Overexpression of Adenovirus-mediated $p27^{kip1}$ Lacking the Jab1-binding Region Enhances Cytotoxicity and Inhibits Xenografted Human Cholangiocarcinoma Growth

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Abstract. *The cyclin-dependent kinase inhibitor (CDK1) $p27^{kip1}$ is a negative regulator of cell cycling and has antitumor effects. In our previous study, the recombinant adenovirus expressing wild-type $p27^{kip1}$ (Adp27-wt) induced cell cycle arrest and apoptosis, and proved that p27 is a tumor suppressor gene like p53. Another adenovirus vector expressing mutant $p27^{kip1}$ (Adp27-mt), which inhibited degradation by the ubiquitin-proteasome system, showed increased protein stability and caused a stronger induction of apoptosis. Recently, the $p27^{kip1}$ protein binding with Jab1 (Jun activating binding protein 1) was found to translocate from the nucleus into the cytosol, and then become degraded by the 26S proteasome system. The inhibition of nuclear-cytoplasmic translocation increases the protein stability of $p27^{kip1}$ and $p27^{kip1}$ with a deletion of the Jab1-binding region (p27-jab-d) is not translocated and not degraded. Therefore, a new recombinant adenovirus (Adp27-jab-d) expressing p27-jab-d was made which was able to induce greater cytotoxicity. Adp27-jab-d inhibited the growth of human cholangiocarcinoma cell line (TFK-1) cells in vitro at 3.3 times (IC_{50}) lower concentration than Adp27-wt. Moreover, in a xenografted severe combined immuno-deficient (SCID) mouse model injected with TFK-1 cells in the subcutaneous tissue, treatment by intratumor injection of Adp27-jab-d once a day for 3 days after the tumor was established, inhibited tumor growth more strongly than Adp27-wt or Adp27-mt and even induced tumor regression. However, the flow cytometric TUNEL assay showed little enhancement of apoptosis. Adp27-jab-d was thought to induce not only apoptosis but also necrosis, which was due to a specific effect of the Adp27-jab-d. Thus, by enhancing the cytotoxicity through inhibiting the translocaton of $p27^{kip1}$, $p27^{kip1}$ lacking the Jab1-binding region might be useful for cancer therapy. The control protein localization might also be a new target not only for cancer treatment, but also other diseases.*

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Based on the rapid progress in understanding the molecular biology of carcinoma, various gene therapies have been applied for clinical use, of which p53 is the most popular. However, the frequency of p53 mutations in cholangiocarcinoma is low (30 to 35%) (1, 2). Therefore, p53-targeted gene therapy against cholangiocarcinoma cannot be widely applied, and a broader and stronger gene therapy is needed.

Cyclin dependent kinase inhibitor (CDKI) $p27^{kip1}$, a member of the Cip/Kip family ($p21^{Waf1/Cip1}$, $p27^{kip1}$ and $p57^{kip2}$), is a negative regulator of the cell cycle progress (3, 4), and $p27^{kip1}$ inhibits the kinase activity of cyclin A-CDK2, cyclin B-CDC2, cyclin D1-CDK4 and cyclin E-CDK2, by preventing CDK activation (3, 5). The importance of $p27^{kip1}$ in regulating the cell cycle is supported by the fact that mice lacking the gene encoding $p27^{kip1}$ have rapid growth, exhibit gross organomegaly and have a higher incidence of pituitary tumors compared to littermate controls (6, 7). Although mutations in $p27^{kip1}$ are rarely found in human tumors (8, 9), reduced expression of the protein correlates well with poor survival among patients with lung (10), breast (11) or colorectal carcinomas(12). $p27^{kip1}$ has to be transported into the nucleus to exert its CDK inhibitory action (13). In quiescent cells, the $p27^{kip1}$ protein located almost exclusively

in the nucleus during G0-phase is increased, but it rapidly decreases on entry into the G1-S-phase triggered by specific mitogenic factors (14, 15). While the level of $p27^{kip1}$ protein fluctuates during the cell cycle, the level of $p27^{kip1}$ mRNA is constant (16, 17). The abundance of $p27^{kip1}$ is controlled both at the level of transcription and by multiple post-translational mechanisms, among which cell-cycle-dependent and substrate-specific proteolysis of $p27^{kip1}$ is the most important (18). The degradation of $p27^{kip1}$ has been reported to involve the phosphorylation of the Thr187 residue by the cyclin E-CDK2 complex (19, 20), nuclear export induced by Jab1 *Jun* activating binding protein 1 (21), ubiquitination mediated by the ligase SCF/Skp2 complex (22, 23), and proteolysis by the 26S proteasome. Skp2 is known as CDK2/cyclin A-associated protein p45. Additionally, signal-transducing adaptor Grb2 facilitates the down-regulation of $p27^{kip1}$ (24).

Jab1 was originally identified as a co-activator of the c-jun transcription factor (25) and is the fifth component of the constitutive photomorphogenesis (COP9) signalosome (CSN) complex, also known as CSN5 (26, 27). A Jab1 binding region is located at amino acids 97-151 of $p27^{kip1}$ and $p27^{kip1}$ binding with Jab1 precedes translocation from the nucleus to the cytoplasm, after which the level of $p27^{kip1}$ is decreased by acceleration of its degradation (21). Jab1 thus causes the degradation of $p27^{kip1}$ and Jab1 expression in breast cancer inversely correlates with the $p27^{kip1}$ level (28).

In our previous study, the overexpression of $p27^{kip1}$ resulted in higher cytotoxicity in human cancer cell lines compared to $p21^{Waf1/Cip1}$, a member of the same family of universal CDK inhibitors (29, 30). We also show that the overexpression of $p27^{kip1}$ triggered apoptosis in several different human cancer cells including the cholangiocarcinoma cell line TFK-1 (31), and recombinant adenovirus expressing wild-type $p27^{kip1}$ (Adp27-wt) had a remarkable tumor inhibitory effect in a xenografted nude mouse model (32) Therefore, $p27^{kip1}$ plays a role as a tumor suppressor gene and the antitumor effect of Adp27-wt could be a useful tool in gene therapy. However, complete repression was not achieved. When Adp27-mt (33) expressing p27-mt protein that had a mutation of Thr-187/Pro-188 to Met-187/Ile-188 stabilizing the $p27^{kip1}$ protein and resisting phosphorylation and therefore ubiquitination was used and subsequent degradation was inhibited. This Adp27-mt showed increased $p27^{kip1}$ expression and induced stronger apoptosis compared to the Adp27-wt (34, 35).

However, Adp27-mt showed a lower antitumor effect on TFK-1 cells compared to its effect MDA-MB-231 human breast cancer cells (36). To enhance the antitumor effect, a more stable $p27^{kip1}$ was required. We hypothesized that $p27^{kip1}$ lacking the Jab1-binding region (p27-jab-d) would be resistant to degradation and have increased activity as a CDK inhibitor, resulting in a more powerful antitumor effect. To confirm this hypothesis, a recombinant adenovirus expressing p27-jab-d protein in which the Jab1 -binding region 97-151 (Adp27-jab-d) was deleted was constructed. The antitumor effect of Adp27-jab-d was compared with Adp27-wt expressing wild-type $p27^{kip1}$ protein and Adp27-mt expressing mutant $p27^{kip1}$ protein that had the mutation of Thr187 described above.

Materials and Methods

Cell culture. Human cholangiocarcinoma cell lines, TFK-1 and HuCCT-1 andhuman breast cancer cell lines, MDA-MB-231 and MCF-7 were cultured at 37˚C in an atmosphere of 5% CO_2 and 95% air in RPMI 1640 medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (JRH Bioscience, Lenexa, KS, USA) and 100 unit/ml penicillin and 100 μg/ml streptomycin sulfate (Gibco BRL, Gaithersburg, MD, USA) (31, 37). A normal human fibroblast cell line, WI-38 was cultured in DMEM medium (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum. All cell lines were provided from Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University.

Construction of Adp27-jab-d and gene transduction. The construction of adenoviral vectors containing human $p27^{kip1}$ (Adp27-wt) or devoid of any transgene (AdNull) has been described previously (35). Adp27-wt is a replication deficient recombinant adenovirus that expresses $p27^{kip1}$. AdNull is a control adenovirus that does not carry any transgene, but has an adenoviral backbone similar to that of Adp27-wt. Adp27-mt expresses p27-mt that has a mutation from Thr-187/Pro-188 (ACGCCC) to Met-187/Ile-188 (ATGATC). Adp27-mt was generated by the same procedure as Adp27-wt. The pCG-N plasmids, which containes SV40 origins of replication under control of CMV promoter, containing mouse $p27^{kip1}$ cDNA inserts lacking the Jab1 interaction domain (p27-jab-d) was kindly provided by Junya Kato, Nara Institute of Science and Technology, Nara, Japan (21). The p27-jab-d, in which the carboxy-terminal amino-acid residues 97-151 were deleted, did not bind to Jab1. The p27-jab-d inserts were cut and inserted into a shuttle vector (pACCMV. pLpA; E1-deleted adenovirus plasmid PJM17). The inserted p27-jab-d in the shuttle vector was sequenced and certified. Then Adp27-jab-d was constructed by co-transfection of the shuttle vector and genomic adenoviral DNA, Ad5CMVlacZΔE1/ΔE3 (Quantum Biotechnologies, QC, Canada). Several adenoviral plaques were screened for the presence of p27-jab-d sequences by polymerase chain reactions. A viral plaque containing p27-jab-d cDNA, but devoid of E1 sequences was used to infect 293 cells. Adp27-jab-d was subsequently purified by double $CsCl_2$ gradient as described previously (29). The adenoviral vectors are illustrated in Figure 1A.

The infection with various adenoviral vectors was accomplished by incubating the cells with various doses of the virus, expressed in pfu (plaque forming unit)/cell, in serum free medium for 2 hours followed by the addition of medium containing 10% serum and further incubation for the required time.

Western blot analysis. TFK-1 cells (2×10^6) were uninfected (control) or infected with 50 pfu/cell of AdNull, Adp27-wt, Adp27-mt, and Adp27-jab-d for 48 h, scraped and then the whole cell lysates were run on SDS-PAGE and subjected to Western blot

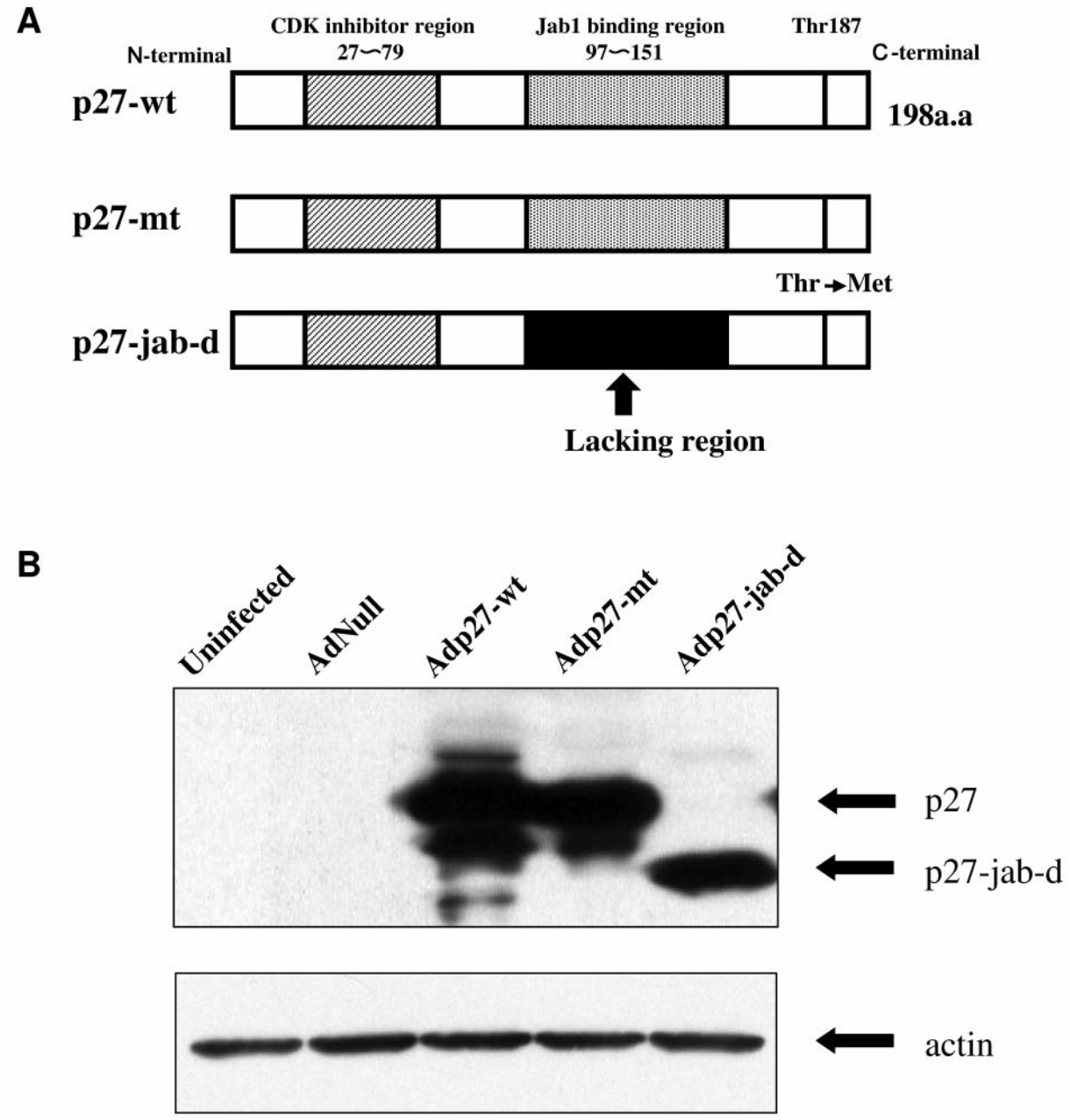

Figure 1. *Construction and expression of p27-wt, p27-mt and p27-jab-d. (A) Schematic representation. (B) Western blot of p27 and p27-jab-d protein expression in adenovirus infected TFK-1 after 48 h incubation.*

analysis as previously described (30, 38). WI-38 cells were infected at 10 pfu/cell of adenovirus vectors for 3 days.

The blots were probed with anti-p27 (sc527, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Jab1 (BD Transduction), anti-actin (sc1615, Santa Cruz Biotechnology), anti-Rb (BD Pharmingen San Diego, CA, USA), anti-cyclinB_1 (sc245, Santa Cruz Biotechnology), anti-cdc2 (Ab-1, Oncogene, Cambridge, MA, USA), anti-cyclinD1 (BD Pharmingen) and anti-PARP (poly ADP-ribose polymerase)(BD Pharmingen).

Cytotoxicity assay. TFK-1, HuCCT-1, MDA-MB-231, MCF-7 and WI-38 cells were plated at 2×10^3 cells/well in triplicate in 96-well plates and incubated for 24 h at 37˚C, and then unaffected (control) or exposed to various doses of AdNull, Adp27-wt, Adp27-mt or Adp27-jab-d for 3 days. The effects were tested by the non-radioactive CellTiter 96 assay (MTT assay) (Promega, Madison, WI, USA), which is a colorimetric method for determining the number of viable cells, according to the manufacturer's directions. These cells were assayed as in a previous paper (29). The IC_{50}

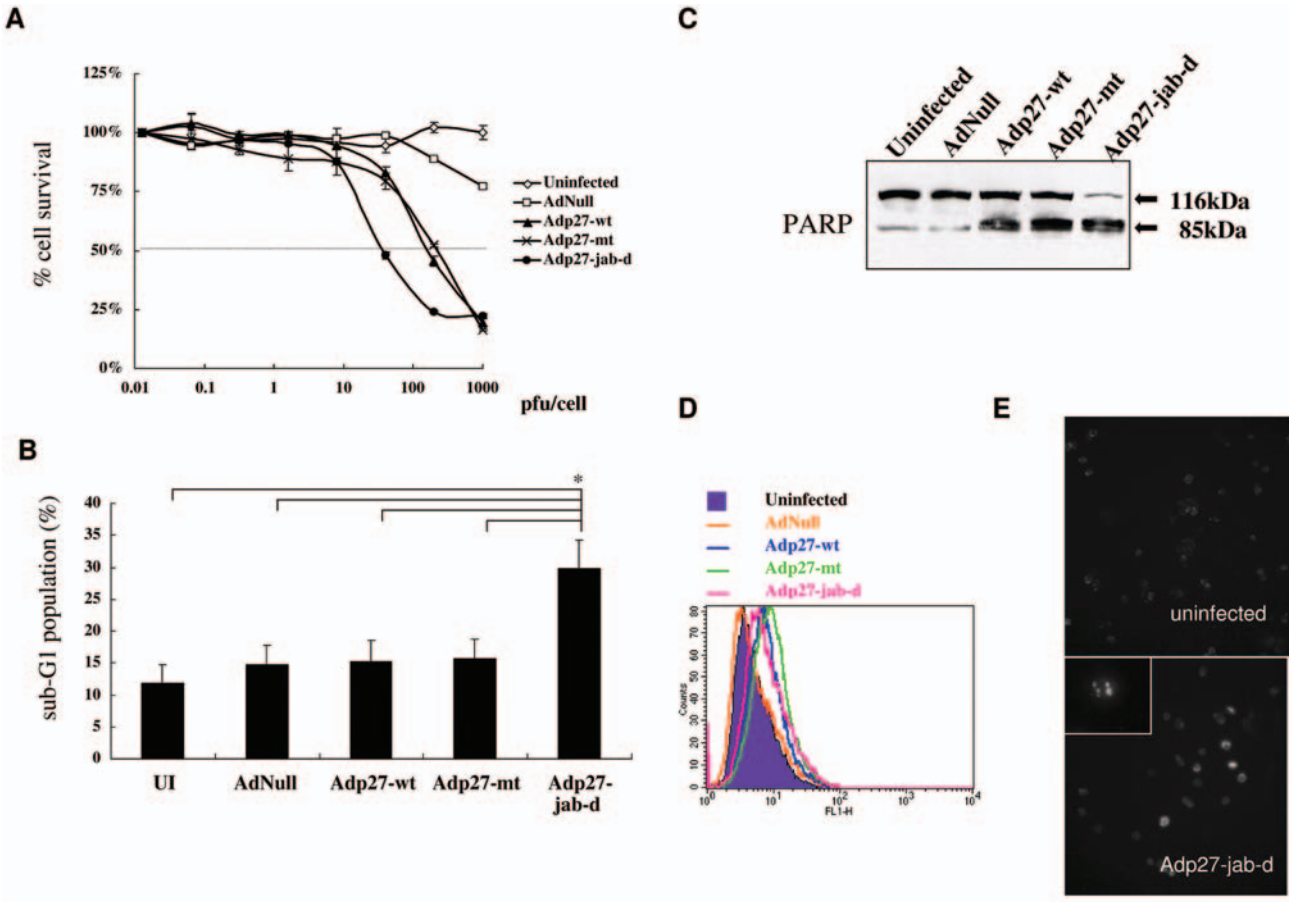

Figure 2. *Cytotoxicity and induction of apoptosis in TFN-1 cells. (A) MTT assay after 48 h incubation with adenoviral vectors. Each value is an average of triplicate wells; bars, SE. (B) Sub-G1 population by propidium iodide staining and flowcytometry 48 h after infection with 50 pfu/cell of adenovirus vectors. Data are the averages of three independent experiments; bars, SE. *p<0. 05 for Adp27-jab-d versus UI (uninfected) AdNull, Adp27-wt and Adp27-mt. (C) Western blot analysis of PARP cleavage 48 h after adenovirus infection 116 kD band, a native band of PARP and 85 kD band, a specific PARP cleavage product. (D) Flow cytometric TUNEL assay and (E) staining (inset panel, one apoptotic cell at higher magnification) 48 h after adenovirus infection.*

(median toxic viral dose) was calculated assuming the survival rate of uninfected cells to be 100%. Fold differences in IC_{50}'s were calculated by dividing the IC_{50} of Adp27-wt by the IC_{50} of Adp27-jab-d or Adp27-mt.

Cell cycle analysis and sub-G1 population. TFK-1 cells were uninfected (control) or infected with 10 or 50 pfu/cell of AdNull, Adp27-wt, Adp27-mt or Adp27-jab-d for 48 h. 50 pfu/cell of AdNull, Adp27-wt, Adp27-mt or Adp27-jab-d. The cell cycle distribution and the subG1 (sub-diploid) population were analyzed in by measuring the fluorescence activity of propidium iodide stained DNA of the permeabilized and fixed cells on a FACSCalibur instrument (Becton Dickinson, Mansfield, MA), using the CycleTest Plus kit protocol (Becton Dickinson, San Jose, CA, USA) % sub-G1 population=number of cells belowG1/total number analyzed (10,000) × 100.

TUNEL method. For the detection of apoptosis by the TUNEL (TdT-mediated dUTP-biotin nick end labeling) method, the 2×10^6 TFK-1 cells (60% confluence of 75 cm^2 flask) were uninfected (control) or infected with the adenoviral vectors, after 48 h incubation the cells were collected by 0.02% EDTA in phosphate buffered saline (PBS) and washed two times with PBS. TUNEL was performed using a Mebstain Apoptosis Kit (Medical and Biological Laboratories Co. Ltd, Nagoya, Japan) according to the manufacturer's directions. The FACS analysis was performed on a FACSCalibur instrument (Becton Dickinson). Cytospin mounted glass slides were viewed under a fluorescence microscope.

Cdc2 kinase assay. TFK-1 cells were cultured in 15 cm dishes (60% confluence) and uninfected or infected with 10 pfu/cell of recombinant adenoviruses for 48 h. Then the cells were harvested and 2×10^7 cells were applied to a Mesacup cdc2 kinase assay kit (Medical and Biological Laboratories Co. Ltd) according to the manufacturer's protocol. This kit is a non-radioisotopic assay kit for measuring cdc2 kinase activity and is based on enzyme linked immunosorbent assay that utilizes a synthetic peptide as a substrate for cdc2 kinase and monoclonal antibody recognizing the phosphorylated form of the peptide substrate. OD at 492 nm was measured and calculated assuming the OD of uninfected TFK-1 cells as 1.

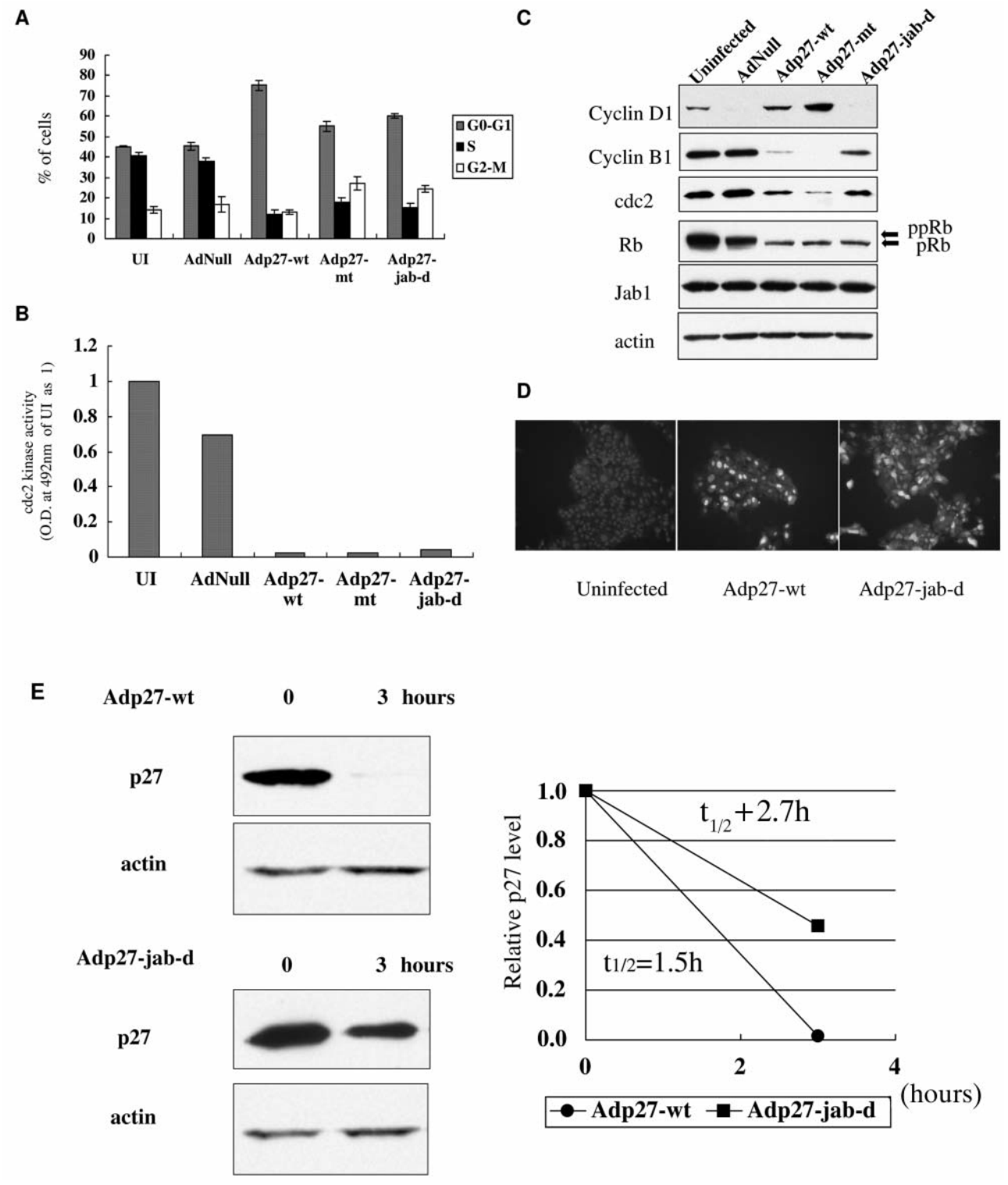

Figure 3. *Characterization of the p27-jab-d protein expressed by Adp27-jab-d in TFK-1 cells. (A) Cell cycle distribution staining and flowcytometry by propidium iodide 48 h after infection with 10 pfu/cell of adenoviral vectors.Data are the mean of three independent experiments; bars, SE UI, uninfected. (B) Cdc2 kinase assay. (C) Western blot analyses of Rb, cyclin B1, cyclin D1, cdc2 and Jab1 protein 48 h after. infection with 50 pfu/cell Actin was used to verify equal loading between wells. (D) Immnofluorescence staining of* $p27^{kip1}$*. 48 h after adenoviraul infection. (E) Protein stability assay. Western blot after the addition of 20 μg/ml cycloheximide. The relative* $p27^{kip1}$ *and actin levels were quantified by NIH image (1.62). The quantified relative p27 and actin level were plotted in a graph and calculated protein half life of p27-wt and p27-jab-d.*

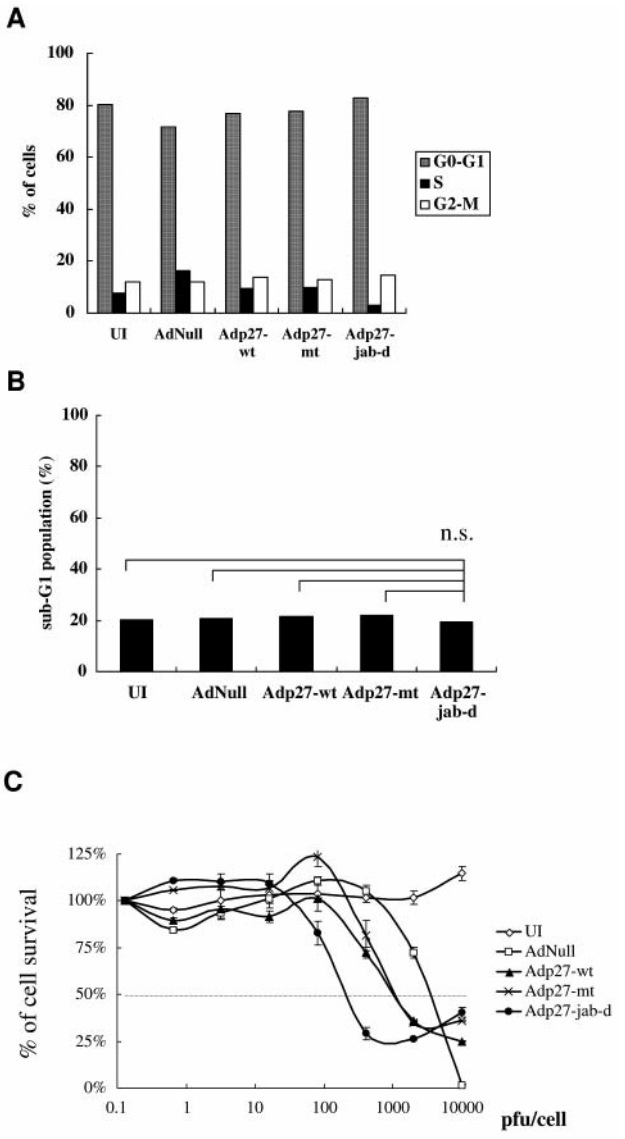

Figure 4. *The effects of recombinant adenoviruses in human fibroblast cell line, WI-38. (A, B)WI-38 cells (80% confluence) were infected at 10 pfu/cell of adenovirus vectors for 3 days. Cell cycle distribution and sub-G1 population measured by flowcytometry. (C) MTT assay. Each value is the mean of triplicate wells ; bars, SE. UI, uninfected.*

Immnofluorescence staining. TFK-1 cells were uninfected (control) or infected with 50 pfu/cell of Adp27-wt or Adp27-jab-d for 48 h. Then the cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0. 5% Triton X-100 in PBS for 5 min and blocked with 1% BSA in PBS for 10 min at room temperature. The cells were then incubated with the primary antibody (anti-p27 antibody) overnight at 4˚C followed by incubation with the secondary antibody, FITC-conjugated goat anti-rabbit IgG (AlexaFlour 488, Molecular Probes, Invitrogen, Carlsbad, CA, USA) (39). The cells samples were viewed by fluorescence microscopy.

Protein stability assay. The 2×10^6 TFK-1 cells (60% confluence of 75 cm^2 flask) were infected with 10 pfu/cell of Adp27-wt or Adp27-jab-d as described above. After 24 h the supernatant was removed, the cells were washed in the plates with PBS and medium including 20 μg/ml cycloheximide was added to inhibit protein synthesis. One set of cultures was harvested immediately and another after 3h incubation and then the whole cell lysates were subjected to Western blot analysis with anti-p27 and anti-actin antibodies. The relative p27 and actin levels were quantified by NIH image (1.62). The quantified relative p27 and actin level were plotted in a graph and calculated protein half life of p27-wt and p27-jab-d.

Treatment of established cholangiocarcinoma xenografts of adenovirus vectors. To investigate the *in vivo* effects of Adp27-jab-d, cholangiocarcinoma xenografts were established. TFK-1 cells (5×10^6) were injected into the subcutaneous tissue of 6-week old female severe combined immune-deficient (SCID) mice purchased from Charles River Laboratories Japan, Inc(Yokohama, Japan). After 2 weeks, when small tumors (3-5 mm in diameter) were visible, intratumoral injections of AdNull, Adp27-wt, Adp27-mt or Adp27-jab-d (5.0×10^8 pfu) were initiated and repeated once a day for 3 days. The tumor sizes were calculated every week by the following formula: tumor volume (mm^3)=1/2×(length)^2×width.

Statictics. The values are given as the mean±SE. Statistical analysis of the data was preformed using Fisher's protected least significant difference test. $P<0.05$ was considered significant.

Results

Effect of Adp27-jab-d on p27-jab-d expression. Western blot assay of the $p27^{kip1}$ in the proteins extracted from Adp27-wt, Adp27-mt and Adp27-jab-d infected TFK-1 cells showed the overexpression of $p27^{kip1}$ protein (Figure 1B). The $p27^{kip1}$ protein mediated by Adp27-jab-d was smaller in size than p27-wt or p27-mt (22 kD), because p27-jab-d was lacking amino acids 97-151 of $p27^{kip1}$. The p27-jab-d expression was less than the p27-wt or p27-mt expression.

Cytotoxicity and induction of apoptosis. The MTT assay showed that the survival rate of the TFK-1 cells was lower with Adp27-jab-d than with Adp27-wt or Adp27-mt (Figure 2A). Adp27-jab-d was 3.3-fold more cytotoxic than Adp27-wt in the TFK-1 cells. Comparisons of the effect of Adp27-jab-d or Adp27-mt infection with Adp-wt in all four cancer cell lines, on the IC_{50} measured by MTT assay are shown in Table I. Adp27-mt had stronger cytotoxicity than Adp27-wt in MDA-MB-231 the as previously reported (33). However, Adp27-mt did not enhance the cytotoxicity in the MCF-7 and TFK-1 cells. Adp27-jab-d showed enhanced cytotoxicity in all of the cell lines.

The confirmation of apoptosis was demonstrated by TUNEL, sub-G1 population by flowcytometry and PARP cleavage by Western blot. A marked increase in the sub-G1 population as detected by propidium iodide staining and

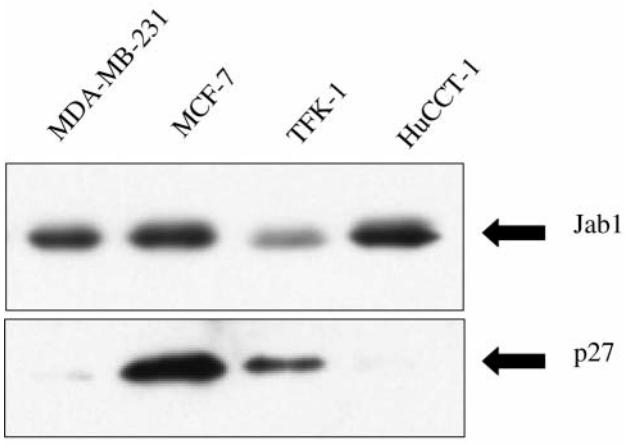

Figure 5. *Western blot of Jab1 and p27^{kip1} expression in several cancer cell lines.*

Table I. *Comparison of fold difference* in IC_{50} by MTT assay in several cancer cell lines.*

	MDA-MB 231	MCF-7	TFK-1	HuCCT-1
Adp27-jab-d	9.9±2.4	4.4±1.2	3.3±0.7	4.5±0.8
Adp27-mt	6.8±2.3	0.7±0.3	1.1±0.2	4.2±0.6

$$\text{*Fold difference} : \frac{IC_{50} \text{ of Adp27-wt}}{IC_{50} \text{ of Adp27-mt or Adp27-jab-d}}$$

Values are the means of three independent experiments ±SE.

flowcytometry analysis was shown in the Adp27-jab-d infected cells (Figure 2B). The sub-G1 population reached 32.3% in the cells infected with Adp27-jab-d, while 14 to 18% of the uninfected and AdNull, Adp27-wt or Adp27-mt infected cells had DNA contents in the sub-G1 population.

Figure 2C shows the increase of a specific 85 kD cleavage product of PARP, a hallmark of apoptosis, in the cells infected with Adp27-wt, Adp27-mt and Adp27-jab-d, compared to the uninfected or AdNull infected cells. PARP is known to be a key substrate and is associated with the activation of the biochemical pathway of apoptosis. This result suggested that Adp27-jab-d induced apoptosis as well as Adp27-wt and Adp27-mt.

The TUNEL assay was performed to analyze DNA fragmentation, another key hallmark of apoptosis. As shown in Figure 2D, infection of TFK-1 cells with recombinant adenoviruses caused apoptosis, however Adp27-jab-d did not enhance apoptosis compared to Adp27-wt or Adp27-mt independently of the sub-G1 population. Figure 2E shows TUNEL nuclear staining of the Adp27-jab-d infected apoptotic cells.

Characterization of Adp27-jab-d pritein. In order to verify the function of the p27-jab-d protein expressed by Adp27-jab-d, the cell cycle distribution and cdc2 kinase activity were analyzed. The TFK-1 cells infected with Adp27-jab-d showed a decrease in the number of cells in the S-phase (Figure 3A). Adp27-jab-d induced G1 arrest as did Adp27-wt and Adp27-mt. The cdc2 kinase activity was obviously decreased by infection with Adp27-jab-d as well as Adp27-wt and Adp27-mt (Figure 3B). Thus the p27-jab-d of Adp27-jab-d inhibited the cdc2 complex and cdc2 kinase. The cell cycle arrest and cdc2 kinase assay comfirmed the function of p27-jab-d protein as a CDK inhibitor.

To confirm whether cyclin B1, D1 and cdc2 were related to the cell cycle arrest induced by Adp27-jab-d, Western blot analysis of the various cyclins and cdc2, Rb and Jab1 proteins was performed in TFK-1 cells infected with 50 pfu/cell of the adenoviral vectors for 48 h (Figure 3C). Dephosphorylation of Rb protein was induced by Adp27-wt, Adp27-mt and Adp27-jab-d. The Adp27-wt and Adp27-mt infected cells showed increased cyclin D1 expression, but the Adp27-jab-d infected cells showed decreased expression. The levels of cdc2 and cyclin B1 protein were decreased in the Adp27-wt, Adp27-mt and Adp27-jab-d infected cells, though the decrease in the Adp27-jab-d infected cells was not as great as that with Adp27-wt or Adp27-mt. The Jab1 protein level was constant in the uninfected and the infected cells.

Immnofluorescence staining showed p27^{kip1} expression in the Adp27-jab-d infected TFK-1 cells as well as in the Adp27-wt infected cells (Figure 3D). No difference in the location of p27^{kip1} expression was distinguished between the Adp27-wt and Adp27-jab-d infected cells. The Adp27-jab-d infected cells tended to have swollen nuclei and distorted/disrupted cell shape, compared to the Adp27-wt infected cells with preserved shape.

The protein stability assay demonstrated that the p27-jab-d protein was more stable than the p27-wt protein (Figure 3E). While the p27-wt induced by Adp27-wt had a half-life of about 1. 5 h, p27-jab-d induced by Adp27-jab-d had a half-life of about 2. 7 h.

The influence of recombinant adenoviruses in a human fibroblast cell line. The cell cycle analysis of the nomal human fibroblast, WI-38 cells infected with the recombinant adenoviruses Figure showed no significant change in the cell cycle distribution (Figure 4A) and sub-G1 population (Figure 4B) between the uninfected cells and the cells infected with AdNull, Adp27-wt, Adp27-mt or Adp27-jab-d. Although the IC-50, shown by MTT assay, of the Adp27-jab-d infected cells was low compared with Adp27-wt or Adp27-mt, infection under 10 pfu/cell did not show cytotoxicity in the WI-38 cells (Figure 4C).

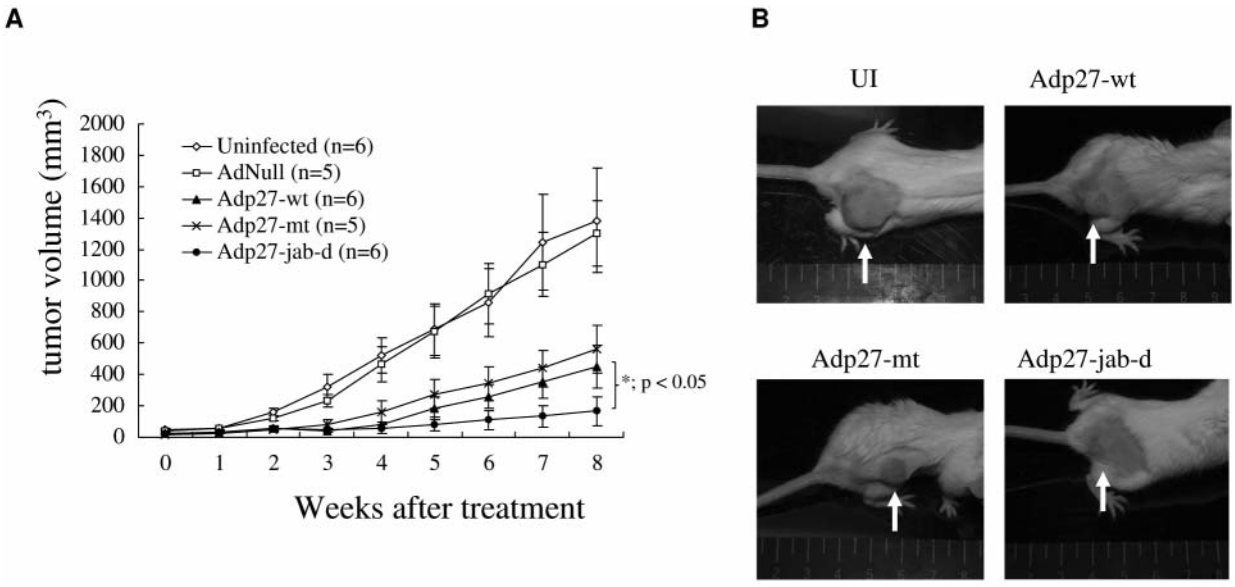

Figure 6. *Intratumoral injection of adenovirus vectors in human cholangiocarcinoma xenografted SCID mouse model. Intratumoral injections of AdNull, Adp27-wt, Adp27-mt or Adp27-jab-d (5.0×10^8 pfu) were given once a day for 3 days 2 weeks after TFK-1 cell (5.0×10^6) infection. (A) Tumor size Each point represents the mean (±SE). *$p<0.05$ for Adp27-jab-d versus Adp27-wt and Adp27-mt by Fisher's protected least significant difference test. (B) Xenografted SCID mice at 8 weeks after treatment UI: uninfected (no adenovirus treatment). White arrow indicates tumor.*

The level of endogenous p27^{kip1} in various cell lines. The level of endogenous p27^{kip1} and Jab1 protein, demonstrated by Western blot analysis in the various cell lines is shown in Figure 5. The endogenous p27^{kip1} protein was detected in the MCF-7 and TFK-1 cells and Jab1 protein was expressed in all of the cell lines. The TFK-1 cells showed relatively low Jab1 expression. Relatively high levels of endogenous p27^{kip1} expression were inversely correlated with Jab1 expression except in the MCF-7 cells.

Effect of intratumoral injection of adenovirus vectors in the human cholangiocarcinoma xenografted SCID mouse model. A s shown in Figure 6, the intratumor injection of Adp27-wt, Adp27-mt or Adp27-jab-d showed tumor growth suppression. At 8 weeks after treatment, Adp27-jab-d had induced a significant reduction in the rate of tumor growth compared with Adp27-mt or Adp27-wt ($p<0.05$). Moreover, 33% of the Adp27-jab-d injected mice showed complete tumor regression.

Discussion

Wild type p27^{kip1} protein is known to have a short half-life (under 2 h) and the protein stability assay demonstrated that the p27-jab-d protein was resistant to degradation and had a more prolonged half-life than wild-type p27^{kip1} protein (Figure 3E). Though the p27-jab-d was lacking amino acids 97-151, the CDK binding region was preserved and p27-jab-d had activity as a CDK inhibitor in the Western blot analysis of Rb protein, the cdc2 kinase assay and the cell cycle analysis (Figure 3A, B, C).

The p27^{kip1} expression induced by Adp27-wt and Adp27-jab-d was confirmed by immunofluorescence staining which also demonstrated that the Adp27-jab-d infected cells tended to have swollen nuclei and destored / disrupted cell shape, compared with the Adp27-wt infected cells, indicating that Adp27-jab-d induced necrosis. Although the p27-jab-d expressed by Adp27-jab-d was thought to inhibit translocation from the nucleus to the cytoplasm, there was no difference in the location of p27^{kip1} expression between Adp27-wt and Adp27-jab-d in this experiment (Figure 3D). This might have been because the p27^{kip1} expression by the adenovirus vector was too great to remain only in the nucleus. In cells expressing ectopic Jab1, p27^{kip1} exported from the nucleus to the cytoplasm is degraded, while p27-jab-d remains in the nucleus (21). Thus, the difference in the location of p27^{kip1} might have been more clear in the present experiment, if the jab1 expression vector had been co-transfected.

Adp27-jab-d showed the strongest cytotoxicity among the three recombinant adenoviruses. In our previous report, the overexpression of p27^{kip1} triggered apoptosis in several cancer cell lines. In this study, the apoptosis assay by TUNEL and PARP cleavage demonstrated that the three recombinant adenoviruses expressing p27-wt, p27-mt or p27-jab-d induced apoptosis. Although Adp27-mt induced stronger apoptosis than Adp27-wt, Adp27-jab-d did not induce apoptosis strongly. These results suggested that

Adp27-jab-d may induce cytotoxicity not only by apoptosis but also by necrosis.

It was reported that Jab1 expression inversely correlated with $p27^{kip1}$ in breast cancer (28). Relatively high levels of endogenous $p27^{kip1}$ expression that were inversely correlated with Jab1 expression were demonstrated in several cancer cell lines (excluding MCF-7) (Figure 5) in the present study. Also as shown in Table I, Adp27-jab-d showed 3.3±0.7 to 9.9±2.4 -fold greater cytotoxicity in (IC_{50}) compared to Adp27-wt in several cancer cell lines, while, there was no significant difference between Adp27-wt and Adp27-mt in the high endogenous $p27^{kip1}$-expressing cell lines MCF-7 and TFK-1. MCF-7 and TFK-1 cells may be able to tolerate high levels of $p27^{kip1}$ expression and therefore $p27^{kip1}$ overexpression by Adp27-mt did not show stronger cytotoxicity. Nevertheless, Adp27-jab-d had stronger cytotoxicity than Adp27-wt or Adp27-mt in these cell lines. These facts support the inference that Adp27-jab-d must have other mechanisms for cytotoxicity in addition to the apoptosis induced by p27-jab-d overexpression. There were differences in the expression level of cyclin B and cyclin D in Western blot among the adenoviruses (Figure 3C), implying the existence of other mechanisms induced by Adp27-jab-d. In our previous study, infection of Adp27-wt induced the expression of the Fas ligand (FasL), suggesting that apoptosis by Adp27-wt was mediated by the Fas pathway (40). There was no difference in Fas and FasL expression by flow cytometric analysis among the adenoviruses. The cytotoxic mechanism of Adp27-jab-d, apart from apoptosis, remains unclear.

The strong antitumor effect of Adp27-jab-d was reconfirmed in the cholangiocarcinoma xenografted SCID mouse model. Intratumor injection of Adp27-jab-d induced tumor regression and inhibited the tumor growth more strongly than Adp27-wt or Adp27-mt (Figure. 6A, B). Moreover, 33% of Adp27-jab-d injected mice showed complete tumor regression.

When the normal human fibroblast, WI-38cells were examined most were in the G0-G1 phase and were little influenced by Adp27-wt, Adp27-mt or Adp27-jab-d when infected at less than 10 pfu/cell (Figure 4).

In conclusion, the new recombinant adenovirus Adp27-jab-d shows enhanced antitumor effects and could achieve the same cytotoxicity as Adp27-wt at a lower dose. Controlled localization of protein may reduce cytotoxicity for normal cells. Therefore, this treatment might be useful for developing new cancer treatments.

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