the dl312-coinfected H358 cells (Fig. 4B). Consistent with p53 overexpression, BAX expression was also upregulated. However, despite the enhanced p53 expression, the expression of p21 and MDM2 proteins was lower in Ad5-coinfected cells than in dl312-coinfected cells. Furthermore, PARP cleavage was only detected in H358 cells 72 h after coinfection of Ad-p53 with Ad5. As expected, OBP-301 infection had no apparent effect of the expression of p53, and p53-downstream target proteins (Supplementary Fig. 2). These results suggest that adenoviral E1A suppresses the expression of p21 and MDM2 thereby enhancing apoptosis through p53-dependent BAX upregulation (Fig. 4C, Supplementary Fig. 3).

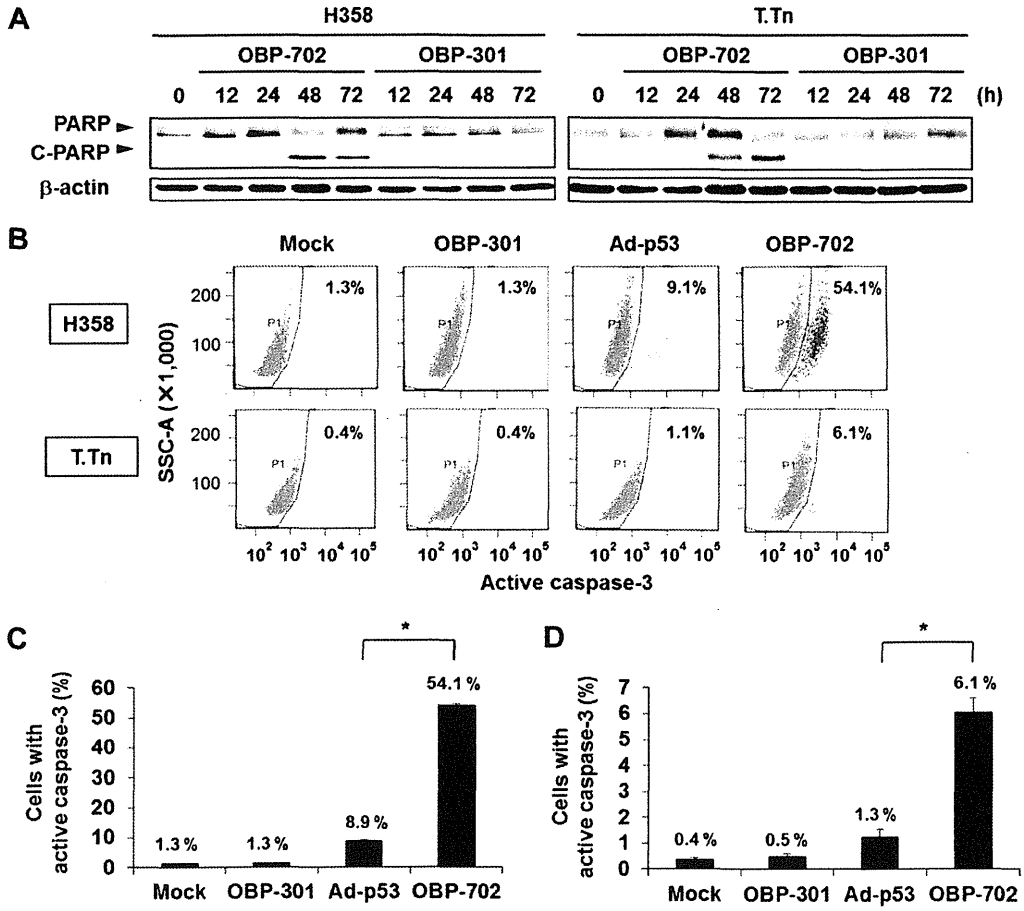

Fig. 3. OBP-702 induces increased apoptosis compared to OBP-301 or Ad-p53. (A) OBP-301-sensitive H358 cells and OBP-301-resistant T.Tn cells were infected with OBP-301 or OBP-702 at an MOI of 10 and 100, respectively, for 48 h. The level of cleaved PARP (C-PARP) and intact PARP in cell lysates was analysed using Western blotting. β-Actin was assayed as a loading control. (B–D), H358 and T.Tn cells were infected with OBP-702, OBP-301 or Ad-p53 at an MOI of 10 and 100, respectively, for 48 h. Mock-infected cells were used as controls. Caspase-3 activation was quantified using flow cytometric analysis. Representative flow cytometric data are shown (B). The mean percentage of H358 cells (C) and T.Tn cells (D) that express active caspase-3 was calculated based on three-independent experiments. Bars, SD. Statistical significance was determined using Student's *t* test. $^{*}P < 0.05$.

3.5. Enhanced antitumour effect of OBP-702 in tumour xenograft animal models

Finally, to assess the *in vivo* antitumour effect of OBP-702, we used subcutaneous H358 and T.Tn tumour xenograft models. OBP-702, OBP-301, Ad-p53 or PBS was intratumourally injected for three cycles every 2 days. OBP-702 administration significantly suppressed tumour growth compared to OBP-301, Ad-p53 or PBS in H358 and T.Tn tumour xenograft models (Fig. 5A). Furthermore, H358 tumour-bearing mice treated with OBP-702 significantly survived longer than those treated with OBP-301 or Ad-p53 (Fig. 5B). Although there was no significant difference in the survival rates between OBP-702-treated and OBP-301-treated mice with T.Tn tumours, OBP-702 treatment significantly increased the survival rate of T.Tn tumour-bearing mice compared to Ad-p53. These results suggest that OBP-702 eliminates tumour tissues more efficiently than OBP-301 or Ad-p53.

4. Discussion

Genetically engineered transgene-expressing armed oncolytic adenoviruses are expected to be a third-generation oncolytic virus for induction of a strong antitumour effect through induction of oncolytic and transgene-induced cell death.[6,13] Although the tumour suppressor *p53* gene is a potent therapeutic transgene for enhancement of an oncolytic adenovirus-mediated antitumour effect,[21–23] the molecular mechanisms by which p53 mediates enhancement of the antitumour effect remain unclear. In this study, we showed that the p53-expressing telomerase-specific oncolytic adenovirus OBP-702 exerted stronger *in vitro* and *in vivo* antitumour effects than OBP-301 or Ad-p53 (Figs. 2 and 5). This enhanced antitumour effect was due to p53-induced apoptosis, and adenoviral E1A enhanced this apoptosis via suppression of the expression of anti-apoptotic p21 and p53-inhibitory MDM2 (Figs. 3 and 4). Although

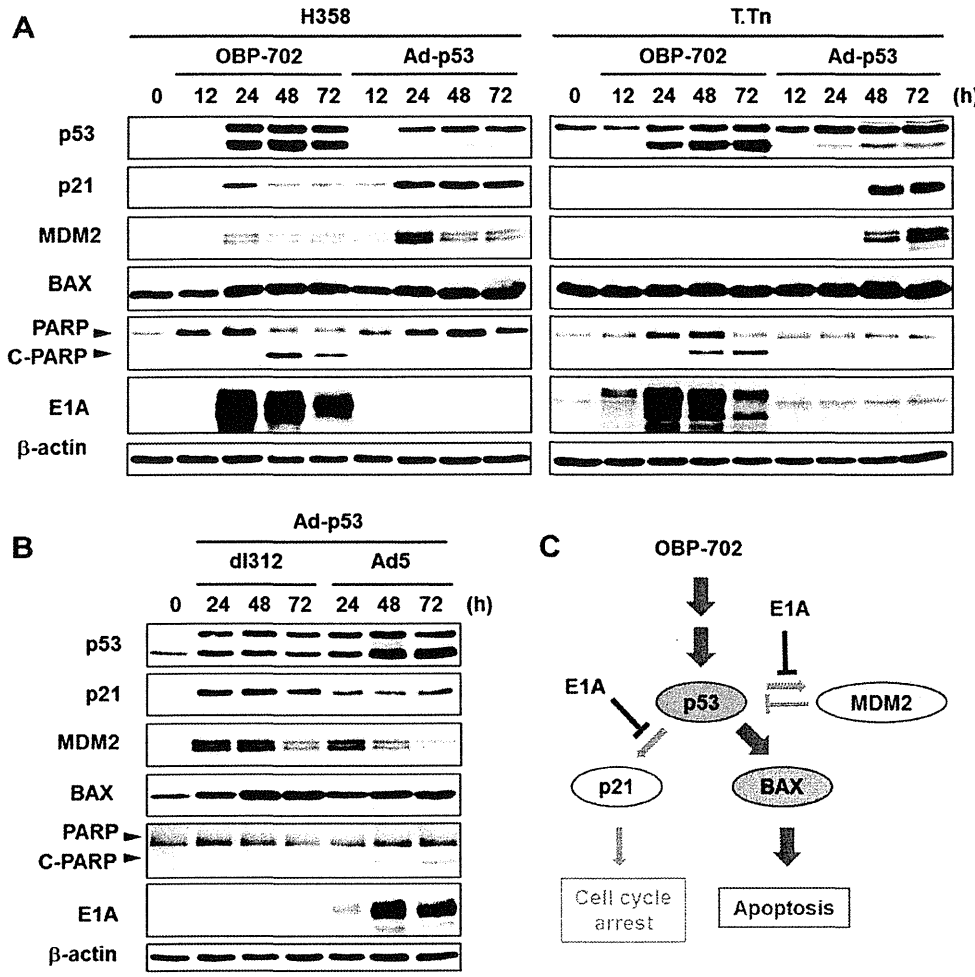

Fig. 4. OBP-702-mediated activation of p53, p53-target proteins and PARP in an E1A-dependent manner. (A) H358 and T.Tn cells were infected with OBP-702 or Ad-p53 at an MOI of 10 and 100, respectively, and infected cells were harvested at the indicated time points. The level of p53, p21, MDM2, BAX, PARP, cleaved PARP (C-PARP) and E1A proteins in cell lysates was analysed by Western blotting. β-Actin was assayed as a loading control. (B) H358 cells were infected with Ad-p53, following which they were coinfected with the E1A-deficient adenovirus (dl312) or an E1A-expressing wild-type adenovirus (Ad5) at the indicated time points. (C) Outline of OBP-702-mediated apoptosis induction through p53-dependent BAX upregulation and E1A-dependent downregulation of p21 and MDM2.

replication-competent adenovirus-mediated *p53* gene transduction has been suggested to exert an increased antitumour effect compared to replication-deficient Ad-p53 through replication-mediated p53 overexpression,[22] adenoviral E1A also enhanced p53-mediated apoptosis through suppression of expression of the p53-downstream targets p21 and MDM2 (Fig. 4). The adenoviral E1A protein has been previously shown to suppress p53-induced p21 and MDM2 expression.[28,29] E1A-mediated p21 and MDM2 suppression has also been shown to induce apoptosis in DNA-damaged cells that overexpress p53.[30,31] These reports support our findings that adenoviral E1A protein enhances p53-induced apoptosis through p21 and MDM2 suppression. It has recently been further shown that replication-deficient Ad-p53 enhances apoptosis through p21 suppression in combination with artificial microRNAs[32] or with OBP-301.[24] Thus, replication-competent oncolytic adenovirus-mediated *p53* gene transfer would strongly induce apoptosis not only through replication-dependent p53 overexpression, but also through E1A-dependent enhancement of p53-mediated apoptosis.

The molecular mechanism by which E1A suppresses p53-mediated upregulation of p21 and MDM2 remains unclear. Since adenoviral E1A has been shown to repress the expression of many target genes through activation of p300/CBP [cyclic adenosine monophosphate response element-binding protein (CREB)-binding protein] histone acetyltransferases that cause global histone modification,[33,34] p300/CBP activation may be involved in E1A-mediated p21 and MDM2 suppression. Indeed, E1A-mediated p21 and MDM2 suppression has been shown to be regulated in a p300/CBP dependent manner.[29,31] A recent report also suggested that an E1B-defective adenovirus activates p53 expression, but

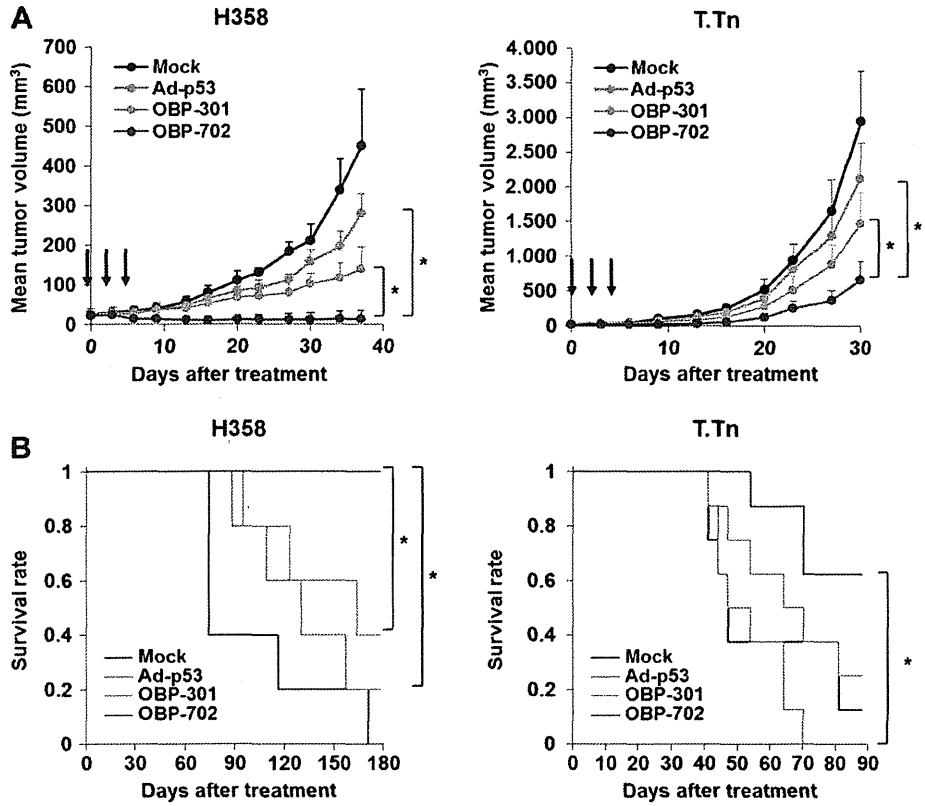

Fig. 5. Strong antitumour effect of OBP-702 on subcutaneous human tumours in xenograft models. (A) H358 or T.Tn cells (5×10^6 cells per site) were inoculated into the flank of 5-week-old female BALB/c *nu/nu* mice. When the tumours reached 3–5 mm in diameter, OBP-702 (10^8 PFU/tumour), OBP-301 (10^8 PFU/tumour), Ad-p53 (10^8 PFU/tumour) or PBS (Mock) was intratumourally injected on days 0, 2 and 4 (Black arrows). Tumour growth is expressed as the mean tumour volume ± SD in each group of H358 tumours ($n = 5$) or T.Tn tumours ($n = 8$). Statistical significance was determined using Student's *t* test. $^*P < 0.05$. The data are representative of three separate experiments. (B) Survival rate in each group of H358 tumours-bearing mice ($n = 5$) or T.Tn tumours-bearing mice ($n = 8$) was shown using the Kaplan–Meier method. Statistical significance was determined using log-rank test. $^*P < 0.05$.

suppresses p21 and MDM2 expression, through the binding of E1A with p300/CBP.[35] However, p300 disruption has also been shown to both increase p53 stability through MDM2 suppression, and to suppress p21 expression, resulting in apoptosis in UV-irradiated human cancer cells.[36] Therefore, the role of p300/CBP in adenoviral E1A-mediated p21 and MDM2 suppression may be cell type-specific.

It has recently been shown that siRNA-mediated p21 suppression enhances the antitumour effect of an oncolytic adenovirus,[37,38] suggesting that p21 suppression further induces oncolytic cell death. Oncolytic adenovirus-mediated cell death has been shown to be associated with autophagy-related cell death, which is distinct from apoptosis.[39,40] Autophagy has been shown to be positively regulated by p53,[14] but negatively regulated by p21.[41] These results suggest that p53 upregulation without p21 activation enhances autophagic cell death. Thus, oncolytic adenovirus-mediated p21 suppression may enhance not only p53-mediated apoptosis, but also autophagic cell death during the OBP-702-mediated antitumour effect.

Telomerase-specific replication-competent OBP-301 that possesses the *hTERT* gene promoter replicates, and induces an antitumour effect in, human cancer cells in a telomerase-dependent manner.[9–11] Previous reports have shown that Ad-p53-mediated p53 overexpression suppresses *hTERT* mRNA expression,[42,43] suggesting possible suppression of OBP-301 and OBP-702 replication by p53 overexpression. However, we previously reported that Ad-p53-mediated p53 overexpression did not suppress OBP-301 replication during combination therapy.[24] Shats et al. previously reported that knockdown of p21 eliminated the p53-dependent repression of *hTERT* mRNA expression.[44] Since OBP-702, or combination therapy of OBP-301 with Ad-p53, induces p53 overexpression together with E1A-mediated p21 downregulation, p53 overexpression may not suppress hTERT expression. Furthermore, we recently demonstrated that OBP-301 infection itself induces a 1.1- to 50-fold increase in *hTERT* mRNA expression in an E1A-dependent manner.[45] Thus, OBP-702-mediated p53 overexpression would induce apoptosis without affecting hTERT expression.

An anti-tumour effect of Ad-p53-mediated gene therapy has been shown in various clinical studies.[17–20] We previously reported that Ad-p53 induces sensitivity to chemotherapeutic drugs, resulting in enhancement of the antitumour effect.[46,47] Since OBP-702-mediated p53 gene transfer has a stronger antitumour effect than Ad-p53 (Fig. 5), combination therapy of OBP-702 with chemotherapeutic agents may be a more effective antitumour therapy than monotherapy of OBP-702. The adenoviral E1A protein has been shown to enhance chemotherapy-induced apoptosis.[48,49] In particular, p21 suppression has been suggested to be involved in E1A-mediated chemosensitisation.[30] Indeed, artificial miRNA-mediated p21 suppression on Ad-p53-induced p53 overexpression enhanced tumour sensitivity to chemotherapeutic agents.[32] Thus, combination therapy of a p53-armed oncolytic adenovirus with chemotherapy may be a more efficient antitumour strategy for eradication of tumour cells through p53 and E1A-mediated chemosensitisation than monotherapy.

In conclusion, we have clearly demonstrated that the p53-expressing oncolytic adenovirus OBP-702 has a much stronger antitumour effect than OBP-301 or Ad-p53 through p53-mediated apoptosis that is enhanced by E1A-dependent p21 and MDM2 suppression. Oncolytic adenovirus-mediated *p53* gene transduction should therefore be a promising antitumour therapy for efficient elimination of tumour cells.

Conflict of interest statement

Yasuo Urata is an employee of Oncolys BioPharma Inc., the manufacturer of OBP-301 (Telomelysin). Other authors declare no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.12.020.

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