Adenovirus-mediated eukaryotic initiation factor 4E binding protein-1 in combination with rapamycin inhibits tumor growth of pancreatic ductal adenocarcinoma *in vivo*

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Abstract. Over-expression of eIF4E indicates a poor prognosis in different tumors. In the present study, we investigated the frequency of eIF4E, 4E-BP1 and phosphorylated 4E-BP1 expression in PDAC cell lines, gastric carcinoma (GC) cell lines and human embryonic pancreatic cells, as well as gene therapy using translation repressor gene 4E-BP1 in combination with the mTOR inhibitor rapamycin. We also assessed the significance of eIF4E expression in 80 PDAC cases. Combination therapy of adenovirus vector-delivered 4E-BP1 gene and rapamycin was administered to determine their growth inhibition effect *in vitro* and *in vivo* in mice. Our study revealed that all PDAC cell lines, GC cell lines and human embryonic pancreas-derived cells expressed the 25-kDa eIF4E protein (MIAPaca-2 cells also expressed the 13-kDa protein 4E-BP1). The 80 PDAC specimens showed a heterogeneous pattern of eIF4E staining. No significant correlation between eIF4E expression and TNM classification was found. Adenovirus vectors Ad-4E-BP1 and Ad-GFP efficiently showed transgenic expression with hyperphosphorylation of 4E-BP1; however, insignificant growth inhibition of the PDAC and GC cell lines was observed. Combination therapy with rapamycin significantly inhibited proliferation and tumor growth *in vitro* as well as *in vivo*. Therefore, combination of Ad 4E-BP1 and rapamycin may be a more effective adjuvant therapy.

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Introduction

Pancreatic cancer is one of the most aggressive and devastating human malignancies, with pancreatic ductal adenocarcinoma (PDAC) being the most common. Difficulties in early diagnosis and less-than-effective chemotherapy have resulted in no reduction in the number of PDAC cases. Every year approximately 0.001% of the population in western countries are diagnosed with PDAC (1). The 5-year survival rate for all stages of PDAC is <4%, so there is an urgent need for more effective therapy for patients with advanced disease (2). In this context, much attention has been paid towards the endogenous tumor proteins responsible for cell growth, proliferation, differentiation, and invasion. The identification of these proteins will provide valuable aids for molecular diagnosis and for developing novel therapeutic tactics for better prognosis of patients. Among these strategies, those employing replication-deficient recombinant adenovirus-delivered translation repressor genes in combination with drugs effective against signal transduction pathways play an important role.

Translation initiation in mammalian cells involves a set of proteins called eukaryotic initiation factors (eIFs). EIF4E is a 25-kDa protein found on chromosome 4q21-q25 in humans, and is responsible for binding the 5'm7GpppN cap structure (where m is a methyl group and N any nucleotide) present in all mRNAs. The process starts with formation of the eIF4F complex, which consists of two other subunits (3). Eukaryotic translation initiation factors have been shown to play an important role in cell growth and proliferation, and over-expression has been documented in breast, head and neck and gastric carcinoma (GC) with unfavorable clinical prognosis (4-8). Therefore, in the present study we screened expression of eIF4E and 4E-BP1 in human PDAC and examined their correlation with clinicopathological features.

Translation initiation is dependent on the phosphorylation status of translation repressor eIF4E-binding protein-1 (4E-BP1), which is regulated by the upstream molecule mammalian target of rapamycin (mTOR) in the PI3K/AKT pathway (9-13). Rapamycin, which is a highly specific inhibitor of mTOR, is currently used as an immunosuppressant and as an antifungal

agent and may be a potential new anti-tumor drug (14-17). This leads to the dissociation of 4E-BP1 from initiation factor eIF4E, permitting an increase in protein translation and mitogenesis. In contrast, dephosphorylated 4E-BP1 interacts with eIF4E and thereby inhibits cap-dependent protein synthesis and cell growth (18). A previous study determined that the mTOR/FRAP-p70s6k pathway is constitutively active in two serum-starved PDAC cell lines (Panc-1 and MiaPaca-2) and express translation repressor 4E-BP1 (19). In contrast, we focused on examining these cell lines under normal growth conditions, and found low 4E-BP1 expression in MiaPaca-2 cells alone. However, cDNA delivered via an adenoviral vector efficiently expressed total and hyperphosphorylated 4E-BP1. Rapamycin forms a complex with FK binding protein (FKBP-12) that then binds strongly with mTOR. Hypophosphorylated 4E-BP1 interacts with translation initiation factor eIF4E and inhibits cap structure-dependent protein synthesis and cell growth (20-24). In the present study, we demonstrated the therapeutic value of replication-deficient recombinant adenovirus delivered translation repressor 4E-BP1 used in combination with the mTOR inhibitor rapamycin.

Materials and methods

Cell lines. The human embryonic pancreas-derived cell lines 1C3D3 and 1B2C6 and the human PDAC cell lines Panc-1, MIAPaca-2 and KP-4 were purchased from RIKEN Cell Bank (Tsukuba, Japan). The PDAC cell lines SUIT-2 and KP1N were from the Health Science Research Resources Bank (Osaka, Japan). The PDAC cell line PK-9 and the human erythromyeloblastoid leukemia cell line K-562 were kindly provided by Tohoku University (Sendai, Japan). The human GC cell lines MKN-1 and MKN-45 were obtained from the Japanese Collection of Research Bio Resources (Tokyo, Japan). All cell lines were cultured in the recommended medium, supplemented with 10% FBS, 1% penicillin and streptomycin (Gibco, Invitrogen Corp., Carlsbad, CA, USA), and maintained in a humidified incubator under 5% CO_2 at 37˚C.

Patients and tissue specimens. Surgically resected specimens from 80 patients with ductal pancreatic adenocarcinomas were examined. All patients underwent surgical resection between 1992 and 1999 at the Second Department of Surgery at Hokkaido University School of Medicine, the Department of Surgery at Teinekeijinkai Hospital, and at the Department of Surgery at Hokkaido Gastroenterology Hospital. Among the 80 patients with cancer, 61 patients underwent pancreaticoduodenectomy (Whipple operation), 14 patients underwent distal pancreatectomy, and 5 patients underwent total pancreatectomy; all patients received extended radical lymphadenectomy. Of the 23 patients with positive portal vein invasion, 17 patients received portal vein resection. Pancreatic resection was not performed in patients presenting with distant site metastases. Any cases of mucinous cystadenocarcinoma or intraductal papillary-mucinous carcinoma were excluded from our study. None of the patients received either radiation or chemotherapy. Ductal pancreatic adenocarcinoma tissue was obtained from 45 men and 35 women with a mean age of 62 years (range 31-83 years). The median duration of follow-up was 95.2 months (range 50.0-141.7 months), and 71 patients (88.8%) died during the follow-up period. Sixty-five cases had recurrence and the types of recurrences were clarified in 48 of them. The site of recurrence was hepatic metastasis in 24 cases, peritoneal recurrence in 12 cases, local or lymph node recurrence in 10 cases, and other types of recurrence in 2 cases. These specimens were from the same cohort as our previously reported study with renewed outcome data for surviving patients (25). All informed consent processes for this study were conducted in accordance with the guidelines of the Hokkaido University Institutional Review Board.

All specimens were fixed in 10% formalin and embedded in paraffin wax. Unstained 4-μm sections were then cut from paraffin blocks for immunohistochemical analysis. Histological classification of tumors was based on the WHO criteria. All tumors were staged according to the pathological Tumor-Node-Metastasis (TNM) Classification of the International Union against Cancer. Seventeen of the tumors were classified as stage II, 32 as stage III, and 31 as stage IV tumors. All immunostained slides were inspected by two independent observers who were blinded to the clinical information.

Mice. For the *in vivo* study, female BALB/c-nu/nu mice, 4-6-weeks old, were purchased from Japan Charles River Laboratory (Tokyo, Japan) and maintained under specific pathogen-free conditions. All animal procedures were conducted according to the Institutional Guidelines for Animal Care of Hokkaido University.

Drugs. Rapamycin isolated from *Streptomyces hygroscopicus* was purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored protected from light at -20˚C. For the *in vitro* experiments, a 1-mg/ml stock solution was made up using dimethyl-sulfoxide (DMSO) (Wako Chemicals, Osaka, Japan). Stock solution was diluted to the desired final concentration with growth medium just before use. For the *in vivo* experiments in mice, rapamycin was dissolved in ethanol to prepare a 10-mg/ml stock solution and then diluted in 5% PEG400, 5% Tween-80 and 4% ethanol as vehicle.

Adenovirus construction. Adenoviruses regulated by the CMV promoter containing the green fluorescent protein (GFP) gene (Ad-GFP) or both GFP and the human 4E-BP1 gene (Ad-BP1) were prepared using the Vira Power adenovirus expression system (Invitrogen) according to the gateway method. In brief, each full-length cDNA was cloned into the pENTR vector, and the cDNA insert then transformed into the pAd/CMV/V5-DEST vector (Invitrogen) by the gateway system and using LR clonase (Invitrogen). The resulting plasmid was purified and digested with PacI (New England BioLabs, Herts, UK). Linearized plasmids (8 μg) were mixed with 32 μl of Lipofectamine 2000 (Invitrogen) in 500 μl of Opti-MEM medium (Invitrogen) and the mixture was added to sub-confluent HEK293 cells cultured in 1 ml of medium in 6-well culture plates. All the viruses were propagated in packaging HEK293 cells, purified by ultracentrifugation in a cesium chloride gradient, filtered through an Amicon Ultra Centrifugal Filter Device (Millipore, Bedford, MA, USA) and stored at -80˚C, according to the manufacturer's protocols

(26,27). The titer for each virus was determined using the Adeno-X rapid titer kit (BD Biosciences Clontech, Palo Alto, CA, USA). Each titer described in this report is expressed as infection-forming unit/milliliter (ifu/ml). The initial viral titer yielded $1.0x10^{10}$ ifu/ml.

Subcutaneous tumor model. KP1N or MIAPaca-2 cells ($5x10^6$ cells/100 μl PBS) were implanted subcutaneously (s.c.) into the left flanks of BALB/c-nude mice. When the resultant tumors reached 3-5 mm in diameter, the mice were randomly placed into six different groups (10 mice in each group). The first group was treated with rapamycin alone, the second group was treated with a combination of rapamycin and Ad 4E-BP1, the third group with rapamycin and Ad-GFP, the fourth group with Ad-GFP (at MOI 100) alone, the fifth group with Ad 4E-BP1 alone (at MOI 100), and the sixth group was the control group (treated as described below). Rapamycin was delivered by intra-peritoneal (i.p.) injection at 1 mg/kg body weight every alternate day for 4 weeks with control animals receiving vehicle alone. The control group received intra-tumoral (i.t.) injection of PBS on days 0 and 14, and i.p. injection of vehicle alone every alternate day. The tumors were monitored every day and measured after a 7-day interval; the tumor volume was calculated as follows: tumor volume = length x width^2/2. For *in vivo* gene transduction efficiency analysis, one mouse from each group was sacrificed 72 h after intra-tumoral injection and tumor tissues snap-frozen in liquid nitrogen and homogenized lysates prepared.

Western blot analysis. Total cell lysates were prepared from all the cell lines mentioned above, either non-treated or treated only with the adenovirus vectors Ad-GFP or Ad-BP1 with a multiplicity of infection (MOI) of 100, or treated with a combination of adenovirus vector and rapamycin at different concentrations. In addition, subcutaneous gene transduction efficiency was determined using lysates prepared as described above. All protein extracts were separated by electrophoresis, transferred onto nitrocellulose membranes (Schleicher and Schüell, Freiburg, Germany) and probed with different antibodies: mouse monoclonal anti-eIF4E (sc-9976) and mouse monoclonal anti-4E-BP1 (sc-9977, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-p4E-BP1 (ser65) (9451, Cell Signaling Technology, Danvers, MA, USA), mouse anti-green fluorescent protein (MAB 3580, Chemicon International, Temecula, CA, USA) and mouse anti-ß actin antibody (MAB 150-1R, Chemicon International). They were then incubated in a 1:10000 dilution of peroxidase-conjugated goat anti-mouse antibody (69779, Jackson Immuno Research Lab, West Grove, PA, USA) and peroxidase-conjugated goat anti-rabbit antibody (72323, Jackson Immuno Research Lab). The immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Aylesbury, UK).

Immunohistochemical staining. Immunohistochemical staining of tumor specimens from the 80 PDAC patients was also performed. Each slide was deparaffinized in xylene and dehydrated in descending concentrations of ethanol and washed three times for 5 min each with double distilled water and treated with pH 6.5 citric acid buffer in a pressure cooker for 2 min.

After blocking in 0.3% H_2O_2 and methanol, the slides were incubated in 10% goat anti-serum for 30 min. Primary eIF4E antibody (sc-9976, Santa Cruz Biotechnology) was applied at a 1:200 dilution in antibody dilutent solutions (Dako-Cytomation, Glostrup, Denmark), and the slides incubated overnight at 4˚C. The anti-eIF4E antibody was detected by adding immuno-peroxidase polymer anti-mouse and anti-rabbit antibody simple stain (MAX-PO MULTI, Nichirei Corp., Tokyo, Japan). The peroxidase activity was visualized with 3'3' diaminobenzidine tetrahydrochloride (Histofine Simple Stain DAB solution; Nichirei Corp.) as the substrate. The sections were then lightly counterstained with hematoxylin. The negative control used mouse isotype IgG1 as the primary antibody.

Although qualitative differences in staining intensity were observed, all positive cases showed heterogeneous staining in the cytoplasm of some normal acinar cells, normal pancreatic ductal cells and some ductal adenocarcinoma cells, at least focally.

In the present study, the level of eIF4E expression, as determined by staining intensity, was categorized into three groups as follows: low expression (cytoplasmic staining evident in ≤20% of the cells), moderate expression (cytoplasmic staining evident in >20-50% of the cells), and high expression (cytoplasmic staining evident in >50% of the cells).

Adenovirus transduction efficiency in vitro. The transduction efficiency of recombinant adenovirus into all the PDAC and GC cell lines *in vitro* was evaluated using Ad-GFP control vector. Cells ($5x10^5$) were seeded in 6-well culture plates in triplicate and incubated at 37˚C for 6 h. After washing with PBS, the cells were infected with Ad-GFP vector at different MOIs ranging from 0 to 800 for 1 h. The infected cells were incubated in complete culture medium at 37˚C for 48 h. The cells were washed twice in PBS and analyzed for GFP expression by flow cytometry (Becton-Dickinson, San Jose, CA, USA). Transduction was considered efficient when 80-90% of cells were GFP-positive.

WST-8 assay. For the cell proliferation and growth inhibition assays, we used the water soluble tetrazolium (WST) assay as described below. The PDAC cell lines (KP-4, KP1N, Panc-1, and MIAPaca-2) ($5x10^3$ cells/well) were seeded in 96-well plates using complete culture medium and incubated at 37˚C for 24, 48, 72, and 96 h. After incubation, cells were incubated with cell counting kit CCK-8 (Dojindo Lab, Kumamoto, Japan) in fresh medium for 4 h at 37˚C. Cell proliferation was measured by spectrophotometric absorbance using a Spectra Max-190 (Molecular Devices, Sunnyvale, CA, USA) plate reader at 450/650 nm.

The cell viability assay was performed in the PDAC cell lines KP-4 and Panc-1 and in the GC cell lines MKN-1 and MKN-45. Cells ($5x10^3$ cells/well) were seeded in 96-well plates, incubated at 37˚C for 6 h, and then treated with different MOIs using Ad-GFP or Ad-BP1GFP or medium alone. Cell proliferation analysis was performed 24, 48, 72 and 96 h post-infection using the WST-8 assay described above.

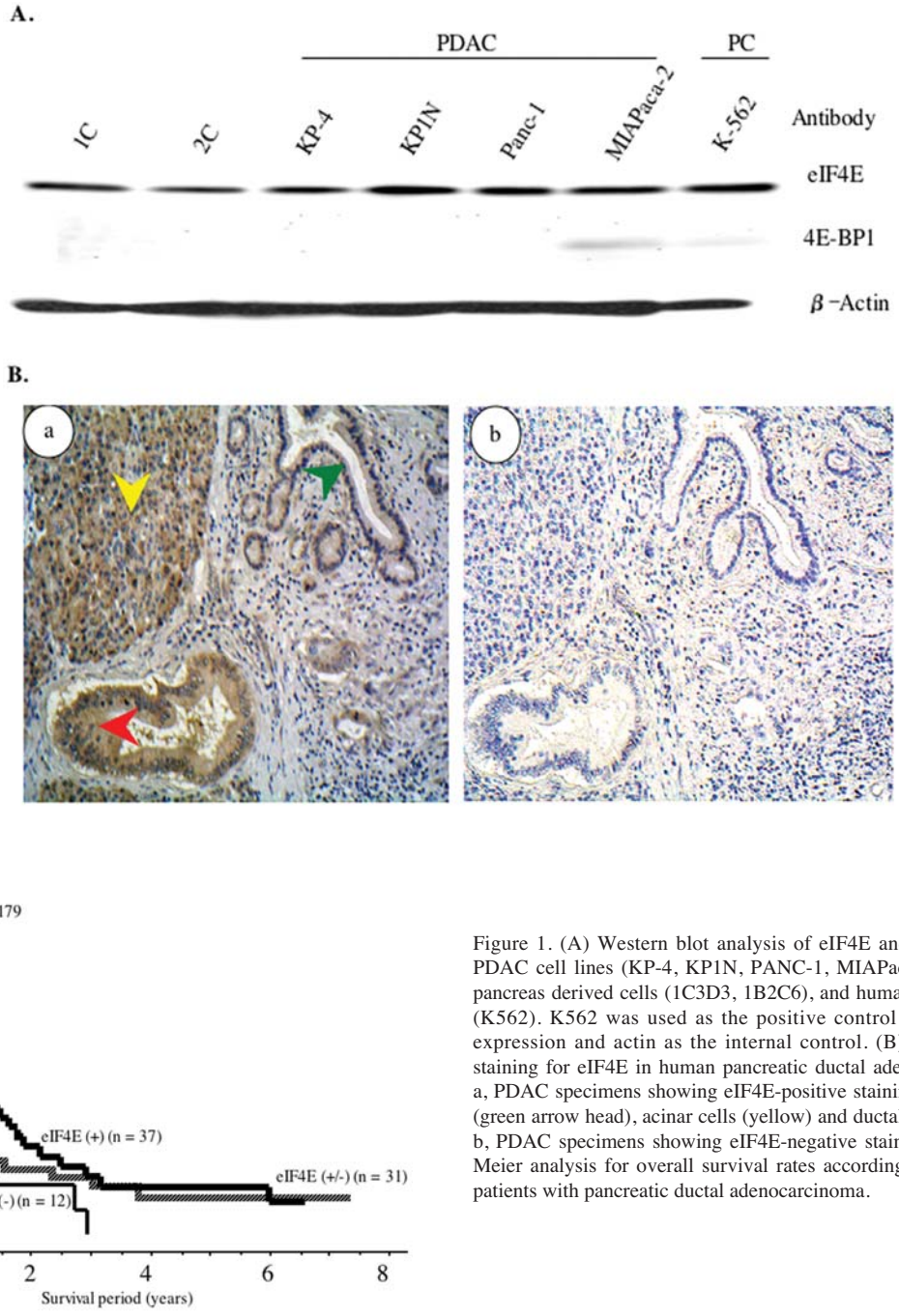

Figure 1. (A) Western blot analysis of eIF4E and 4E-BP1 expression in PDAC cell lines (KP-4, KP1N, PANC-1, MIAPaca-2), human embryonic pancreas derived cells (1C3D3, 1B2C6), and human chronic leukemia cells (K562). K562 was used as the positive control for eIF4E and 4E-BP1 expression and actin as the internal control. (B) Immunohistochemical staining for eIF4E in human pancreatic ductal adenocarcinoma specimens. a, PDAC specimens showing eIF4E-positive staining in normal ductal cells (green arrow head), acinar cells (yellow) and ductal carcinoma (red) (x200). b, PDAC specimens showing eIF4E-negative staining (x200). (C) Kaplan-Meier analysis for overall survival rates according to eIF4E expression in patients with pancreatic ductal adenocarcinoma.

For the growth inhibition assay, four PDAC cell lines (KP-4, KP1N, Panc-1, and MIAPaca-2) and two GC cell lines (MKN-1 and MKN-45) were seeded in 96-well plates and treated with Ad-GFP or Ad-BP at an MOI of 100 or medium alone as described above. After 24 h, the cell lines were treated with different concentrations of rapamycin ranging from 1-10^4 nmol/l and the infected cells were then incubated in complete culture medium at 37˚C for 48 h. Cell proliferation was analyzed 72 h post-infection using the WST-8 assay described above.

Statistical analysis. Statistical analyses, except the extended Fisher's exact test, were performed using StatView Software Ver 5.0, (SAS Institute, Inc., Cary, NC, USA). The extended Fisher's exact test was used to determine correlation between eIF4E expression and clinicopathological variables using the web page http://aoki2.si.gunma-u.ac.jp/exact/fisher/getpar.html. Survival rates were estimated using the Kaplan-Meier method. Survival differences according to eIF4E expression were analyzed by the log-rank test. The Mann-Whitney U test was used to analyze differences between two groups. Each experiment was performed at least three times independently. P-values <0.05 were considered to represent statistical significance.

Results

Screening for eIF4E and 4E-BP1 in the PDAC and GC cell lines by Wstern blot analysis. All of the PDAC and GC cell lines (data not shown for the GC lines) as well as the human

Table I. Association between eIF4E expression and clinicopathological variables in surgical specimens of ductal pancreatic adenocarcinoma.

Variables	No. of cases	eIF4E (+) n=37	eIF4E (+/-) n=31	eIF4E (-) n=12	p-value
Gender					0.2246
Male	45	17	20	8	
Female	35	20	11	4	
Age (years)					0.4826[a]
<65	47	20	18	9	
≥65	33	17	13	3	
p-T classification					0.4793
T2, T3	48	21	21	6	
T4	32	16	10	6	
Lymph node metastasis					0.0810[a]
Negative	23	9	13	1	
Positive	57	28	18	11	
Stage					0.5257
II, III	49	22	21	6	
IV	31	15	10	6	
Grade					0.6959
G1	27	12	12	3	
G2, G3	53	25	19	9	

[a]Extended Fisher's exact test.

embryonic pancreas-derived cells expressed the 25-kDa eIF4E protein. In addition, the MIAPaca-2 cells also expressed the 13-kDa translation repressor protein 4E-BP1 (Fig. 1A).

Evaluation of eIF4E expression in normal and carcinoma tissue. The level of eIF4E expression in pancreatic cancer tissue was evaluated for the first time in the present study. In the human PDAC specimens, cytoplasmic expression of eIF4E was found in normal acinar cells and normal ductal cells as well as in the pancreatic ductal adenocarcinoma cells (Fig. 1Ba). The same specimens stained with IgG served as the negative controls (Fig. 1Bb). Although qualitative differences in staining intensity were observed, all positive cases showed heterogeneous staining in the cytoplasm of some normal acinar cells, normal pancreas ductal cells and some ductal adenocarcinoma cells, at least focally. Of the 80 specimens, 42 (52.5%) showed acinar cell staining of eIF4E, 3 (3.7%) showed normal ductal cell staining of eIF4E and 68 (85%) showed high eIF4E expression in cancer tissue. Among all patients, 12 showed low, 31 moderate, and 37 high eIF4E expression.

Correlation between eIF4E expression and clinicopathological variables. No significant correlation was found between eIF4E expression and age, gender, histopathological grading, lymphatic invasion or lymph node metastasis (Table I).

Survival analysis. Of the 80 cases, 37 were classified as high, 31 as moderate, and 12 as low eIF4E-expressing tumors. The overall survival rate of the moderate eIF4E group tended to be higher, but not significantly, than that of the low eIF4E group, in which patients did not survive >3 years (log-rank test, p=0.1179) (Fig. 1C). There were no significant differences between the high eIF4E expressing group and either the low or moderate eIF4E expressing groups.

In vitro transduction efficiency of adenovirus vector. The efficiency of adenovirus-mediated gene transfer into the PDAC cell lines and GC cell lines was assessed by counting GFP-positive cells using fluorescence-activated cell sorting analysis. The MIAPaca-2 and KP-4 cell lines showed 80% GFP expression with MOI 50, while Panc-1, KP1N, SUIT-2 and PK-9 achieved 80% efficacy at MOI 100, 200 and 400, respectively. GC cell lines MKN-1 and MKN-45 showed similar efficiency at MOI 100. MOI of 100 was chosen for subsequent *in vitro* analyses because it provided maximum transduction efficacy in these cell lines.

In vitro translation repressor 4E-BP1 and p4EBP1(ser65) expression in PDAC and GC cell lines. Translation repressor 4E-BP1 and its phosphorylation status were detected using Western blot analysis in four PDAC cell lines (KP-4, KP1N, Panc-1, and MIAPaca-2) and in the two GC cell lines. A high level of expression of 4E-BP1 was observed when the six cell lines were infected with Ad-BP1 at MOI 100 for 72 h. All six cell lines efficiently expressed 4E-BP1; however, MIAPaca-2 cells expressing endogenous 4E-BP1 showed hypophosphorylated status compared with the other PDAC and GC cell lines (Fig. 2).

In vitro proliferation effect of Ad4E-BP1GFP. The effects of Ad-BP1 expression on cell growth and viability were evaluated at 24-h intervals over a period of 3 days after Ad-BP1 and Ad-GFP infection in two GC cell lines (MKN-1 at MOI 100 and MKN-45 at MOI 200) and two PDAC cell lines (Panc-1 at MOI 200 and KP-4 at MOI 100). Cell lines infected at high MOI showed marked growth inhibition compared to uninfected cell lines (Fig. 3A). No significant difference was observed between 4E-BP1 and GFP. Examination of the growth of four non-treated PDAC cell lines (KP-4, KP1N, Panc-1, and MIAPaca-2) at 24-h intervals for 4 days showed that cell lines expressing eIF4E alone had a lower proliferation rate than those expressing both eIF4E and 4E-BP1 (data not shown).

Combination of rapamycin with 4E-BP1 enhanced the inhibitory effect on growth of PDAC cell lines and GC cell lines in vitro. We examined the effects of rapamycin alone and in combination with 4E-BP1 or GFP on the growth of four PDAC cell lines (KP-4, KP1N, Panc-1, and MIAPaca-2) and the two GC cell lines using different concentrations of rapamycin and adenoviral vectors. Significant growth inhibition

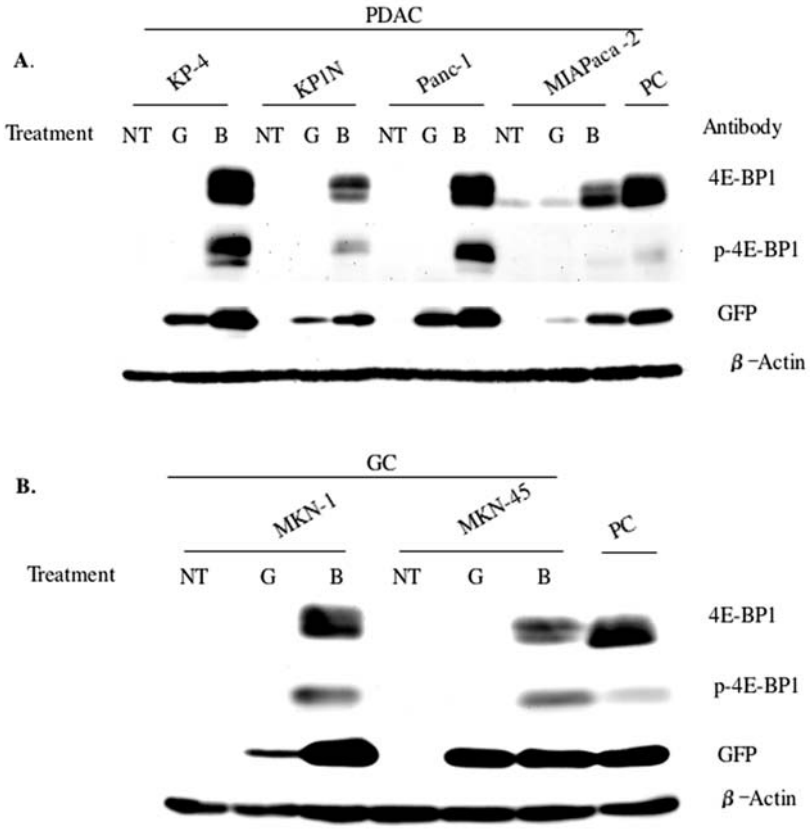

Figure 2. Western blot analysis of 4E-BP1, p4E-BP1(ser65) and GFP in PDAC and GC cell lines. Analysis was performed using cell lysate from each cell line infected with Ad vectors at MOI 100. NT, non-treatment; G, GFP; B, 4E-BP1; PC, positive control.

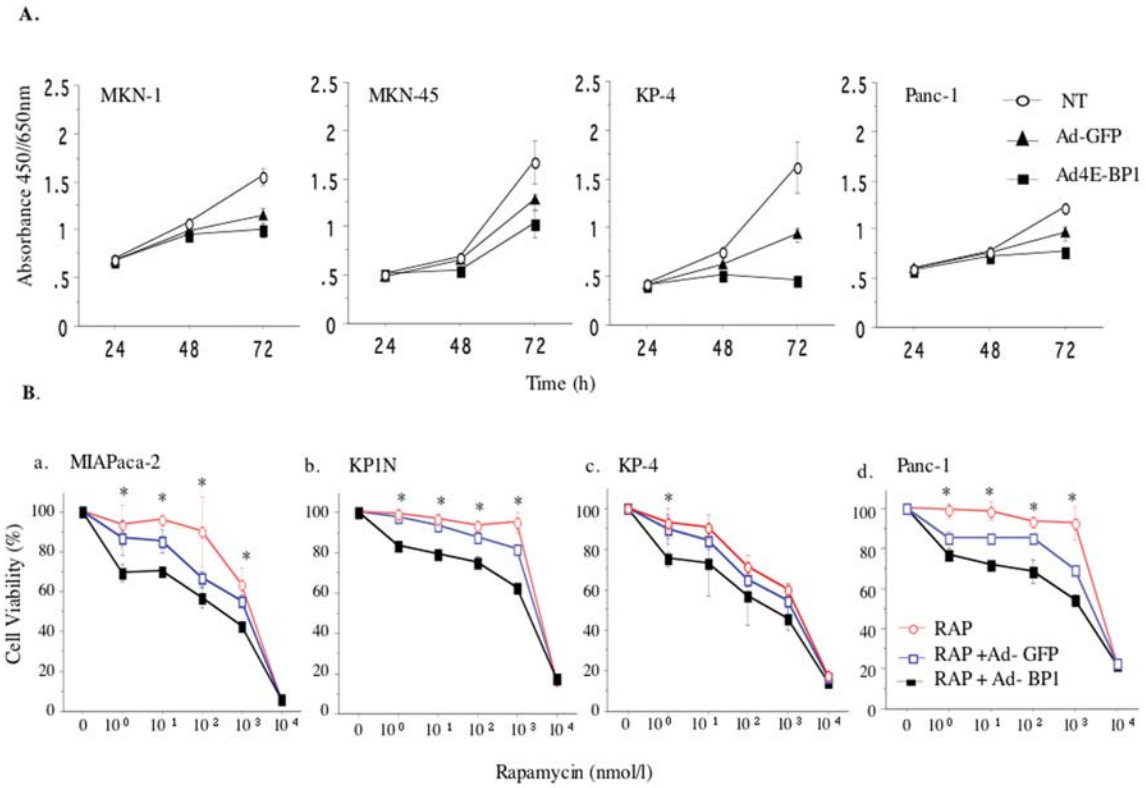

Figure 3. (A) WST-8 assay to analyze the gene transfer effects on the proliferation of PDAC and GC cell lines. Gastric carcinoma cells (MKN-1 at MOI 100 and MKN-45 at MOI 200) and PDAC cell lines (KP-4 at MOI 100 and Panc-1 at MOI 200) were treated with Ad 4E-BP1 or Ad-GFP or medium alone (NT) for the indicated times before evaluation of cell proliferation. Data are expressed as absorbance units standardized against medium alone for each assay and indicate mean ± SD (standard deviation) of three replicate assays. (B) WST-8 assay to analyze the growth inhibition effects of combination therapy on proliferating PDAC cell lines. Cell lines treated with different concentrations of rapamycin alone and in combination with Ad-GFP or Ad 4E-BP1 at MOI 100 showed significant growth inhibition after 72 h. Data are expressed as the surviving percentage of cells relative to non-infected cells. Data represent the mean ± SD of three replicate assays.

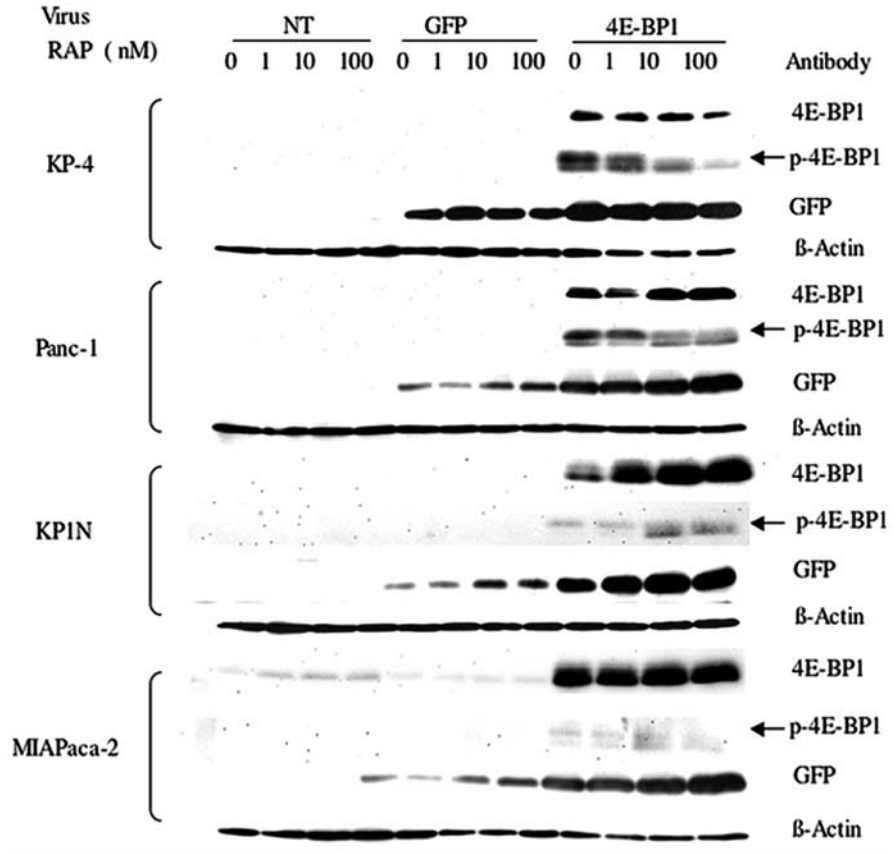

Figure 4. Western blot analysis for inhibition of mTOR pathway-mediated phosphorylation of translation repressor protein 4E-BP1. The indicated cell lines were treated with 1-100 nmol//l rapamycin alone and in combination with Ad-GFP or Ad 4E-BP1 at MOI 100 for 72 h and whole cell lysates were then examined by Western blot analysis. Rapamycin in combination with Ad 4E-BP1 inhibited mTOR pathway-mediated phosphorylation of translation repressor protein 4E-BP. Arrow indicates active form of phosphorylated 4E-BP1.

was caused by the combination therapy and this inhibition was greater than that caused by rapamycin alone. For example, in the MIAPaca-2 cell line, 40% growth inhibition was obtained by combination therapy compared to rapamycin alone at 100 nmol/l; however, the Panc-1 cell line showed around 30% inhibition by combination therapy at a similar dose (Fig. 3B). Similar growth inhibition effects were seen among the GC cell lines (data not shown).

Rapamycin inhibits hyperphosphorylated 4E-BP1 in combination therapy. The growth inhibition effects seen *in vitro* are identical to the Western blot analysis results showing inhibition by rapamycin in combination with 4E-BP1. Among all the PDAC, as well as GC cell lines (data not shown), the hyperphosphorylated gamma form of 4E-BP1 (inactive) was strongly inhibited by rapamycin in a dose-dependent manner, while the active form of the protein was present in all cell lines (Fig. 4).

Combination of rapamycin with 4E-BP1 suppressed subcutaneous PDAC tumor growth. The treatment efficacy of the adenoviral vectors or rapamycin alone, or the combination of both, was assessed in two PDAC cell lines (KP1N and MIAPaca-2) *in vivo*. Each vector was injected on the third day of tumor cell implantation directly into tumors 3-5 mm in diameter, with the second injection 2 weeks later. Rapamycin 1 mg/kg in combination with 4E-BP1 showed significant tumor-growth suppression compared with rapamycin combined with GFP in two different cell lines (p=0.005 for KP1N and p=0.009 for MIAPaca-2 xenograft model) (Fig. 5A). Intratumoral injection of Ad-BP1 and Ad-GFP efficiently showed transgenic expression of these vectors when checked by Western blot analysis using homogenized protein samples of mice sacrificed 72 h after adenoviral vector injection. However, endogenous 4E-BP1 expression was not detected in the non-treatment group of mice for the MIAPaca-2 cell line (Fig. 5B).

Discussion

Malignant transformation is a multi-step process that involves the initiation of unregulated cell growth and division. Malignant tissue consists of dividing cells, which require sufficient protein synthesis to achieve adequate proliferation rates (10-12). Protein translation is enhanced by the activation of mTOR via phosphorylation of 4E-BP1 and the 70-kDa s6-kinase (S6K1) which belongs to the downstream target of mTOR in the phosphatidylinositol 3-kinase (PI3K/AKT) and mitogen activated protein-kinase (MAPK) cell signaling pathway (3,9,11,14). Thus, translation factor eIF4E may be critical for tumorigenesis in PDAC.

Our Western blot analysis revealed eIF4E expression in non-cancerous pancreatic cells in all the PDAC cell lines and GC cell lines analyzed; however, one PDAC cell line,

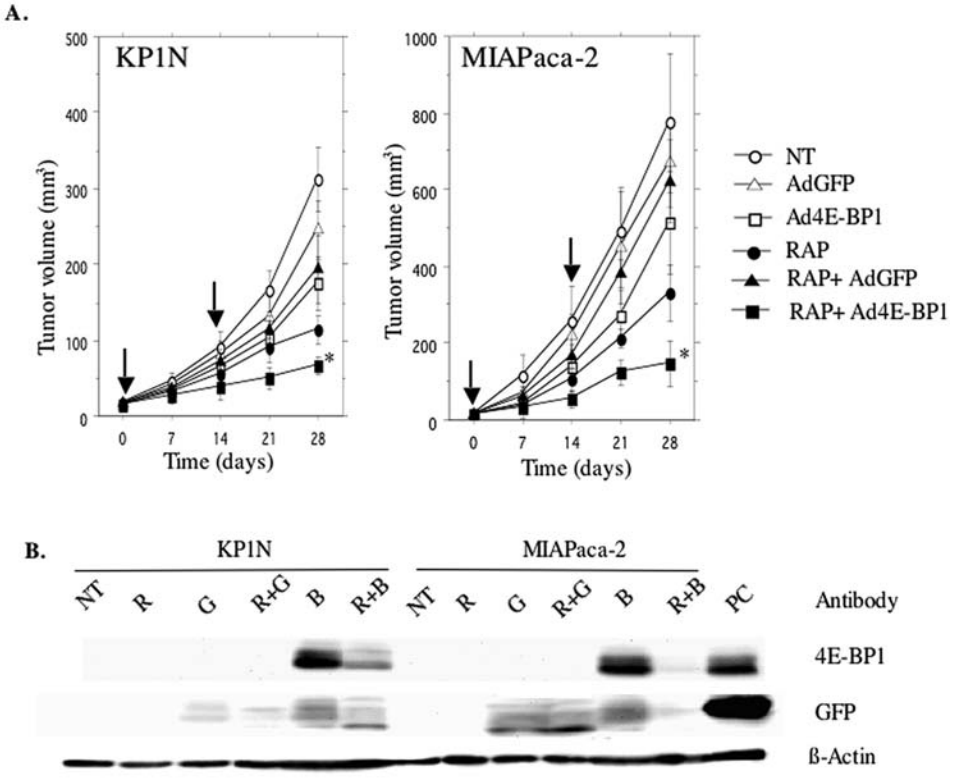

Figure 5. (A) *In vivo* combination therapy using Ad 4E-BP1 and rapamycin-controlled tumor growth. Subcutaneously transplanted KP1N- and MIAPaca-2-induced tumors were injected with Ad 4E-BP1 or Ad-GFP or PBS alone on days 0 and 14 intra-tumorally (i.t.) and with 1 mg/kg rapamycin (i.p.) every alternate day for 4 weeks. Arrow point indicates Ad vector injections. Tumor volume was measured using calipers over 4 weeks, shown as the mean ± SD from 10 mice in each group. *Indicates significant p-value for Rap + G vs. Rap + B ($p<0.005$ for KP1N and $p<0.009$ for MIAPaca-2). (B) Transduction efficiency of Ad-GFP and Ad 4E-BP1 in the KP1N and MIAPaca-2 cell lines *in vivo*. Three days after subcutaneous inoculation of tumor cells ($5x10^6$), mice were treated with Ad-GFP or Ad 4E-BP1 or PBS alone (i.t. injection) at $0.2x10^{10}$ ifu/ml, or with 1 mg/kg rapamycin alone or vehicle alone (i.p injection), or rapamycin in combination with 4E-BP1 or GFP vector. After 72 h, the mice were sacrificed and protein was extracted from homogenized tumor tissue. Western blot analysis was performed for the following groups: NT, non treatment group; R, rapamycin group; G, GFP group; B, 4E-BP1 group; R+G, rapamycin in combination with GFP; and R+B, rapamycin in combination with 4E-BP1-treated group.

MIAPaca-2, also expressed translation repression protein 4E-BP1. In contrast to a previous study, in which two PDAC cell lines (Panc-1 and MiaPaca-2) cultured under serum-starved conditions both efficiently expressed 4E-BP1 (19), we found lower or minimal 4E-BP1 expression using a monoclonal antibody. Tumor growth, progression and transformation are highly affected by different growth stimuli, of which further detailed investigation is still required to elucidate the exact mechanism by which this protein expression pattern varies according to the culture conditions. The details of the mechanism of cellular expression of this protein also need to be determined. In addition, the 1C3D3 and 1B2C6 cell lines were derived from normal pancreatic ductal cells supposed to have low eIF4E expression, and their contradictory high expression of eIF4E may have been due to immortalization causing them to behave as cancer cells.

Interestingly, the human PDAC specimens showed a heterogeneous pattern of eIF4E expression. Most of the normal ductal cells had low eIF4E expression (78.8%) compared to the ductal adenocarcinoma cells expressing moderate as well as high eIF4E expression (85%), suggesting that eIF4E is upregulated in PDAC cases.

We found no correlation between eIF4E expression and TNM classification, indicating that eIF4E expression level may not be a critical factor in tumor progression in PDAC, contrary to our hypothesis. However, we found a better survival tendency in moderate eIF4E expression in PDAC, although, unexpectedly, patients with low eIF4E expression had a poor survival rate. These contradictory results suggest that moderate and high eIF4E expression are a favorable prognostic factor; however, the low survival rate (20%) among the high eIF4E-expression cases and the less than 3-year survival among the low eIF4E-expression cases, which is different from other cancers, do not agree with this suggestion. In contrast to other cancers, we suggest that other factors play a much stronger role in PDAC than eIF4E expression (22,23), resulting in severe progression with poor prognosis in low eIF4E-expressing cases, which would agree with our findings. Thus, eIF4E alone appears not to be of significant prognostic value in PDAC cases. Moreover, previous studies have shown that high expression of eIF4E is of unfavorable prognostic value in various solid tumors, and increased expression and function of initiation factors, and consequently a constitutive increase in protein synthesis, has been suggested as a key tumorigenic event (4-8). The search for therapeutic approaches to inhibit eIF4E-initiated protein translation has therefore focused on adenovirus delivery of translation repressor gene 4E-BP1.

Adenovirus vectors are widely used for desired transgenic expression to obtain high effective infection titer with low toxicity in therapeutic models (27). Our CMV promoter-mediated adenovirus vectors efficiently expressed translation repressor gene 4E-BP1 and control gene GFP; however, the

growth inhibition effect on KP-4, Panc-1 and the two GC cell lines may be due to adenovirus vector effect, which needed a high MOI to obtain more efficient transduction in these cell lines compared with the other cell lines. At the same time, similar growth inhibition by 4E-BP1 and control vector GFP was observed. Exogenously delivered 4E-BP1 is hyper-phosphorylated among PDAC cell lines as well as GC cell lines, which indicates that mono-therapeutic approach does no significantly inhibit translation and growth. The phosphorylation status of 4E-BP1 is regulated by the upstream signaling effector mTOR, which belongs to the PI3K/AKT pathway (9-11). The hyperphosphorylated form of 4E-BP1 is selectively inhibited by mTOR inhibitor rapamycin (21,22). mTOR activation is observed in most cancers, including pancreatic cancer, and its specific inhibitor rapamycin, a widely used immunosuppressant and antifungal drug (28-31), significantly inhibited proliferation of PDAC and GC cell lines in combination with adenovirus-delivered therapeutic gene 4E-BP1 compared to rapamycin alone, which also showed growth inhibition at a higher dose, indicating the effects of the drug.

Phosphorylated 4E-BP1 is inhibited by the combination of mTOR inhibitor rapamycin with 4E-BP1 vector compared to cells treated with 4E-BP1 alone in a dose-dependent manner. However, rapamycin appears to be enhancing 4E-BP1 and GFP expression in Panc-1 and KP1N cells compared to actin, while GFP appears to decrease endogenous 4E-BP1 expression in MIAPaca-2 as revealed by our Western blot analysis. This completely coincides with the synergistic growth inhibition effects on the PDAC and GC cell lines. Rapamycin at 10-100 nmol/l in combination with Ad4E-BP1 inhibited growth rapidly, and effectively suppressed the phosphorylation of 4E-BP1, indicating that rapamycin indeed blocks mTOR signaling.

The immunosuppressant drug rapamycin, in combination with translation repressor gene 4E-BP1, showed significant growth inhibition of tumors in nude mice. The anti-tumor effects of rapamycin alone and in combination with Ad4E-BP1 suggests that hyperphosphorylated 4E-BP1 is inhibited by rapamycin not only *in vitro* but also *in vivo*. Furthermore, the therapeutic gene delivered alone showed similar tumor growth inhibition compared to that of the control; however, in combination with rapamycin, much more significant growth inhibition was achieved.

Thus, our results suggest that adenoviral vector delivery of translation repressor 4E-BP1 could be a suitable therapeutic approach. However, its combination with mTOR inhibitor rapamycin at low dose may effectively control pancreatic cancer growth, which is an aggressive cancer with poor prognosis.

In conclusion, eIF4E expression alone is not a prognostic factor in PDAC cases; however, translation repressor 4E-BP1 delivered by adenovirus vector in combination with mTOR inhibitor rapamycin may provide more effective adjuvant therapy for aggressive pancreatic cancer.

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References

1. Jemal A, Murray T, Ward T, *et al*: Cancer Statistics. CA Cancer J Clin 55: 10-30, 2005.
2. Kleef J, Michalski C, Friess H and Büchler MW: Pancreatic cancer: from bench to 5-year survival. Pancreas 33: 111-118, 2006.
3. Sonenberg N and Gingras AC: The mRNA 5'cap-binding protein eIF4E and control of cell growth. Curr Opin Cell Biol 10: 268-275, 1998.
4. Kerekatte V, Smiley K, Hu B, Smith A, Gelder F and De Benedetti A: The proto-oncogene/translation factor eIF4E: a survey of its expression in breast carcinomas. Int J Cancer 64: 27-31, 1995.
5. Li BDL, McDonald JC, Nassar R and De Benedetti A: Clinical outcome in stage I to III breast carcinoma and eIF4E over expression. Ann Surg 227: 756-763, 1998.
6. Nathan CO, Franklin S, Abreo FW, Nassar R, De Benedetti A and Glass J: Analysis of surgical margins with the molecular marker eIF4E: a prognostic factor in patients with head and neck cancer. J Clin Oncol 17: 2909-2914, 1999.
7. Franklin S, Pho T, Abreo FW, Nassar R, De Benedetti A, Stucker FJ and Nathan CO: Detection of the proto-oncogene eIF4E in larynx and hypopharynx cancers. Arch Otolaryngol 125: 177-182, 1999.
8. Chen CN, Hsieh FJ, Cheng YM, Lee PH and Chang KJ: Expression of eukaryotic initiation factor 4E in gastric adeno-carcinoma and its association with clinical outcome. J Surg Oncol 86: 22-27, 2004.
9. Gingras AC, Gygi SP, Raught B, *et al*: Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev 13: 1422-1437, 1999.
10. Vogelstein B and Kinzler KW: Cancer genes and the pathways they control. Nat Med 10: 789-799, 2004.
11. Adjei AA and Hidalgo M: Intracellular signal transduction pathway proteins as targets for cancer therapy. J Clin Oncol 23: 5386-5403, 2005.
12. De Benedetti A and Graff JR: eIF-4E expression and its role in malignancies and metastases. Oncogene 23: 3189-3199, 2004.
13. Schmelzle T and Hall MN: TOR, a central controller of cell growth. Cell 103: 253-262, 2000.
14. Huang S and Houghton PJ: Targeting mTOR signaling for cancer therapy. Curr Opin Pharmacol 3: 371-377, 2003.
15. Bjornsti MA and Houghton PJ: The TOR pathway: a target for cancer therapy. Nat Rev Cancer 4: 335-348, 2004.
16. Rubio-Viqueira B and Hidalgo M: Targeting mTOR for cancer treatment. Curr Opin Investig Drugs 7: 501-512, 2006.
17. Abraham RT and Gibbons JJ: The mammalian target of rapamycin signaling pathway: twist and turns in the road to cancer therapy. Clin Cancer Res 13: 3109-3114, 2007.
18. Rousseau D, Gingras AC, Pause A and Sonenberg N: The eIF4E - binding proteins 1 and 2 are negative regulators of cell growth. Oncogene 13: 2415-2420, 1996.
19. Martin G, Gansauge F, Schmid MR, Adler G and Seufferlein T: Regulation of cell growth and cyclin D1 expression by the constitutively active FRAP-p70s6k pathway in human pancreatic cancer cells. Cancer Res 59: 3581-3587, 1999.
20. Dancey JE: Inhibitors of the mammalian target of rapamycin. Expert Opin Investig Drugs 14: 313-328, 2005.
21. Hidalgo M and Rowinsky EK: The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. Oncogene 27: 6680-6686, 2000.
22. Huang S, Bjornsti MA and Houghton PJ: Rapamycin: mechanism of action and cellular resistance. Cancer Biol Ther 2: 222-232, 2003.
23. Shaw RJ and Cantley LC: Ras. PI(3)K and mTOR signaling controls tumor cell growth. Nature 441: 424-430, 2006.
24. Petroulakis E, Mamane Y, Le Bacquer O, Shahbazian D and Sonenberg N: mTOR signaling: implication for cancer and anticancer therapy. Br J Cancer 94: 195-199, 2007.
25. Fukunaga A, Miyamoto M, Cho Y, *et al*: $CD8^+$ tumor-infiltrating lymphocytes together with $CD4^+$ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma. Pancreas 28: 26-31, 2004.

26. He TC, Zhou S, Da Costa LT, Yu J, Kinzler KW and Vogelstein B: A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA 95: 2509-2514, 1998.
27. Ugai H, Yamasaki T, Hirose M, *et al*: Purification of infectious adenovirus in two hours by ultracentrifugation and tangential flow filtration. Biochem Biophys Res Commun 331: 1053-1060, 2005.
28. Guba M, Breitenbuch P, Steinbauer M, *et al*: Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med 8: 128-135, 2002.
29. Jacobson BA, Alter MD, Kratzke MG, *et al*: Repression of cap-dependent translation attenuates the transformed phenotype in non-small cell lung cancer both *in vitro* and *in vivo*. Cancer Res 66: 4256-4262, 2006.
30. Stephan S, Datta K, Wang E, *et al*: Effect of rapamycin alone and in combination with antiangiogenesis therapy in an orthotopic model of human pancreatic cancer. Clin Cancer Res 10: 6993-7000, 2004.
31. Ito D, Fujimoto K, Mori T, *et al*: *In vivo* antitumor effect of the mTOR inhibitor CCI-779 and gemcitabin in xenograft models of human pancreatic cancer. Int J Cancer 118: 2337-2343, 2006.