Overexpression of eukaryotic elongation factor eEF2 in gastrointestinal cancers and its involvement in G2/M progression in the cell cycle

JUNYA NAKAMURA[1], SAYAKA AOYAGI[1], ISAMU NANCHI[1], SHIN-ICHI NAKATSUKA[8], ERIKA HIRATA[1], SHOHEI SHIBATA[1], MARI FUKUDA[1], YUMIKO YAMAMOTO[1], IKUYO FUKUDA[1], NAOYA TATSUMI[1], TAZU UEDA[1], FUMIHIRO FUJIKI[1], MASAYA NOMURA[9], SUMIYUKI NISHIDA[4], TOSHIAKI SHIRAKATA[2], NAOKI HOSEN[3], AKIHORO TSUBOI[4], YOSHIHIRO OKA[5], RIICHIRO NEZU[10], MASAKI MORI[6], YUICHIRO DOKI[6], KATSUYUKI AOZASA[7], HARUO SUGIYAMA[1] and YUSUKE OJI[2]

Departments of [1]Functional Diagnostic Science, [2]Biomedical Informatics, and [3]Cancer Stem Cell Biology, Osaka University Graduate School of Medicine, 1-7 Yamada-Oka; Departments of [4]Cancer Immunotherapy, [5]Respiratory Medicine and Allergy, Rheumatic Disease, [6]Surgery, and [7]Pathology, Osaka University Graduate School of Medicine, 2-2 Yamada-Oka, Suita, Osaka 565-0871; [8]Department of Pathology, Sumitomo Hospital, 5-3-20 Nakanoshima, Kita-ku, Osaka, Osaka 530-0005; [9]Department of Surgery, Osaka General Medical Center, 3-1-56 Mandaihigashi, Sumiyoshi-ku, Osaka, Osaka 558-8558; [10]Department of Surgery, Osaka Rosai Hospital, 1179-3 Nagasone-cho, Sakai, Osaka 591-8025, Japan

Received October 29, 2008; Accepted December 18, 2008

DOI: 10.3892/ijo_00000246

Abstract. A high level protein synthesis is one of the characteristics of cancer cells. The aim of this study is to show the contribution of eukaryotic elongation factor 2 (eEF2), which plays an essential role in the polypeptide chain elongation step, in the tumorigenesis of gastrointestinal cancers. In the present study, we demonstrated by using immunohistochemistry that eEF2 protein was overexpressed in 92.9% (13 of 14) of gastric and 91.7% (22 of 24) of colorectal cancers. No mutations were found in any of the exons of the *eEF2* gene in six gastric and six colorectal cancers. Knockdown of eEF2 by eEF2-specific short-hairpin RNA (shEF2) inhibited cancer cell growth in two gastric cancer cell lines, AZ-521 and MKN28, and one colon cancer cell line, SW620. Flow cytometric analysis showed that knockdown of eEF2 induced G2/M arrest and resulted in inactivation of Akt and cdc2 (a G2/M regulator) and activation of eEF2 kinase (a negative regulator of eEF2) in these cancer cells. Conversely, forced expression of eEF2 in AZ-521 cells significantly enhanced the cell growth through promotion of G2/M progression in cell cycle, activated Akt and cdc2, and inactivated eEF2 kinase. Furthermore, forced expression of eEF2 in these cancer cells enhanced *in vivo* tumorigenicity in a mouse xenograft model. These results showed that overexpressed eEF2 in gastrointestinal cancers promoted G2/M progression and enhanced their cell growth *in vitro* and *in vivo*. These results also suggested a novel linkage between translational elongation and cell cycle mechanisms, implying that the linkage might play an important role to orchestrate the deregulated translation and cell cycle mechanisms for promotion of the development of gastrointestinal cancers.

Correspondence to: Dr Yusuke Oji, Department of Biomedical Informatics, Osaka University Graduate School of Medicine, 1-7 Yamada-oka, Suita, Osaka 565-0871, Japan
E-mail: oji@sahs.med.osaka-u.ac.jp

Key words: eEF2, gastric cancer, colorectal cancer, Akt, cdc2

Introduction

Gastrointestinal cancers are common malignancies and leading causes of cancer death in the world. Clinical outcome of these cancers still remains unsatisfactory despite recent progress in diagnosis and medical treatments. A wide range of alterations in gene expression have been identified in gastrointestinal cancers. In gastric cancer, these include altered expression of p53 gene (1), loss of the FHIT gene (2), and decreased expression of E-cadherin (1,3). In colon cancer, loss of APC gene (4), Ras mutation (5), upregulated expression of COX2 (6), and altered expression of p53 (7) were reported. However, molecular mechanisms by which disease development, progression, and resistance to chemotherapies occur in gastrointestinal cancers are not yet fully understood.

High level protein biosynthesis is one of the characteristics of cancer cell metabolism. Mounting evidence links deregulated protein synthesis to tumorigenesis of human cancers (8). Protein biosynthesis (translation) consists of three general steps, initiation, elongation, and termination. Although all the three steps are tightly controlled, the initiation step appears to be the most commonly targeted by signal transduction pathways deregulated in cancer. Eukaryotic initiation factor 4E (eIF4E) that is overexpressed in various kinds of human

tumors such as colon, lung, liver, bladder, and breast cancers, was the first component of the translation initiation machinery known to cause deregulated cell growth and malignant transformation (9,10). Overexpression of eIF4E does not increase global translation rates, but rather selectively stimulates translation of a subset of poorly translated mRNAs of oncogenic genes such as c-Myc (11), ODC (12), VEGF (13), or cyclin D1 (14).

Some studies suggest that deregulation of elongation step in translation is also involved in tumorigenesis. Overexpression of eukaryotic elongation factor 1A that catalyzes the first step of elongation was reported in ovarian cancer (15) and breast cancer (16). Recently, it was reported that eukaryotic elongation factor 2 (eEF2), which catalyses the translocation of the elongated peptidyl-tRNA from A to P sites of the ribosome, was inactivated by treatment with chemotherapeutic agent doxorubicin (17) or taxol (18), which was associated with induction of apoptosis in cancer cells or inhibition of translation in NIH-3T3 cells.

In the present study, we demonstrate that the wild-type *eEF2* gene is overexpressed in the majority of gastric and colorectal cancers. The overexpression of eEF2 promotes G2/M progression in cell cycle in association with activation of Akt and a G2/M regulator, cdc2 proteins and enhances *in vitro* and *in vivo* the growth of cancer cells.

Materials and methods

Sample tissues. Tissue samples were obtained from 14 patients with gastric cancer (11 adenocarcinoma, two scirrhous carcinoma, and one small cell carcinoma; ages 19-81) and 24 patients with colorectal cancer (eight colon adenocarcinoma and 16 rectal adenocarcinoma; ages 37-88) at Osaka University Hospital and Nissay Hospital under informed consent. For RT-PCR, tissue samples were stocked in RNA*later* (Qiagen) at -80˚C until use.

Cells. Gastric cancer cell lines, AZ-521 and MKN28 and colon cancer cell line, SW620 were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For culture of AZ-521 cells, 1X Eagle's MEM amino acids and vitamins (Nissui, Tokyo, Japan) were added to the medium.

Immunohistochemistry. Formalin-fixed tissue sections of 3-μm thickness were cut from paraffin-blocks. After dewaxing with xylene and rehydration through a graded series of ethanol, the sections were antigen retrieved using Pascal apparatus (Dako Cytomation, Carpinteria, CA) in 10 mM citrate buffer (pH 6.0). These sections were reacted with anti-eEF2 Ab (H-118, Santa Cruz Biotechnology, Santa Cruz, CA) at 4˚C overnight and then reacted with Envision kit/HRP (Dako Cytomation) at room temperature for 30 min. After treatment with 0.7% H_2O_2 solution to reduce endogenous peroxidase activity, immunoreactive eEF2 protein was visualized using DAB tablet Wako, Osaka, Japan. The sections were then counterstained with hematoxylin. Immunostaining was evaluated by two observers including a pathologist and considered as positive if appropriate brown staining was seen in the cytoplasm in >25% of cancer cells in the visual field.

Sequencing. Total RNA was isolated using Isogen (Wako) and reverse transcribed using murine Moloney leukemia virus (M-MLV) reverse transcriptase accoding to the manufacturer's instructions (Promega, Madison, WI). The 5' (eEF2F, 84-1334 nt) and 3' (eEF2L, 1314-2660 nt) sequences of the *eEF2* gene (Ref Seq NM_001961, coding sequence: 84-2660 nt) were amplified by PCR, which was performed for 35 cycles (94˚C for 30 sec/65˚C for eEF2F, 62˚C for eEF2L, and 55˚C for GAPDH for 30 sec/72˚C for 60 sec) using ExTaq (Takara, Shiga, Japan). Sequences of the primers for eEF2 and GAPDH amplification were as follows: eEF2F-F, 5'-tcagtgaattccatggtgaacttcacggtagac-3'; eEF2F-R, 5'-agtctgaa ttctca gaagactcgtccaaaggcgta-3'; eEF2L-F, 5'-tcagtgaattcctacg cctttggacgagtcttc-3'; eEF2L-R, 5'-agtctgaattcctacaatttgtccagg aagttg-3'; GAPDH-F, 5'-gccaaaagggtcatccatctc-3'; and GAPDH-R, 5'-gtagaggcagggatgatgttc-3'.

After electrophoresis on agarose gels, the PCR-amplified eEF2 DNA fragments were recovered by freeze-phenol method and directly sequenced in both directions by an ABI PRISM 377 sequencer (Perkin-Elmer Life Science, Boston, MA) using a Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Life Science). Besides the primers for amplification of eEF2 sequence described above, the following primers were used as sequencing primers: eEF2-1 (241-260 nt), 5'-ggtatcagtgaagcgtgtct-3'; eEF2-2 (1042-1061 nt), 5'-acatcaa actggacagcgag-3'; eEF2-3 (1558-1577 nt), 5'-cttcatcacccgcat gttgt-3'; and eEF2-4 (2428-2447 nt), 5'-ttgtggtcaaggcctatctg-3'.

Construction of shRNA targeting eEF2 and its transient expression (transfection). To prepare shRNA vector targeting eEF2 mRNA (shEF2), chemically synthesized sense and antisense single stranded DNAs targeting 5'-caugggc aacaucaugaucgauccuguccu-3' in eEF2 mRNA (Fasmac, Kanagawa, Japan) were annealed, and inserted into tRNA-shRNA expression vector piGENE tRNA Pur (Clontech, Palo Alto, CA). For transient expression of shRNA vector, AZ-521, MKN28, and SW620 cells ($5x10^5$ cells) were washed three times with PBS and incubated with 10 μg of shEF2 or control empty shRNA vector (shMock) in 300 μl DMEM without FBS in a 4-mm cuvette. Then, shRNA vector was transfected into the cells by electroporation (165 V, 1000 μF) using Gene Pulser Xcell™ system (Bio-Rad, Hercules, CA).

Construction of eEF2 expression vector and its stable expression (transduction). The cDNA was prepared from mRNA isolated from AZ-521 cells, and the sequences of the eEF2 gene were PCR-amplified using Pfx Taq polymerase (Invitrogen, Carlsbad, CA, USA). The sequence of the *eEF2* gene was inserted into a pcDNA 3.1(+) vector (Invitrogen). Vector with or without the eEF2 sequence (pcEF2 and pcMock, respectively) was linealized with PvuI, and AZ-521 cells were transfected with the vector by electroporation using Gene Pulsor II (Bio-Rad). The cell clones that stably expressed the vectors were isolated by the resistance to G418 at the concentrations of 750 μg/ml.

Western blot analysis. Cells were washed twice with PBS and lysed with 2X Laemmli SDS sample buffer. Proteins were separated by SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford,

Table I. Overexpression of eEF2 in gastrointestinal cancers.

Gastrointestinal cancers	Positive (%)	Negative (%)
Gastric cancer	92.9 (13/14)	7.1 (1/14)
Adenocarcinoma	90.9 (10/11)	9.1 (1/11)
Scirrhous carcinoma	100 (2/2)	0
Small cell carcinoma	100 (1/1)	0
Colorectal cancer	91.7 (22/24)	8.3 (2/24)
Colon adenocarcinoma	87.5 (7/8)	12.5 (1/8)
Rectal adenocarcinoma	93.7 (15/16)	6.3 (1/16)

Expression of eEF2 protein was examined in 38 gastrointestinal cancers by immunohistochemistry. Expression of eEF2 protein was considered as positive if appropriate brown staining was seen in the cytoplasm in >25% of cancer cells in the visual field.

MA). After blocking of non-specific binding, the membranes were incubated with first antibody, followed by incubation with appropriate anti-rabbit or anti-mouse IgG antibody conjugated with alkaline phosphatase, and visualized using BCIP/NBT kit (Nacalai Tesque, Kyoto, Japan). Polyclonal anti-EF2 (Santa Cruz Biotechnology), P-eEF2, p-eEF2K, p-Akt(thr308), p-Akt (ser473), Akt, Cdc2, p-Cdc2(Tyr15), (Cell Signaling Technology, Danvers, MA), and anti-GAPDH (Chemicon International, Temecula, CA) were used as first antibodies.

Flow cytometric analysis of apoptosis and cell cycle distribution. For analysis of apoptosis, $1.0x10^5$ cells were washed with PBS and stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) at room temperature for 15 min in the dark using MebCyto Apoptosis Kit (Medical and Biological Laboratories Co., Ltd., Aichi, Japan) according to the manufacturer's instructions. Then, the stained cells were analyzed by FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Frequency of Annexin V-positive apoptotic cells is shown as a percentage of apoptotic cells to the total number of counted cells.

For analysis of cell cycle distribution, $1x10^5$ cells were harvested at the indicated time-points, washed with PBS once, resuspended in 100 μl of ice-cooled PBS followed by adding 1 ml of ice-cooled 80% ethanol with pipetting and fixed at -20˚C overnight. After incubation at room temperature for 10 min, cells were resuspended in 400 μl of PBS containing 5 μg/ml of propidium iodide (PI, Nacalai Tesque, Kyoto, Japan) and 200 μg/ml of RNase A (Nacalai Tesque) and incubated at 37˚C for 30 min in the dark. Flow cytometry was performed on a FACSort (Beckton Dickinson), and cell cycle distribution was analyzed using Mod Fit ver.2 software (Verity Software Home, Topsham, ME).

Animal experiments. The animal study was performed in accordance with the guidelines for animal experiments of Osaka University. To examine *in vivo* tumorigenicity of AZ-521 cells with forced expression of eEF2, BALB/cAJcl-nu/nu female mice (5-7weeks old, CLEA Japan, Inc., Tokyo, Japan) were subcutaneously inoculated in each side of flank with AZ-521 cells ($5x10^6$ cells) transduced with eEF2 or pcMock vector in 150 μl of PBS containing 33.3% Matrigel (Becton-Dickinson). Tumor size was measured weekly with calipers and the volume was calculated by the following formula: tumor volume = (length x width^2)/2.

Statistical analyses. The statistical significance in a difference between arithmetical means of test groups was assessed by unpaired t-test.

Results

Overexpression of eEF2 in gastric and colorectal cancers. Expression of eEF2 protein was examined in 14 gastric and 24 colorectal cancers by immunohistochemistry. Immuno-staining was evaluated by two observers including a pathologist and considered as positive if appropriate brown staining was seen in the cytoplasm in >25% of cancer cells in the visual field.

eEF2 protein expression was positive in 13 (92.9%) and negative in one (7.1%), of 14 patients with gastric cancer, and it was positive in 22 (91.7%) and negative in two (8.3%), of 24 patients with colorectal cancer (Table I and Fig. 1). In

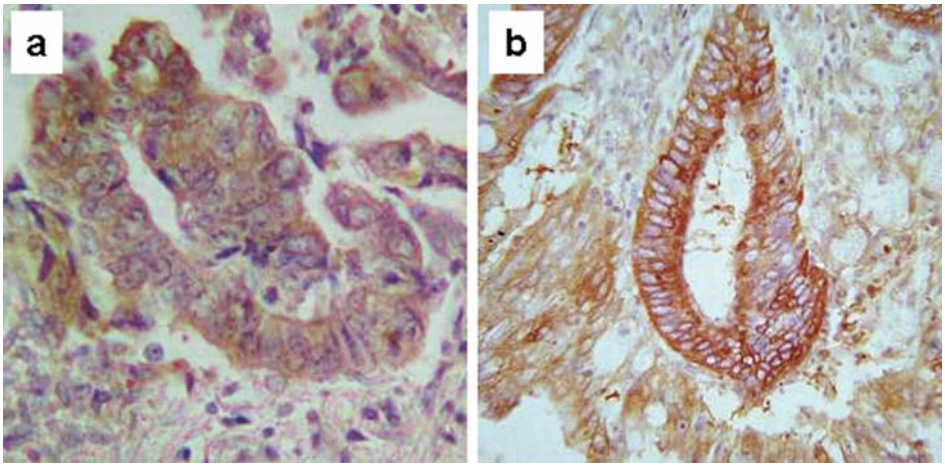

Figure 1. Overexpression of eEF2 in gastric and colorectal cancers. Expression of eEF2 protein was examined by immunohistochemistry. Representative positive results with gastric (a) and colon (b) cancers are shown. eEF2 protein was detected in brown in the cytoplasm of cancer cells.

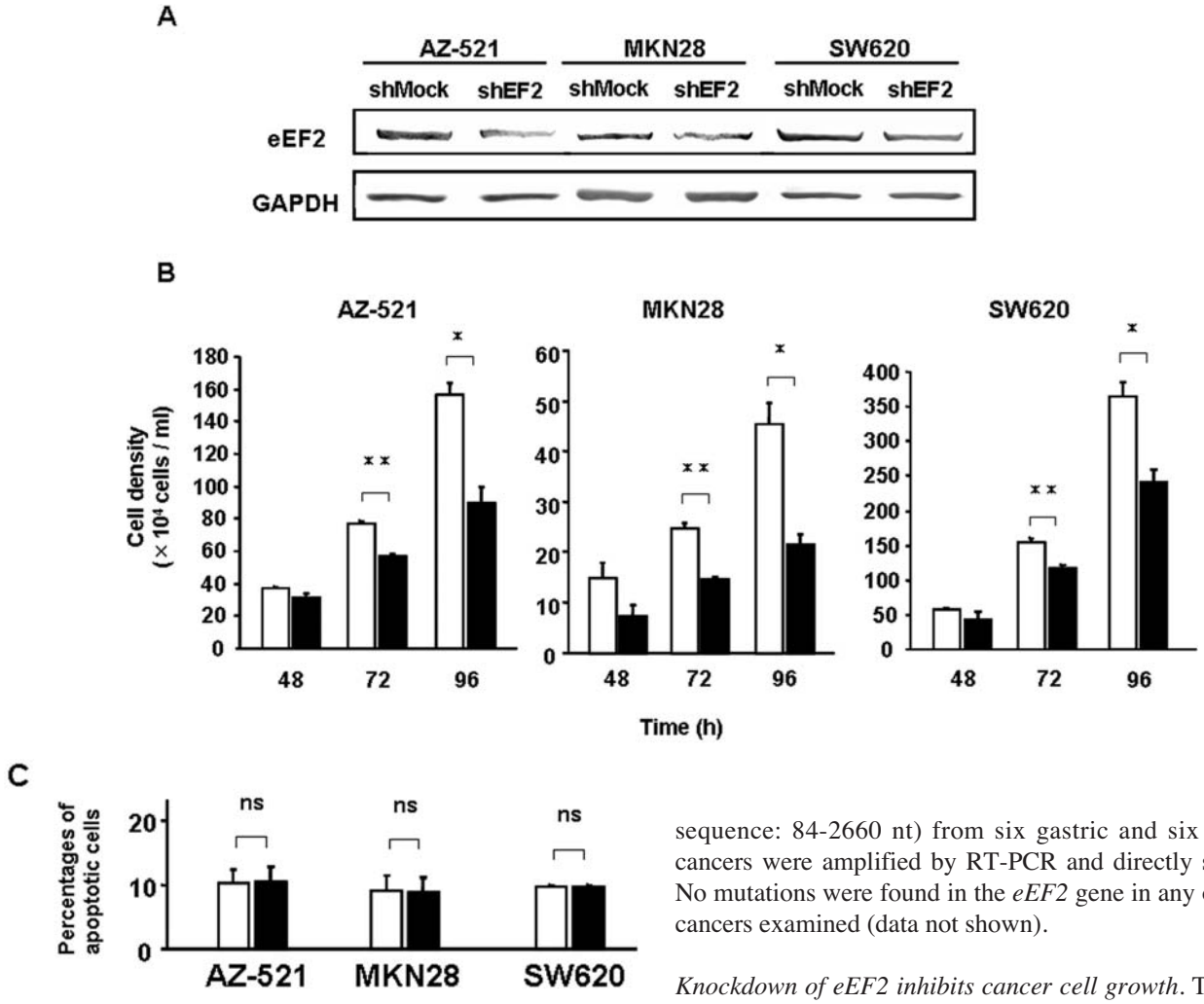

Figure 2. Knockdown of eEF2 inhibits cancer cell growth. Two gastric cancer cell lines, AZ-521 and MKN28 and one colorectal cancer cell line SW620 ($5x10^5$ cells) were transfected with 10 μg of shEF2 or shMock vector, cultured for 72 h, and then examined for eEF2 protein levels (A) and their cell growth (B). (A) Western blot analysis of eEF2 protein expression. GAPDH was used as an internal control. To normalize the difference in protein loading for Western blot analysis in individual samples, the density of eEF2 band was divided by that of GAPDH band, and the relative expression levels of eEF2 in shEF2-transfected cells to those in shMock-transfected ones are shown. Open columns, shMock-transfected cells; closed columns, shEF2-transfected cells. (B) Effect of knockdown of eEF2 on cell growth rate. Cells were transfected with shEF2 or shMock vector and cultured, and viable cell number was counted at the indicated time-points. $^{*}P<0.05$; $^{**}P<0.01$. (C) shEF2- and shMock-transfected cells were analyzed for apoptosis by Annexin V-PI two-color flow cytometry. Percentages of apoptotic cells after 24 h of culture are shown. ns, not significant. (B and C) Experiments were performed independently three times. Open columns, shMock-transfected cells; closed columns, shEF2-transfected cells.

positive cases, eEF2 protein was diffusely detected in the cytoplasm of cancer cells. No or faint immunostaining of eEF2 protein was observed in the neighboring normal cells. Thus, eEF2 protein was overexpressed in the majority of gastric and colorectal cancers.

No mutation of eEF2 overexpressed in gastric and colorectal cancers. To examine whether the *eEF2* gene expressed in gastrointestinal cancers was wild-type, the 5' (84-1334 nt) and the 3' (1314-2660 nt) sequences of eEF2 mRNA (coding sequence: 84-2660 nt) from six gastric and six colorectal cancers were amplified by RT-PCR and directly sequenced. No mutations were found in the *eEF2* gene in any of these 12 cancers examined (data not shown).

Knockdown of eEF2 inhibits cancer cell growth. To examine whether or not eEF2 was involved in growth of gastrointestinal cancer cells, shRNA targeting eEF2 (shEF2) or empty shRNA vector (shMock) was transfected into two gastric cancer cell lines, AZ-521 and MKN28 and one colon cancer cell line SW620. shEF2 reduced expression of eEF2 protein in these cells (Fig. 2A), and cell growth was significantly inhibited by shEF2 in all the three cell lines examined (Fig. 2B). Furthermore, whether or not the growth inhibition induced by shEF2 was due to apoptosis was examined. The three cancer cell lines transfected with shEF2 or shMock were cultured for 24 h and analyzed for apoptosis. PI-Annexin V two-color flow cytometry showed that knockdown of eEF2 by shEF2 did not induce apoptosis in any of the three cancer cells examined (Fig. 2C).

eEF2 promotes G2/M phase progression in the cell cycle. Since knockdown of eEF2 did not induce apoptosis, it was probable that the growth inhibition by knockdown of eEF2 was attributed to delay in cell cycle progression. To examine whether or not eEF2 was involved in cell cycle progression, three cancer cell lines, AZ-521, MKN28, and SW620 were transfected with shEF2 or shMock, cultured for 48 h, and then analyzed for cell cycle by flow cytometry. Knockdown of eEF2 by shEF2 significantly accumulated cells at G2/M phase of the cell cycle in all the three cell lines examined (Fig. 3).

To show that eEF2 was involved in G2/M progression of the cell cycle, the effects of forced expression of eEF2

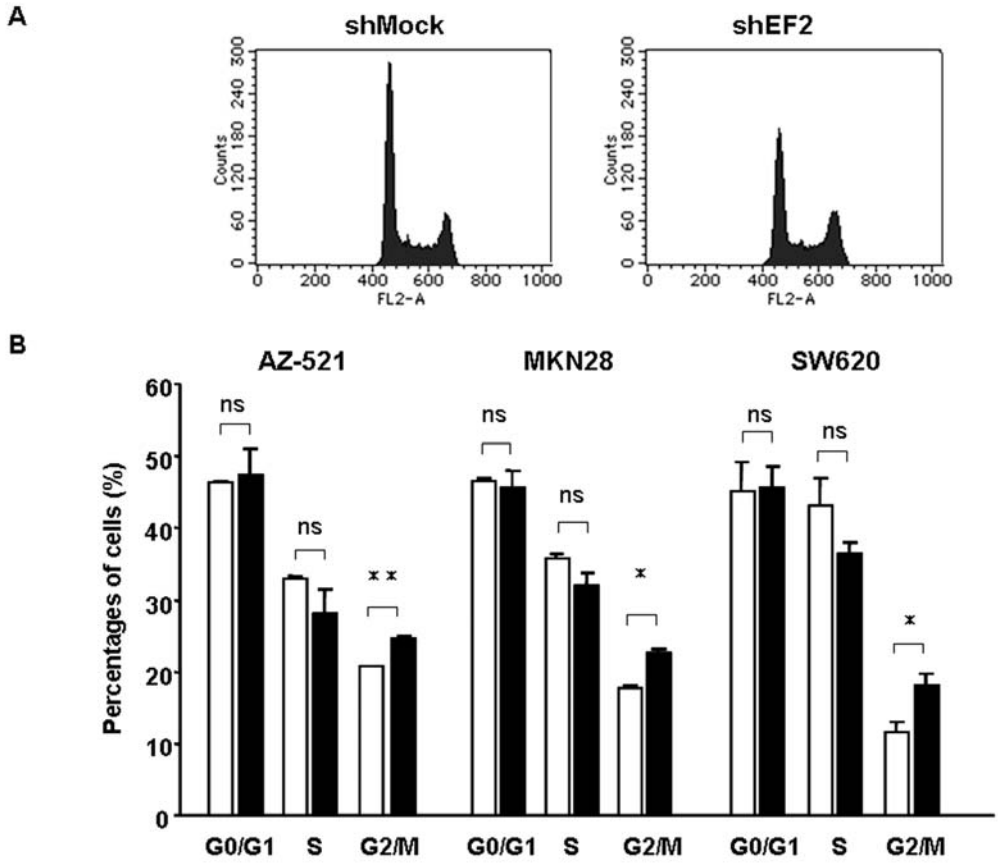

Figure 3. Knockdown of eEF2 induces G2/M arrest. Three cell lines, AZ-521, MKN28, and SW620 ($5x10^5$ cells) were transfected with 10 μg of shEF2 or shMock vector. After 48 h of culture, the cells were stained with PI for measurement of DNA content and analyzed for cell cycle distribution by flow cytometry. (A) Representative dot plots of AZ-521 cells transfected with shMock or shEF2. Vertical and horizontal axes represent cell count and intensity of fluorescence (DNA content), respectively. (B) Stages of cell cycle were determined by the DNA contents and percentages of each stage are shown. Experiments were performed independently three times. Open columns, shMock-transfected cells; closed columns, shEF2-transfected cells. $^{*}P<0.05$; $^{**}P<<0.01$; ns, not significant.

on cell cycle progression was examined in AZ-521 gastric cancer cells. First, two EF2-transduced AZ-521 cell clones with high expression levels of eEF2 and two pcMock-transduced AZ-521 cell clones were isolated (Fig. 4A), and the growth rate of these cells was examined (Fig. 4B). Doubling time of the cells was calculated as 9.6 and 12.7 h in eEF2-transduced and pcMock-transduced AZ-521 cells, respectively. Cell cycle distribution of the asynchronously growing cells was analyzed by flow cytometry. Forced expression of eEF2 significantly decreased cells in G2/M phase and increased those in the S phase (Fig. 4C). Then, progression time of each phase of the cell cycle was determined by multiplying doubling time with percentages of each phase in the cell cycle in each cell clone (Fig. 4D). G2/M progression time was significantly shortened by 39.7% in eEF2-transduced AZ-521 clones compared to mock vector-transduced ones, whereas there was no significant difference in G1 and S phase progression time between eEF2-transduced and pcMock vector-transduced cells. These results indicated that eEF2 was involved in cancer cell growth through promotion of cell cycle progression at G2/M phase.

eEF2 regulates function of Akt and cdc2. Since knockdown of eEF2 by shEF2 induced G2/M arrest in the cell cycle in gastrointestinal cancer cells, the expression and activation status of cdc2 protein (a G2/M regulator) were examined in AZ-521 and SW620 cells transfected with shEF2 or shMock vector (Fig. 5A). Western blot analysis showed that expression levels of total cdc2 protein were similar between shEF2- and shMock-transfected cells, but expression levels of inactivated phosphorylated cdc2 protein were higher in shEF2-transfected cells compared to shMock-transfected cells in AZ-521 cells (Fig. 5A). Then, the expression and activation status of Akt protein that played an important role in regulation of cell proliferation and translation were also analyzed. Western blot analysis showed that expression levels of total Akt protein were similar between shEF2- and shMock-transfected cells, but expression levels of activated phosphorylated Akt protein were decreased in shEF2-transfected cells compared to shMock-transfected cells (Fig. 5A). These results showed that knockdown of eEF2 by shEF2 increased inactivated cdc2 protein and decreased activated form of Akt protein regardless of no or little change in total amount of cdc2 and Akt proteins, resulting in the suppression of the function of both cdc2 and Akt proteins. Since previous reports showed that cdc2 and Akt could regulate the activation status of eEF2 kinase that was a negative regulator of eEF2, activation status of eEF2 kinase was examined (Fig. 5A). Inactivated phosphorylated eEF2 kinase was decreased in shEF2-transfected cells compared to shMock-transfected ones. Consistent

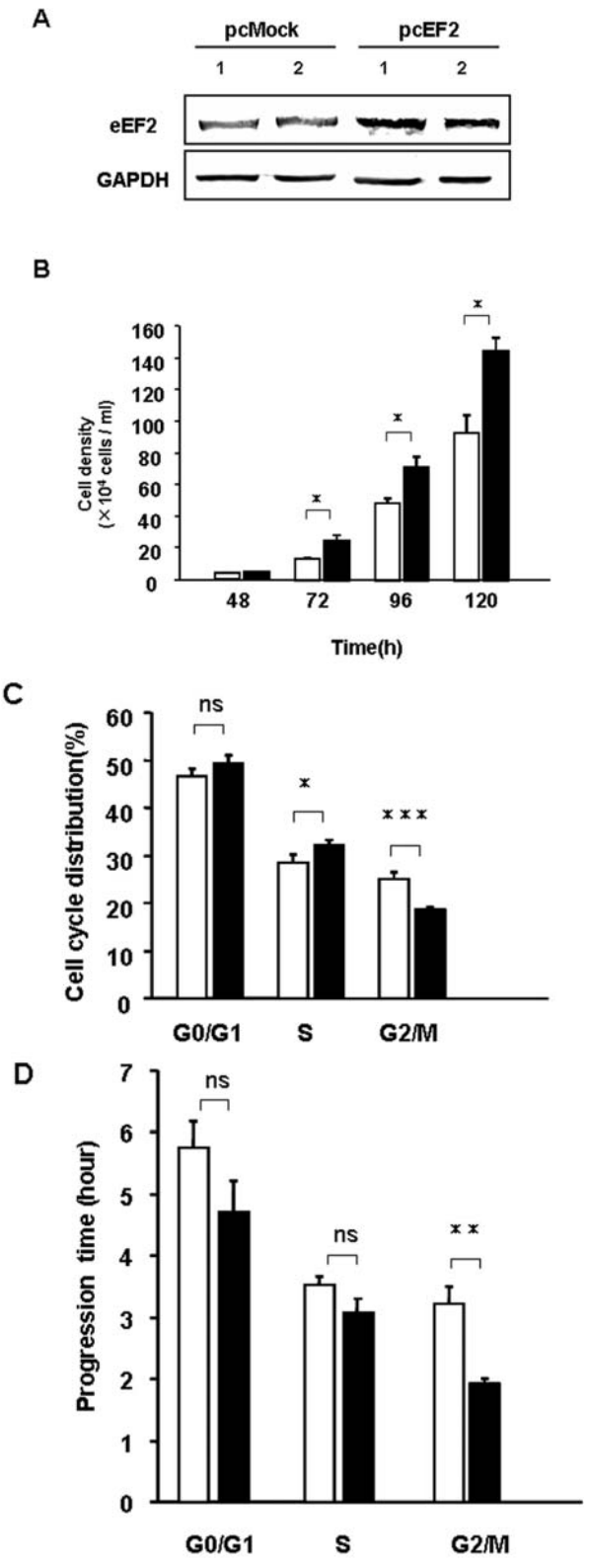

Figure 4. Forced expression of eEF2 promotes G2/M progression. Asynchronously growing AZ-521 cell clones were transduced with pcEF2 (n=2) or pcMock vector (n=2), cultured, and then analyzed for cell cycle distribution by flow cytometry. (A) Representative results of Western blot analysis for forced expression of eEF2 protein. (B) Cell growth rates. Viable cells were counted at the indicated time-points. (C) Cell cycle distribution. Asynchronously growing cells were stained with PI and FACS-analysed for DNA content, and cell cycle distribution was determined by the DNA content. (D) Progression time of each phase of cell cycle was determined as described in Results. (B-D) Open columns, shMock-transfected cells; closed columns, shEF2-transfected cells. $^{*}P<0.05$; $^{**}P<0.01$; $^{**}P<0.001$; ns, not significant. Results were obtained from three independent experiments using two cell clones for each test group.

with this, inactivated phosphorylated eEF2 was increased in shEF2-transfected cells compared to shMock-transfected ones regardless of decrease in expression levels of total eEF2 protein (Fig. 5A). Similar results were obtained from SW620 cancer cells (data not shown). These results indicated that eEF2 protein whose expression was suppressed by shEF2 was further inactivated by activated eEF2 kinase, resulting in the decrease in total activity of eEF2.

To confirm the above results concerning the regulation of cdc2 and Akt by eEF2, the expression and activation status of cdc2 and Akt proteins were examined under the conditions of the forced expression of eEF2 using the EF2- and pcMock-transduced AZ-521 cell clones (Fig. 5B). Expression levels of total cdc2 protein were similar between EF2- and pcMock-transduced AZ-521 cells, but expression levels of inactivated phosphorylated cdc2 protein was decreased in EF2-transduced cells compared to pcMock-transduced ones. Expression levels of total Akt protein were similar between EF2- and pcMock-transduced AZ-521 cells, but expression levels of activated phosphorylated Akt protein were increased in EF2-transduced cells compared to pcMock-transduced ones. Furthermore, inactivated phosphorylated eEF2 kinase was increased, and inactivated phosphorylated eEF2 was reasonably decreased in EF2-transduced AZ-521 cells (Fig. 5B) despite an increase in expression levels of total eEF2. These results indicated that the forced expression of eEF2 increased the activity of eEF2 through the decrease in inactivated form of eEF2 due to inactivation of eEF2 kinase and resulted in the increase in the activity of both the cdc2 and Akt proteins.

Taken together, the results presented herein indicated that knockdown of eEF2 suppressed cell cycle progression in G2/M through inactivation of cdc2 and Akt proteins. Conversely, forced expression of eEF2 promoted G2/M progression through the activation of cdc2 and Akt proteins. Therefore, eEF2 may be involved in the G2/M progression of cell cycle in gastrointestinal cancer through regulation of the activity of cdc2 and Akt proteins.

Forced expression of eEF2 enhances in vivo tumorigenicity of gastric cancer cells. To examine whether or not forced expression of eEF2 promotes *in vivo* tumorigenicity of gastrointestinal cancer cells, the eEF2- or pcMock-transduced AZ-521 cell clones (Fig. 4A) were inoculated into both flanks of female BALB/c Nu/Nu mice and tumor volume was measured weekly. As shown in Fig. 6, forced expression of eEF2 significantly enhanced *in vivo* tumor growth of AZ-521 gastric cancer cells. These results indicated that forced expression of eEF2 significantly enhanced *in vivo* tumorigenicity of gastrointestinal cancer cells, implying the important role of eEF2 in tumorigenesis.

Discussion

Both mechanisms of translation and cell cycle are essential systems for cell proliferation. A previous study reported a link between translational initiation and G1 phase progression of the cell cycle. Overexpression of eukaryotic initiation factor 4E (eIF4E) markedly increased the amount of a G1-specific cyclin, cyclin D1 protein in NIH 3T3 cells (19). However, whether or not the disrupted regulation of the elongation

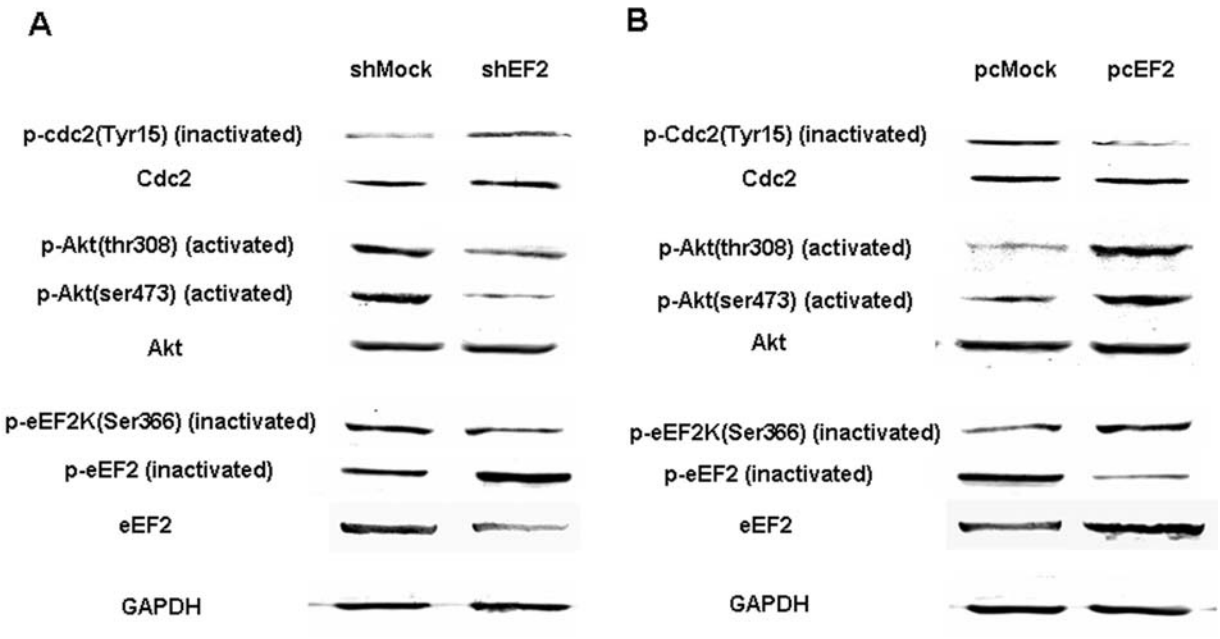

Figure 5. eEF2 regulates activities of cdc2 and Akt. (A) Knockdown of eEF2 by shEF2. AZ-521 cancer cells were transfected with shEF2 or shMock vector, and protein expression levels of cdc2, Akt, and eEF2 kinase were analyzed by Western blotting. Similar results were also obtained from SW620 cancer cells. (B) Forced expression of eEF2. AZ-521 cancer cells were transduced with pcEF2 or pcMpck vector, and protein expression levels of cdc2, Akt, and eEF2 kinase were analyzed by Western blotting. GAPDH was used as an internal control.

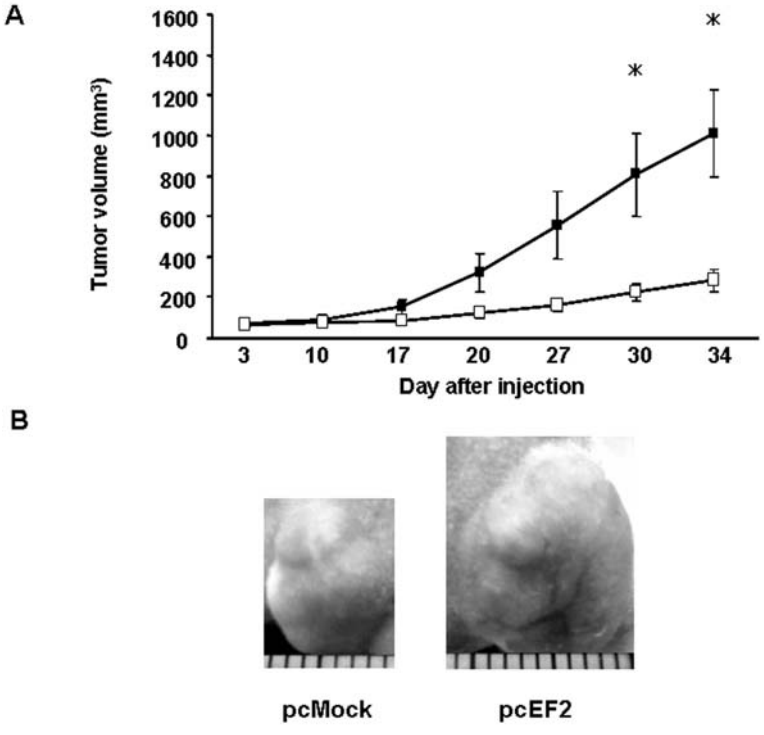

Figure 6. Forced expression of eEF2 enhances *in vivo* tumorigenicity. (A) *In vivo* growth curves of AZ-521 cells with or without forced expression of eEF2 are shown. AZ-521 cells transduced with pcEF2 (closed squares) or pcMock (open squares) were subcutaneously inoculated in each side of flank of BALB/cAJcl-nu/nu female mice. Tumor volumes were weekly measured from 3 to 34 days after the inoculation. Shown are average and standard error of three independent experiments using two cell clones for each test group (Fig. 4A). (B) Representative *in vivo* tumors (day 34) of AZ-521 cells transduced with pcEF2 (right) or pcMock (left) vector are shown.

step of translation was concerned with tumorigenesis remained undetermined although mounting evidence suggest a linkage between disrupted translation and tumorigenesis. Herein we showed for the first time that the wild-type *eEF2* gene, which played an essential role in the polypeptide chain elongation step, was overexpressed in the majority of human

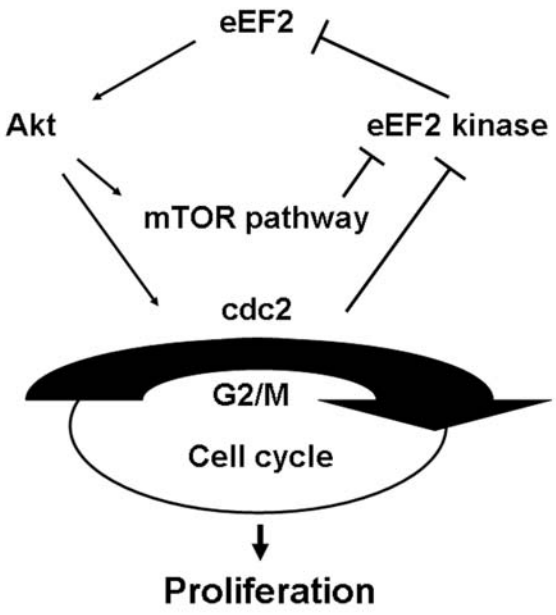

Figure 7. Schematic representation of eEF2 signaling pathway. ↓, activation; ⊣, inactivation.

gastric and colorectal cancers and that its overexpression promoted *in vitro* and *in vivo* the growth of these cancer cells through the promotion of progression of G2/M phase in the cell cycle. These results indicated that aberrant upregulation of translational elongation step played an important role in the tumorigenesis of gastrointestinal cancers.

Function of eEF2 as clarified in the present study is summarized in Fig. 7. Akt is a serine/threonine kinase that mediates a variety of biological processes. In gastrointestinal cancers, increased expression of activated phosphorylated Akt was associated with disease progression in gastric (20) and colon (21) cancers and with resistance to chemotherapy in gastric (22) and colorectal cancers (23), and thus activation of Akt was considered to play an important role in their tumorigenesis. In the present study, we demonstrated that eEF2 regulated the activity of Akt, which could play at least two important roles as a downstream molecule of eEF2 (Figs. 5 and 7). First, Akt activates the G2/M regulator cdc2 by its dephosphorylation (24), implying that Akt mediates the function of eEF2 for promotion of G2/M progression by activation of cdc2. Second, Akt activates the mTOR signaling pathway through inactivation of an upstream regulator of mTOR, TSC2 (25), resulting in activation of eEF2 through the inactivation of a negative regulator of eEF2, the eEF2 kinase. Taken together, Akt might be an important molecule in eEF2 signaling pathway that mediates promotion of cancer cell growth induced by eEF2 and activation of eEF2 in a positive feedback manner. These findings might provide a novel oncogenic role to Akt in the tumorigenesis.

We showed that overexpression of eEF2 significantly promoted G2/M progression in cancer cells in association with the activation of the G2/M regulator, cdc2 protein. It was reported that cdc2/cylin B1 directly phosphorylated eEF2 kinase that negatively regulated the activity of eEF2 through its phosphorylation (26). Thus, activation of cdc2 by eEF2 results in activation of eEF2 itself through inactivation of eEF2 kinase due to its phosphorylation (Figs. 5 and 7). In fact, overexpression of eEF2 decreased inactivated phosphorylated eEF2 protein, and conversely, knockdown of eEF2 expression by shEF2 increased inactivated phosphorylated eEF2 protein. Our results presented herein suggested a novel linkage between translational elongation and cell cycle machineries. Since eEF2 can be activated in a positive feedback manner through inactivation of eEF2 kinase by cdc2, the novel linkage can mediate a bidirectional crosstalk between translation and cell cycle machineries. Thus, the linkage may play an important role in orchestrating the deregulated translational elongation and cell cycle machineries to promote the development of gastrointestinal cancers.

We showed that eEF2 promoted cancer cell growth *in vitro* and enhanced *in vivo* tumorigenicity in a mouse xenograft model. eEF2 signaling pathway could form a linkage between the translational elongation step and the cell cycle machineries and might play an important role in orchestrating the deregulated translational elongation and cell cycle machineries. Knockdown of eEF2 by shEF2 significantly inhibited growth of the three gastrointestinal cancer cell lines examined. Knockdown of eEF2 could break this linkage and inhibit cancer cell growth. These results suggested that eEF2 might be a promising target molecule in targeted cancer therapy. Therefore, eEF2-targeted therapy could provide a novel therapeutic strategy for gastrointestinal cancers.

Acknowledgements

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology-Japan and the Ministry of Health, Labour, and Welfare-Japan.

References

1. Mingchao M, Devereux TR, Stockton P, Sun K, Sills RC, Clayton N, Portier M and Flake G: Loss of E-cadherin expression in gastric intestinal metaplasia and later stage p53 altered expression in gastric carcinogenesis. Exp Toxicol Pathol 53: 237-246, 2001.
2. Zheng H, Takahashi H, Murai Y, Cui Z, Nomoto K, Tsuneyama K and Takano Y: Low expression of FHIT and PTEN correlates with malignancy of gastric carcinomas: tissue-array findings. Appl Immunohistochem Mol Morphol 122: 432-440, 2007.
3. Chan AO, Wong BC, Lan HY, Loke SL, Chan WK, Hui WM, Yuen YH, Ng I, Hou L, Wong WM, Yuen MF, Luk JM and Lam SK: Deregulation of E-cadherin-catenin complex in pre-cancerous lesions of gastric adenocarcinoma. J Gastroenterol Hepatol 18: 534-539, 2003.
4. Boynton RF, Blount PL, Yin J, Brown VL, Huang Y, Tong Y, McDaniel T, Newkirk C, Resau JH and Raskind WH: Loss of heterozygosity involving the APC and MCC genetic loci occurs in the majority of human esophageal cancers. Proc Natl Acad Sci USA 89: 3385-3388, 1992.
5. Nagasaka T, Sasamoto H, Notohara K, Cullings HM, Takeda M, Kimura K, Kambara T, MacPhee DG, Young J, Leggett BA, Jass JR, Tanaka N and Matsubara N: Colorectal cancer with mutation in BRAF, KRAS, and wild-type with respect to both oncogenes showing different patterns of DNA methylation. J Clin Oncol 22: 4584-4594, 2004.
6. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S and DuBois RN: Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107: 1183-1188, 1994.
7. Munro AJ, Lain S and Lane DP: P53 abnormalities and outcomes in colorectal cancer: a systematic review. Br J Cancer 92: 434-444, 2005.

8. Bilanges B and Stokoe D: Mechanism of translational deregulation in human tumors and therapeutic intervention strategies. Oncogene 26: 5973-5990, 2007.
9. De Benedetti A and Graff JR: eIF-4E expression and its role in malignancies and metastases. Oncogene 23: 3189-3199, 2004.
10. De Benedetti A and Harris AL: eIF4E expression in tumors: its possible role in progression of malignancies. Int J Biochem Cell Biol 31: 59-72, 1994.
11. Ruggero D, Montanaro L, Ma L, Xu W, Londei P, Cordon-Cardo C and Pandolfi PP: The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. Nat Med 10: 484-486, 2004.
12. Shantz LM, Hu RH and Pegg AE: Regulation of ornithine decarboxylase in a transformed cell line that overexpresses translation initiation factor eIF-4E. Cancer Res 56: 3265-3269, 1996.
13. Zhou S, Wang GP, Liu C and Zhou M: Eukaryotic initiation factor 4E (eIF4E) and angiogenesis: prognostic markers for breast cancer. BMC Cancer 6: 231, 2006.
14. Yang SX, Hewitt SM, Steinberg SM, Liewehr DJ and Swain SM: Expression levels of eIF4E, VEGF, and cyclin D1, and correlation of eIF4E with VEGF and cyclin D1 in multi-tumor tissue microarray. Oncol Rep 17: 281-287, 2007.
15. Tash JS, Chakrasali R, Jakkaraj SR, Hughes J, Smith SK, Hornbaker K Heckert LL, Ozturk SB, Hadden MK, Kinzy TG, Blagg BS and Georg GI: Gamendazole, an orally active indazole carboxylic acid male contraceptive agent, targets HSP90AB1 (HSP90BETA) and EEF1A1 (eEF1A), and stimulates Il1a transcription in rat sertoli cells. Biol Reprod 78: 1139-1152, 2008.
16. Tomlison VA, Newbery HJ, Wray NR, Jackson J, Larionov A, Miller WR, Dixon JM and Abbott CM: Translation elongation factor eEF1A2 is a potential oncoprotein that is overespressed in two-thirds of breast tumors. BMC Cancer 5: 113, 2005.
17. White SJ, Kasman LM, Kelly MM, Lu P, Spruill L, McDermott PJ and Voelkel-Johnson C: Doxorubicin generates a proapoptotic phenotype by phosphorylation of EF-2. Free Radic Biol Med 43: 1313-1321, 2007.
18. Pineiro D, Gonzalez VM, Hernandez-Jimenez M, Salinas M and Martin ME: Translation regulation after taxol treatment in NIH3T3 cells involves the elongation factor (eEF) 2. Exp Cell Res 313: 3694-3706, 2007.
19. Rosenwald IB, Lazaris-Karatzas A, Sonenberg N and Schmidt EV: Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E. Mol Cell Biol 13: 7358-7363, 1993.
20. Han Z, Wu K, Shen H, Li C, Han S, Hong L, Shi Y, Liu N, Guo C, Xue Y, Qiao T and Fan D: Akt1/protein kinase Balpha is involved in gastric cancer progression and cell proliferation. Dig Dis Sci 53: 1801-1810, 2008.
21. Saglam O, Garrett CR, Boulware D, Sayegh Z, Shibata D, Malafa M, Yeatman T, Cheng JQ, Sebti S and Coppola D: Activation of the serine/threonine protein kinase AKT during the progression of colorectal neoplasia. Clin Colorectal Cancer 6: 652-656, 2007.
22. Yu HG, Ai YW, Yu LL, Zhou XD, Liu J, Li JH, Xu XM, Liu S, Chen J, Liu F, Qi YL, Deng Q, Cao J, Liu SQ, Luo HS and Yu JP: Phosphoinositide 3-kinase/Akt pathway plays an important role in chemoresistance of gastric cancer cells against etoposide and doxorubicin induced cell death. Int J Cancer 15: 433-443, 2008.
23. Bates RC, Edwards NS, Burns GF and Fisher DE: A CD44 survival pathway triggers chemoresistance via lyn kinase and phosphoinositide 3-kinase/Akt in colon carcinoma cells. Cancer Res 61: 5275-5283, 2001.
24. Kuo PL, Hsu YL and Cho CY: Plumbagin induces G2-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells. Mol Cancer Ther 5: 3209-3221, 2006.
25. Manning BD, Logsdon MN, Lipovsky AI, Abbott D, Kwiatkowski DJ and Cantley LC: Feedback inhibition of Akt signaling limits the growth of tumors lacking Tsc2. Genes 19: 1773-1778, 2005.
26. Smith EM and Proud CG: Cdc2-cyclin B regulates eEF2 kinase activity in a cell cycle- and amino acid-dependent manner. EMBO J 27: 1005-1016, 2008.